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| Title | Nuclear and cytoplasmic effects of human CRM1 on HIV-1 production in rat cells |
| Author(s) | Nagai-Fukataki, Mika; Ohashi, Takashi; Hashimoto, Iwao et al. |
| Citation | Genes to Cells, 16(2), 203-216<br>https://doi.org/10.1111/j.1365-2443.2010.01476.x |
| Issue Date | 2011-02 |
| Doc URL | https://hdl.handle.net/2115/46874 |
| Rights | The definitive version is available at wileyonlinelibrary.com |
| Type | journal article |
| File Information | GtC16-2_203-216.pdf |

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# Nuclear and Cytoplasmic Effects of Human CRM1 on HIV-1 Production in Rat Cells

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Sub-title: Human CRM1 on HIV-1 Production in Rat Cells

Character count in text: 52,916

Word count in abstract: 180

**Abstract**

The human immunodeficiency virus type 1 (HIV-1) regulatory protein, Rev, mediates the nuclear export of unspliced *gag* and singly-spliced *env* mRNAs by bridging viral RNA and the export receptor, CRM1. Recently, rat CRM1 was found to be less efficient than human CRM1 in supporting Rev function in rats. In this study, to understand the role of CRM1 in HIV propagation, the mechanism underlying the function of human and rat CRM1 in HIV-1 replication was investigated in rat cells. The production of viral particles, represented by the p24 Gag protein, was greatly enhanced by hCRM1 expression in rat cells; however, this effect was not simply due to the enhanced export of *gag* mRNA. The translation initiation rate of *gag* mRNA was not increased, nor was the Gag protein stabilized in the presence of hCRM1. However, the processing of the p55 Gag precursor and the release of viral particles were facilitated. These results indicated that hCRM1 exports *gag* mRNA to the cytoplasm, not only more efficiently than rCRM1, but also correctly, leading to efficient processing of Gag proteins and particle formation.

**Introduction**

Appropriate animal models for viral infection allow the analysis of viral pathogenesis and oncogenesis, which in turn assist the development of therapeutic and preventative measures. Current animal models of HIV-1 (human immunodeficiency virus-1) disease have used non-human primates (Giuffre *et al.* 2003; Hazuda *et al.* 2004; Hu 2005; Veazey *et al.* 2005) and severe combined immunodeficiency (SCID) mice with transplanted fetal human tissues (Shultz *et al.* 2007; Watanabe *et al.* 2007). These models have made significant contributions to our understanding of lentiviral pathogenesis and have assisted in the development of therapeutic strategies. However, these models have significant shortcomings, such as the limited availability and high cost of nonhuman primates, the absence of or delay in disease progression, permissivity only for related retroviruses, and insufficient viral propagation. For these reasons, new animal models are needed. If viral infection is possible, it would be convenient to use small animal models, in particular mice and rats, since their inbred strains are well characterized and can be genetically manipulated.

Studies on rodent cell-specific defects in the HIV-1 life cycle have facilitated

the identification and characterization of the host cell gene products that are essential for viral replication, and these may provide a molecular basis for generating a fully permissive small-animal model. It has been suggested that the major block to HIV-1 replication is at the level of entry and that this hurdle could be overcome by introducing human CD4 and CCR5, which serve as receptors for HIV-1 (Keppler *et al.* 2001; Keppler *et al.* 2002). Transgenic (Tg) rats, expressing human CD4 and CCR5, have been reported to support some replication of HIV-1, albeit very poorly (Keppler *et al.* 2002; Goffinet *et al.* 2007). In contrast, Tg mice expressing human CD4/CCR5 do not allow the propagation of HIV-1 (Browning *et al.* 1997). These results support the rat as a promising small-animal model candidate.

Analyses have revealed novel interactions between rodent and viral factors. A notable example is CRM1 (chromosome region maintenance 1; exportin 1), a member of the karyopherin family of nucleocytoplasmic-transport factors (Neville *et al.* 1997; Cullen 2003) that is involved in nuclear export. In the nucleus, CRM1 binds its cargo in the presence of a GTP-bound form of the Ran GTPase (RanGTP), and following nuclear export, hydrolysis of GTP to GDP causes a conformational shift that

induces cytoplasmic cargo release, providing directionality for this export pathway (Fornerod *et al.* 1997; Neville *et al.* 1997; Nakielny & Dreyfuss 1999; Cullen 2003). Human CRM1 cooperates with HIV-1 Rev and the human T cell leukemia virus type 1 (HTLV-1) Rex proteins to transport 4 kb of partially-spliced and 9 kb of unspliced mRNAs encoding viral structural proteins and genomes (Cullen 1998). Rev or Rex multimerizes on viral RNA to recruit several CRM1 molecules; furthermore, the recruitment of CRM1 enhances Rev or Rex interactions vice versa (Hakata *et al.* 2002). Rat CRM1, which exhibits 98% amino acid identity to its human counterpart, is able to bind efficiently to Rex, but cannot induce the multimerization of Rex proteins on its cognate RNA. This results in the breakdown of HTLV-1 RNA transport in rat cells (Hakata *et al.* 2001). Thus, rat CRM1 represents a major factor in the human-rat species barrier to HTLV-1 (Zhang *et al.* 2006; Takayanagi *et al.* 2007). Further detailed comparisons of human and rat CRM1 proteins identified the domains essential for the induction of Rex multimerization and the binding of RanBP3, another component involved in RNA export (Hakata *et al.* 2003). These analyses suggested that CRM1 functions not only in RNA export out of the nucleus, but also in the formation of the

transport complex that includes viral RNA and various cellular components (Hakata *et al.* 2003).

In contrast to the definitive results obtained for rat CRM1-Rex interactions, the existence of a profound block in Rev function in rodent cells remains controversial, although a reduced level of the 9 kb transcript has been generally reported (Bieniasz & Cullen 2000; Keppler *et al.* 2001). Some studies have found impaired Rev activity (Marques *et al.* 2003), whereas others have ascribed the reduced transcript level to oversplicing or to the reduced stability of unspliced transcripts in rodent cells compared to human cells (Malim *et al.* 1991), a problem that is corrected by the expression of the human p32 protein (Zheng *et al.* 2003). These contradictory results seem to stem partly from the use of different cells and reporters for evaluating Rev activity and partly from the limited investigation of cellular cofactors. However, recently, we found that Gag production was increased by hCRM1 in rat macrophages in contrast to its marginal effect in rat T cells (Okada *et al.* 2009).

In this study, we have compared the molecular mechanisms underlying the function of human and rat CRM1 proteins in HIV propagation in rat cells. We show that

the expression of hCRM1 in rat cells not only enhances the export of HIV *gag* mRNA, but also restores the processing of the precursor Gag protein and release of viral particles, leading to efficient virus production.

## Results

### Enhanced p24 production in hCRM1-transfected rat cells

Recently, we found that hCRM1 enhanced Gag production in rat macrophages (Okada *et al.* 2009); therefore, it was decided to examine the molecular mechanism involved in this process using convenient rat cell lines. ER1/neo1 cells are particularly useful because they, like rat macrophages, support the efficient expression of HIV-1 genes without the need for human cyclin T1 (unpublished results; [Okada *et al.* 2009]). The initial step involved transfecting the hCRM1-expressing plasmid into ER1/neo1 cells along with the reporter pCRRE (Kimura *et al.* 1996), which harbors the HIV-1 genome, including the intact *gag* and *rev* sequences (Fig. 1A). Next, the production of the Gap protein, p24, in the medium was measured by ELISA. In addition, cells were simultaneously co-transfected with pCDMβ-gal, which was used to normalize the transfection efficiency, and pH1-luc, which acted as a surrogate marker for total gene expression driven by the HIV-1 LTR. In comparison with untransfected rat cells, hCRM1-transfected cells produced increasing levels of p24 in the culture supernatant with increasing amounts of pSRαhCRM1. On the other hand, β-gal and luc activities

remained at similar levels in both hCRM1-transfected and untransfected rat cells, indicating similar transfection efficiencies and HIV LTR-mediated transcription, irrespective of hCRM1 expression (Fig. 1B). These results demonstrated the enhancement of p24 production following transfection with hCRM1.

Next, we compared the effect of hCRM1 and rCRM1 in HeLa and ER1/neo1 cells by measuring p24 production. Following transfection of either of the CRM1-expressing plasmids into HeLa cells, a small enhancement of p24 production was observed, although the differences between rat and human CRM1 were not significant (Fig. 1C). In addition, the same results were obtained in other human cell lines, including 293T and HOS cells (data not shown). In contrast, p24 production increased markedly in ER1/neo1 cells following transfection with the hCRM1-expressing plasmid, whereas expression of rCRM1 had no detectable effect (Fig. 1C). As expected, no significant changes in β-gal or luc activities were observed following transfection with human and rat CRM1-expressing plasmids (data not shown). In addition, hCRM1 enhanced expression of Gag in other rat cell lines (REF52, W31, FPM-SV, and NR8383 cells [Okada *et al.* 2009]), whereas rCRM1 did not (data not

shown). These results suggested that rCRM1 is a poor cofactor for Rev.

**Effect of hCRM1 on the export of *gag* mRNA**

The above results indicated that hCRM1 enhanced p24 production in rat cells, thus the next step was to examine the mechanism underlying this phenomenon. It is conceivable that hCRM1 may export *gag* mRNA more efficiently than rCRM1. To investigate this possibility, hCRM1- or mock-transfected cells were separated into their nuclear and cytoplasmic fractions, and *gag* mRNA levels in the cytoplasmic fractions were compared using quantitative RT-PCR. To confirm augmented levels of Gag protein in these hCRM1-transfected cells, the medium was collected and subjected to a p24 ELISA. In the cytoplasm of hCRM1-transfected rat cells, the increase in *gag* mRNA levels was two to three times that of the mock-transfectants. A similar increase in *gag* mRNA was also observed in the nuclear fraction in the presence of hCRM1 (data not shown). On the other hand, hCRM1 expression had no effect on *tat* mRNA levels, since CRM1 is not involved in that particular export pathway (Fig. 1D and E). No signals were detected in the samples that had not been subjected to a reverse

transcriptase (RT) reaction (data not shown). Efficient separation of the cytosolic and nuclear fractions was confirmed by amplification of *β-actin* pre-mRNA, which was detected exclusively in the nuclear fraction (data not shown). In contrast to the small increase in *gag* mRNA levels in the cytoplasm, at its peak, the production of p24 from hCRM1-transfected rat cells was nine times greater than in the mock-transfectants (Fig. 1E). p24 Gag production and the efficiency of gag mRNA export was decreased by transfection of 0.6μg hCRM1 expression plasmids (Fig. 1D and E). Too much hCRM1 may affect nuclear export adversely due to imbalance with other factors. These results revealed that hCRM1 modestly enhanced *gag* mRNA export in rat cells but greatly increased Gag protein production, suggesting that hCRM1 may function in the cytoplasm.

