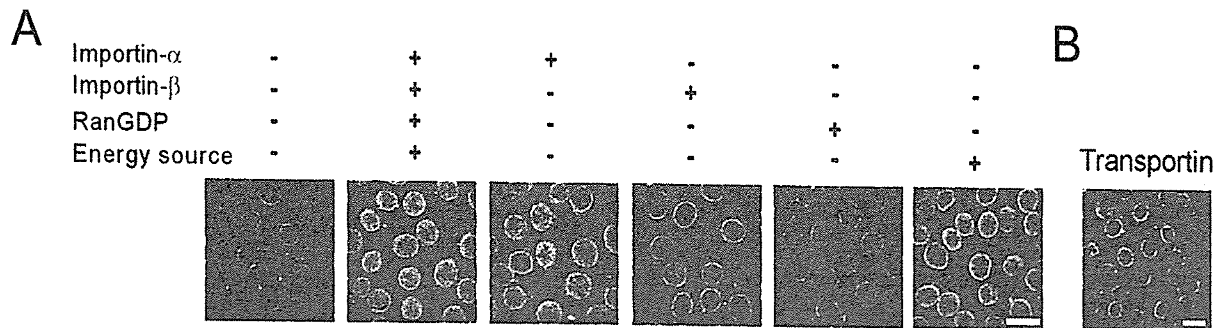

FIG. 2. Entry into the nucleus of the N17C74 chimeric protein is promoted by importin-α or energy. (A and B) HeLa cells were permeabilized by treatment with 50 μg of digitonin per ml and were treated with 10 U of apyrase per ml at 30°C for 5 min. The cells were incubated with GST- and GFP-tagged N17C74 in the absence (−) or presence (+) of soluble factors, as indicated at the top of the photographs. Then they were analyzed by confocal laser-scanning microscopy. The respective fields show the signals from GFP. Soluble factors were included at the concentrations given in the legend to Fig. 1. Transportin was used at a concentration of 1 μM with 1 μM RanGDP. Bar = 20 μm.

RanGDP, importin-β, and transportin (43) hardly had an effect on import into the nucleus. The effects of importin-α and the energy source were considerably diminished in the presence of WGA (Fig. 3), also indicating that N17C74 entered into the nucleus through the NPC. To confirm that importin-α could support the passage through the NPC of N17C74, we labeled digitonin-permeabilized HeLa cells with the GFP-specific MAb and Cy3-conjugated antibody against mouse IgG after incubation of these cells with the chimeric protein in the absence of soluble factors. Then we incubated the cells for a further 15 min in the presence of importin-α. The chimeric protein that had been labeled with Cy3 clearly entered the nucleus by upon incubation with importin-α. No signal due to Cy3 was detected in the intranuclear region without these factors, indicating that importin-α promoted nuclear translocation of N17C74 through the NPC (data not shown). These results also indicated that N17C74 could mediate entry into the nucleus of large molecules, such as an antigen-antibody complex.

The carboxyl terminus of importin-α is required for the nuclear import of Vpr. It has been suggested that Vpr interacts with importin-α (α1/Rch1 and α5/NPI-1) (2, 27, 48, 54). We confirmed whether Vpr directly interacts with importin-α in a pull-down assay using the N17C74 chimeric protein and a series of derivatives of importin-α (Fig. 4). We produced seven different derivatives of importin-α as a fusion protein with MBP (Fig. 4A). The construct with full-length importin-α (positions 1 to 529; right panel, lane 15) bound to N17C74 (Fig. 4B). Similar binding was observed with the protein that lacked the carboxy-terminal sequence (positions 1 to 392; right panel, lane 13). Such binding was reduced considerably by truncation of the IBB domain, but weak binding that did not depend on the IBB domain was still observed (ΔIBB 1-392 and ΔIBB 1-529; right panel, lanes 14 and 16, respectively). Moreover,

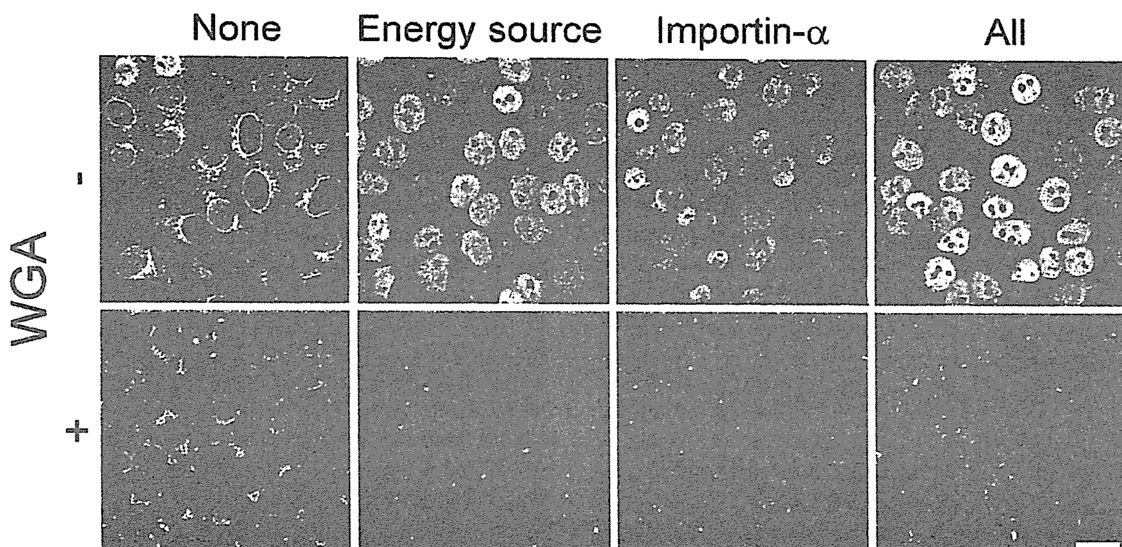

FIG. 3. Nuclear import of Vpr is performed in an NPC-dependent manner. Digitonin-permeabilized HeLa cells were incubated with (+) or without (−) 200 μg of WGA per ml and then incubated with GST- and GFP-tagged N17C74 in the presence of an energy source, 1 μM importin-α, or the mixture of soluble factors (All) described in the legend to Fig. 1. After fixation, cells were analyzed by confocal laser-scanning microscopy. Bar = 20 μm.

