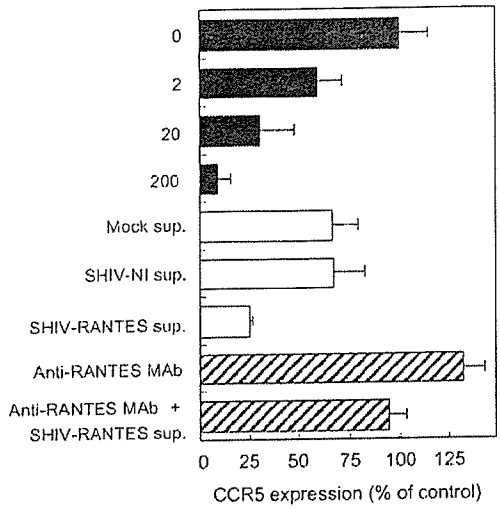

Fig. 3. Down-modulation of CCR5 on the surface of PM1 cells by RANTES. White bars, CCR5 expression on PM1 cells exposed to supernatant from cells infected with SHIV-RANTES, SHIV-NI, and uninfected cells (Mock). Black bars, PM1 cells treated with recombinant human RANTES at the indicated concentrations (in ng/ml). Hatched bars, cells treated with anti-RANTES antibody (Anti-RANTES MAb). These data are expressed as the mean of triplicate experiments ± S.E.M.

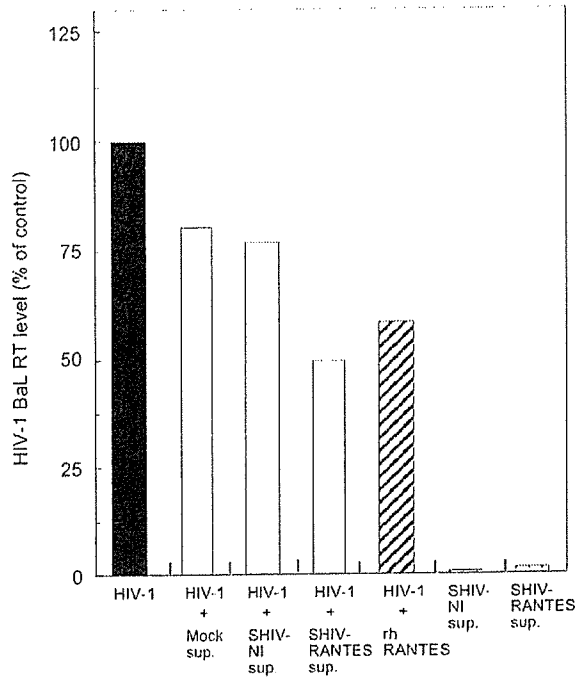

Fig. 4. Inhibition of HIV-1 BaL by UV-irradiated culture supernatants from SHIV-RANTES-infected cells. Virus replication of HIV-1 BaL was monitored by RT activity from the culture supernatants of virus-infected PM1 cells at 12 d.p.i. As control, cells treated with the same concentrations of UV-irradiated recombinant human RANTES were monitored (HIV-1 + rh RANTES). UV-irradiated supernatants from SHIV-RANTES and SHIV-NI-infected cells without HIV-1 BaL infection were used as negative controls. These data show one of three independent experiments with similar results.

completely abolished the down-modulation of CCR5. Thus, the reduction of the cell surface CCR5 correlates with the level of the expressed RANTES from SHIV-RANTES-inoculated cells.

3.3. Inhibition of R5-tropic HIV-1 BaL infection by RANTES from SHIV-RANTES-inoculated cells

In this study, the levels of RANTES in the UV-irradiated samples from the cultures infected with SHIV-RANTES, SHIV-NI, and Mock were 14, 0.8, and 0.8 ng/ml, respectively. In the case of the culture supernatants from SHIV-RANTES-infected cells, approximately 51% of the inhibition of HIV-1 BaL infection was consistently observed at 12 d.p.i., when the viral loads of the HIV-1 BaL peaked (Fig. 4). A reduction of the HIV-1 BaL RT activity was also observed for the culture supernatants from the SHIV-NI-inoculated cells and that of the Mock-inoculated cells, but the reductions were only about 23% and 20%, respectively. Interestingly, inhibition of HIV-1 BaL infection with the 14 ng/ml of the produced RANTES from the SHIV-RANTES-infected cells (51% inhibition) was slightly greater than inhibition with an equal concentration of recombinant human RANTES (42% inhibition). These results suggest that the produced RANTES efficiently inhibits R5-tropic HIV-1 infection.

3.4. In vitro challenge experiments of the SHIV-RANTES inoculated cells with R5-tropic HIV-1

A previous finding in the SIV-macaque models suggested that the induction of a CC-chemokine by a recombinant SIV vaccine was associated with protective immunity to mucosal infection with SIV [34]. We assessed whether the pre-inoculated cells with SHIV-RANTES were protected against the challenge with R5-tropic HIV-1 in in vitro experiments. In this assay, C8166-CCR5 cells were used as CCR5 positive cells, since the ability of virus replication in C8166-CCR5 cells was better than that in PM1 cells. As shown in Fig. 5A,B, the SHIV-RANTES-inoculated C8166-CCR5 cells produced RANTES, reaching a maximum level of 66.7 ng/ml at 6 days post challenge (d.p.c.) (at 12 d.p.i.). At the time of the challenge with HIV-1 BaL, the RANTES level in the culture supernatant of SHIV-RANTES-infected cells was already 16.9 ng/ml, while that of the SHIV-NI-infected cells was less than the cut-off value (2.5 ng/ml) during the experiment.

As shown in Fig. 5B, the viral loads of HIV-1 in the naive HIV-1 BaL-infected C8166-CCR5 cells increased to above 10^7 copies per ml at 9 d.p.c., and remained at a high level. Pre-inoculation of SHIV-RANTES suppressed the challenge of HIV-1 BaL. The peak virus loads of HIV-1 BaL in the SHIV-RANTES pre-inoculated C8166-CCR5 cells were two orders of magnitude lower than that of the naive HIV-1 BaL-inoculated cells. The viral loads of HIV-1 BaL in the culture supernatants of the SHIV-RANTES pre-inoculated cells plateaued at 10^4 copies per ml during the observation period. Viral loads of HIV-1 BaL in the SHIV-NI pre-infected cells reached between 10^5 and 10^6 copies per ml, which is higher

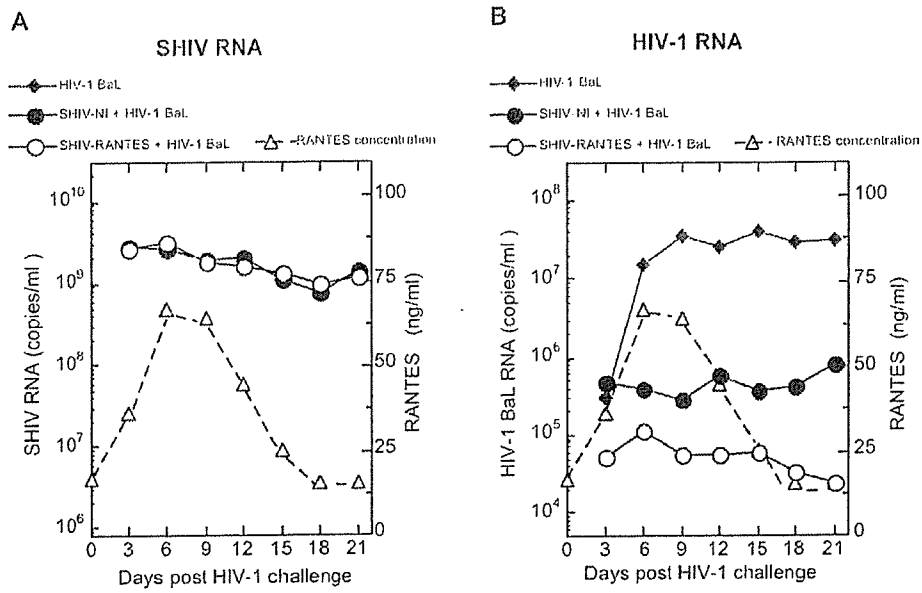

Fig. 5. Suppression of HIV-1 BaL by the pre-inoculation of SHIV-RANTES. Cells were pre-inoculated with SHIV-RANTES (open circles) and SHIV-NI (closed circles) at 6 days before the challenge of HIV-1 BaL. The HIV-1 BaL-infected cells without a pre-inoculation of SHIVs were used as a naive control (closed diamonds). Viral RNA levels of HIV-1 BaL and SHIVs were independently monitored by real-time RT-PCR using specific primer sets for either SIV *gag* region (A) or HIV *gag* region (B). Open triangles indicate RANTES levels produced by SHIV-RANTES, as determined by ELISA. (The RNA level of HIV-1 BaL detected by the primer for the SIV *gag* region was below 10^6 copies per ml).

than that in the SHIV-RANTES pre-inoculated cells. These results show that pre-inoculation of C8166-CCR5 with SHIV-RANTES partially protected against the challenge with HIV-1 BaL in vitro.

3.5. Stability of inserted RANTES gene in SHIV

Insertion of an additional gene may be a burden for the virus itself. In other studies, some cytokine genes such as IFN-γ, IL-2, and IL-12 inserted into either SHIV or SIV were found to be deleted during long-term experiments [19,35,36]. The stability of the inserted RANTES fragments in SHIV-RANTES after serial passage was examined by PCR of proviral DNA. These experiments were done more than three times. As shown in Fig. 6A, the inserted RANTES gene was found to be stable for at least 10 passages. The peaks of RANTES production in the culture supernatants from passage 1, 5, and 10 in the SHIV-RANTES-infected cells were 78.9, 76.8, and 82.2 ng/ml, respectively (Fig. 6B). These results show that SHIV-RANTES retained the ability to produce RANTES after serial passages. Furthermore, the expressed RANTES retained its ability to down-modulate CCR5 and to protect against HIV-1 BaL infection after 10 passages (Fig. 6C,D). These results show that the inserted RANTES gene in SHIV-RANTES was functional and stable until at least the 10th passage.

4. Discussion

In this paper, we compare the in vitro properties of SHIV-RANTES with those of its parental SHIV-NI. SIV/SHIV vectors containing a cytokine gene appear to be appropriate tools for observing the effect of local production of a cytokine on virus replication, pathogenesis, and immunogenicity, especially because the inserted cytokine gene is expressed in the region where the SIV/SHIV vector replicates. Because SIV dominantly utilized CCR5 for the virus entry and RANTES would suppress its replicate, we used the SHIV-NM3rN having the Env of X4-tropic HIV-1 NL432 as a vector that expresses RANTES genes. As expected, the replication of SHIV-RANTES, X4-tropic SHIV, was not suppressed by the expressed RANTES in this experiment.

SHIV-RANTES replicated well not only in the human $CD4^+$ T cell line M8166 but also in the monkey $CD4^+$ T cell line HSC-F. In addition, SHIV-RANTES successfully replicated in monkey PBMCs (data not shown). The expression level of RANTES in human M8166 cells was very high (up to 98.5 ng/ml). The high level of expression of RANTES may be partly due to the introduction of an effective ribosomal binding sequence at the flanking region of the RANTES ORF [21] and partly due to inserting the gene at the *nef* region [37]. We previously constructed the SHIV-*vpr* vector, named SHIV-3sj, from the same parental SHIV-NM3rN. Insertion of the RANTES gene into SHIV-3sj resulted in the expression of RANTES at a maximum level of 47.4 ng/ml in the culture supernatants of virus-infected M8166 cells [20]. The expression of RANTES with the SHIV-*nef* vector was about two times higher than that with the SHIV-*vpr* vector. A high level of expression of RANTES is advantageous to study the adjuvant effect of RANTES in vivo.