**Stability of Gag protein**

Next, we examined whether hCRM1 has an effect on Gag protein stability. A [$^{35}$S]-methionine/cysteine pulse-chase analysis of the Gag protein was performed in rat cells transfected with pCRRE. An inhibitor against HIV-1 protease was added to

prevent processing of the Gag precursor, which would hamper the assessment of Gag stability. Since Gag proteins are released as viral particles into the medium, the total amount of Gag protein in both the cell and the medium should be quantified to assess its stability. Thus, the total cell culture including both cell lysates and medium was dissolved with the detergent solution and then immunoprecipitated with an anti-p24 monoclonal antibody (mAb), followed by SDS-PAGE and autoradiography. The Gag protein was highly stable, irrespective of the presence or absence of hCRM1 (Fig. 1F). The stability of the Gag protein was ascertained using another plasmid, pNLmyr(-)pro(-)Flag, which exclusively expresses intracellular, nonmyristoylated p55 Gag due to a G2A mutation at the 5' region of the *gag* gene (Kawada *et al.* 2008) (Fig. 1G). These results excluded the involvement of hCRM1 in Gag protein stability.

The small increase of intensity of p55 Gag bands in the presence of hCRM1 (Fig. 1F and G) corresponded to the enhancement of gag mRNA in the cytoplasm (Fig. 1D and E), suggesting that the efficiency of *gag* mRNA translation was not elevated by hCRM1 expression. It is consistent with the results of polyribosome analysis that indicated a constant rate of translation initiation irrespective of hCRM1 expression

(Supplementary Materials Fig. S1).

**Effect of hCRM1 on the export of *gag* mRNA and p24 production using pCMVΔR8.2**

Next, the effect of hCRM1 was examined further using the plasmid, pCMVΔR8.2, which generates higher levels of Gag protein in the medium of ER1/neo1 cells, compared to other reporters, such as pCRRE. The results showed that pCMVΔR8.2 synthesized Gag at 20- to 30-fold higher levels in the presence of hCRM1 than in its absence (Fig. 2A). As with the other reporters, a slight increase in cytoplasmic *gag* mRNA levels was observed in the presence of hCRM1 (Fig. 2B), while *tat* and *β-gal* mRNA levels were not affected (Fig. 2C and D). A similar increase in *gag* mRNA was also observed in the nuclear fraction in the presence of hCRM1 (data not shown). Efficient separation of the cytosolic and nuclear fractions was confirmed by the amplification of *β-actin* pre-mRNA, which was detected exclusively in the nuclear fraction (Fig. 2E), and *β*-galactosidase (as a cytoplasmic marker), of which approximately 95% was detected in the cytoplasmic fraction (Fig. 2F).

**FISH analysis of *gag* mRNA**

As part of our further investigation into the effect of hCRM1 on the export of *gag* mRNA in rat cells, the intracellular distribution of *gag* mRNA was analyzed using a fluorescence *in situ* hybridization assay (FISH). Since this method does not require the physical separation of the cytoplasmic and the nuclear fractions, it was possible to rule out the leakage of mRNAs from the nuclear fraction during the process. ER1/neo1 cells were cotransfected with pCMVΔR8.2 and pSR αhCRM1, pSRαrCRM1 or the pSRα296 control vector. The intracellular distribution of *gag* mRNA was visualized using a DIG-labeled minus strand DNA probe (nucleotides [nts] 964 to 798 of HIV-$1_{NL4-3}$ provirus), which is complementary to the *gag* coding region. The *gag* mRNA was distributed throughout the nucleus and cytoplasm of some cells, but was confined to the nucleus of other cells, irrespective of hCRM1 or rCRM1 expression. Both patterns of *gag* mRNA distribution were observed in HeLa and ER1/hCRM1-7 cells, which constitutively express hCRM1 (Fig. 3A). Control experiments demonstrated that the signals detected were specific for HIV-1 *gag*

mRNAs; firstly, mock-transfected cells exhibited no signal after hybridization with the same probe, and secondly, hybridization with the plus strand DNA probe (nts 798 to 964 of the HIV-$1_{NL4-3}$ provirus) failed to reveal any signals in the transfected cells (data not shown). Fig. 3B shows the percentage of cells positive for the nuclear or cytoplasmic accumulation of *gag* mRNA. An approximately three-fold increase in the cytoplasmic distribution of *gag* mRNA was observed in cells with constitutive or transient expression of hCRM1. These results were consistent with the quantification of cytoplasmic *gag* mRNA using RT-PCR (see Fig. 2B).

**Effect of hCRM1 on the processing of Gag protein**

We examined the processing of the Gag precursor and the release of viral particles using Western blot analysis. ER1/neo1 cells were transfected with pCMVΔR8.2, pCDMβ-gal and pSRαhCRM1-HA or pSRαrCRM1-HA as described above, and cell lysates and virus-like particle (VLP) fractions were prepared from the culture medium (Fig. 4A). The hCRM1 construct enhanced expression of the p55 Gag precursor approximately three-fold, whereas it had a remarkable effect on the efficient processing

of Gag (increasing it by approximately 10-fold) and on the formation of viral particles. The amount of VLP was evaluated by the p24 ELISA assay because the p24 bands of the VLP fractions, prepared from pSRα296 and pSRαrCRM1-transfected cells, were too faint in the Western blot profile to be quantified. The ELISA revealed a 70-fold increase in VLP in response to the expression of hCRM1, while rCRM1 had no effect (data not shown). Less hCRM1 protein was expressed than rCRM1 protein (Fig. 4A), and it is possible that the hCRM1-HA protein may be unstable in ER1/neo1 cells, as shown previously (Okada *et al.* 2009). The slight increase in p55 Gag protein in the presence of hCRM1 is consistent with the slight increase in *gag* mRNA observed in the cytoplasm (see Figs. 1D, E and 2B). In addition, the remarkable increase in VLP is consistent with the increase in p24 detected by ELISA in the culture supernatant, as described above (Figs. 1B and 2A). In human HeLa cells, which support high levels of HIV-1 production, both efficient processing and particle release were observed (Fig. 4A).

Next, to investigate whether the amount of Gag protein in the cells affected the processing of Gag and the release of viral particles, cells were transfected with

various amounts of pCMVΔR8.2, together with pSRαhCRM1-HA or pSRαrCRM1-HA. The proportions of the p55 and p24 Gag proteins were similar, irrespective of the amount of p55 in the hCRM1-transfected cells (Fig. 4B). In contrast, the ratio of p24/p55 in the rCRM1-transfected cells was less than in the hCRM1-expressing cells, even when the rCRM1-transfected cells contained more p55. Moreover, the bands of p24 were clearly detected in the VLP fractions that were prepared from hCRM1-transfected cells, but not in the fractions from rCRM1-transfected cells (Fig. 4B). The relative amounts of p24, as quantified by ELISA, were 4.6, 59 and 1 in the VLP fractions of 0.8 μg pCMVΔR8.2/pSRαhCRM1-, 1.6 μg pCMVΔR8.2/pSRαhCRM1-, and 0.8 μg pCMVΔR8.2/pSRαrCRM1-transfected cells, respectively (data not shown), consistent with the Western blotting profiles (Fig. 4B). These results suggested that the efficiency of Gag protein processing was enhanced by hCRM1-mediated *gag* gene expression but not by the amount of intracellular Gag protein.

**Effect of hCRM1 on membrane trafficking and the release of p55 Gag**

To examine the effect of hCRM1 on the destination of the Gag protein, in the absence of Gag processing, the p55 Gag protein was expressed using pNLmyr(-)pro(-)Flag and pNLmyr(+)pro(-)Flag because these vectors express only uncleaved p55, while the FLAG tag supports clear images in immunofluorescent assays. Moreover, the amount of p55 expressed by pNLmyr(-)pro(-)Flag may reflect the amount of *gag* mRNA in the cytoplasm more accurately since it is not released into the medium due to the lack of myristoylation.

When pNLmyr(-)pro(-)Flag was transfected into ER1/neo1 cells, hCRM1 expression augmented the amount of intracellular p55 by 3.3- or 2.5-fold more than the empty vector or rCRM1 expression, respectively (Fig. 5A). This moderate increase in p55 Gag protein in the presence of hCRM1 reflected the small enhancement of *gag* mRNA observed in the cytoplasm (See Fig. 1D and E, and Fig. 2B), consistent with the results described in Fig. 1F, 1G, and S1. When pNLmyr(+)pro(-)Flag was transfected into ER1/neo1 cells, hCRM1 expression enhanced the intracellular p55 level by two-fold compared to the empty vector or rCRM1 expression. In contrast, hCRM1 expression augmented VLP production by 19- or 8-fold compared to the empty vector

or rCRM1 expression, respectively (Fig. 5B). These results suggested that hCRM1 promotes the release of viral particles independent of the processing of p55.