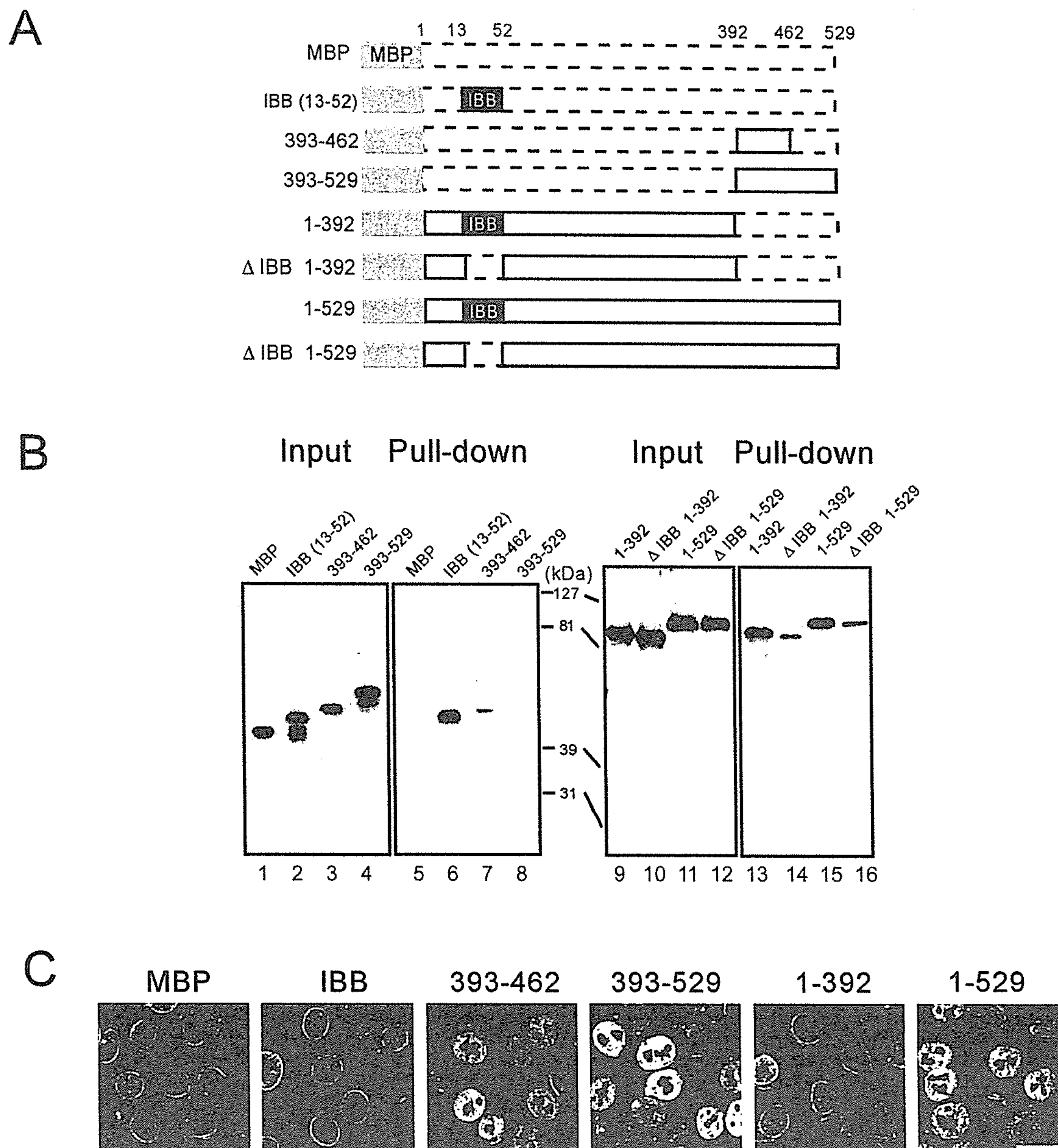

FIG. 4. Promotion of entry of Vpr into the nucleus requires the carboxyl terminus of importin-α. (A) Deletion mutants of importin-α used in this study. All mutants were expressed as an MBP fusion protein. (B) Glutathione-Sepharose beads coupled with GST- and GFP-tagged N17C74 were incubated with MBP-tagged derivatives of importin-α. The bound fractions were analyzed by Western blotting with MBP-specific antibody. In the "Input" lanes, 2% of each of the proteins was used for each respective reaction. (C) Transport assays were performed in the presence of 3 μM MBP-tagged derivatives of importin-α. After fixation, cells were analyzed by confocal laser-scanning microscopy. Bar = 20 μm.

the fragment that corresponded to the IBB domain bound to N17C74 as did as full-length importin-α [IBB (13-52); left panel, lane 6]. These results indicated that the IBB domain was the main participant in the interaction between N17C74 and importin-α.