The results of many studies indicate that RANTES and its analogues suppress the infection of R5-tropic lentiviruses in

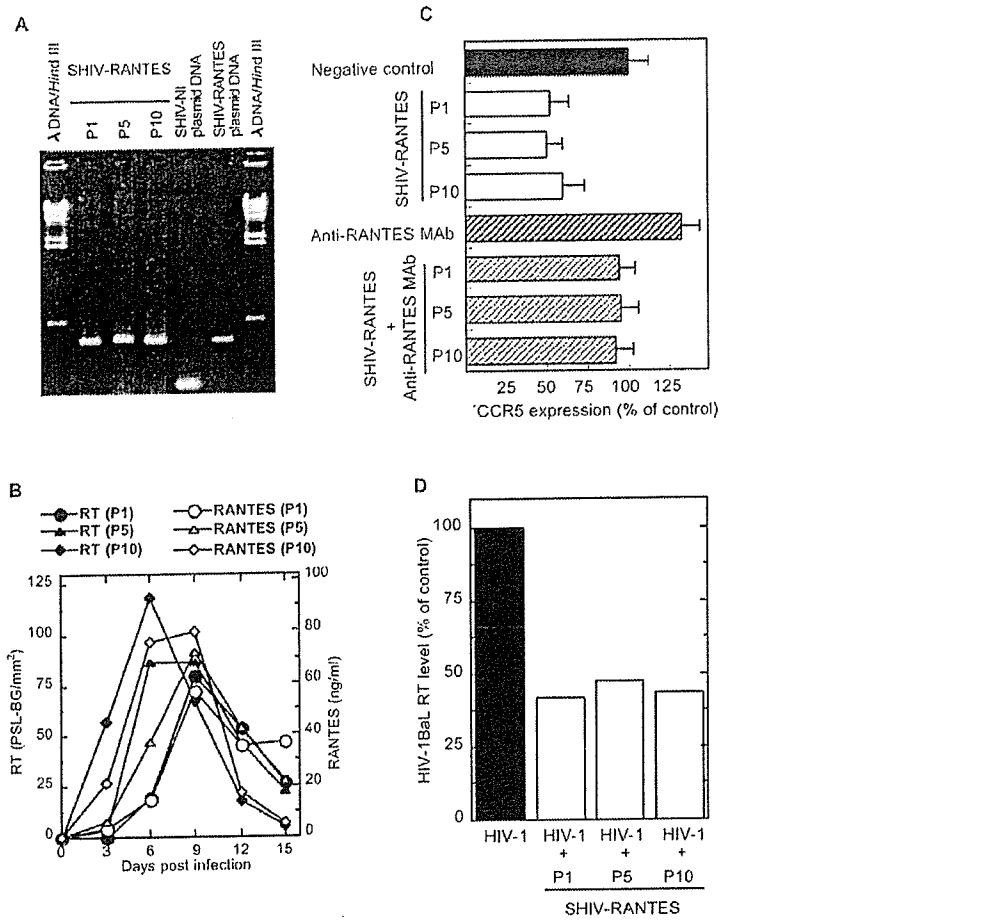

Fig. 6. Stability of SHIV-RANTES with serial passage. Culture supernatants from cells infected with SHIV-RANTES at passage 1 (P1), 5 (P5), and 10 (P10) were used for the experiments. (A) Stability of the inserted RANTES fragment in SHIV-RANTES was examined by PCR. DNA plasmids of SHIV-NI and SHIV-RANTES were used as the templates of the control. A λ DNA digested with *Hind* III was used as a size marker. (B) Kinetics of the RT activity and expression of RANTES at different passages. The unit for RT activity is PSL-BG, photo-stimulated luminescence minus background (FLA-3000, Fuji Film, Japan). The unit for RANTES (ng/ml) was determined by ELISA. (C) Down-modulation of CCR5 by RANTES expressed at different passages. The samples were also treated with anti-RANTES antibody to confirm the specificity of RANTES. (D) Inhibitory effect of R5-tripoc HIV-1 BaL replication by RANTES produced by a serial passage of SHIV-RANTES.

vitro. However, little information is available on the role of RANTES on X4-tropic lentivirus replication. Previous studies reported that RANTES enhanced X4-tropic and R5X4-tropic HIV-1 replication, both of which depend on a signal transduction and the enhanced HIV-1 replication is associated with increased colocalization of CD4 and CXCR4 [38]. The over-expression of RANTES may increase the replication of X4-tropic SHIV. A safety concern about live-attenuated viruses is that their replication rate may increase. In vitro, however, we did not observe increased SHIV replication in either human or monkey cell lines infected with the RANTES-producing virus.

Although the goal of this study is to test the immune-modulating effect of RANTES against HIV-1-related virus infection, immune responses are difficult to assess in in vitro experiments. In vitro, the inhibitory effects of RANTES on the entry of R5-tropic strains of lentivirus are well established. RANTES blocks or down-modulates CCR5 in vitro, which suppresses HIV-1 infections [29,32]. The down-modulation of CCR5 is a biological activity of RANTES. Our findings that the produced RANTES down-modulated CCR5 expression on the cell surface of PM1 cells and that UV-treated samples from SHIV-RANTES-infected cells partially protected the PM1 cells from the R5-tropic HIV-1 BaL infection suggest that RANTES expressed by SHIV-RANTES-infected cells is the factor protecting against R5-tropic HIV-1 infection. In this study, the biological activity of RANTES was confirmed by its ability to down-modulate expression of CCR5 and to inhibit the R5-tropic HIV-1 BaL infection.

Our finding that the viral growth kinetics of HIV-1 BaL was more strongly inhibited by the RANTES produced by SHIV-RANTES-infected cells than by the recombinant RANTES may be due to the presence of inactivated virus particles and other inhibitory factors in the culture supernatants of the SHIV-RANTES-inoculated cells. Another reason

is that the RANTES produced by mammalian cells is structurally different from the recombinant RANTES. RANTES is secreted from some cells as a macromolecular complex containing sulfated proteoglycans (with molecular sizes of 400–600 kDa), whereas the molecular size of recombinant RANTES is approximately 7.8 kDa [39,40]. The interaction of chemokines with proteoglycans enhances their anti-HIV-1 activity [40,41]. In this experiment, after the separation into < 100 kDa fractions by ultrafiltration (UF) membranes, the concentration of the produced RANTES was reduced to 1.6% of the pre-filtered levels (from 39.3 to 0.61 ng/ml) (data not shown). Many studies have used recombinant RANTES to determine the role of RANTES in HIV-1 infection. In our SHIV-*nef* vector system, the RANTES is produced in a natural, secreted form, which may be an advantage for studying the effect of RANTES in vivo.

The immune responses induced by an attenuated virus can increase the immunity to a pathogenic virus. Compared with the immunostimulatory cytokines produced by other *nef* deletion mutants, the RANTES produced by SHIV-RANTES is expected to not only boost the primitive non-specific immunity and virus-specific immune response but also directly inhibit the entry of R5-tropic virus to susceptible cells. SHIV-RANTES has an ability to protect against challenge with a pathogenic virus when inoculated to monkeys. Indeed, we found that HIV-1 BaL replication was two orders of magnitude lower in the cells pre-inoculated with SHIV-RANTES than in the naive HIV-1 BaL-inoculated cells.

In conclusion, we have established a SHIV-*nef* vector system that stably expresses a high level of biologically active RANTES. Inoculation of macaque monkeys with SHIV-RANTES should provide further information on the immune-modulating effects of RANTES against lentiviral infections.

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References

[1] T. Kuwata, T. Igarashi, E. Ido, M. Jin, A. Mizuno, J. Chen, M. Hayami, Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their in vitro and in vivo replication, J. Gen. Virol. 76 (Pt. 9) (1995) 2181–2191.

[2] Y. Enose, M. Ui, A. Miyake, H. Suzuki, H. Uesaka, T. Kuwata, J. Kunisawa, H. Kiyono, H. Takahashi, T. Miura, M. Hayami, Protection by intranasal immunization of a nef-deleted, nonpathogenic SHIV against intravaginal challenge with a heterologous pathogenic SHIV, Virology 298 (2002) 306–316.

[3] M. Ui, T. Kuwata, T. Igarashi, K. Ibuki, Y. Miyazaki, I.L. Kozyrev, Y. Enose, T. Shimada, H. Uesaka, H. Yamamoto, T. Miura, M. Hayami, Protection of macaques against a SHIV with a homologous HIV-1 Env and a pathogenic SHIV-89.6P with a heterologous Env by vaccination with multiple gene-deleted SHIVs, Virology 265 (1999) 252–263.

[4] T.W. Baba, V. Liska, A.H. Khimani, N.B. Ray, P.J. Dailey, D. Penninck, R. Bronson, M.F. Greene, H.M. McClure, L.N. Martin, R.M. Ruprecht, Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques, Nat. Med. 5 (1999) 194–203.

[5] B.R. Gundlach, M.G. Lewis, S. Sopper, T. Schnell, J. Sodroski, C. Stahl-Hennig, K. Uberla, Evidence for recombination of live, attenuated immunodeficiency virus vaccine with challenge virus to a more virulent strain, J. Virol. 74 (2000) 3537–3542.

[6] R.P. Johnson, R.C. Desrosiers, Protective immunity induced by live attenuated simian immunodeficiency virus, Curr. Opin. Immunol. 10 (1998) 436–443.

[7] L. Giavedoni, S. Ahmad, L. Jones, T. Yilma, Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces postchallenge virus load in vaccinated rhesus macaques, J. Virol. 71 (1997) 866–872.

[8] C. Stahl-Hennig, B.R. Gundlach, U. Dittmer, P. ten Haaft, J. Heeney, W. Zou, D. Emilie, S. Sopper, K. Uberla, Replication, immunogenicity, and protective properties of live-attenuated simian immunodeficiency viruses expressing interleukin-4 or interferon-gamma, Virology 305 (2003) 473–485.

[9] F. Sallusto, A. Lanzavecchia, C.R. Mackay, Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses, Immunol. Today 19 (1998) 568–574.

[10] A. Frauenschuh, A.L. DeVico, S.P. Lim, R.C. Gallo, A. Garzino-Demo, Differential polarization of immune responses by co-administration of antigens with chemokines, Vaccine 23 (2004) 546–554.

[11] J.J. Kim, L.K. Nottingham, J.I. Sin, A. Tsai, L. Morrison, J. Oh, K. Dang, Y. Hu, K. Kazahaya, M. Bennett, T. Dentchev, D.M. Wilson, A.A. Chalian, J.D. Boyer, M.G. Agadjanyan, D.B. Weiner, CD8 positive T cells influence antigen-specific immune responses through the expression of chemokines, J. Clin. Invest. 102 (1998) 1112–1124.

[12] P.M. Waterman, M. Kitabwalla, G.S. Hatfield, P.S. Evans, Y. Lu, I. Tikhonov, J.L. Bryant, C.D. Pauza, Effects of virus burden and chemokine expression on immunity to SHIV in nonhuman primates, Viral Immunol. 17 (2004) 545–557.

[13] K.Q. Xin, Y. Lu, K. Hamajima, J. Fukushima, J. Yang, K. Inamura, K. Okuda, Immunization of RANTES expression plasmid with a DNA vaccine enhances HIV-1-specific immunity, Clin. Immunol. 92 (1999) 90–96.

[14] R.K. Ahmed, C. Nilsson, Y. Wang, T. Lehner, G. Biberfeld, R. Thorstensson, Beta-chemokine production in macaques vaccinated with live attenuated virus correlates with protection against simian immunodeficiency virus (SIVsm) challenge, J. Gen. Virol. 80 (Pt. 7) (1999) 1569–1574.

[15] M.C. Gauduin, R.L. Glickman, S. Ahmad, T. Yilma, R.P. Johnson, Immunization with live attenuated simian immunodeficiency virus induces strong type 1T helper responses and beta-chemokine production, Proc. Natl. Acad. Sci. USA 96 (1999) 14031–14036 (B).