Next, immunofluorescence was used to examine the localization of p55 Gag in the presence or absence of hCRM1. Unmyristoylated p55 was distributed diffusely throughout the cytoplasm of both HeLa and hCRM1-expressing ER1/neo1 cells transfected with pNLmyr(-)Gag-Flag (Fig. 5C panels a and c). On the other hand, myristoylated Gag was characterized by its punctate state on the plasma membranes of HeLa and ER1/neo1 cells transfected with pNLmyr(+)Gag-Flag, irrespective of the expression of human or rat CRM1 (Fig. 5C panels b, d, e, and f). The ratio (20 to 30%) of rat cells that showed the punctate Gag profile was not increased by the expression of hCRM1, whereas almost HeLa cells showed the punctate profiles. These results suggest that the membrane trafficking of p55 Gag occurred in the rat cells inefficiently compared to the human cells, and it was not restored by hCRM1.

**Region of hCRM1 responsible for the upregulation of p24 production**

To identify the region of hCRM1 responsible for the production of p24, we

compared the ability of the two chimeric CRM1s to produce p24: hrCRM1 consisted of the N-terminal half of hCRM1 (amino acids [aa] 1 to 679) and the C-terminal half of rCRM1 (aa 680 to 1072), while rhCRM1 was its reverse chimera (Fig. 6A). Expression of hrCRM1-HA increased p24 production, whereas there was little enhancement of p24 production by rhCRM1-HA. It should be noted that hCRM1, when expressed at the same level as rCRM1, augmented p24 production but that rCRM1 had no effect, which ruled out the possibility that overexpression of rCRM1 inhibited p24 production. No significant changes in $\beta$-gal activity were observed following transfection with CRM1-expressing plasmids (Fig. 6B). These results indicated that the N-terminal region of hCRM1 was involved in the enhanced production of p24.

**Discussion**

Although initial reports suggested that only fully-spliced viral transcripts are detectable and that the effects of Rev are diminished in murine cells (Trono & Baltimore 1990), these findings were not reproduced (Malim & Cullen 1991; Bieniasz & Cullen 2000; Keppler *et al.* 2001). Instead, it was proposed that excessive splicing was responsible for a significant reduction in the levels of viral unspliced RNA species and Gag protein in rodent cells (Zheng *et al.* 2003). Moreover, it has been reported that some rat cells exhibit largely unimpaired expression of Env, a Rev-dependent HIV-1 gene product that is translated from a partially spliced viral mRNA (Keppler *et al.* 2001). One reason for these inconsistent conclusions may have been due to lack to examine the cellular cofactors of Rev, such as CRM1. In both our recent studies and the present study, we found that expression of hCRM1, but not rCRM1, enhanced production of the Gag protein in various rat cells, including macrophages (Okada *et al.* 2009), supporting previous findings of poor Rev activity in these cells.

Although significant export of *gag* mRNA into the cytoplasm of rat cells was observed, we found that hCRM1 enhanced *gag* mRNA export two- to three-fold as

indicated by quantitative RT-PCR after cell fractionation and *in situ* hybridization. This increase was in sharp contrast to the 10- to 30-fold augmentation of the Gag protein in the medium. This phenomenon is quite interesting since it has been firmly established that CRM1 is an export factor for Rev, which bridges CRM1 and the viral *gag*/*env* mRNAs (Cullen 1998, 2003). The above results suggested that, in addition to simple nucleocytoplasmic transport, CRM1 is somehow involved in regulation of gene expression in the cytoplasm, which affects destination of the cognate protein.

To determine the step during which the activity of hCRM1 differs from that of rCRM1, we examined the stability of the Gag protein using a pulse label and chase experiment. However, the results suggested that hCRM1 does not play a role in prolonging the life of the Gag protein. Neither polyribosome profiling analysis nor the quantification of p55 Gag supported the enhancement of *gag* mRNA translation efficiency by hCRM1. Instead, the data showed that the p55 Gag precursor that was synthesized in the presence of hCRM1, but not rCRM1, underwent efficient processing to the mature Gag protein and budding of virus particles into the culture medium. The results that hCRM1 affects the efficient processing and budding of Gag proteins in rat

cells explain the marked enhancement of p24 accumulation in the medium in spite of the minor increase of *gag* mRNA in the cytoplasm in the presence of hCRM1.

Our finding is reminiscent of HIV-1 replication in murine cells where *gag* mRNA, exported through the CTE/Tap pathway, subsequently restored efficient trafficking of the Gag protein to the plasma membrane, thus underlying the efficient processing and budding of HIV Gag, whereas Gag, translated from mRNA exported via the RRE/Rev/CRM1 pathway, was not targeted to the plasma membrane (Swanson *et al.* 2004). The inefficiency of HIV-1 assembly in murine cells likely reflects a cellular deficiency in the RRE/Rev-dependent nuclear export pathway that is specifically linked to the activation of Gag membrane targeting by the regulatory MA globular-head domain (Sherer *et al.* 2009). Similarly, the trafficking of Gag to the plasma membrane was less efficient in rat cells than human cells, which was substantiated by the immunofluorescent localization of p55 Gag protein to the plasma membrane in rat cells, and was not restored by the presence of hCRM1. Thus, hCRM1 may improve budding process but not membrane trafficking of Gag protein in rat cells.

The results suggest that rCRM1 may export *gag* mRNA into the cytoplasm

improperly, resulting in the inefficient processing of Gag proteins. It is most likely that CRM1 has an indirect effect on *gag* mRNA in the cytoplasm, since, upon reaching the cytoplasm, it dissociates from the transport complex containing *gag* mRNA, Rev, Ran and other cellular factors via a mechanism in which RanGTP converts to RanGDP at the periphery of the nuclear pore (Fornerod *et al.* 1997; Fukuda *et al.* 1997; Kudo *et al.* 1997; Neville *et al.* 1997; Ossareh-Nazari *et al.* 1997; Stade *et al.* 1997). Another cellular factor, Rev-interacting protein (hRIP), also known as hRab or Hrb, which is likely bridged by CRM1, has been reported to function in releasing *gag* mRNA, which would otherwise be trapped at the nuclear periphery, to the cytoplasm (Sanchez-Velar *et al.* 2004; Yu *et al.* 2005). Although, in our hands, *in situ* hybridization did not reveal improper localization of *gag* mRNA in the cytoplasm of rat cells, it is still possible that aberrant construction of the *gag* mRNA transport complex containing rCRM1 might prevent rat Rip and other cellular factors from functional interactions, finally leading to the inefficient processing of Gag proteins.

Alternatively, cytoplasmic effect of hCRM1 could be attributable to the properties of Gag proteins. Gag has capacities for nuclear entry and RNA binding

through its NC region, and RNA promotes multimerization of Gag proteins (D'Souza *et al*, 2005；Muriaux *et al*, 2001) that may be an important step of viral particle formation. Thus it is also conceivable that aberrant RNA complex in rat nucleus may cause inefficient Gag multimerization, leading to reduced viral production.

It is possible that hCRM1 may be needed for correct formation of the complex, which can then interact with rat factors and/or Gag proteins appropriately. Our results indicated that hCRM1 exports *gag* mRNA not only more efficiently than rat CRM1, but also correctly, to the cytoplasm in rat cells, leading to the efficient processing of Gag proteins and HIV-1 particle formation. Our finding that HIV-1 production in rat cells was rescued efficiently by the expression of hCRM1 may have important implications for the development of rat animal models of HIV replication and pathogenesis.

**Experimental procedures**

**Cells**

Human cell lines, including HeLa, HOS and 293T, and the rat cell lines, ER1/neo1, ER1/hCRM1-7 (Zhang *et al.* 2006), REF52 and W31 (Kanki *et al.* 2000), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in the absence or presence of 300 μg/ml G418 in 10% $CO_2$ at 37°C.

**Plasmids**

The following plasmids were used in this study: pCMVΔR8.2 (Naldini *et al.* 1996); pCRRE (Kimura *et al.* 1996); pSRαhCRM1 and pSRαrCRM1 (Hakata *et al.* 1998; Hakata *et al.* 2001); pSRα296 (Takebe *et al.* 1988); pSRαtat (Takebe *et al.* 1988); pCDM*β*-gal (Hakata *et al.* 1998); hCRM1-HA and rCRM1-HA, which carried CRM1, fused at the C terminus to an HA tag (Okada *et al.* 2009); pH1-luc (a kind gift from Dr. A. Adachi), which harbors the luciferase gene downstream of the HIV-1 LTR; and pNLmyr(-)pro(-)Flag and pNLmyr(+)pro(-)Flag (a kind gift from Dr. Y. Morikawa) (Kawada *et al.* 2008).

To construct pSRαhrCRM1-HA and pSRαrhCRM1-HA, the hrCRM1 and rhCRM1 coding regions in pSRαhrCRM1 and pSRαrhCRM1, respectively (Hakata *et al.* 2003), were amplified by PCR using the primer pair for hrCRM1-HA of 5'-GGT CAA TAC CCA CGT TTT TTG AGA GCT CAC TGG A-3' (the underlined sequence is a *Sac*I site) and 5'-TAT GGT ACC TTA AGC ATA ATC AGG AAC ATC GTA TGG GTA GTC ACA CAT TTC TTC TGG GAT TTC-3' (the underlined sequence is a *Kpn*I site), and the primer pair for rhCRM1-HA of 5'-CTG GAA TCA CTT GGC AGC TGA GCT CTA CAG AGA GAG TCC A-3' (the underlined sequence is a *Sac*I site) and 5'-TAT GGT ACC TTA AGC ATA ATC AGG AAC ATC GTA TGG GTA ATC ACA CAT TTC TTC TGG AAT CTC-3' (the underlined sequence is a *Kpn*I site.), which encodes the HA tag. The PCR reaction was performed using 10 ng of the plasmid as a template, which was denatured at 94ºC for 2 min and was then followed by 20 cycles of amplification: denaturation (94ºC, 30 s), annealing (62ºC, 1 min), and extension (68ºC, 2 min). A final extension was performed at 68ºC for 10 min. The amplified DNA was digested with *Sac*I and *Kpn*I, and inserted into these sites in pSRαhrCRM1 or pSRαrhCRM1, as appropriate.