To clarify whether binding between N17C74 and importin-α is necessary for entry of the N17C74 chimeric protein into the nucleus, we performed an in vitro transport assay using our MBP-tagged fragments of importin-α. All fragments containing residues 393 to 462 promoted entry of the N17C74 chimeric protein into the nucleus, as did as full-length importin-α (Fig. 4C, positions 393 to 462, 393 to 529, and 1 to 529). Fragments without residues 393 to 462 no longer promoted nuclear entry, even if they were able to bind to the N17C74 chimeric protein (Fig. 4C, IBB and positions 1 to 392). Furthermore, the mutant form of importin-α that lacked the IBB domain (ΔIBB 1-529) still promoted a nuclear entry of N17C74 (data not shown). Taken together, the results indicate that the carboxyl terminus of importin-α, between residues 393 and 462, functions to promote entry of N17C74 into the nucleus and this ability is unrelated to the interaction between N17C74 and importin-α that involves the IBB domain.

The αH1 region of Vpr is indispensable for nuclear import. To identify the region within N17C74 that is responsible for

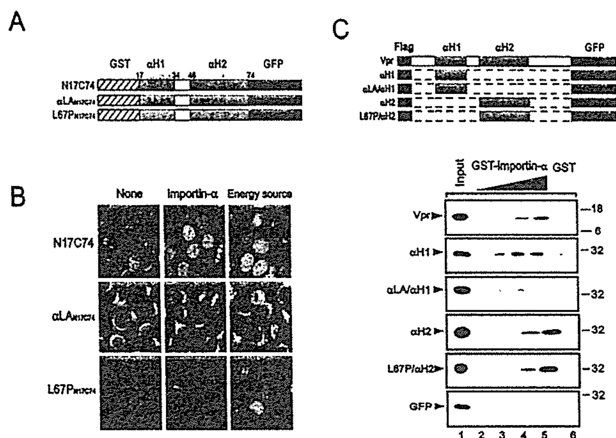

FIG. 5. The αH1 and αH2 regions of Vpr play different roles in nuclear import. (A) Construction of plasmids that encoded GST- and GFP-tagged N17C74 and similarly tagged substitution mutants of N17C74. (B) Digitonin-permeabilized HeLa cells were incubated with GST- and GFP-tagged N17C74, αLA_{N17C74}, and $L67P_{N17C74}$. After fixation, cells were analyzed by confocal laser-scanning microscopy. Bar = 20 μm. (C) The lysates of HeLa cells that had been transfected with vectors that encoded Flag- and GFP-tagged full-length Vpr, αH1, αLA/αH1, αH2, and L67P/αH2, and Flag-tagged GFP, were incubated with glutathione-Sepharose beads coupled with GST–importin-α (lanes 2 to 5; 37.5, 75, 150, and 300 pmol, respectively) or GST (300 pmol), as indicated at the top of each panel. The proteins recovered on beads were separated by SDS-PAGE and detected by Western blotting with a Flag-specific MAb.

nuclear localization, we examined the effects of substitutions within αH1 or αH2 of N17C74 (Fig. 5). In the absence of soluble factors, the mutant of αH1, αLA_{N17C74}, was localized in the perinuclear region. In contrast, the αH2 mutant, $L67P_{N17C74}$, was barely detectable in the perinuclear localization. These mutants had lost the capacity for nuclear entry promoted by importin-α. That import depended on an energy source, however, was observed on the αLA_{N17C74} mutant, even if it was faint. Collectively, these findings indicate that both αH1 and αH2 are indispensable for the nuclear translocation of N17C74 and that each domain plays a different role in the nuclear import.

To clarify the region in Vpr that contributes to the interactions with importin-α, we examined various mutant forms of Vpr in a pull-down assay using GST-tagged importin-α (Fig. 5C). As protein sources, we used lysates of HeLa cells that has been transfected with vectors that encoded, respectively, Flag- and GFP-tagged chimeric proteins that included αH1, αLA/αH1, αH2, L67P/αH2, or full-length Vpr, which we had used in previous studies (29). The chimeric proteins that included αH1 or αH2 clearly interacted with importin-α. The interaction via αH1 was, however, apparently attenuated by the mutation, and the ability to enter the nucleus that was promoted by importin-α (αLA/αH1) was lost. In contrast, the interaction via αH2 was not affected by the mutation that destroyed the capacity for nuclear import (L67P/αH2). Similar results were obtained when we used MBP-fused importin-α (data not shown). Taken together, these results indicate that the interaction between importin-α and Vpr through αH1, but not through αH2, is indispensable for the entry of Vpr into nucleus.

DISCUSSION

In this study, we first confirmed the region that corresponds to the NLS in Vpr by using in vivo and in vitro assays. The N17C74 fragment was sufficient for entry into the nuclei of microinjected MDBK cells and digitonin-permeabilized HeLa cells. Import in each case was completely inhibited by WGA and by low temperature, suggesting that N17C74 migrates into the nucleus through the gated channels of the NPC. Furthermore, the nuclear entry of the N17C74 chimeric protein was not affected by addition of a fusion protein that consisted of the SV40 NLS and β-galactosidase (data not shown). These results confirmed all the features of the nuclear import of Vpr reported previously (28) and allowed us to conclude that this region corresponds to the actual NLS of Vpr.