[16] J.L. Heeney, V.J. Teeuwsen, M. van Gils, W.M. Bogers, C. De Giuli Morghen, A. Radaelli, S. Barnett, B. Morein, L. Akerblom, Y. Wang, T. Lehner, D. Davis, Beta-chemokines and neutralizing antibody titers correlate with sterilizing immunity generated in HIV-1 vaccinated macaques, Proc. Natl. Acad. Sci. USA 95 (1998) 10803–10808.

[17] F. Cocchi, A.L. DeVico, A. Garzino-Demo, S.K. Arya, R.C. Gallo, P. Lusso, Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells, Science 270 (1995) 1811–1815.

[18] G. Alkhatib, C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy, E.A. Berger, CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1, Science 272 (1996) 1955–1958.

[19] T. Kuwata, T. Miura, T. Haga, I. Kozyrev, M. Hayami, Construction of chimeric simian and human immunodeficiency viruses that produce interleukin 12, AIDS Res. Hum. Retroviruses 16 (2000) 465–470.

[20] T. Haga, M. Okoba, N. Yamazaki, S. Kumabe, Y. Shimizu, Y. Goto, T. Kuwata, I.L. Kozyrev, M. Hayami, T. Miura, Characterization of vpr vector constructed from chimeric simian and human immunodeficiency virus, J. Vet. Med. Sci. 65 (2003) 633–636.

[21] M. Kozak, Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles, Microbiol. Rev. 47 (1983) 1–45.

[22] T. Haga, Y. Shimizu, M. Okoba, S. Kumabe, Y. Goto, T. Shinjo, H. Ichimura, T. Kuwata, M. Hayami, T. Miura, Construction and in vitro properties of chimeric simian and human immunodeficiency virus with the human TNF-alpha gene, Microbiol. Immunol. 46 (2002) 849–855.

[23] P.R. Clapham, R.A. Weiss, A.G. Dalgleish, M. Exley, D. Whitby, N. Hogg, Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester, Virology 158 (1987) 44–51.

[24] H. Akari, K. Mori, K. Terao, I. Otani, M. Fukasawa, R. Mukai, Y. Yoshikawa, In vitro immortalization of Old World monkey T lymphocytes with Herpesvirus saimiri: its susceptibility to infection with simian immunodeficiency viruses, Virology 218 (1996) 382–388.

[25] P. Lusso, F. Cocchi, C. Balotta, P.D. Markham, A. Louie, P. Farci, R. Pal, R.C. Gallo, M.S. Reitz Jr., Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1, J. Virol. 69 (1995) 3712–3720.

[26] Y. Soda, N. Shimizu, A. Jinno, H.Y. Liu, K. Kanbe, T. Kitamura, H. Hoshino, Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line, Biochem. Biophys. Res. Commun. 258 (1999) 313–321.

[27] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, Am. J. Hyg. 27 (1938) 493–497.

[28] S. Gartner, P. Markovits, D.M. Markovitz, M.H. Kaplan, R.C. Gallo, M. Popovic, The role of mononuclear phagocytes in HTLV-III/LAV infection, Science 233 (1986) 215–219.

[29] M. Mack, B. Luckow, P.J. Nelson, J. Cihak, G. Simmons, P.R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, T.N. Wells, D. Schlondorff, A.E. Proudfoot, Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity, J. Exp. Med. 187 (1998) 1215–1224.

[30] K. Suryanarayana, T.A. Wiltrout, G.M. Vasquez, V.M. Hirsch, J.D. Lifson, Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction, AIDS Res. Hum. Retroviruses 14 (1998) 183–189.

[31] T. Haga, T. Kuwata, I. Kozyrev, T.B. Kwofie, M. Hayami, T. Miura, Construction of an SIV/HIV type 1 chimeric virus with the human interleukin 6 gene and its production of interleukin 6 in monkey and human cells, AIDS Res. Hum. Retroviruses 16 (2000) 577–582.

[32] N. Signoret, A. Pelchen-Matthews, M. Mack, A.E. Proudfoot, M. Marsh, Endocytosis and recycling of the HIV coreceptor CCR5, J. Cell Biol. 151 (2000) 1281–1294.

[33] L. Wu, G. LaRosa, N. Kassam, C.J. Gordon, H. Heath, N. Ruffing, H. Chen, J. Humblias, M. Samson, M. Parmentier, J.P. Moore, C.R. Mackay, Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding, J. Exp. Med. 186 (1997) 1373–1381.

[34] T. Lehner, Y. Wang, M. Cranage, L.A. Bergmeier, E. Mitchell, L. Tao, G. Hall, M. Dennis, N. Cook, R. Brookes, L. Klavinskis, I. Jones, C. Doyle, R. Ward, Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques, Nat. Med. 2 (1996) 767–775.

[35] L.D. Giavedoni, T. Yilma, Construction and characterization of replication-competent simian immunodeficiency virus vectors that express gamma interferon, J. Virol. 70 (1996) 2247–2251.

[36] B.R. Gundlach, H. Linhart, U. Dittmer, S. Sopper, S. Reiprich, D. Fuchs, B. Fleckenstein, G. Hunsmann, C. Stahl-Hennig, K. Uberla, Construction, replication, and immunogenic properties of a simian immunodeficiency virus expressing interleukin-2, J. Virol. 71 (1997) 2225–2232.

[37] M. Robert-Guroff, M. Popovic, S. Gartner, P. Markham, R.C. Gallo, M.S. Reitz, Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages, J. Virol. 64 (1990) 3391–3398.

[38] A. Kinter, A. Catanzaro, J. Monaco, M. Ruiz, J. Justement, S. Moir, J. Arthos, A. Oliva, L. Ehler, S. Mizell, R. Jackson, M. Ostrowski, J. Hoxie, R. Offord, A.S. Fauci, CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: role of signal transduction, Proc. Natl. Acad. Sci. USA 95 (1998) 11880–11885.

[39] T.J. Schall, J. Jongstra, B.J. Dyer, J. Jorgensen, C. Clayberger, M.M. Davis, A.M. Krensky, A human T cell-specific molecule is a member of a new gene family, J. Immunol. 141 (1988) 1018–1025.

[40] L. Wagner, O.O. Yang, E.A. Garcia-Zepeda, Y. Ge, S.A. Kalams, B.D. Walker, M.S. Pasternack, A.D. Luster, Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans, Nature 391 (1998) 908–911.

[41] T. Oravecz, M. Pall, J. Wang, G. Roderiquez, M. Ditto, M.A. Norcross, Regulation of anti-HIV-1 activity of RANTES by heparan sulfate proteoglycans, J. Immunol. 159 (1997) 4587–4592.

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Isolation of the Feline α1,3-Galactosyltransferase Gene, Expression in Transfected Human Cells and its Phylogenetic Analysis

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ABSTRACT The enzyme alpha 1,3-galactosyltransferase (α1,3-GT), which catalyzes synthesis of terminal α-galactosyl epitopes (Gal α1,3Gal β1-4GlcNAc-R), is produced in non-primate mammals, prosimians and new-world monkeys, but not in old-world monkeys, apes and humans. We cloned and sequenced a cDNA that contains the coding sequence of the feline α1,3-GT gene. Flow cytometric analysis demonstrated that the α-galactosyl epitope was expressed on the surface of a human cell line transduced with an expression vector containing this cDNA, and this α-galactosyl epitope expression subsided by α-galactosidase treatment. The open reading frame of the feline α1,3-GT cDNA is 1,113 base pairs in length and encodes 371 amino acids. The nucleotide sequence and its deduced amino acid sequence of the feline α1,3-GT gene are 88–90% and 85–87%, respectively, similar to the reported sequences of the bovine, porcine, marmoset and cebus monkey α1,3-GT genes, while they are 88% and 82–83%, respectively, similar to those of the orangutan and human α1,3-GT pseudogenes, and 81% and 77%, respectively, similar to the murine α1,3-GT gene. Thus, the α1,3-GT genes and pseudogenes of mammals are highly similar. Ratios of non-synonymous nucleotide changes among the primate pseudogenes as well as the primate genes are still higher than the ratios of non-primates, suggesting that the primate α1,3-GT genes tend to be divergent. *J. Exp. Zool. (Mol. Dev. Evol.) 306B:59–69, 2006.*

Alpha 1,3-galactosyltransferase (α1,3-GT) (EC 2.4.1.151) is responsible for the synthesis of terminal α-galactosyl epitopes (Gal α1,3Gal β1-4GlcNAc-R) in sugar chains. These epitopes are produced by non-primate mammals, prosimians and new-world monkeys and are found on the cell surface ($>10^6$ epitopes/cell) as well as in secreted glycoproteins. The full-length α1,3-GT sequences of human and orangutan (Koike et al., 2002) and some partial sequences, similar to α1,3-GT genes of non-primate mammals, have been detected in humans and higher primates (Larsen et al., '90; Joziasse et al., '91). These sequences have been judged to be pseudogenes because of the generation of premature stop codons due to multiple base deletions (Larsen et al., '90; Joziasse et al., '91; Koike et al., 2002). The expression of α1,3-GT protein is not detected in humans and other catarrhines (Spiro and Bhoyroo, '84; Galili et al., '87, '88; Thall and Galili, '90), thus resulting in the production of large amounts of a natural antibody against the α-galactosyl epitope (Galili et al., '84, '87).

The α1,3-GT is a Golgi membrane-bound enzyme that catalyzes the addition of α-galactosyl epitopes to existing β-galactose terminals accord-

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The nucleotide sequence reported in this paper has been submitted to the GenBank™ with the accession number AY167024 (feline α1,3-GT).

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ing to the following reaction (Basu and Basu, '73; Blanken and Van den Eijnden, '85; Elices et al., '86): UDP galactose+β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-R→ UDP+α-D-galactosyl-1,3-β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-R, in which R may be a glycoprotein or a glycolipid. α1,3-Galactose and less abundant α1,6-linked galactose can be cleaved by the enzyme α-galactosidase (EC 3.2.1.22).

The whole mRNA for α1,3-GT has been found in cells of non-primate mammals and marmoset (Joziasse et al., '89; Larsen et al., '89; Henion et al., '94; Strahan et al., '95; Koike et al., 2002), but only partial mRNAs have been detected in those of old-world monkeys (OWM) (rhesus monkey, green monkey and patas monkey) or human cells (Joziasse et al., '89; Joziasse, '92), indicating that the regulatory sequences can still be functional. Several homologs of the α1,3-GT gene have been described in the human genome, but all of them contain several frame-shift mutations that lead to the generation of premature internal stop codons (Larsen et al., '90; Joziasse et al., '91). One homolog is thought to correspond to the original α1,3-GT gene because it contains intronic sequences (Joziasse et al., '91) as well as one exonic sequence corresponding to the largest part of the catalytic domain of the enzyme (Larsen et al., '90), and this gene has been localized on chromosome 9. Another one, which does not contain any intronic sequences and is located on chromosome 12, corresponds to a copy of the α1,3-GT gene (Larsen et al., '90; Joziasse et al., '91). This pseudogene has also been found in apes and OWM (Galili and Swanson, '91).

By Northern blot analysis, 3.6–3.9 kb α1,3-GT transcripts are detected in bovine and marmoset cells, but not detected in human and OWN cells (Joziasse et al., '89). For the first time, Koike et al. (2002) have detected the full-length sequences of α1,3-GT of orangutans and humans using sensitive PCR-based methods. Comparison of the deduced amino acid sequences with the marmoset gene sequence has revealed that in the human α1,3-GT pseudogene sequence, three single-nucleotide deletions are present at the site corresponding to the amino acid positions 81, 256 and 284 of marmoset α1,3-GT protein, and result in the appearance of the stop codons at positions 268 and 362. Thus, only non-functional proteins are made even when the full-length mRNA is overexpressed and translated in human cells.