**Real-time RT-PCR**

To separate the cytoplasmic and nuclear fractions, lysis buffer (0.5% NP40, 10 mM Tris-HCl [pH 7.4], 0.14 M NaCl, 1.5 mM $MgCl_2$, 1 U/μl RNase Inhibitor (TOYOBO), 1 mM DTT) was added to the cell pellets and the lysates were centrifuged in a microcentrifuge at 3000 rpm for 5 min at 4°C. Total RNA in the cytoplasm (supernatant) and nucleus (pellet) was extracted using the Absolutely RNA Miniprep Kit (Stratagene). The ThermoScript RT-PCR System (Invitrogen) was used to synthesize cDNAs from 150 to 500 ng of RNA and the abundance was quantified using real-time PCR with a Light Cycler PCR machine (Roche). PCR reactions were performed using the following primer pairs: *gag* forward (5'-AGA GAA GGC TTT CAG CCC AGA AGT-3') and reverse (5'-GGA TTT GTT ACT TGG CTC ATT GCT-3'); *tat* forward (5'-GTG GAA GCA TCC AGG AAG TCA GCC-3') and reverse (5'-CTA TTC CTT CGG GCC TGT CGG GTC-3'); and *β-gal* forward (5'-GCG AAT ACC TGT TCC G-3') and reverse (5'-GCG TCA CAC TGA GGT T-3'). PCR mixtures (20 μl) were prepared in a capillary tube containing 1/16 of the volume of the RT reaction mixture, each primer pair (0.5 μM), and 1x Light Cycler-FastStart SYBR

Green PCR Master Mix. Following an initial Taq polymerase activation step (95°C, 15 min) the *gag* and *tat* mRNA amplification reactions were performed using 40 cycles of denaturation (95°C, 10 s), annealing (68°C, 10 s) and extension (72°C, 15 s), whereas the *β-gal* mRNA was amplified using 40 cycles of denaturation (95°C, 15 s), annealing (60°C, 5 s) and extension (72°C, 11 s). The abundance of each mRNA was estimated with a standard regression curve using the Light Cycler Software v. 3 (Roche). The standard curve was obtained by PCR amplification of 0.0064 to 500 pg of pCMVΔR8.2 (for *gag* mRNA), pSRαtat and pCDM*β*-gal.

To ascertain the efficiency of fractionation, *β-actin* pre-mRNA was amplified by PCR using the primers *β-actin*-F (5'-TCG ATC GCC TTT CTG ACT AGG TG-3') and *β-actin*-R (5'-GGT CAG GAT CTT CAT GAG GTA GTC TG-3'); the former targets the intron and the latter targets the exon of *β-actin* pre-mRNA. Reactions were performed with 25 cycles of denaturation at 94°C for 30 s, followed by annealing at 59°C for 1 min and extension at 72°C for 30 s.

**Pulse chase assay**

At 36 to 48 h post transfection, cells were metabolically labeled with an

[$^{35}$S]-methionine/cysteine mixture (GE Healthcare UK Ltd.) for 30 min. Cells were washed once and then chased for 0, 4, 8 and 24 hours in 1 ml of medium supplemented with 10 μM methionine, 10 μM cysteine and 100 nM KNI-272 (an HIV-1 protease inhibitor, the kind gift of Dr. Y. Kiso). After chasing, 0.25 ml of 5x detergent buffer containing 2.5% deoxycholate (DOC), 2.5% Triton X100, 250 mM Tris HCl (pH 7.6), 0.1 M NaCl, 0.1 M EDTA, 100 nM KNI-272, and a protease inhibitor cocktail (Roche) was added to the culture to dissolve Gag proteins in both the medium and cells. The lysates were centrifuged in a microcentrifuge at 12,000 rpm for 15 min and the supernatant was incubated with anti Gag mAb V107 (Ikuta *et al.* 1989) (a kind gift from Dr. K. Ikuta) for 1 hour at 4°C, followed by incubation with protein G Sepharose for 1 hour at 4°C. After three washes with detergent buffer, the precipitated complexes were analyzed by SDS-PAGE and autoradiography.

At 40 hours post transfection with pNLmyr(-)pro(-)Flag, cells were metabolically labeled with an [$^{35}$S]-methionine/cysteine mixture for 15 min, washed, and then chased for 0, 4 and 7 hours as described above. The detergent buffer (0.5 ml) was added to the cells and centrifuged in a microcentrifuge at 12,000 rpm for 15 min. The

supernatant was incubated with anti-Flag M2 antibody (Sigma) conjugated to protein G Sepharose for 3 hours at 4°C, followed by three washes with the detergent buffer.

***In situ* hybridization**

At 48 to 50 hours after transfection, cells were subjected to an *in situ* hybridization assay, which has been described previously (Tautz & Pfeifle 1989; Kimura *et al.* 1996). Probes were prepared by PCR amplification of a 167-bp fragment from HIV-1 *gag* using the primers T66 (5'-GAGAGCGTCGGTATTAAG-3'; HIV-$1_{NL4-3}$ nts 798 to 815) and T67 (5'-GTCTACAGCCTTCTGATG-3'; HIV-$1_{NL4-3}$ nts 964 to 947). The fragment generated was then used as a template for a second PCR, using either T67 or T66 primers and the digoxigenin (DIG)-nucleotide mixture (Roche), essentially as described by Tautz et al (Tautz & Pfeifle 1989). Cells were examined under a confocal laser-scanning microscope (Olympus).

**Western blotting**

The transfected cells were dissolved in TMN buffer containing 10 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.5% NP40, and a protease inhibitor cocktail. After measuring the β-gal activity and protein levels, the proteins were solubilized with

sample buffer and resolved by SDS-PAGE, and then transferred to a nitrocellulose filter. V107, a rat anti-HA mAb (Roche), a rabbit anti-β-gal Ab, a mouse anti-actin mAb, and anti-FLAG M2 Ab were used as primary Abs. Horseradish peroxidase-conjugated anti-IgG Abs (Promega) were used as secondary Abs. Immunoreactive bands were visualized using ECL+plus (GE Health care) followed by the LAS-1000 Plus system (Fujifilm) and were evaluated by Image Gauge (version 3.4) software (Fujifilm).

**Immunofluorescence**

Subconfluent ER1/neo1 cells, seeded onto coverslips (Fisher Scientific) in six-well plates, were transfected using Polyethylenimine "Max"（PEI）(Polyscience) and fixed 18 hours post-transfection with 2% paraformaldehyde dissolved in PBS. The cells were then permeabilized with 0.5% NP40 in PBS and incubated with blocking solution (5% skim milk in PBS) for 1 hour at RT. The cells were stained with Alexa Fluor488-conjugated anti-HA-Tag (6E2) mouse mAb and Alexa Fluor® 555-conjugated anti DYKDDDDK (FLAG) Tag antibody (Cell Signaling Technology) for 1 hour at RT, washed, and then mounted with VECTASHIELD Hard set Mounting Medium (Vector Laboratories). The stained cells were examined under a confocal laser-scanning

microscope.

**Preparation of VLP**

To prepare the viral-like particle (VLP) fraction, medium from the cell cultures was centrifuged at 2000 rpm for 10 min and the supernatant was filtered through a Millex HV 0.45 μm Filter Unit (Millipore). The HIV-1 particles were pelleted through a 20% (W/V) sucrose solution by centrifugation at 27,000 rpm in an SW28 rotor for 2 hours at 4°C.

**Statistical analysis**

Comparisons between individual data points were made using a Student's *t*-test. Two-sided P values < 0.05 were considered statistically significant.

**Acknowledgements**

We thank Dr. J. Fujisawa for kind administration, and K. Ofuji, A. Hirano, N. Mizuno and S. Yamanouchi for excellent technical assistance. V107, pH1-luc, pNLmyr(-)pro(-)Flag, and KNI-272 were kind gifts from Dr. K. Ikuta (University of Osaka), Dr. A. Adachi (Tokushima University), Dr. Y. Morikawa (Kitazato University), and Dr. Y. Kiso (Kyoto Pharmaceutical University), respectively. This study was supported by grants from the Ministry of Sports and Culture (Japan) and the Ministry of Health and Welfare (Japan).

**References**

Bieniasz, P.D. & Cullen, B.R. (2000) Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. *J. Virol.* **74**, 9868-9877.

Browning, J., Horner, J.W., Pettoello-Mantovani, M., Raker, C., Yurasov, S., DePinho, R.A. & Goldstein, H. (1997) Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl Acad. Sci. USA* **94**, 14637-14641.

Cullen, B.R. (1998) Retroviruses as model systems for the study of nuclear RNA export pathways. *Virology* **249**, 203-210.

Cullen, B.R. (2003) Nuclear RNA export. *Journal of cell science* **116**, 587-597.

D'Souza, V. & Summers, M. F. (2005) How retroviruses select their genomes. *Nat. Rev. Microbiol.* **3**, 643-655

Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. & Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311.

Giuffre, A.C., Higgins, J., Buckheit, R.W., Jr. & North, T.W. (2003) Susceptibilities of simian immunodeficiency virus to protease inhibitors. *Antimicrob. Agents Chemother* **47**, 1756-1759.

Goffinet, C., Michel, N., Allespach, I., Tervo, H.M., Hermann, V., Krausslich, H.G., Greene, W.C. & Keppler, O.T. (2007) Primary T-cells from human CD4/CCR5-transgenic rats support all early steps of HIV-1 replication including integration, but display impaired viral gene expression. *Retrovirology* **4**, 53.

Hakata, Y., Umemoto, T., Matsushita, S. & Shida, H. (1998) Involvement of human CRM1 (exportin 1) in the export and multimerization of the Rex protein of human T-cell leukemia virus type 1. *J. Virol.* **72**, 6602-6607.

Hakata, Y., Yamada, M., Mabuchi, N. & Shida, H. (2002) The carboxy-terminal region of the human immunodeficiency virus type 1 protein Rev has multiple roles in mediating CRM1-related Rev functions. *J. Virol.* **76**, 8079-8089.

Hakata, Y., Yamada, M. & Shida, H. (2001) Rat CRM1 is responsible for the poor activity of human T-cell leukemia virus type 1 Rex protein in rat cells. *J. Virol.* 15-11525.