Our results described in this paper lead to three major conclusions. First, the nuclear translocation of Vpr requires importin-α or energy; to our knowledge, there have been no previous reports of an NLS that utilizes importin-α as a mediator of nuclear import. About the mechanisms for the promotion of Vpr-nuclear import by importin-α or energy source, we are currently inferring that those differ from each other, because the nuclear import of the αLA_{N17C74} mutant was partially promoted by the addition of an energy source but not by that of importin-α (Fig. 5B). We have also observed that the chimeric protein with only either the αH1 region or the αH2 region entered the nucleus in response to the addition of an energy source, not importin-α (manuscript in preparation). More refined studies to examine this possibility are in progress. Second, the NLS of Vpr contains two independent functional regions, αH1 and αH2, and each region plays different roles and is indispensable for the nuclear entry of Vpr. Similar results have been reported by Mahalingam et al. (34). An in vitro transport assay using N17C74 mutants showed that a mutant form of αH1 (αLA_{N17C74}) was localized in the perinuclear region but lacked the capacity for nuclear entry promoted by importin-α. Meanwhile, the mutant form of αH2 ($L67P_{N17C74}$) lacked the capacity for both activities, and addition of importin-α was not able to supplement the disability of this mutant to target the perinuclear region, indicating that importin-α itself did not affect the perinuclear localization of Vpr. These observations indicate that the αH1 region contributes to nuclear entry, while the αH2 region is necessary for targeting to the perinuclear region, and suggest that Vpr seems first to be targeted to the NPC via interaction with its αH2 region and then enters the nucleus in a process that involves the αH1 region. Third, an interaction between Vpr and importin-α via the αH1 region, and not the αH2 region, is required for the nuclear entry of Vpr that depends on importin-α. Binding via the αH1 region disappeared upon mutation of leucine to an alanine residue (αLA), and the nuclear entry that depended on importin-α disappeared concomitantly. In contrast, binding with importin-α via the αH2 region was not affected by the mutation L67P, even if it destroyed the ability to enter the nucleus that was dependent on importin-α. Thus, an interaction between αH1 region and importin-α seems likely to require for the nuclear entry of Vpr.

The region in importin-α required for facilitation of nuclear translocation of N17C74 was located between residues 393 and 462. This region overlaps with the binding region for CAS,

which is the nuclear export factor for importin-α (23). It has been also reported that the carboxyl terminus of importin-α, which partially or completely includes the region between the residues 392 and 462, binds to several nucleoporins, such as Nup1p (4), Nup2p (4, 52), Npap60/Nup50 (33), and Nup153 (41), which are FG repeat nucleoporins. These facts are supposed that the region between the residues 392 and 462 may contribute to the localization of importin-α at the perinulear region. Moreover, there is the possibility that these factors are involved in the nuclear import of Vpr or that Vpr disturbs their functions, which are needed for cellular homeostasis, by competition against importin-α. Further studies will be required to explore these possibilities and should yield new insights into this novel nuclear import mechanism.

Importin-α bound strongly to N17C74 via the IBB domain, but this binding was not essential for the nuclear entry of Vpr (Fig. 4). The IBB domain contains an NLS-like sequence (49-KRRNV-53) and it binds to autologous NLS-binding sites in a similar way to the NLS of SV40. Thus, importin-α appears to be prevented from the binding to a classical type NLS by an internal NLS until importin-β binds to the IBB domain (30). These facts suggest that Vpr might modulate the interaction between a classical NLS-bearing protein and importin-α, as does importin-β. Indeed, Popov et al. (49) reported that Vpr increases the affinity of binding between importin-α and HIV-1 MA, which is one of the components of the PIC and has a basic type NLS. Vpr might accelerate a nuclear import of the PIC through interaction with the IBB domain.

We reported here that Vpr has a novel NLS and that it requires importin-α for effective passage through the NPC. There are no similar reports of such an NLS, and the detailed mechanism is now unknown. We have reported recently that importin-α migrates into the nucleus by itself without addition of importin-β, Ran, or any other soluble factors (38). This import was totally energy independent and observed in the presence of apyrase. Furthermore, the region between residues 393 and 462 was required for this import. These profiles observed on the nuclear import of importin-a by itself are consistent with that of Vpr observed in the present study, suggesting the possibility that Vpr utilizes a nuclear import of importin-α by itself. Further investigations are essential for a full understanding of the mechanism of nuclear entry promoted by importin-α. The understanding of the import mechanism is important if we are to determine the biological significance of this novel nuclear import mechanism. Since it appears that viruses have evolved via incorporation and assimilation of cellular functions, it is possible that molecules that utilize importin-α as a mediator of nuclear import might also exist in uninfected mammalian cells.

ACKNOWLEDGMENTS

We appreciate the excellent assistance of Terue Kurosawa.

This work was supported in part by a grant for AIDS Research from the Japan Health Sciences Foundation (KA21502); by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan (Research on HIV/AIDS 13110201 and 16150301); by a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (1402113, 15019115, and 16017304); and by a President's Special Research Grant from RIKEN.