A study on the murine α1,3-GT gene has revealed that it is distributed over nine exons that span at least 35 kb of the genomic sequence (Joziasse, '92). Transcription of this gene results in the production of four distinct mRNAs that are generated by the alternative splicing. Translation of these individual mRNAs produces four related isoforms of α1,3-GT consisting of 337, 349, 359 and 371 amino acids.

In this study, we have described the isolation and characterization of a cDNA clone comprising the complete coding sequence of feline (cat) α1,3-GT that is capable of catalyzing the synthesis of α-galactosyl epitopes on human cells. Phylogenetic analysis was performed to evaluate the evolutionary relationship among the six different full coding sequences of α1,3-GT genes and two pseudogenes.

MATERIALS AND METHODS

Cell culture

8C feline kidney cells (Fischinger et al., '73) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) at 37°C under 5% CO_2 in humidified air. A human osteosarcoma cell line, HOS, infected with a Melanesian strain of human T-cell leukemia virus type I (HTLV-I), termed HOS/HTLV-I_{MEL5}, was cultured in Dulbecco's modified EMEM (Hoshino et al., '93).

Reverse transcriptase-polymerase chain reaction

When 8C cells became confluent, they were collected in an Eppendorf tube using a scraper. Total RNA was isolated from 8C cells using an RNA extraction kit, SepaGene (Sanko-Junyaku Co. Ltd., Tokyo, Japan), according to the manufacturer's protocol. cDNA was synthesized using total cellular RNA as the template, using the SuperScript™ Preamplification System for First-Strand cDNA Synthesis kit (GibcoBRL, Life Technologies™, Carlsbad, CA) in accordance with the manufacturer's protocol. PCR was used to amplify the α1,3galactosyltransferase gene in cDNA preparation. Two sets of primers were used for PCR. One set was made according to the porcine α1,3-GT sequence as follows: 5′-ATGAATGTCAAAGGAAGAGTGGTTCTGTCA-3′ as a forward primer and 5′-TCAGATGTTATTTCTAACCAAATTATACTC-3′ as a reverse primer. The other set was designed using DNA sequences of α1,3-GT of pig (Strahan et al., '95), mouse (Larsen et al., '89), cow (Joziasse et al., '89) and

marmoset (Galili et al., '88). Namely, the regions where the nucleotide sequences of α1,3-GT are well conserved were selected and used to make PCR primers as follows: 5′-GGAGAAAATAAT GAATGTCAA-3′ as a forward primer and 5′-TCAGATGTTATTTCTAACCAAATT-3′ as a reverse primer. The initiation and stop codons in the primers are underlined.

Cloning of the feline α1,3-GT and sequencing

The PCR product was cloned into a TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA), and the ligated product was used to transform DH5α *E. coli* strain. *E. coli* colonies were tested for mini-scale plasmid preparation, and colonies containing a 1.1 kb insert after multiple enzyme digestion were selected. Clones containing inserts were sequenced using Texas Red-labeled M13 forward and reverse primers for sequencing DNA. A Hitachi SQ-5500 automated DNA sequencer and software (Hitachi, Tokyo, Japan) were used to determine DNA sequences as described previously (Jinno et al., '98). The plasmid vector pCR2.1 containing the insert and the expression vector pcDNA3 (Clontech Co., Palo Alto, CA) were digested with *BamHI* and *Not*I and purified. The insert in the pCR2.1 vectors was then re-ligated to the digested pcDNA3 vector DNA. After transformation of competent cells, DH5α, by this ligation product, plasmids purified from colonies contained an insert of about 1.1 kb. It was confirmed by enzyme digestion of the plasmid DNA with *Stu*I, *Spe*I, *BamHI* and *Stu*I that the inserts were in a correct direction (data not shown).

Transfection of the feline α1,3-GT gene and detection of its expression

The mammalian expression vector pcDNA3 harboring the gene of the feline α1,3-GT was transfected into a clonal line of HTLV-I-infected HOS cells using LipofectAMINE (GibcoBRL) to examine the effects of α1,3-GT expression on HTLV-I infection, and neomycin-resistant cells were selected as described elsewhere (Jinno et al., '98). In short, the cells were seeded at 4×10^5 cells/ml/well into 12-well plates and incubated overnight. The cells in nine wells were independently transfected with pcDNA3 DNA harboring the gene of the feline α1,3-GT or pcDNA3 DNA alone using LipofectAMINE (GibcoBRL). The cells were selected with neomycin after 24 hr incubation and maintained for 3 weeks. The expression of α-galactosyl epitope on the surface of HOS and HOS/HTLV-I cells and feline cells was detected by flow cytometry (FCM) (Cyto ACE-100, Auto cell screener, Japan Spectroscopic Co., Ltd.) after treatment with fluorescent isothiocyanate labeled *Bandeiraea simplicifolia* Isolectin B_4 (FITC/BS-IB_4) (Sigma Chemical Co., St. Louis, MO) (Wood et al., '79; Azimzadeh et al., '97; Bracy et al., '98). Briefly, cells were collected into Eppendorf tubes from cultured plates, washed with washing solution (cold PBS with 1% FCS and 0.01% NaN3), pelleted by centrifugation at 5,000 rpm for 5 min and treated for 1 hr with diluted FITC/BS-IB_4 (3.3 pg/ml) on ice. The cells were washed and fixed with 1% paraformaldehyde and analyzed by FCM. In seven (#1–#7) out of nine wells seeded with the feline α1,3-GT-transduced cells, 20–45% of cells were positively stained with FITC/BS-IB_4 (Table 1). These cells were cloned using 96-well plates by seeding 1 cell/well and thus obtained clones were screened again by FCM.

α-Galactosidase treatment

Cells were treated with α-galactosidase as described by Bracy et al. ('98). Briefly, cells were seeded at 4×10^5 cells/well into 6-well plates in 2 ml and incubated overnight. The culture medium was replaced with fresh medium and the cells were incubated for another day. The cells were collected into two Eppendorf tubes from each cultured plate, washed with the washing solution

TABLE 1. Flow cytometry of the cells transfected with the feline α1,3-GT gene

Cell	BS-IB4-positive cells (%)
HOS/HTLV-I	4
HOS/HTLV-I/pcDNA3	5
HOS/HTLV-I/fGT	
#1	45
#2	44
#3	38
#4	32
#5	27
#6	27
#7	22
#8	4
#9	4

Human HOS/HTLV-I cells in nine culture wells were transfected with the feline α1,3-GT gene independently. Neomycin-resistant cells were selected. These HOS cells were processed for FCM using FITC/BS-IB4 lectin and examined for the expression of α-galactosyl epitope on the cell surface. HOS/HTLV-I/pcDNA3 cells were used as a control negative for terminal α1,3-galactose. The cut-off value for FCM was set at the fluorescence intensity that gave 5% positive for the control cells as shown in Figs. 1 and 2.

and pelleted by centrifugation at 5,000 rpm for 5 min. One of them was treated with α-galactosidase dissolved in sodium citrate/phosphate buffer, and the other tube was treated only with buffer for 1 hr at 37 °C. The cells were washed with the washing solution and treated for 1 hr with FITC/BS-IB_4 on ice. The cells were washed and fixed with 1% paraformaldehyde and analyzed by FCM.

Synonymous (K_S) and non-synonymous (K_A) nucleotide substitutions per site

The number of synonymous substitutions per possible synonymous site (K_S) and the number of non-synonymous substitutions per possible non-synonymous site (K_A) (Li et al., '85; Li, '93) were calculated for codons of full-length α1,3-GT gene and pseudogene sequences using MEGA 2.1 (Kumar et al., 2001). K_A/K_S ratios less than 1.0 are generally considered as evidence that the proteins have evolved under negative or purifying selection; pairwise comparisons between active genes show this pattern (Wolfe and Sharp, '93; Endo et al., '96). K_A/K_S ratios over 1.0 suggest positive or directional selection.

Phylogenetic trees for the α1,3-GT isolates

Two phylogenetic trees using the amino acid sequences and nucleotide sequences of the α1,3-GT cDNAs were constructed using the N–J method (Saitou and Nei, '87), and the reliability of the clusters obtained was evaluated by means of 1,000 bootstrap replicates. The MEGA 2.1 software was used to make the trees.

RESULTS

Isolation of feline α1,3-GT cDNA

A feline kidney cell line, 8C, was used to make α1,3-GT cDNA. Cellular RNA was isolated and reverse transcribed. PCR with the two sets of primers described above was used to amplify cDNA. The PCR product was then ligated with the cloning vector pCR2.1 and the construct was used to transform *E. coli* cells. Plasmid DNAs were isolated from *E. coli* colonies that harbored DNA with the expected length of the insert and used for DNA sequencing. We sequenced seven different clones, and six out of seven clones gave overlapping nucleotide sequences. The full-length coding sequence of the feline α1,3-GT gene was 1,113-bp long (GenBank accession number AY167024) and the deduced amino acid sequence contained 371 amino acids. Its molecular mass was calculated to be 43,568 Da.

Expression of α-galactosyl epitope on the cell surface

The expression vector pcDNA3 with the feline α1,3-GT insert was made and transfected into a cloned human osteosarcoma cell line, HOS, infected with HTLV-I, i.e., HOS/HTLV-I (Hoshino et al., '93) in nine culture wells, and neomycin selection was done. The cells were maintained about 3 weeks in neomycin-containing medium and examined by FCM using fluorescent isothiocyanate-labeled *B. simplicifolia* Isolectin B_4 (FITC/BS-IB_4). In the first screening, FCM results showed that 20–45% cells derived from seven (#1–#7) out of nine wells containing the feline α1,3-GT plasmid-transfected cells were positively stained with FITC/BS-IB_4 (Table 1). The cells derived from wells #1 and #2 were single-cell cloned by seeding them into 96-well plates at a density of one cell per well, and thus obtained two of 19 clones were about 98% positive by FCM (Fig. 1). The clones derived from initial #1 and #2 wells were designated as HOS/HTLV-I/fGT#1-a and #2-a. The surface expression of α-galactosyl epitopes was stable as it was detected by FCM even after 20 cell passages (data not shown). In contrast, the surface expression of α-galactosyl epitopes was not detected upon FCM of untransfected or only pcDNA3 vector-transfected cells. When the feline α1,3-GT-transduced HOS/HTLV-I cells were treated with α-galactosidase, specific reduction of α-galactosyl epitope expression was detected (Fig. 2) by staining with FITC/BS-IB_4 as compared with results of these cells treated with buffer alone. These findings indicated that the cDNA cloned from 8C cells really coded for the feline α1,3-GT. The cells for highly positive α-galactosyl epitopes grew as well as untransfected HOS/HTLV-I cells, and their morphology was indistinguishable from that of the untransfected HOS/HTLV-I cells.