Hakata, Y., Yamada, M. & Shida, H. (2003) A multifunctional domain in human CRM1 (exportin 1) mediates RanBP3 binding and multimerization of human T-cell leukemia virus type 1 Rex protein. *Mol. Cell. Biol.* **23**, 8751-8761.

Hazuda, D.J., Young, S.D., Guare, J.P. *et al.* (2004) Integrase inhibitors and cellular immunity suppress retroviral replication in rhesus macaques. *Science* **305**, 528-532.

Hu, S.L. (2005) Non-human primate models for AIDS vaccine research. *Curr. Drug Targets Infect. Disord.* **5**, 193-201.

Ikuta, K., Morita, C., Miyake, S., Ito, T., Okabayashi, M., Sano, K., Nakai, M., Hirai, K. & Kato, S. (1989) Expression of human immunodeficiency virus type 1 (HIV-1) gag antigens on the surface of a cell line persistently infected with HIV-1 that highly expresses HIV-1 antigens. *Virology* **170**, 408-417.

Kanki, K., Torigoe, T., Hirai, I., Sahara, H., Kamiguchi, K., Tamura, Y., Yagihashi, A. & Sato, N. (2000) Molecular cloning of rat NK target structure--the possibility of CD44 involvement in NK cell-mediated lysis. *Microbiol. Immunol.* **44**, 1051-1061.

Kawada, S., Goto, T., Haraguchi, H., Ono, A. & Morikawa, Y. (2008) Dominant negative inhibition of human immunodeficiency virus particle production by the

nonmyristoylated form of gag. *J. Virol.* **82**, 4384-4399.

Keppler, O.T., Welte, F.J., Ngo, T.A. *et al.* (2002) Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J. Exp. Med.* **195**, 719-736.

Keppler, O.T., Yonemoto, W., Welte, F.J., Patton, K.S., Iacovides, D., Atchison, R.E., Ngo, T., Hirschberg, D.L., Speck, R.F. & Goldsmith, M.A. (2001) Susceptibility of rat-derived cells to replication by human immunodeficiency virus type 1. *J. Virol.* **5**, 8063-8073.

Kimura, T., Hashimoto, I., Nishikawa, M. & Fujisawa, J.I. (1996) A role for Rev in the association of HIV-1 gag mRNA with cytoskeletal beta-actin and viral protein expression. *Biochimie* **78**, 1075-1080.

Kudo, N., Khochbin, S., Nishi, K., Kitano, K., Yanagida, M., Yoshida, M. & Horinouchi, S. (1997) Molecular cloning and cell cycle-dependent expression of mammalian CRM1, a protein involved in nuclear export of proteins. *J. Biol. Chem.* **272**, 29742-29751.

Malim, M.H. & Cullen, B.R. (1991) HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency.

*Cell* **65**, 241-248.

Malim, M.H., McCarn, D.F., Tiley, L.S. & Cullen, B.R. (1991) Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. *J. Virol.* **65**, 4248-4254.

Marques, S.M., Veyrune, J.L., Shukla, R.R. & Kumar, A. (2003) Restriction of human immunodeficiency virus type 1 Rev function in murine A9 cells involves the Rev C-terminal domain. *J. Virol.* **77**, 3084-3090.

Muriaux, D., Mirro, J., Harvin, D. & Rein, A. (2001) RNA is a structural element in retrovirus particles. *Proc. Natl. Acad. Sci. USA* **98,** 5246-5251

Nakielny, S. & Dreyfuss, G. (1999) Transport of proteins and RNAs in and out of the nucleus. *Cell* **99**, 677-690.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M. & Trono, D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263-267.

Neville, M., Stutz, F., Lee, L., Davis, L.I. & Rosbash, M. (1997) The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore

complex during nuclear export. *Curr. Biol.* **7**, 767-775.

Okada, H., Zhang, X., Fofana, I.B., Nagai, M., Suzuki, H., Ohashi, T. & Shida, H. (2009) Synergistic effect of human CycT1 and CRM1 on HIV-1 propagation in rat T cells and macrophages. *Retrovirology* **6**, 43.

Ossareh-Nazari, B., Bachelerie, F. & Dargemont, C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278**, 141-144.

Sanchez-Velar, N., Udofia, E.B., Yu, Z. & Zapp, M.L. (2004) hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region. *Genes Dev.* **18**, 23-34.

Sherer, N.M., Swanson, C.M., Papaioannou, S. & Malim, M.H. (2009) Matrix mediates the functional link between human immunodeficiency virus type 1 RNA nuclear export elements and the assembly competency of Gag in murine cells. *J. Virol.* **83**, 8525-8535.

Shultz, L.D., Ishikawa, F. & Greiner, D.L. (2007) Humanized mice in translational biomedical research. *Nat. Rev. Immunol.* **7**, 118-130.

Stade, K., Ford, C.S., Guthrie, C. & Weis, K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050.

Swanson, C.M., Puffer, B.A., Ahmad, K.M., Doms, R.W. & Malim, M.H. (2004) Retroviral mRNA nuclear export elements regulate protein function and virion assembly. *The EMBO J.* **23**, 2632-2640.

Takayanagi, R., Ohashi, T., Yamashita, E., Kurosaki, Y., Tanaka, K., Hakata, Y., Komoda, Y., Ikeda, S., Tsunetsugu-Yokota, Y., Tanaka, Y. & Shida, H. (2007) 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. *J. Virol.* **81**, 5908-5918.

Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. & Arai, N. (1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**, 466-472.

Tautz, D. & Pfeifle, C. (1989) A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

Trono, D. & Baltimore, D. (1990) A human cell factor is essential for HIV-1 Rev action. *The EMBO J.* **9**, 4155-4160.

Veazey, R.S., Klasse, P.J., Schader, S.M., Hu, Q., Ketas, T.J., Lu, M., Marx, P.A., Dufour, J., Colonno, R.J., Shattock, R.J., Springer, M.S. & Moore, J.P. (2005) Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. *Nature* **438**, 99-102.

Watanabe, S., Terashima, K., Ohta, S., Horibata, S., Yajima, M., Shiozawa, Y., Dewan, M.Z., Yu, Z., Ito, M., Morio, T., Shimizu, N., Honda, M. & Yamamoto, N. (2007) Hematopoietic stem cell-engrafted NOD/SCID/IL2Rgamma null mice develop human lymphoid systems and induce long-lasting HIV-1 infection with specific humoral immune responses. *Blood* **109**, 212-218.

Yu, Z., Sanchez-Velar, N., Catrina, I.E., Kittler, E.L., Udofia, E.B. & Zapp, M.L. (2005) The cellular HIV-1 Rev cofactor hRIP is required for viral replication. *Proc. Natl Acad. Sci. USA* **102**, 4027-4032.

Zhang, X., Hakata, Y., Tanaka, Y. & Shida, H. (2006) CRM1, an RNA transporter, is a major species-specific restriction factor of human T cell leukemia virus type 1 (HTLV-1) in rat cells. *Microbes Infect.* **8**, 851-859.

Zheng, Y.H., Yu, H.F. & Peterlin, B.M. (2003) Human p32 protein relieves a

post-transcriptional block to HIV replication in murine cells. *Nat. Cell Biol.* **5**, 611-618.

**Figure Legends**

**Figure 1.** Effect of CRM1 on p24 production. (A) The structure of pCRRE. (B) ER1/neo1 cells ($1x10^5$) were transfected with 1.6 μg of pCRRE and pSRαhCRM1 as indicated, 0.1 μg of pCDM*β*-gal, and 0.3 μg of pH1-luc using TransIT-LT1 (Mirus) reagent. The total abundance of transfected plasmids was adjusted with pSRα296. Two days after transfection, p24 was quantified from the cell culture medium using a RETRO-TEK HIV-1 p24 Antigen ELISA kit (ZeptoMetrix). Cytoplasmic β-gal activity was measured using standard colorimetric methods (β-gal assay) (Hakata *et al.* 2001), and luciferase activities were also quantified. (C) ER1/neo1 and HeLa cells were transfected with pCRRE, pCDM*β*-gal and pH1-luc along with pSRαhCRM1 or pSRαrCRM1 as described above. The amount of p24 in the medium was quantified as in (B). The value of the control sample without CRM1 was set at 1. The *β*-galactosidase and luciferase activities were similar in all samples (data not shown). These data are representative of three independent experiments. (D) The amount of mRNA in cytoplasmic fraction in ER1/neo1 cells was indicated. ER1/neo1 cells were

transfected with pCRRE, pSRαhCRM1, pCDM*β*-gal and pH1-luc, and then separated into nuclear and cytoplasmic fractions using an NP40 detergent-containing buffer, as described in Materials and Methods. Quantitative RT-PCR was used to determine the abundance of *gag*, *tat* and *β-gal* mRNAs in each fraction. The ratios of *gag* mRNA/*β-gal* mRNA and *tat* mRNA/*β-gal* mRNA were calculated. The value of the control sample without hCRM1 was set at 1. These values are the means of three independent sets of experiments and the SD was calculated. (E) Gag protein (p24) in the medium and *β*-gal activity were quantified and the Gag/*β*-gal ratio was plotted and compared to the ratio of cytoplasmic *gag* mRNA/*β-gal* mRNA. These data are representative of three independent experiments. (F) Stability of Gag protein in rat cells. At 48 hours post transfection, cells were pulse-labeled with [$^{35}$S]-methionine/cysteine and chased for 0, 4, 8 and 24 hours. The medium and cells were solubilized with detergent-containing buffer and combined. Then, Gag proteins were immunoprecipitated using anti-Gag antibodies and protein-G Sepharose, and analyzed by SDS-PAGE followed by autoradiography. (G) ER1/neo1 cells were transfected with pNLmyr(-)pro(-)Flag, pSRαhCRM1, and pCDM*β*-gal. At 40 hours

post transfection, cells were pulse-labeled with [$^{35}$S]-methionine/cysteine and chased for 0, 4 and 7 hours. Gag proteins in the cell lysates were immunoprecipitated using anti-FLAG antibodies and protein-G Sepharose.