REFERENCES

1. **Adam, S. A., R. S. Marr, and L. Gerace.** 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. **111**:807–816.
2. **Agostini, I., S. Popov, J. Li, L. Dubrovsky, T. Hao, and M. Bukrinsky.** 2000. Heat-shock protein 70 can replace viral protein R of HIV-1 during nuclear import of the viral preintegration complex. Exp. Cell Res. **259**:398–403.
3. **Aitchison, J. D., G. Blobel, and M. P. Rout.** 1996. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. Science **274**:624–627.
4. **Belanger, K. D., M. A. Kenna, S. Wei, and L. I. Davis.** 1994. Genetic and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. J. Cell Biol. **126**:619–630.
5. **Bischoff, F. R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl.** 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. Proc. Natl. Acad. Sci. USA **91**:2587–2591.
6. **Bukrinsky, M., and A. Adzhubei.** 1999. Viral protein R of HIV-1. Rev. Med. Virol. **9**:39–49.
7. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature **365**:666–669.
8. **Bukrinsky, M. I., N. Sharova, M. P. Dempsey, T. L. Stanwick, A. G. Bukrinskaya, S. Haggerty, and M. Stevenson.** 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc. Natl. Acad. Sci. USA **89**:6580–6584.
9. **Chi, N. C., E. J. Adam, and S. A. Adam.** 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. J. Cell Biol. **130**:265–274.
10. **Cortes, P., Z. S. Ye, and D. Baltimore.** 1994. RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. Proc. Natl. Acad. Sci. USA **91**:7633–7637.
11. **Cuomo, C. A., S. A. Kirch, J. Gyuris, R. Brent, and M. A. Oettinger.** 1994. Rch1, a protein that specifically interacts with the RAG-1 recombination-activating protein. Proc. Natl. Acad. Sci. USA **91**:6156–6160.
12. **Emerman, M.** 1996. HIV-1, Vpr and the cell cycle. Curr. Biol. **6**:1096–1103.
13. **Enenkel, C., G. Blobel, and M. Rexach.** 1995. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. J. Biol. Chem. **270**:16499–16502.
14. **Fassati, A., D. Görlich, I. Harrison, L. Zaytseva, and J. M. Mingot.** 2003. Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin 7. EMBO J. **22**:3675–3685.
15. **Finlay, D. R., D. D. Newmeyer, T. M. Price, and D. J. Forbes.** 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. **104**:189–200.
16. **Fouchier, R. A., and M. H. Malim.** 1999. Nuclear import of human immunodeficiency virus type-1 preintegration complexes. Adv. Virus Res. **52**:275–299.
17. **Fouchier, R. A. M., B. E. Meyer, J. H. M. Simon, U. Fischer, A. V. Albright, F. González-Scarano, and M. H. Malim.** 1998. Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex. J. Virol. **72**:6004–6013.
18. **Gallay, P., T. Hope, D. Chin, and D. Trono.** 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. USA **94**:9825–9830.
19. **Gallay, P., V. Stitt, C. Mundy, M. Oettinger, and D. Trono.** 1996. Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. J. Virol. **70**:1027–1032.
20. **Görlich, D.** 1998. Transport into and out of the cell nucleus. EMBO J. **17**:2721–2727.
21. **Görlich, D., S. Kostka, R. Kraft, C. Dingwall, R. A. Laskey, E. Hartmann, and S. Prehn.** 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. Curr. Biol. **5**:383–392.
22. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91**:7311–7315.
23. **Herold, A., R. Truant, H. Wiegand, and B. R. Cullen.** 1998. Determination of the functional domain organization of the importin α nuclear import factor. J. Cell Biol. **143**:309–318.
24. **Iijima, S., Y. Nitahara-Kasahara, K. Kimata, W. Zhong Zhuang, M. Kamata, M. Isogai, M. Miwa, Y. Tsunetsugu-Yokota, and Y. Aida.** 2004. Nuclear localization of Vpr is crucial for the efficient replication of HIV-1 in primary $CD4^+$ T cells. Virology **327**:249–261.
25. **Imamoto, N., T. Shimamoto, S. Kose, T. Takao, T. Tachibana, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda.** 1995. The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile. FEBS Lett. **368**:415–419.
26. **Imamoto, N., T. Shimamoto, T. Takao, T. Tachibana, S. Kose, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda.** 1995. In vivo evidence for

involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. EMBO J. **14**:3617–3626.