Analysis of the feline α1,3-GT gene sequence

We aligned the deduced amino acid sequences of the α1,3-GT genes of feline, porcine, murine, bovine, cebus and marmoset origins and the human and orangutan α1,3-GT pseudogenes using software for multiple alignments, Clustal W (Thompson et al., '94) (Fig. 3). In our alignment of the amino acid sequences of the eight α1,3-GT genes, the feline sequence was 85–87% similar to

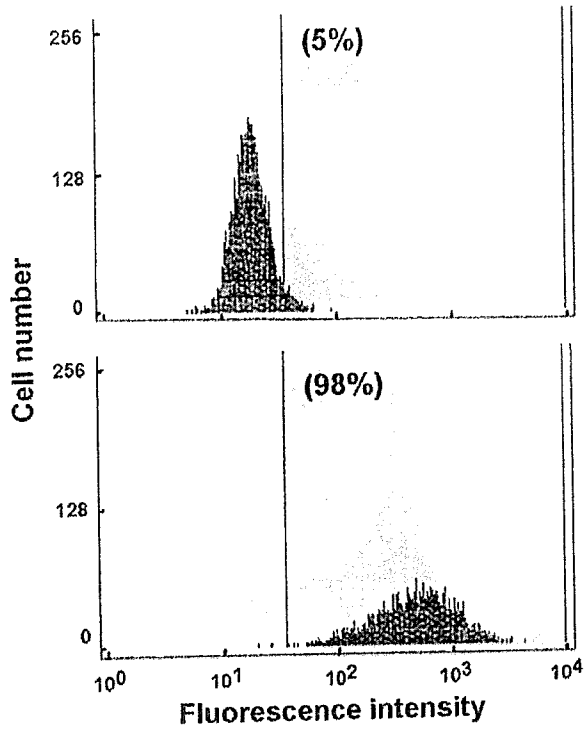

Fig. 1. Detection of α-galactosyl epitope expression. The pcDNA3 control vector-transfected HOS/HTLV-I cells (A) and the feline α1,3-GT gene-transfected HOS/HTLV-I cells (B) were processed for FCM using FITC/BS-IB_4 and examined for the expression of α-galactosyl epitope on the cell surface. The cut-off value for fluorescence intensity was set as 5% of control cells were scored positive (A); the cells that showed stronger intensity than the cut-off value were considered to be positive (B).

those of bovine, porcine, cebus and marmoset origins; the human and orangutan sequences were 82–83% and the murine sequence was 77% similar to the feline sequence. The nucleotide sequences of the bovine, porcine, marmoset and cebus α1, 3-GT genes and of the orangutan and human α1, 3-GT pseudogenes showed a high similarity (88–90%) to the feline sequence, while the murine sequence was only 81% similar to it (Table 2).

Figure 3 shows that there is an especially high similarity among all eight α1,3-GT sequences after amino acid number 84 (feline), 85 (porcine), 85 (murine), 81 (bovine), 89 (human), 89 (cebus), 89 (orangutan) and 90 (marmoset): there was 82–91% similarity in the domain corresponding to amino acid numbers 84–370 of the feline sequence, while in the domain 1–83 the similarity was much lower and between 65% and 72% (Table 2). The highly similar domain has been thought to encode catalytic activities (Henion et al., '94). Although the human and orangutan α1,3-GT genes are inactive, we still noticed the high similarity in their nucleotide sequences with those of other animal α1,3-GT genes. The murine sequence showed a slightly lower similarity: 82% in the 84–370 domain and 62% in the 1–89 domain (Table 2).

To study the evolutionary forces that have been operated among α1,3-GT genes, we analyzed their sequences for synonymous substitution per site (K_S) and non-synonymous substitution per site (K_A) between paired species using the modified Nei–Gojobori method (Kumar et al., 2001). The total number of possible synonymous sites in an individual sequence among the eight α1,3-GT gene sequences was between 287 and 296 (standard error between 6 and 8) with an average of 292, and the total number of possible non-synonymous sites was between 795 and 810 (standard error between 7 and 16) with an average of 803 (data not shown). The total number of possible synonymous and non-synonymous sites in an individual sequence was between 1,082 and 1,104 with an average of 1,095. The total synonymous-site differences between paired samples were between 8 and 121 (standard error between 3 and 9) with a mean of 65 (Table 3), and the non-synonymous-site differences were between 12 and 129 (standard error between 3 and 12) with a mean of 69 (Table 4). We found that the range of the paired samples K_S (calculated using MEGA 2.1 software) was 0.028–0.410 (standard error between 0.009 and 0.028) with a mean of 0.223, and the K_A range was 0.015–0.161 (standard error between 0.006 and 0.015) with a mean of 0.086 (data not shown). The K_S values are significantly higher than the K_A values ($P<0.0005$, according to Student's t-test). Table 5 shows the K_A/K_S ratios where all the values are within a range of 0.219–0.763, indicating that the α1,3-GT genes are in a direction from negative, purifying selection to neutrality. The ratios between the species that express the active enzyme are around 0.2–0.4 and those of primates, including cebus, orangutan and human but not marmoset, are around 0.7–0.8 irrespective of whether the enzyme is active (cebus) or inactive (orangutan and human). The marmoset α1,3-GT gene shows an intermediate type: 0.273 with that of cebus and about 0.7–0.8 with that of orangutan and human (Table 5).

Phylogenetic trees of the α1,3-GT genes

To analyze the evolutionary distance among α1,3-GT genes of feline, murine, bovine, porcine,

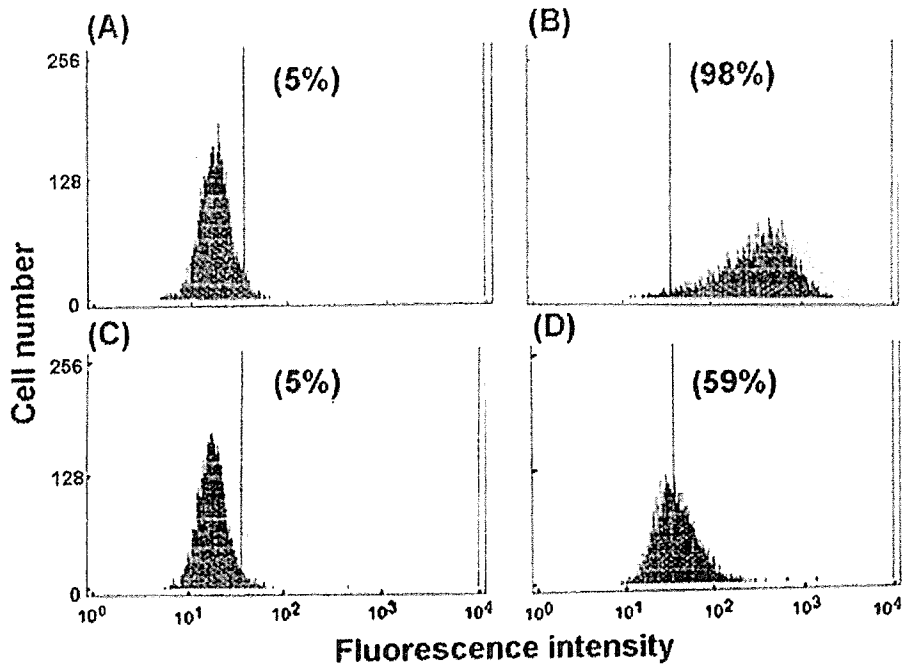

Fig. 2. Detection of α-galactosyl epitope expression. The pcDNA3 vector-transfected HOS/HTLV-I and the feline α1,3-GT plasmid-transfected HOS/HTLV-I cells were initially treated with buffer (**A** and **B**) alone or α-galactosidase (**C** and **D**). The cells were then processed for FCM (as described in Fig. 1) and examined for the expression of α-galactosyl epitope on the cell surface.

cebus and marmoset origins and the α1,3-GT pseudogenes in orangutan and human species, we constructed phylogenetic trees for these α1,3-GT genes and pseudogenes by the N–J method (Saitou and Nei, '87) using the total coding nucleotide sequences (date not shown) and the amino acid sequences (Fig. 4). When the feline α1,3-GT nucleotide sequence was aligned with the other α1,3-GT nucleotide sequences and a phylogenetic tree was made with Poisson correction, it was closely related with the other mammalian α1,3-GT nucleotide sequences. The phylogenetic tree showed that the feline α1,3-GT gene clustered with all other mammalian α1,3-GT genes, especially with the porcine and bovine genes, with a bootstrap support of 93% (date not shown).

The phylogenetic tree constructed using the deduced amino acid sequences shown in Fig. 4 reveals a markedly similar pattern to that constructed using the nucleotide sequences. We also aligned 1–83 and 84–370 amino acid sequences of the feline α1,3-GT with all other α1,3-GT amino acid sequences to make two other phylogenetic trees (data not shown). Both of the phylogenetic trees show almost the same pattern as shown in Fig. 4.

DISCUSSION

When HOS/HTLV-I human cells were transfected with the cDNA encoding the feline α1, 3-GT, they became positive for the expression of α-galactosyl epitopes. In addition, α-galactosidase treatment of HOS/HTLV-I cells transfected with the feline α1,3-GT led to specific reduction of α-galactosyl epitope expression (Fig. 2) as detected by staining with FITC/BS-IB_4. The enzymatic removal of α-galactosyl epitopes was, however, incomplete, probably because the reaction was performed at sub-optimal pH to preserve the cell viability as described by Bracy et al. ('98). Thus, newly cloned human cells showed the stable expression of the feline α1,3-GT (Fig. 1), which catalyzes the addition of α-galactosyl epitopes to existing carbohydrate side chains.

According to the general topology of glycosyltransferases (Paulson and Colley, '89; Joziasse et al., '92), it has been reported that there are three domains in the sequences of glycosyltransferases: a cytoplasmic domain, a transmembrane domain and a luminal domain. Henion et al. ('94) have shown that the 67 amino acids from positions

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Feline     MNVKGRVVLSMLVVSTVIVVFWEYINSPEGSFLWIYHSKNPEVGDSSTQKGWWFPSWFNN : 60
Murine     .....K.I.L..I....V......V...D........T.I....ENRW..D.......K. : 60
Bovine     .....K.I..................H.....LF..NP.R.....G..I.....L.R.... : 60
Porcine    ............L....M.............LF...Q.......-..A.R.......... : 59
Marmoset   .....K.I.....................................D...A..D....G.... : 60
Cebus      .....K.I.....................................D.-.A.......G.... : 59
Orangutan  .....K.I............I......T.............D....A.....L..... : 60
Human      .....K.I..............F...T.............D...A......L..... : 60

Feline     RTHSYPEEEAVD----EGDEQRKENSE--ELQLSDWFNPQKRPDVVTVTEWKAPVVWEGT :114
Murine     G....Q.DNVEG-----RR.KGRNGDRIE.P..W.....KN....L...P....I..... :115
Bovine     ---G.H..DGDI----NEEKEQRNED.-SK.K.......F...E...M.K.......... :112
Porcine    G....H...DAI----GNEKEQRKEDNRG..P.V.....E...E...I.R.......... :115
Marmoset   GI.N.QQ..EDTDKEKGRE.EQ.KEDDTT..R.W.....K...E.M...Q.......... :120
Cebus      GI.N.QQ..EDIDKEKGRE.EQRKEDDTT....W.....K...E.....K.......... :119
Orangutan  GI.N.QQR.EDIDKEKGRE.-QRKENDTT..R.W.....K...K.....R.......... :119
Human      GI.N.QQG.EDIDKEKGRE.-QRKENDTT..R.W.....K.H.E.....R.......... :119

Feline     YNKAILENYYARQKITVGLTVFAVGRYIEHYLEEFLISANRYFMVGHKVIFYIMVDDVSK :174
Murine     .DT.L..K...T..L..........K.......D..E..DM......R....V.I..T.R :175
Bovine     ..R.V.D....K.............................KH.....P..........R :172
Porcine    ..R.V.D....K.............................T..............I.R :175
Marmoset   ..........K...........I............VT..............V....... :180
Cebus      ..........K...........I............VT..............V....... :179
Orangutan  F.....G....K.......M...I...NG......IT..................... :179
Human      F.....G....K.....R.M...I...ND......IT..................... :179

Feline     MPLIELGPLRSFKVFEIKPEKRWQDISMMRMKIIGEHIVAHIQHEVDFLFCMDVDQVFQD :234
Murine     ..VVH.N..H.LQ....RS.............T.....L..................... :235
Bovine     ..............K..................T.......................... :232
Porcine    ..................S.............T.....L.....................N :235
Marmoset   A.F.............V..............T.....L..................... :240
Cebus      V.F.............V..............T.....L..................... :239
Orangutan  V.F......H......V..............T....L..................... :239
Human      L.F......H...M..V..............T....L..................... :239