**Figure 2.** Effects of rat and human CRM1 on the production of Gag protein and *gag* mRNA derived from pCMVΔR8.2 in rat cells. (A-D) ER1/neo1 cells were transfected with pCMVΔR8.2 (1.6 μg) and pCDM*β*-gal (0.1 μg) along with pSRαhCRM1 (0.4 μg) or pSRαrCRM1 (0.4 μg). The level of p24 in the medium and cytoplasmic *β*-gal activity, *gag* mRNA, *tat* mRNA and *β-gal* mRNA were quantified. The average and SD were calculated based on three independent sets of experiments. (E) PCR fragments of *β*-actin pre-mRNA in the nucleus (lanes 1, 3 and 5) and cytoplasm (lanes 2, 4 and 6) of cells without (lanes 1 and 2) or with (lanes 3 and 4) hCRM1 and (lane 5 and 6) rCRM1. (F) *β*-gal activity in the nuclear and cytoplasmic fractions was quantified, and the ratios of the *β*-gal activities in each fraction were calculated.

**Figure 3.** Fluorescence *in situ* hybridization (FISH) profiles of *gag* mRNA distribution in cells. (A) ER1/neo1 cells (panels a-f) were transfected with

pCMVΔR8.2, along with pSRα296 (panels a and b), pSRαhCRM1 (panels c and d), or pSRαrCRM1 (panels e and f). ER1/hCRM1-7 (panels g and h) and HeLa cells (panels i and j) were transfected with pCMVΔR8.2. Cells were subjected to *in situ* hybridization. In all panels, the upper left image is phase-contrast and the upper right shows DAPI-stained cells. The lower left image shows the FISH profile and the lower right is a merged image. (B) The number of cells (designated N) in which *gag* mRNA is localized exclusively to the nucleus and those (designated C), which contain *gag* mRNA distributed throughout the cytoplasm and nucleus. More than 150 cells were counted and their ratios are presented. These data represent the total number of cells counted in three independent experiments.

**Figure 4.** Western blotting of Gag proteins expressed in ER1/neo1 cells. (A) ER1/neo1 and HeLa cells were transfected with pCMVΔR8.2 and pCDM *β*-gal along with the empty vector, pSRα296, and pSRαhCRM1-HA or pSRαrCRM1-HA as described above. Fractions corresponding to approximately $1.5 \times 10^4$ and $5 \times 10^5$ ER1/neo1 cells were subjected to Western blotting to analyze Gag protein profiles in the

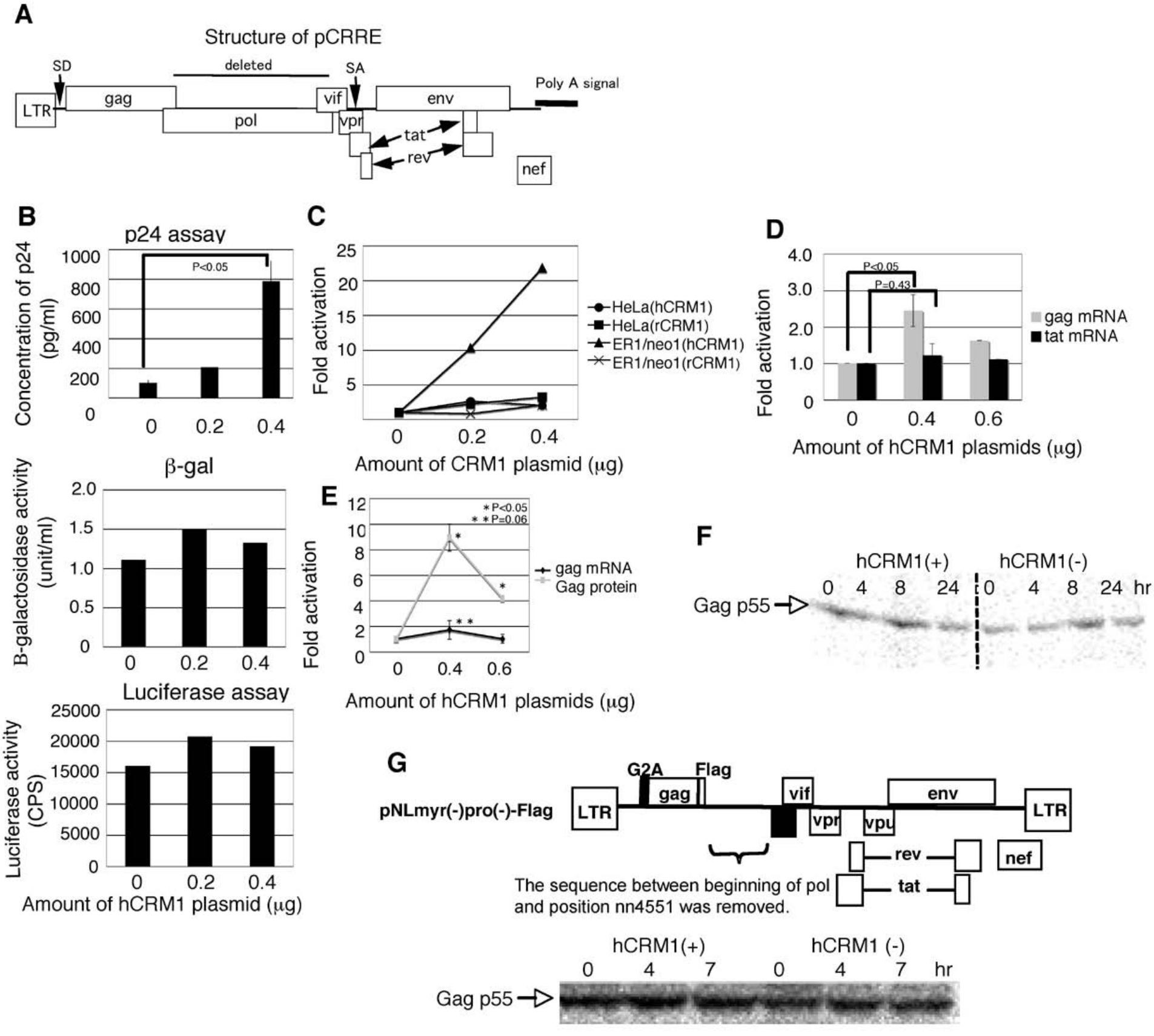
cell and VLP fractions, respectively. Fractions corresponding to approximately $5x10^3$ and $5x10^4$ HeLa cells were subjected to SDS-PAGE for analysis of the cell and VLP fractions, respectively. The intensities of the Gag proteins were quantified by LAS-1000. The amount of p55 Gag in pSRα296-transfected ER1/neo1 cells was set at 1.0, and the relative amounts of Gag protein were calculated. The ratio of p24/p55 expressed in HeLa was also presented. (B) ER1/neo1 cells were transfected with pCMVΔR8.2 (0.4 to 1.6 μg) and pCDM*β*-gal (0.1 μg) along with pSRαhCRM1-HA (0.4 μg) or pSRαrCRM1 (0.4 μg). Fractions corresponding to approximately $2x10^4$ and $5x10^5$ cells were subjected to Western blotting to analyze Gag protein profiles in the cell and VLP fractions, respectively. The intensity of the p55 Gag band in the lane representing ER1/neo1 cells, transfected with 0.8 μg of pCMVΔR8.2 and pSRαhCRRM1, was quantified and set at 1.0.

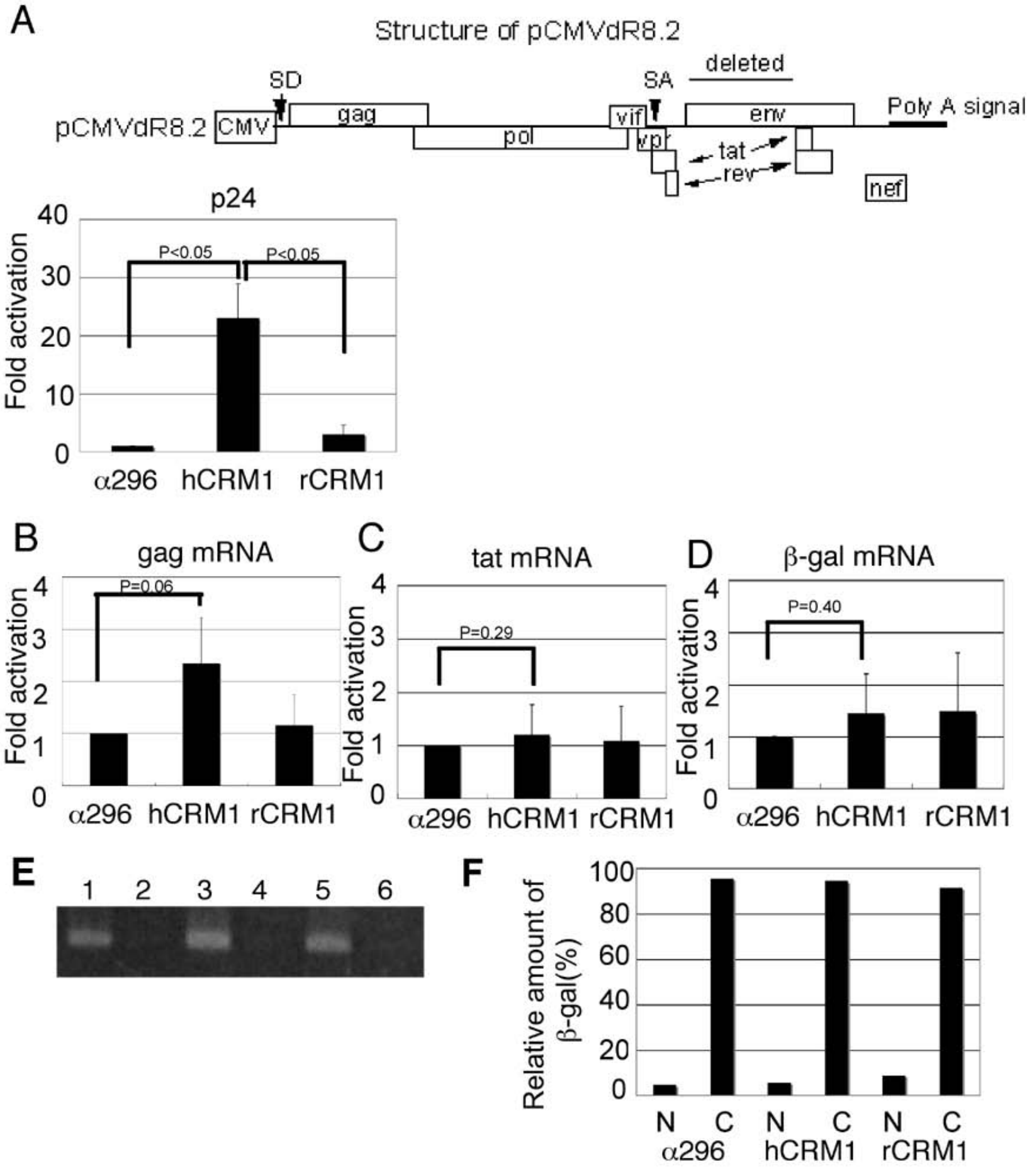
**Figure 5.** Effect of hCRM1 on membrane trafficking and release of virus particles using pNLmyr(-)pro(-)Flag and pNLmyr(+)pro(-)Flag. Approximately $2x10^4$ ER1/neo1 cells transfected with pNLmyr(-)pro(-)Flag along with pSRα296, pSRαhCRM1 or