27. **Jans, D. A., P. Jans, T. Julich, L. J. Briggs, C. Y. Xiao, and S. C. Piller.** 2000. Intranuclear binding by the HIV-1 regulatory protein VPR is dependent on cytosolic factors. Biochem. Biophys. Res. Commun. **270**:1055–1062.
28. **Jenkins, Y., M. McEntee, K. Weis, and W. C. Greene.** 1998. Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. J. Cell Biol. **143**:875–885.
29. **Kamata, M., and Y. Aida.** 2000. Two putative α-helical domains of human immunodeficiency virus type 1 Vpr mediate nuclear localization by at least two mechanisms. J. Virol. **74**:7179–7186.
30. **Kobe, B.** 1999. Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin α. Nat. Struct. Biol. **6**:388–397.
31. **Kohler, M., S. Ansieau, S. Prehn, A. Leutz, H. Haller, and E. Hartmann.** 1997. Cloning of two novel human importin-α subunits and analysis of the expression pattern of the importin-α protein family. FEBS Lett. **417**:104–108.
32. **Lewis, P., M. Hensel, and M. Emerman.** 1992. Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J. **11**:3053–3058.
33. **Lindsay, M. E., K. Plafker, A. E. Smith, B. E. Clurman, and I. G. Macara.** 2002. Npap60/Nup50 is a tri-stable switch that stimulates importin-α:β-mediated nuclear protein import. Cell **110**:349–360.
34. **Mahalingam, S., V. Ayyavoo, M. Patel, T. Kieber-Emmons, and D. B. Weiner.** 1997. Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. J. Virol. **71**:6339–6347.
35. **Mahalingam, S., R. G. Collman, M. Patel, C. E. Monken, and A. Srinivasan.** 1995. Functional analysis of HIV-1 Vpr: identification of determinants essential for subcellular localization. Virology **212**:331–339.
36. **Melchior, F., B. Paschal, J. Evans, and L. Gerace.** 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J. Cell Biol. **123**:1649–1659.
37. **Melchior, F., D. J. Sweet, and L. Gerace.** 1995. Analysis of Ran/TC4 function in nuclear protein import. Methods Enzymol. **257**:279–291.
38. **Miyamoto, Y., M. Hieda, M. T. Harreman, M. Fukumoto, T. Saiwaki, A. E. Hodel, A. H. Corbett, and Y. Yoneda.** 2002. Importin alpha can migrate into the nucleus in an importin beta- and Ran-independent manner. EMBO. J. **21**:5833–5842.
39. **Moore, M. S., and G. Blobel.** 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature **365**:661–663.
40. **Moore, M. S., and G. Blobel.** 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. Proc. Natl. Acad. Sci. USA **91**:10212–10216.
41. **Moroianu, J., G. Blobel, and A. Radu.** 1997. RanGTP-mediated nuclear export of karyopherin α involves its interaction with the nucleoporin Nup153. Proc. Natl. Acad. Sci. USA **94**:9699–9704.
42. **Nachury, M. V., U. W. Ryder, A. I. Lamond, and K. Weis.** 1998. Cloning and characterization of hSRP1 γ, a tissue-specific nuclear transport factor. Proc. Natl. Acad. Sci. USA **95**:582–587.
43. **Nakielny, S., M. C. Siomi, H. Siomi, W. M. Michael, V. Pollard, and G. Dreyfuss.** 1996. Transportin: nuclear transport receptor of a novel nuclear protein import pathway. Exp. Cell Res. **229**:261–266.
44. **Nie, Z., D. Bergeron, R. A. Subbramanian, X.-J. Yao, F. Checroune, N. Rougeau, and E. A. Cohen.** 1998. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. J. Virol. **72**:4104–4115.
45. **Nishino, Y., T. Myojin, M. Kamata, and Y. Aida.** 1997. Human immunodeficiency virus type 1 vpr gene product prevents cell proliferation on mouse NIH3T3 cells without the G_2 arrest of the cell cycle. Biochem. Biophys. Res. Commun. **232**:550–554.
46. **Paschal, B. M., and L. Gerace.** 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. J. Cell Biol. **129**:925–937.
47. **Pollard, V. W., W. M. Michael, S. Nakielny, M. C. Siomi, F. Wang, and G. Dreyfuss.** 1996. A novel receptor-mediated nuclear protein import pathway. Cell **86**:985–994.
48. **Popov, S., M. Rexach, L. Ratner, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. J. Biol. Chem. **273**:13347–13352.
49. **Popov, S., M. Rexach, G. Zybarth, N. Reiling, M. A. Lee, L. Ratner, C. M. Lane, M. S. Moore, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J. **17**:909–917.
50. **Rouzic, F. L., A. Mousnier, C. Rustum, F. Stutz, E. Hallberg, C. Dargemont, and C. Benichou.** 2002. Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. J. Biol. Chem. **277**:45091–45098.
51. **Seki, T., S. Tada, T. Katada, and T. Enomoto.** 1997. Cloning of a cDNA encoding a novel importin-α homologue, Qip1: discrimination of Qip1 and Rch1 from hSrp1 by their ability to interact with DNA helicase Q1/RecQL. Biochem. Biophys. Res. Commun. **234**:48–53.
52. **Solsbacher, J., P. Maurer, F. Vogel, and G. Schlenstedt.** 2000. Nup2p, a yeast nucleoporin, functions in bidirectional transport of importin α. Mol. Cell. Biol. **20**:8468–8479.
53. **Tachibana, T., M. Hieda, T. Sekimoto, and Y. Yoneda.** 1996. Exogenously injected nuclear import factor p10/NTF2 inhibits signal-mediated nuclear import and export of proteins in living cells. FEBS Lett. **397**:177–182.
54. **Vodicka, M. A., D. M. Koepp, P. A. Silver, and M. Emerman.** 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. **12**:175–185.
55. **von Schwedler, U., R. S. Kornbluth, and D. Trono.** 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc. Natl. Acad. Sci. USA **91**:6992–6996.
56. **Weis, K., I. W. Mattaj, and A. I. Lamond.** 1995. Identification of hSRP1 α as a functional receptor for nuclear localization sequences. Science **268**:1049–1053.
57. **Whittaker, G. R., M. Kann, and A. Helenius.** 2000. Viral entry into the nucleus. Annu. Rev. Cell Dev. Biol. **16**:627–651.
58. **Yoneda, Y., N. Imamoto-Sonobe, M. Yamaizumi, and T. Uchida.** 1987. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Exp. Cell Res. **173**:586–595.
59. **Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier, and P. Charneau.** 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell **101**:173–185.

Microbiol. Immunol., 49(2), 155–165, 2005

Editor-Communicated Paper

Interleukin-4 Up-Regulates T-Tropic Human Immunodeficiency Virus Type 1 Transcription in Primary CD4$^+$ CD38$^+$ T-Lymphocyte Subset

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Communicated by Dr. Koki Taniguchi: Received November 4, 2004. Accepted November 24, 2004

Abstract: **The capacity of human immunodeficiency virus type 1 (HIV-1) to infect resting cells and to produce progeny particles may contribute significantly to its pathogenicity *in vivo*. We previously reported that primary culture of resting CD4$^+$ CD38$^+$ T-lymphocyte subset had higher production rate of CXCR4-using (X4) HIV-1 than CD4$^+$ CD38$^-$ subset. Interleukin (IL)-4 highly contributed to the up-regulation of the X4 virus production in the CD38$^+$ subset. Here, we show evidences that IL-4 treatment of both resting CD38$^+$ and CD38$^-$ subsets allowed the adsorption, entry, and integration of X4 virus at similar rates, while the following viral transcription rate was significantly lower in the CD38$^-$ than CD38$^+$ subset. Treatment of the CD38 subsets with IL-4 or phytohemagglutinin revealed no association of X4 virus replication ability in the subsets with classic T-cell activation or proliferation. Interestingly, the activator protein (AP)-1 was significantly activated in the CD38$^+$ subset after IL-4 treatment, while both nuclear factor (NF)-κB and signal transducers and activator of transcription (STAT)-6 were activated in the IL-4-treated CD38$^+$ and CD38$^-$ subsets at similar levels. Thus, IL-4-dependent X4 HIV-1 transcription occurs efficiently in the CD38$^+$ but not CD38$^-$ subset of CD4$^+$ population and AP-1 could play a significant role on viral transcription, leading to the up-regulated X4 virus production in the CD38$^+$ subset.**

Key words: **HIV-1, Transcription, CD38, IL-4, AP-1**

Disease progression after infection with human immunodeficiency virus type 1 (HIV-1) is correlated with virus burden (24, 33), which seems to be derived from the efficiency of HIV-1 progeny particle production from CD4$^+$ T lymphocytes, the major target for HIV-1.