Feline     SFGVETLGQSVAQLQAWWYKADPDEFTYERRKESAAYIPFGEGDFYYHAAIFGGTPTQVL :294
Murine     N........L..........S.EK......EL.......................HI. :295
Bovine     K.......E.............ND..................................... :292
Porcine    N....................H.....................Q.................. :295
Marmoset   H.....................D.....................Q.............I... :300
Cebus      H.....................D......R.........Q........V.....I... :299
Orangutan  H..............R......D..*...E.........Q.-.................. :298
Human      H.............-.R.....YD..*..W....G.....Q.-.......S....I... :297

Feline     NITQECFKGILQDKKNDIEAEWHDESHLNKYFLLNKPTKILSPEYCWDYHIGLPSDIKIV :354
Murine     .L.R..........H....Q...........F.............Q........S. :355
Bovine     ..........K........Q.................................A...L. :352
Porcine    ............E.....................................MSV..R.. :355
Marmoset   ..........L.......................S.....................T. :360
Cebus      ..........L.......................S.....................T. :359
Orangutan  ...R...N...L.....V.................S....LK.............T. :358
Human      ..........L........K.............S....LK.............T. :357

Feline     KISWQTKEYNLVRNNI :370
Murine     .VA...........V :371
Bovine     .M........V....V :368
Porcine    ..A..K.......... :371
Marmoset   .L..........K.V :376
Cebus      .L.............. :375
Orangutan  .R.R..........V :374
Human      .*............V :373
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Fig. 3. Alignment of deduced amino acid sequences of different α1,3-GT genes. The amino acid sequences of the feline, porcine, murine, bovine, cebus and marmoset α1,3-GT genes and human and orangutan α1,3-GT pseudogenes are aligned using CLUSTAL W algorithm. Numbers at the right side indicate amino acid positions. Dots (.) represent amino acids identical to those in the feline gene sequence. Amino acid deletions in each gene are indicated by dash marks (-). In the human sequence, the star marks (*) indicate premature stop codons.

TABLE 2. Nucleotide and amino acid similarity between the feline and other α1,3-GT genes

Species	Nucleotide similarity (%)	Amino acid sequence				
		1–83 domain		84–370 domain		Total
		Similarity (%)	Gap	Similarity (%)	Gap	Similarity (%)
Murine	81	62	1[1]	82	0	77
Bovine	88	65	3	90	0	85
Porcine	90	72	1	91	0	87
Marmoset	89	72	0	91	0	87
Cebus	89	72	1	91	0	87
Orangutan	88	70	1	86	1	83
Human	88	70	1	86	2	82

The total coding sequences of the α1,3-GT genes were aligned, including gaps. Identical nucleotides and amino acids at each position, excluding gaps, were counted and a similarity rate (%) was calculated between the feline α1,3-GT gene and each α1,3-GT gene or pseudogene.
[1]Number of amino acid gaps placed when compared with the 1–83 or 84–370 domains of the feline α1,3-GT gene.

TABLE 3. Total number of synonymous differences between paired species among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	107	—						
Bovine	79	120	—					
Porcine	64	113	61	—				
Marmoset	76	95	74	63	—			
Cebus	71	94	77	61	16	—		
Orangutan	71	87	68	56	17	15	—	
Human	72	91	73	59	21	17	8	—

Total possible synonymous sites were between 287 and 296 with an average of 292.

TABLE 4. Total number of non-synonymous differences between paired species among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	100	—						
Bovine	62	123	—					
Porcine	51	110	65	—				
Marmoset	48	107	68	64	—			
Cebus	43	106	63	60	12	—		
Orangutan	60	123	83	79	37	34	—	
Human	60	129	83	79	40	36	18	—

Total possible non-synonymous sites were between 795 and 810 with an average of 803.

23 to 89 in the marmoset α1,3-GT gene, which they called a stem region, have little effect on enzymatic activity, but the sequence between 90 and 376 can show an almost full catalytic activity. The presence of stop codons in human and orangutan α1,3-GT genes at the site corresponding to the 268th amino acid of the marmoset gene (Fig. 3) will lead to the loss of about 100 amino acids at the C-terminus. This loss is expected to be sufficient to lose the entire catalytic activity

TABLE 5. K_A/K_S ratios of paired samples among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	0.343[1]	—						
Bovine	0.264	0.374	—					
Porcine	0.289	0.355	0.386	—				
Marmoset	0.228	0.411	0.335	0.367	—			
Cebus	0.220	0.414	0.298	0.354	0.273	—		
Orangutan	0.306	0.517	0.445	0.510	0.780	0.808	—	
Human	0.304	0.516	0.411	0.485	0.694	0.763	0.786	—

[1]K_A/K_S ratios calculated using MEGA 2.1 software (Kumar et al., 2001).

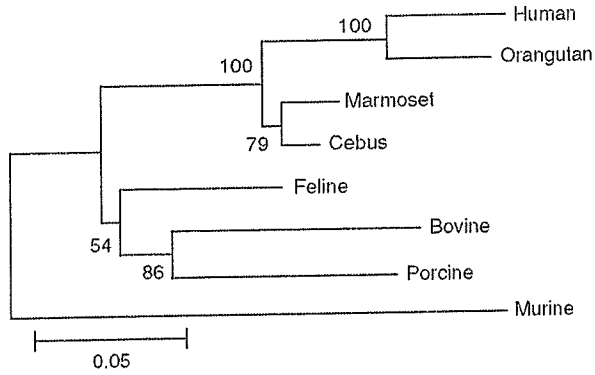

Fig. 4. A phylogenetic tree of the α1,3-GT enzyme. A phylogenetic tree of the amino acid sequences deduced from the nucleotide sequences for murine, feline, porcine, borine, marmoset and cebus α1,3-GT genes and the human and orangutan α1,3-GT pseudogenes was constructed by the N–J method using the MEGA 2.1 software. The phylogenetic relationships of the amino acid sequences are represented as unrooted cladograms. The numbers at branch nodes indicate the bootstrap support level (BSL), which is the percentage of how often each branch presents exactly the same topology in all the resampled trees. The scale bars indicate the number of substitutions per site.

even when α1,3-GT mRNA of human or orangutan is translated.

In our sequence alignment, it appeared that the amino acid residues 61–83 in the feline α1,3-GT gene form a highly variable region and the residues between 84 and 371 form a highly conserved region. This finding also suggests that the catalytic domain of the feline α1,3-GT gene is located in the highly conserved 84–370 domain. In the corresponding domains of animal sequences, including the human and orangutan pseudogenes, the functional constraints appear to have restricted mutations throughout evolution by Wilson et al. ('77). Since most of the amino acid gaps were located between positions 45 and 84 of the feline α1,3-GT sequence, this portion is unlikely to contain catalytic activity.

The α1,3-GT gene is transcribed in a small amount but the enzyme encoded is not active in higher primates (humans, apes and OWM) (Koike et al., 2002): the reason for loss of this enzyme activity in higher primates is unknown. There are several reports on production of α1,3-GT gene-deficient mice (knock-out mice). These mice can grow, live and age normally (Tearle et al., '96; Pearse et al., '98), indicating that the α1,3-GT gene can be dispensable for rodents, although this gene has been evolutionarily conserved as shown in Table 2. On the contrary, the expression of the α1,3-GT gene in human cells did not affect the growth and morphology of human cells, as we could isolate HOS/HTLV-I cell lines highly expressing α1,3-GT (Table 1 and Fig. 1). Thus, its expression did not exert adverse effects on human cells at least in tissue culture. It is intriguing for us that the α1,3-GT genes of marmoset and cebus and the pseudogenes of orangutan and human show similar degrees of substitution rates in their nucleotide sequences as well as in their amino acid sequences to the murine, bovine and porcine genes.

The rate of synonymous substitution (K_S) is usually much higher than that of non-synonymous substitution (K_A) for a normally functioning gene. Synonymous substitution may be used as a molecular clock for dating the evolutionary time of closely related species (Kafatos et al., '77; Kimura, '77; Miyata and Yasunaga, '80; Perler et al., '80). K_A/K_S ratios less than 1.0 are generally taken as negative or purifying selection, and, conversely, K_A/K_S ratios significantly greater than 1.0 are considered to be proper evidence of

directional or positive selection for amino acid replacement (Li et al., '85; Li, '93). That is, when the K_A/K_S value for a given gene is less than 1.0, the encoded protein sequence has been conserved during evolution. For example, the histone H4 gene family protein sequences are under purifying selection (Piontkivska et al., 2002) and some lineages of the primate lysozyme protein sequences are under directional selection (Messier and Stewart, '97). Among the α1,3-GT genes, the synonymous substitution rate is significantly higher than the non-synonymous substitution rate between the paired sequences ($P<0.0005$). Generally, the values of possible non-synonymous substitutions are much higher than the values of possible synonymous substitutions. Among the α1,3-GT genes we examined, the ranges of possible non-synonymous and synonymous substitution sites were 795–810 and 287–296, respectively.

Pairwise K_A/K_S ratios are high (0.7–0.8) in the species where the enzyme had lost its activity (human or orangutan), but are much lower (0.2–0.4) in the species where the enzyme is active. Although the α1,3-GT gene in marmoset and cebus is active and the K_A/K_S value between them is 0.273, their pairwise K_A/K_S values with human and orangutan pseudogenes are as high as 0.7–0.8. High K_A/K_S values (among human, orangutan and cebus) may indicate that these genes have evolved rapidly at the protein level or tend to evolve free of constraint, although their pairwise K_A/K_S ratios are not greater than 1. It is noteworthy that most of the non-synonymous substitutions are concentrated in a region that locates outside of the catalytic domain of the enzyme (Fig. 3).

Humans are known to carry natural antibodies against α1,3-galactosyl epitope at high titers and these antibodies are also known to markedly affect transplantation of xenografts derived from porcine organs and will lead to rejection (Sandrin and McKenzie, '94). A pig strain with knock-out of this gene has already been made (Dor et al., 2004). These antibodies should, however, be beneficial for survival of higher primates. These natural antibodies have been thought to exert prophylactic effects on development of cancer or infection with certain pathogens bearing α-galactosyl epitope (Gollogly and Castronovo, '96; Welsh et al., '98). It remains to be elucidated why α1,3-GT genes should have been inactivated in humans, apes and OWM and why the inactivated gene sequences still have a high degree of similarity to those of the active genes.

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LITERATURE CITED

Azimzadeh A, Wolf P, Thibaudeau K, Cinqualbre J, Soulillou JP, Anegon I. 1997. Comparative study of target antigens for primate xenoreactive natural antibodies in pig and rat endothelial cells. Transplantation 64:1166–1174.

Basu M, Basu S. 1973. Enzymatic synthesis of a blood group B-related pentaglycosylceramide by an alpha-galactosyltransferase from rabbit bone marrow. J Biol Chem 248:1700–1706.

Blanken WM, Van den Eijnden DH. 1985. Biosynthesis of terminal Gal alpha 1-3Gal beta 1-4GlcNAc-R oligosaccharide sequences on glycoconjugates. Purification and acceptor specificity of a UDP-Gal:N-acetyllactosaminide alpha 1, 3-galactosyltransferase from calf thymus. J Biol Chem 260: 12927–12934.

Bracy JL, Sachs DH, Iacomini J. 1998. Inhibition of xenoreactive natural antibody production by retroviral gene therapy. Science 281:1845–1847.

Dor FJ, Tseng YL, Cheng J, Moran K, Sanderson TM, Lancos CJ, Shimizu A, Yamada K, Awwad M, Sachs DH, Hawley RJ, Schuurman HJ, Cooper DK. 2004. Alpha1,3-galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies. Transplantation 78:15–20.

Elices MJ, Blake DA, Goldstein IJ. 1986. Purification and characterization of a UDP-Gal:beta-D-Gal(1,4)-D-GlcNAc alpha(1,3)-galactosyltransferase from Ehrlich ascites tumor cells. J Biol Chem 261:6064–6072.