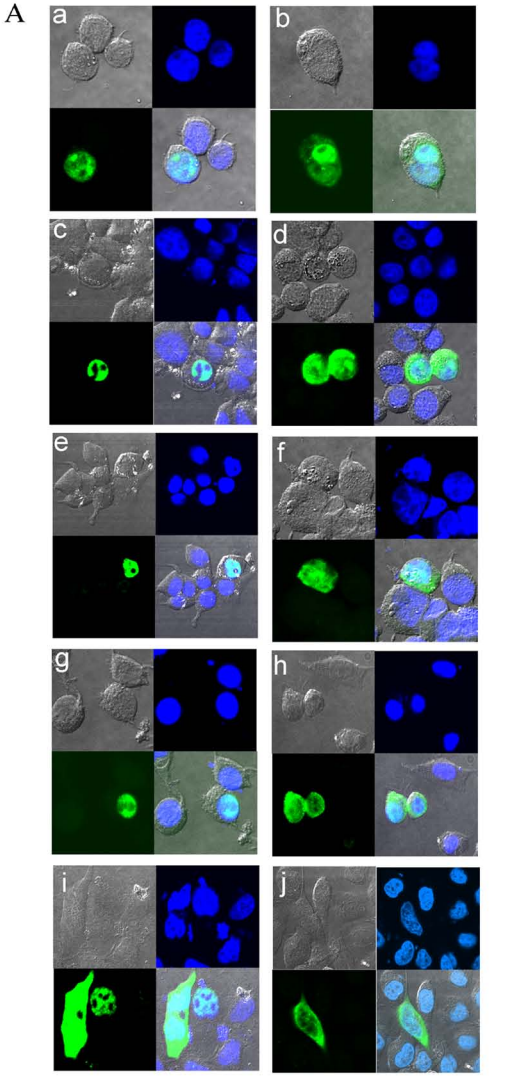
pSRαrCRM1 were subjected to Western blotting (A). The cells and VLP fractions corresponding to approximately $5x10^3$ and $5x10^5$ cells transfected with pNLmyr(+)pro(-)Flag along with pSRα296, pSRαhCRM1 or pSRαrCRM1, were subjected to Western blotting, respectively (B). The band intensity of p55 Gag expression in pSRα296-transfected cells was set at 1.0. (C) HeLa cells were transfected with pNLmyr(-)pro(-)Flag (panel a) or pNLmyr(+)pro(-)Flag (panel b). ER1/neo1 cells were transfected with pNLmyr(-)pro(-)Flag and pSRαhCRM1 (panel c), or pNLmyr(+)pro(-)Flag along with pSRα296 (panel d), pSRαhCRM1 (panel e), or pSRαrCRM1 (panel f). Cells were subjected to direct immunofluorescence assay. In all panels, the left image is a merged picture of DAPI (blue) and p55 Gag protein (red), and the right-hand panels show exogenous CRM1 (green).

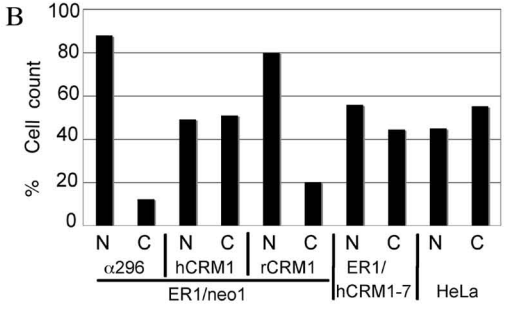
**Figure 6.** Analysis of the region of hCRM1 responsible for the upregulation of p24 production in ER1/neo1 cells. (A) Structure of CRM1. (B) ER1/neo1 cells were transfected with pCMVΔR8.2, pCDMβ-gal and various CRM1-expressing plasmids. HIV-1 p24 in the medium was quantified by ELISA. The protein expression of CRM1,

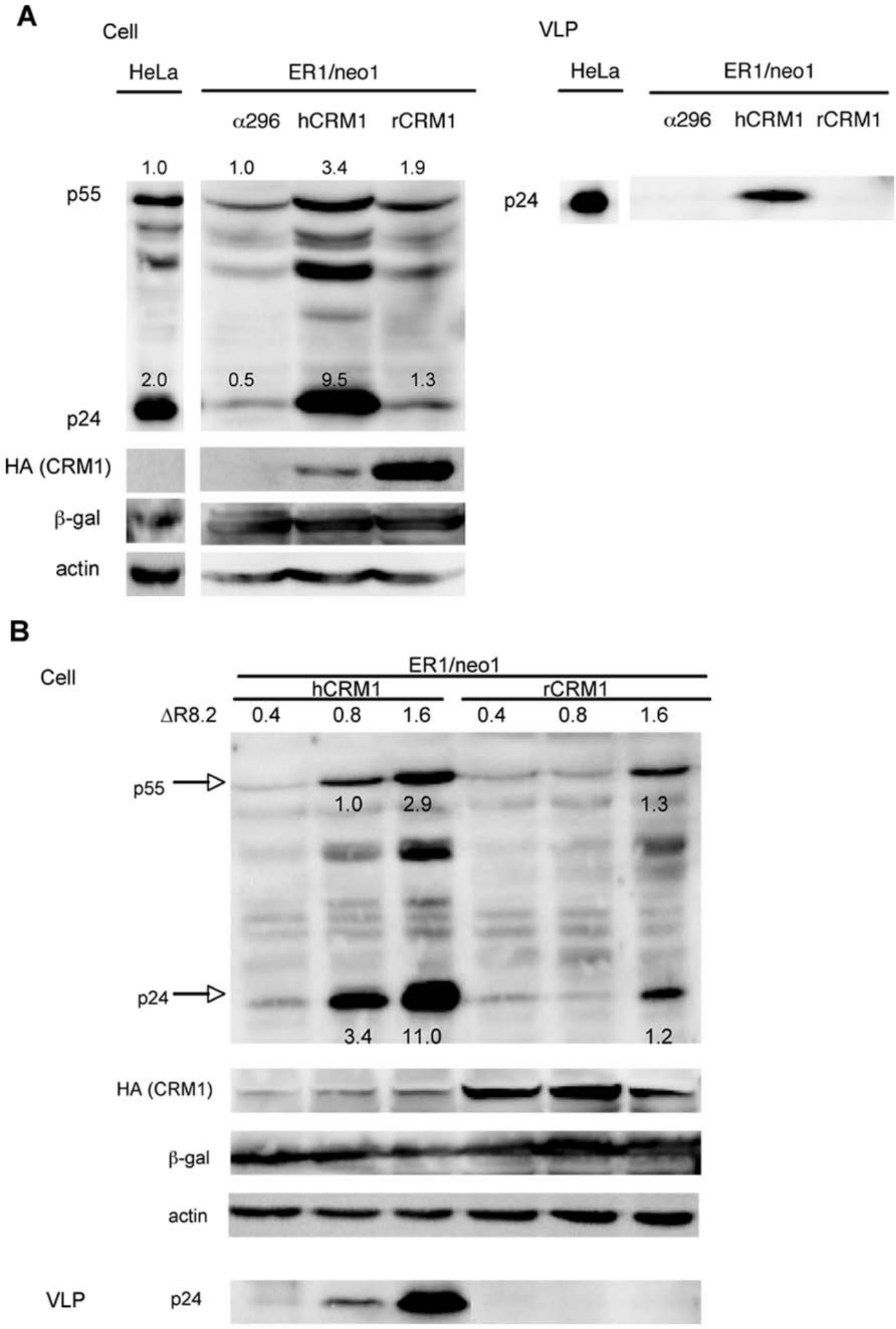
β-gal and actin was analyzed by Western blotting.