The CD4$^+$ T lymphocytes mature into functionally heterogeneous subsets from a common lineage by a process of division, migration, selection, differentiation, and proliferation. Such subsets are, in part, classified by differentiation or activation markers. Therefore, studies have focused on the differences in the susceptibility of the T-cell subsets to HIV-1 infection. Most of the data obtained were derived from the up- or down-regulation of coreceptors, CC and CXC chemokine receptor molecules, or from the enhanced production of CC and CXC chemokine ligands (2, 9, 19, 31, 41, 43). The T-helper type 1 (Th1) and Th2 subsets showed differential

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Abbreviations: Alu-PCR, Alu-polymerase chain reaction; AP, activation protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; HIV-1, human immunodeficiency virus type 1; IL, interleukin; LTR, long-terminal repeat; MAb, monoclonal antibody; NF, nuclear factor; PE, phycoerythrin; PHA, phytohemagglutinin; PMSF, phenylmethylsulfonyl fluoride; R5, CCR5-using; rIL-2, recombinant IL-2; STAT, signal transducers and activator of transcription; Th1, T-helper type 1; VSV-G, vesicular stomatitis virus glycoprotein; X4, CXCR4-using.

expression of chemokine receptors (43), resulting in preferential replication of macrophage-tropic CCR5-using (R5) and T lymphocyte-tropic CXCR4-using (X4) HIV-1s, respectively (34). On the other hand, the *in vitro* studies using primary cultures of CD4$^+$ naïve and memory subsets from healthy donors showed controversial results. This seems, at least in part, to be due to the culture conditions, such as HIV-1 infection before or after cell stimulation by phytohemagglutinin (PHA), monoclonal antibody (MAb) to CD3 or to CD3/CD28 (30, 32, 41). The ability of HIV-1 to establish a state of latent infection is higher in resting memory than naïve CD4$^+$ T-cell subset (30, 32). On the other hand, it was also revealed that although resting naïve T cells have been believed to be less sensitive to HIV-1 productive infection, the endogenous microenvironment of human lymphoid tissues supports *de novo* infection and depletion of this population (6). Similarly, the change in T-cell subsets in patients with primary HIV-1 infection was characterized by depletion of CD4$^+$ naïve T cells (46). In addition, a significant decrease in the CD4$^+$ naïve T cells was much more marked in infected children than in infected adults (10). Thus, the differences between naïve and memory subsets leading to productive and latent infections are still not yet clear.

Generally, it has been believed from the *in vitro* studies that HIV-1 requires activated T cells for productive infection, although the number of circulating T cells in the activated state *in vivo* is extremely low. Therefore, activation of latently infected or quiescent T cells during HIV-1 infection has become another interesting significant area in the study of AIDS pathogenesis. The levels of CD4$^+$ and/or CD38$^+$ T cells expressing the activation markers such as HLA-DR (16), CD25 (7), or CD38 (13, 16, 22), are elevated in HIV-1 infection. It was shown that the up-regulation of the activation markers such as CD38 is in strong correlation with the frequency of cycling CD4$^+$ T cells with Ki-67 expression (33). Therefore, such activation markers could be useful in predicting the clinical outcome of HIV-1 disease, such as CD38 expression on CD38$^+$ T cells, which was shown to be a strong marker for disease progression to AIDS and death in a Multicenter AIDS Cohort Study (20). However, the mechanism of such T-cell activation in HIV-1-infected individuals is not yet clearly understood; e.g., it could reflect antigen-induced T-cell activation *in vivo* or may be secondary to HIV-1-induced cytokine production (12, 13).

The lymphocyte differentiation antigen, CD38, is a type II transmembrane glycoprotein that is extensively expressed on parts of cells of hematopoietic and non-hematopoietic lineage (21). CD38 is expressed on T cells during the early and terminal stages of differentiation, but not during intermediate stages (35), although it has been considered to be one of the T-cell activation marker antigens along with CD25 and HLA-DR, as mentioned above. In adults, immune activation correlates with an increase in CD4$^+$ T cells with CD38 and HLA-DR (27), while in children, the CD38$^+$ marker means a maturation step, rather than an activation stage, since 75% of all CD4$^+$ T cells normally co-express CD38 (29), that is in contrast with the normal adult values of around 30% (10, 25). Therefore, this increase of CD38$^+$ T-cell population may be due to the increase of newly generated immature cells in infected individuals.

Characterization of healthy donor-derived CD38 subsets purified from the CD4$^+$ T-lymphocyte population revealed that CD38$^+$ and CD38$^-$ subsets, about 2/3 of which are overlapped with naïve and memory T-cell populations, are more sensitive to the infection with X4 and R5 HIV-1s, respectively (9). Especially in case of X4 HIV-1 infection, interleukin (IL)-4 was shown to play a key role for the higher replication in the CD38$^+$ subset (8). In this study, we aimed to characterize the mechanisms by which X4 HIV-1 replication rate in CD4$^+$ CD38$^+$ subset was significantly higher compared to CD4$^+$ CD38$^-$ subset after IL-4 stimulation. Our results showed that the subsequent transcription step was greatly different between the two subsets and the activator protein (AP)-1 was IL-4-dependently activated only in the CD38$^+$ subset. This AP-1 activation seems to be involved in the up-regulated virus production in this subset.

Materials and Methods

Viruses. HIV-1 laboratory strain, CXCR4-tropic NL4-3, was obtained from the culture medium of 293T cells transfected with pNL4-3 (1). For the preparation of vesicular stomatitis virus glycoprotein (VSV-G) pseudovirus with NL4-3 background containing deletion at *env* and a luciferase gene in place of *nef*, 293T cells were co-transfected with pNL-ΔEnvΔNef/Luc and the VSV-G expression vector pHIT/G (37) using the calcium phosphate transfection kit (Invitrogen). The cell supernatants were harvested 48 hr after transfection, filtered, then centrifuged at 100,000$\times g$ for 30 min. The precipitate was suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (complete medium) and used as virus stocks for subsequent infection experiments.