Endo T, Ikeo K, Gojobori T. 1996. Large-scale search for genes on which positive selection may operate. Mol Biol Evol 13: 685–690.

Fischinger PJ, Peebles PT, Nomura S, Haapala DK. 1973. Isolation of RD-114-like oncornavirus from a cat cell line. J Virol 11:978–985.

Galili U, Swanson K. 1991. Gene sequences suggest inactivation of alpha-1,3-galactosyltransferase in catarrhines after the divergence of apes from monkeys. Proc Natl Acad Sci USA 88:7401–7404.

Galili U, Rachmilewitz EA, Peleg A, Flechner I. 1984. A unique natural human IgG antibody with anti-alpha-galactosyl specificity. J Exp Med 160:1519–1531.

Galili U, Clark MR, Shohet SB, Buehler J, Macher BA. 1987. Evolutionary relationship between the natural anti-Gal antibody and the Gal alpha 1,3Gal epitope in primates. Proc Natl Acad Sci USA 84:1369–1373.

Galili U, Shohet SB, Kobrin E, Stults CL, Macher BA. 1988. Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. J Biol Chem 263:17755–17762.

Gollogly L, Castronovo V. 1996. A possible role for the alpha 1–>3galactosyl epitope and the natural anti-gal antibody in oncogenesis. Neoplasma 43:285–289.

Henion TR, Macher BA, Anaraki F, Galili U. 1994. Defining the minimal size of catalytically active primate alpha 1,3 galactosyltransferase: structure-function studies on the recombinant truncated enzyme. Glycobiology 4:193–201.

Hoshino H, Nakamura T, Tanaka Y, Miyoshi I, Yanagihara R. 1993. Functional conservation of the neutralizing domains on the external envelope glycoprotein of cosmopolitan and melanesian strains of human T cell leukemia/lymphoma virus type I. J Infect Dis 168:1368–1373.

Jinno A, Shimizu N, Soda Y, Haraguchi Y, Kitamura T, Hoshino H. 1998. Identification of the chemokine receptor TER1/CCR8 expressed in brain-derived cells and T cells as a new coréceptor for HIV-1 infection. Biochem Biophys Res Commun 243:497–502.

Joziasse DH. 1992. Mammalian glycosyltransferases: genomic organization and protein structure. Glycobiology 2: 271–277.

Joziasse DH, Shaper JH, Van den Eijnden DH, Van Tunen AJ, Shaper NL. 1989. Bovine alpha 1,3-galactosyltransferase: isolation and characterization of a cDNA clone. Identification of homologous sequences in human genomic DNA. J Biol Chem 264:14290–14297.

Joziasse DH, Shaper JH, Jabs EW, Shaper NL. 1991. Characterization of an alpha 1,3-galactosyltransferase homologue on human chromosome 12 that is organized as a processed pseudogene. J Biol Chem 266:6991–6998.

Joziasse DH, Shaper NL, Kim D, Van den Eijnden DH, Shaper JH. 1992. Murine alpha 1,3-galactosyltransferase. A single gene locus specifies four isoforms of the enzyme by alternative splicing. J Biol Chem 267:5534–5541.

Kafatos FC, Efstratiadis A, Forget BG, Weissman SM. 1977. Molecular evolution of human and rabbit beta-globin mRNAs. Proc Natl Acad Sci USA 74:5618–5622.

Kimura M. 1977. Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution. Nature 267:275–276.

Koike C, Fung JJ, Geller DA, Kannagi R, Libert T, Luppi P, Nakashima I, Profozich J, Rudert W, Sharma SB, Starzl TE, Trucco M. 2002. Molecular basis of evolutionary loss of the alpha 1,3-galactosyltransferase gene in higher primates. J Biol Chem 277:10114–10120.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245.

Larsen RD, Rajan VP, Ruff MM, Kukowska-Latallo J, Cummings RD, Lowe JB. 1989. Isolation of a cDNA encoding a murine UDPgalactose:beta-D-galactosyl-1, 4-*N*-acetyl-D-glucosaminide alpha-1,3-galactosyltransferase: expression cloning by gene transfer. Proc Natl Acad Sci USA 86:8227–8231.

Larsen RD, Rivera-Marrero CA, Ernst LK, Cummings RD, Lowe JB. 1990. Frameshift and nonsense mutations in a human genomic sequence homologous to a murine UDP-Gal:beta-D-Gal(1,4)-D-GlcNAc alpha(1,3)-galactosyltransferase cDNA. J Biol Chem 265:7055–7061.

Li WH. 1993. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. J Mol Evol 36:96–99.

Li WH, Wu CI, Luo CC. 1985. A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. Mol Biol Evol 2:150–174.

Messier W, Stewart CB. 1997. Episodic adaptive evolution of primate lysozymes. Nature 385:151–154.

Miyata T, Yasunaga T. 1980. Molecular evolution of mRNA: a method for estimating evolutionary rates of synonymous and amino acid substitutions from homologous nucleotide sequences and its application. J Mol Evol 16:23–36.

Paulson JC, Colley KJ. 1989. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. J Biol Chem 264:17615–17618.

Pearse MJ, Witort E, Mottram P, Han W, Murray-Segal L, Romanella M, Salvaris E, Shinkel TA, Goodman DJ, d'Apice AJ. 1998. Anti-Gal antibody-mediated allograft rejection in alpha1,3-galactosyltransferase gene knockout mice: a model of delayed xenograft rejection. Transplantation 66:748–754.

Perler F, Efstratiadis A, Lomedico P, Gilbert W, Kolodner R, Dodgson J. 1980. The evolution of genes: the chicken preproinsulin gene. Cell 20:555–566.

Piontkivska H, Rooney AP, Nei M. 2002. Purifying selection and birth-and-death evolution in the histone H4 gene family. Mol Biol Evol 19:689–697.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Sandrin MS, McKenzie IF. 1994. Gal alpha (1,3)Gal, the major xenoantigen(s) recognised in pigs by human natural antibodies. Immunol Rev 141:169–190.

Spiro RG, Bhoyroo VD. 1984. Occurrence of alpha-D-galactosyl residues in the thyroglobulins from several species. Localization in the saccharide chains of the complex carbohydrate units. J Biol Chem 259:9858–9866.

Strahan KM, Gu F, Preece AF, Gustavsson I, Andersson L, Gustafsson K. 1995. cDNA sequence and chromosome localization of pig alpha 1,3-galactosyltransferase. Immunogenetics 41:101–105.

Tearle RG, Tange MJ, Zannettino ZL, Katerelos M, Shinkel TA, Van Denderen BJ, Lonie AJ, Lyons I, Nottle MB, Cox T, Becker C, Peura AM, Wigley PL, Crawford RJ, Robins AJ, Pearse MJ, d'Apice AJ. 1996. The alpha-1,3-galactosyltransferase knockout mouse. Implications for xenotransplantation. Transplantation 61:13–19.

Thall A, Galili U. 1990. Distribution of Gal alpha 1,3Gal beta 1, 4GlcNAc residues on secreted mammalian glycoproteins (thyroglobulin, fibrinogen, and immunoglobulin G) as measured by a sensitive solid-phase radioimmunoassay. Biochemistry 29:3959–3965.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Welsh RM, O'Donnell CL, Reed DJ, Rother RP. 1998. Evaluation of the Galalpha1-3Gal epitope as a host modification factor eliciting natural humoral immunity to enveloped viruses. J Virol 72:4650–4656.

Wilson AC, Carlson SS, White TJ. 1977. Biochemical evolution. Annu Rev Biochem 46:573–639.

Wolfe KH, Sharp PM. 1993. Mammalian gene evolution: nucleotide sequence divergence between mouse and rat. J Mol Evol 37:441–456.

Wood C, Kabat EA, Murphy LA, Goldstein IJ. 1979. Immunochemical studies of the combining sites of the two isolectins, A4 and B4, isolated from *Bandeiraea simplicifolia*. Arch Biochem Biophys 198:1–11.

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Editor-Communicated Paper

Tandem Repeats of Lactoferrin-Derived Anti-Hepatitis C Virus Peptide Enhance Antiviral Activity in Cultured Human Hepatocytes

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Abstract: **Previously, we found that bovine and human lactoferrin (LF) specifically inhibited hepatitis C virus (HCV) infection in cultured non-neoplastic human hepatocyte-derived PH5CH8 cells, and we identified 33 amino acid residues (termed C-s3-33; amino acid 600–632) from human LF that were primarily responsible for the binding activity to the HCV E2 envelope protein and for the inhibiting activity against HCV infection. Since the anti-HCV activity of C-s3-33 was weaker than that of human LF, we speculated that an increase of E2 protein-binding activity might contribute to the enhancement of anti-HCV activity. To test this possibility, we made two repeats [$(C\text{-}s3\text{-}33)_2$] and three repeats [$(C\text{-}s3\text{-}33)_3$] of C-s3-33 and characterized them. Far-Western blot analysis revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and that the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$. Using an HCV infection system in PH5CH8 cells, we demonstrated that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33. Furthermore, using a recently developed infection system with a VSV pseudotype harboring the green fluorescent protein gene and the native E1 and E2 genes, we demonstrated that the antiviral activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33. These results suggest that tandem repeats of LF-derived anti-HCV peptide are useful as anti-HCV reagents.**

Key words: **Hepatitis C virus, Lactoferrin, Anti-HCV peptide, E2 protein-binding activity**

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (28). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae*. The HCV genome encodes a large polyprotein precursor of about 3,000 amino acids (aa), which is cleaved by the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (7, 8, 18). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (1, 17).

Approximately 170 million people worldwide are infected with HCV (32). The combination of a pegylated interferon with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (6). This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma.

Although the entry mechanism of HCV remains unclear, to date, several candidates for HCV receptors

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Abbreviations: aa, amino acids; DMEM, Dulbecco's modified Eagle's medium; E2, envelope 2; GFP, green fluorescent protein; HCV, hepatitis C virus; LF, lactoferrin; MBP, maltose-binding protein; NS2, non-structural 2; TF, transferrin; VSV, vesicular stomatitis virus.

have been reported: CD81, the scavenger receptor class B type I, the mannose-binding lectins DC-SIGN and L-SIGN, low-density lipoprotein receptors, etc. (4). Most of them have been identified as interacting materials with a soluble and truncated form of the HCV E2 protein, because of the lack of efficient HCV proliferation in cell cultures, although several culture systems using PCR for detection of HCV infection have been reported (20). However, a major advance in investigating HCV entry has been achieved by the development of pseudotype viruses bearing HCV E1 and E2 proteins assembled onto retrovirus particles (2, 9) or vesicular stomatitis virus (VSV) particles (3, 23, 30). Extensive characterization of the pseudotype viruses has shown that these mimic the early steps of the HCV life cycle. This system has allowed the study of the role of candidate receptors in the early steps of HCV infection (4).

We previously found that bovine and human lactoferrins (LFs) specifically prevented HCV infection in cultured human non-neoplastic hepatocyte PH5CH8 cells using the PCR method for detection of HCV infection (10, 12). Regarding these findings, some clinical studies have demonstrated that monotherapy with bovine LF improves the serum HCV RNA and/or alanine aminotransferase levels in patients with chronic hepatitis C (15, 16, 27, 31).

LF is an 80-kDa mammalian iron-binding glycoprotein and consists of two homologous globular lobes (an N-lobe and a C-lobe), each with a single iron (Fe^{3+}) binding site. It is structurally related to the plasma iron-transport protein transferrin (TF). LF's biological roles include activities in the host defense mechanism as well as in iron metabolism (21, 22). Unlike TF, LF is a primary defense protein against microbial infection. LF possesses strong bacteriostatic and bactericidal activities against pathogenic bacteria, as well as inhibitory activity against pathogenic viruses (5, 21, 22, 33).