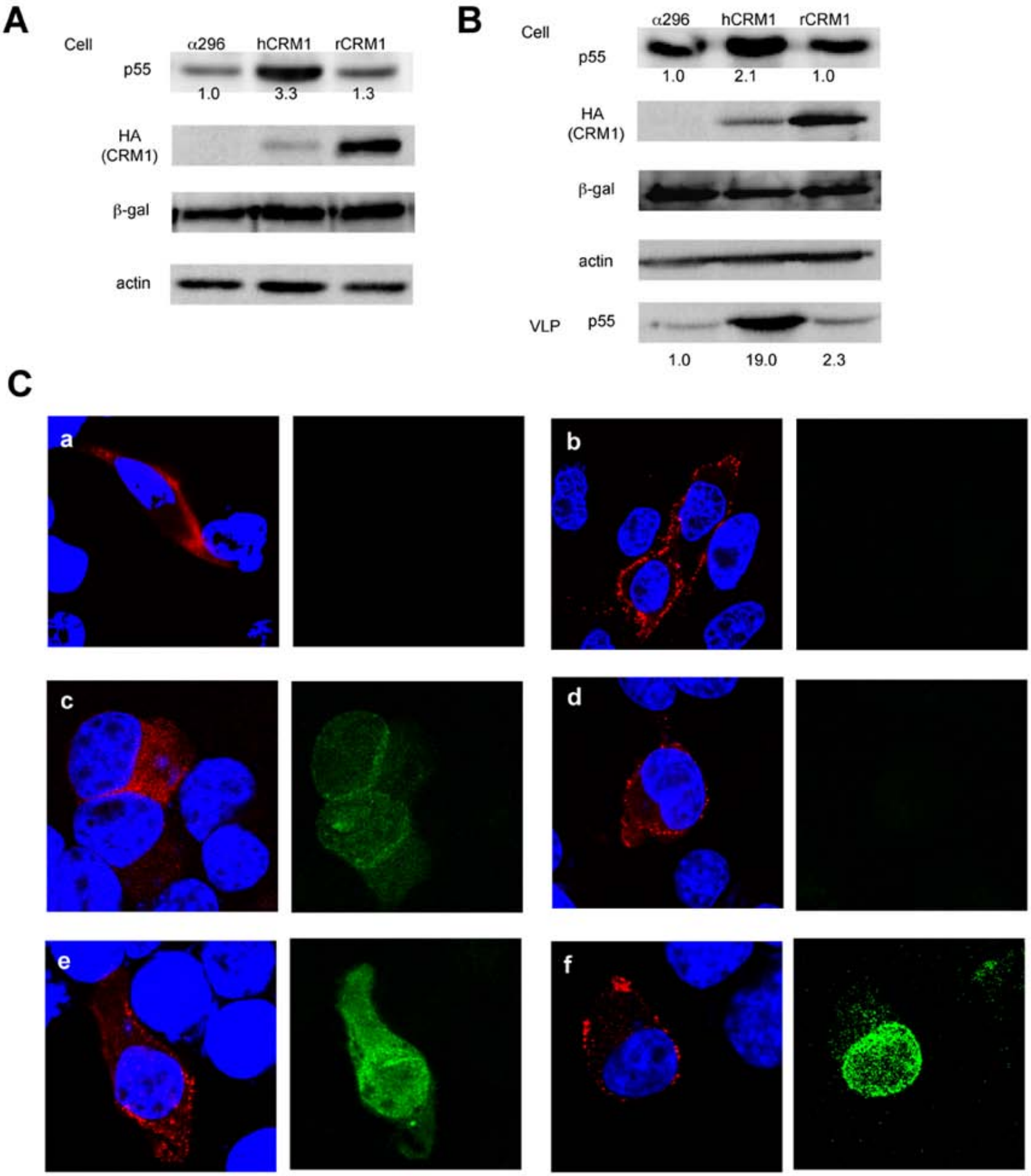

A
Structure of pCRRE
SD
deleted
SA
Poly A signal
LTR
gag
pol
vif
vpr
env
tat
rev
nef
B
p24 assay
Concentration of p24 (pg/ml)
P<0.05
β-gal
B-galactosidase activity (unit/ml)
Luciferase assay
Luciferase activity (CPS)
Amount of hCRM1 plasmid (μg)
C
Fold activation
HeLa(hCRM1)
HeLa(rCRM1)
ER1/neo1(hCRM1)
ER1/neo1(rCRM1)
Amount of CRM1 plasmid (μg)
D
P<0.05
P=0.43
gag mRNA
tat mRNA
Fold activation
Amount of hCRM1 plasmids (μg)
E
*P<0.05
**P=0.06
gag mRNA
Gag protein
Fold activation
Amount of hCRM1 plasmids (μg)
F
hCRM1(+)
hCRM1(-)
0 4 8 24 0 4 8 24 hr
Gag p55
G
pNLmyr(-)pro(-)-Flag
G2A
Flag
gag
vif
env
LTR
vpr
vpu
rev
tat
nef
The sequence between beginning of pol and position nn4551 was removed.
hCRM1(+)
hCRM1 (-)
0 4 7 0 4 7 hr
Gag p55

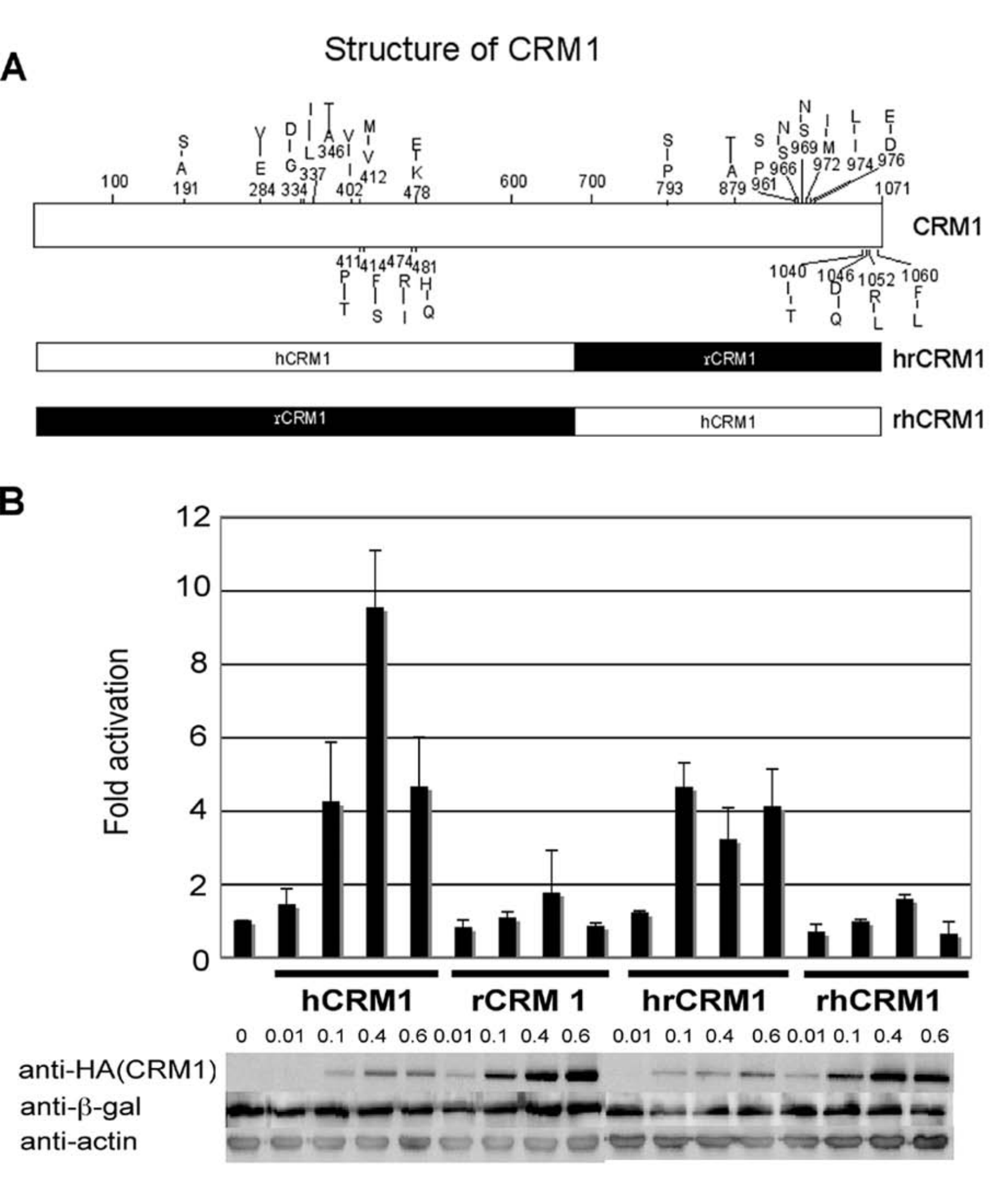

A
Structure of pCMVdR8.2
SD
SA
deleted
pCMVdR8.2
CMV
gag
pol
vif
vpr
env
Poly A signal
tat
rev
nef
p24
Fold activation
P<0.05
P<0.05
α296
hCRM1
rCRM1
B
gag mRNA
P=0.06
C
tat mRNA
P=0.29
D
β-gal mRNA
P=0.40
E
1 2 3 4 5 6
F
Relative amount of β-gal(%)
N C N C N C
α296 hCRM1 rCRM1


A
a
b
c
d
e
f
g
h
i
j


B
100
80
60
40
20
0
% Cell count
N
C
α296
hCRM1
rCRM1
ER1/
hCRM1-7
HeLa
ER1/neo1


A
Cell
VLP
HeLa
ER1/neo1
α296
hCRM1
rCRM1
1.0
1.0
3.4
1.9
p55
2.0
0.5
9.5
1.3
p24
HA (CRM1)
β-gal
actin
B
Cell
ER1/neo1
hCRM1
rCRM1
ΔR8.2
0.4
0.8
1.6
0.4
0.8
1.6
p55
1.0
2.9
1.3
p24
3.4
11.0
1.2
HA (CRM1)
β-gal
actin
VLP
p24


A
Cell
α296
hCRM1
rCRM1
p55
1.0
3.3
1.3
HA
(CRM1)
β-gal
actin
B
Cell
α296
hCRM1
rCRM1
p55
1.0
2.1
1.0
HA
(CRM1)
β-gal
actin
VLP
p55
1.0
19.0
2.3
C
a
b
c
d
e
f


A
Structure of CRM1
100
191
284
334
337
346
402
412
478
600
700
793
879
961
966
969
972
974
976
1071
CRM1
411
414
474
481
1040
1046
1052
1060
hCRM1
rCRM1
hrCRM1
rhCRM1
B
Fold activation
0
2
4
6
8
10
12
hCRM1
rCRM 1
hrCRM1
rhCRM1
0 0.01 0.1 0.4 0.6 0.01 0.1 0.4 0.6 0.01 0.1 0.4 0.6 0.01 0.1 0.4 0.6
anti-HA(CRM1)
anti-β-gal
anti-actin