LF's preventive mechanism against HCV infection has been thought to be the direct interaction between LF and HCV; indeed, by Far-Western blot analysis using thioredoxin-fused LF fragments expressed in *Escherichia coli* (*E. coli*) and the soluble E2 protein expressed in Chinese hamster ovary cells, we demonstrated that the 93 carboxyl aa of LF (human, bovine, and horse), termed C-s3, specifically bound to the E2 protein (25). On the other hand, Yi et al. (34) independently reported that the E1 and E2 proteins could bind to human and bovine LFs, although the binding region of LF was not identified. Furthermore, we identified the 33 aa of human LF (termed C-s3-33; aa 600–632), which was primarily responsible for the E2 protein-binding activity, and demonstrated that maltose-binding protein (MBP)-fused C-s3-33 prevented HCV infection in PH5CH8 hepatocyte cells (25). However, the E2 protein-binding activity and the anti-HCV activity of C-s3-33 were obviously weaker than those of human LF. Therefore, we presumed that the increase of the E2 protein-binding activity would lead to the enhancement of anti-HCV activity.

To evaluate this idea, we made tandem repeats of C-s3-33, and compared their E2 protein-binding activities and anti-HCV activities with those of the C-s3-33. Here, we report our findings that the anti-HCV activity of the tandem repeats were stronger than that of the monomer when accompanied by the enhancement of the E2 protein-binding activity, by analyses using not only the HCV infection system but also the infection system of a VSV pseudotype bearing the native forms of HCV E1 and E2 proteins.

Materials and Methods

Cell cultures. Simian virus 40 large T antigen-immortalized non-neoplastic human PH5CH8 hepatocytes were maintained as described previously (11, 24). Human hepatoblastoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of expression plasmids for E. coli. The pMAL-c2X (hLF600–632) (25) expression plasmid for the MBP-fused C-s3-33 LF fragment, was used as a template for the PCR using a primer set of hLFB6 5'-TGATAGGATCCGTGGTGTCTCGGATGATAAGG-3' containing the *Bam*HI recognition site (underlined) (25) and 632R6A 5'-ATCCATCCGAGACACCACAAACTTGTCCGGGCAGTCAGATCC-3' containing an extra 18 nts (underlined) encoding the amino-terminal 6 aa of the C-s3-33 LF fragment. After PCR (20 cycles) using KOD-plus DNA polymerase (Toyobo, Osaka, Japan), the amplified PCR product was used as a template for a second PCR using the primer set of hLFB6 and 632R 5'-TAATAAAGCTTTTAAAACTTGTCCGGGCAGTCAGATCC-3' containing the *Hin*dIII recognition site (underlined) (25). After PCR (35 cycles) using KOD-plus DNA polymerase, amplified PCR products (approximately 200 bp for the two-repeat form and approximately 300 bp for the three-repeat form) were subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and were used as expression plasmids for the production of the MBP-fused $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$.

To prepare an expression plasmid for the production of the MBP-fused C-s3-33-relevant fragment (aa 587–619) of human TF, pCXbsr/huTF (29) encoding full-length human TF was used as a template for the PCR using a primer set of hTF587F 5'-TGATAG-

GATCCGTGGTCACACGG-3' containing the *Bam*HI recognition site (underlined) and hTF619R 5'-TAATAAAGCTTTTAAAAGTTGCCCG-3' containing the *Hin*dIII recognition site (underlined). After PCR (35 cycles) using KOD-plus DNA polymerase, the amplified PCR product was subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and was used as the expression plasmid.

Expression and purification of the MBP-fused protein. Expression and purification of the MBP-fused LF fragment [C-s3-33, $(C\text{-}s3\text{-}33)_2$, or $(C\text{-}s3\text{-}33)_3$] or the MBP-fused C-s3-33-relevant fragment of human TF were carried out as described previously (25). Briefly, the expression plasmid for MBP-fused protein was transformed into the *E. coli* strain JM109. The transformants were cultured at 37 C for several hours, and the harvested cells were sonicated. After removal of insoluble cellular debris by centrifugation, the supernatant obtained as the soluble fraction was applied onto an amylose resin affinity column (New England Biolabs) to obtain the MBP-fused protein. The purity of the obtained MBP-fused protein was evaluated to be more than 95% by electrophoresis on 10% SDS-PAGE gels. The concentration of the purified MBP-fused protein was determined by using Coomassie protein assay reagent (Pierce). The MBP2 (43 kDa) produced from the pMAL-c2X with a stop codon inserted into the *Xmn*I site was used as a control protein.

Far-Western blot analysis. Far-Western blot analysis was carried out as described previously (25). Briefly, 0.5 μg of human LF, MBP2, and MBP-fused LF fragments were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with N-buffer (25), a binding reaction was carried out using the secreted form of the E2 protein (E2-681) consisting of aa 384–681 expressed in Chinese hamster ovary cells as a probe (14), and then rat monoclonal antibody, MO-12 (13), against E2 protein was used for the detection of E2 protein-bound MBP-fused LF fragments.

Assay for anti-HCV activity of MBP-fused protein. An assay for anti-HCV activity of the MBP-fused LF fragment was carried out by the method described previously (25). Briefly, 2 μl (2×10^4 HCV) of the HCV-positive serum HCV-O (previously described as 1B-2 (19)) (genotype 1b) and the MBP-fused LF fragment (final concentration, 0.5, 1.0, and 2.0 mg/ml) were pre-incubated for 60 min at 4 C and then inoculated onto to the PH5CH8 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed three times with PBS and further cultured for 1 day at 32 C. Cellular RNA (0.5 μg) prepared by ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) was used for the quantitative analysis of HCV RNA using LightCycler PCR as described previously (26). As the positive and negative controls for anti-HCV activity, human LF and MBP2, respectively, were used.

Assay for anti-VSV pseudotype activity of MBP-fused protein. For this assay, the VSV pseudotype VSVΔG*(HCV), bearing the native forms of HCV E1 and E2 proteins from the O strain (19), was used. VSVΔG*(HCV) was prepared by introducing the native form of E1 and E2 proteins into recombinant VSV, VSVΔG*, which harbors the green fluorescent protein (GFP) gene instead of the VSV G envelope protein gene (30). An assay for the anti-VSV pseudotype activity of the MBP-fused LF fragment was carried out by a method described previously (30). Briefly, VSVΔG*(HCV) (Approximately 100 IU/assay) was pre-incubated with the MBP-fused LF fragment (final concentration, 0.1–1.0 mg/ml) at 37 C for 60 min and inoculated onto PH5CH8 or HepG2 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed with DMEM three times and incubated with fresh culture medium. VSVΔG*G was used as a control in this assay. After 24 hr of incubation, each infectious titer was determined by counting the number of GFP-expressing cells under a fluorescence microscope. As the positive and negative controls for the assay, human LF and MBP2 were used, respectively. Human TF and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay.

Results

Two and Three Repeats of the Human LF Fragment (C-s3-33) Strengthened the E2 Protein-Binding Activity

Previously we found that bovine and human LFs prevented HCV infection in PH5CH8 cells via direct interaction between LF and HCV (10, 12), and we further identified 33 aa residues (C-s3-33; aa 600–632 of human LF) as an essential and minimum domain possessing binding activity for the HCV E2 protein (secreted form consisting of aa 384–681) and inhibiting activity against HCV infection (25). This result suggested that the E2 protein-binding activity contributes to the anti-HCV activity. However, the E2 protein-binding activity of C-s3-33 was somewhat weaker than that of human LF (25), and the anti-HCV activity of C-s3-33 (IC_{50}=20 μM) in the infection system using PH5CH8 cells was also weaker than that of human LF (IC_{50}=5 μM) (25). To improve these points, we first tried to

enhance the E2 protein-binding activity of C-s3-33 by the multiplication of C-s3-33. Initially, we made pMAL-c2X-based expression vectors encoding two, three, and four repeats of C-s3-33 as MBP-fused proteins, and then expressed them in *E. coli*. We successfully purified two repeats $(C\text{-}s3\text{-}33)_2$ and three repeats $(C\text{-}s3\text{-}33)_3$ of C-s3-33 as soluble forms of the MBP-fused protein; the purification of the four repeats of C-s3-33 failed due to problems with solubility. Using the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, we performed Far-Western blot analysis to compare their E2 protein-binding activities. The result revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$ (Fig. 1). Although the E2 protein-binding activity of C-s3-33 was weaker than that of human LF, the binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$

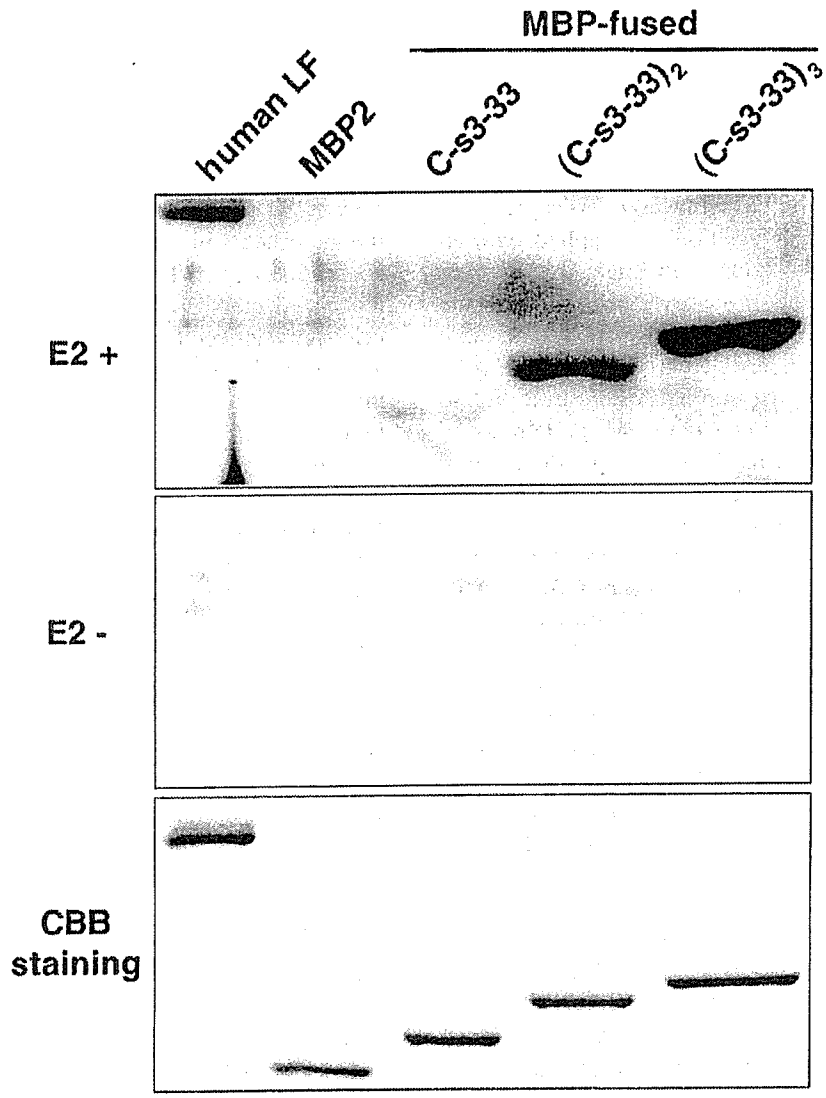

Fig. 1. Comparison of the E2 protein-binding activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$. MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ (0.5 μg each) were resolved by 10% SDS-PAGE. Human LF and MBP2 (0.5 μg each) were also used for the assay as control materials. Far-Western blot analysis using the E2 protein expressed in Chinese hamster ovary cells (14) as a probe was performed as described under "Materials and Methods." Rat monoclonal antibody MO-12 (13) against the E2 protein was used for the detection of the E2 protein bound to MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ as well as human LF. Far-Western blot analysis in the absence of the E2 protein was also performed. The bottom panel shows the results for human LF, MBP2, and MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ detected by staining with Coomassie Brilliant Blue.