Preparation of primary T-cell subsets. Isolation of CD4$^+$ T-cell fraction from healthy donor-derived PBMCs was performed by depletion of non-CD4$^+$ T cells, as described previously (8). Briefly, PBMCs prepared by centrifugation through Ficoll-Paque (Pharma-

cia Biotech, Uppsala, Sweden) were cultured overnight in complete medium. Then, the cells were reacted with cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and MACS Microbeads coupled to an anti-hapten MAb (Miltenyi Biotec). The resulting cells were reacted with Microbeads-conjugated CD25 MAb (Miltenyi Biotec) and separated as above. This final fraction obtained as resting $CD4^+$ T-cell population was further separated into CD38 T-cell subsets by panning with MAb to CD38 (Leu-17; Becton-Dickinson Immunocytometry Systems) followed by addition of goat anti-mouse IgG Microbeads (Miltenyi Biotec). The obtained $CD4^+$ $CD38^+$ and $CD4^+$ $CD38^-$ subsets at resting conditions were stimulated with PHA at 2 μg/ml or IL-4 at 10 ng/ml in complete medium.

HIV-1 infection of primary T-cell culture. The $CD38^+$ and $CD38^-$ subsets of the $CD4^+$ T-cell population were infected with HIV-1 NL4-3 at the same amounts, which were adjusted by HIV-1 Gag p24 antigen-capture enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix Corporation, Buffalo, U.S.A.). After adsorption for 1 hr at 37 C, the cells were washed with PBS and cultured at 1×10^6/ml in complete medium containing recombinant IL-2 (rIL-2) (50 U/ml; Becton Dickinson, Mountain View, U.S.A.). The cells were adjusted to 1×10^6/ml every 3 days and cultured in fresh complete medium containing 50 U/ml of rIL-2.

Flow cytometry. The prepared PBMCs unstimulated or stimulated with IL-4 or PHA were reacted with phycoerythrin (PE)-conjugated MAbs to CD25, CD4, CD8, CD19, and CD14 (PharMingen) for 30 min at 4 C, then subjected to flow cytometry. As a control, PE-conjugated normal mouse IgG_1 (Becton-Dickinson Immunocytometry Systems) was used. For quantification of Ki-67, the cells were fixed with paraformaldehyde-lysine-phosphate buffer (pH 7.4) for 20 min at 4 C, then suspended in a solution containing 0.4 mg/ml saponin and 10% goat normal serum. After 1 hr incubation at room temperature, the cells were reacted with 10 μg/ml of anti-Ki-67 (purified mouse anti-human MAb, BD Biosciences) for 1 hr at 4 C, then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG in a solution containing saponin for 30 min at room temperature. As a control antibody, the same amount of normal mouse IgG_1 was used.

Semi-quantitation of integrated provirus by Alu-PCR. The integration rate of HIV-1 in infected subsets was determined by a preamplification step with primers that bind genomic Alu elements and HIV-1 long-terminal repeat (LTR) sequences, so-called Alu-polymerase chain reaction (Alu-PCR) (3). Briefly, the DNA fractions extracted from infected T-cell subsets were serially 4-fold diluted, then the individual dilutions were subjected to Alu-PCR with Klen*Taq*-1 DNA polymerase (0.1 unit/μl; Sigma), 200 μM dNTP, and 1 μM of primers. The sequences of the preamplification primers are as follows: genomic Alu forward, 5′-TCCCAGCTACTCGGGAGGCTGAGG-3′ and HIV-1 LTR reverse, 5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′. The first round reaction was run according to the following program: 1 min at 95 C; 25 cycles of 30 sec at 94 C and 30 sec at 66 C; and 4 min at 72 C, then 10 min at 72 C. The second round nested PCR was carried out by using 1 μl of the first reaction product. The second PCR was run using the same conditions, except for the extension time of 1 min and the following primer pair: LTR forward, 5′-CACACACAAGGCTACTTCCCT-3′ and LTR reverse, 5′-GCCACTCCCCIGTCCCGCCC-3′.

Northern blotting for viral mRNA. Northern blot hybridization was performed as described previously (11). Total RNA was extracted from infected CD38 subsets by using Isogen (Nippon Gene Co., Tokyo). The RNA (5 μg) was separated in 1% agarose gel containing formaldehyde and transferred to GeneScreen Plus Hybridization Transfer Membranes (NEN Life Sciences, Boston, Mass., U.S.A.). After being baked at 80 C for 2 hr, the membrane was prehybridized in hybridization solution (5× SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml of sheared salmon sperm DNA) at 42 C for 3 hr. HIV-1-specific probe was synthesized using full-length HIV-1 *nef* gene as a template by Random Prime Labeling System (Amersham Biosciences) and Redivue [α-^{32}P]dCTP (Amersham Biosciences) according to manufacturer's instruction and was hybridized at 42 C for 20 hr. After washing, the membrane was exposed to X-ray film at −70 C.

Detection of the active forms of the transcription factors by western blotting. For STAT-6, the nuclear extracts from IL-4-untreated or-treated cells, were subjected to 7.5% SDS-PAGE followed by western blotting with Phospho-STAT-6 (Tyr641) MAb (Cell Signaling Technology, Inc., Beverly, U.S.A.), as described previously (36). The nuclear extracts (10 μg) from $CD38^+$ and $CD38^-$ subset cells untreated or treated with PHA or IL-4 were analyzed by western blotting with MAb to Jun B (C-11, Santa Cruz Biotechnology, Inc.) and MAb to C-Jun (KM-1; Santa Cruz Biotechnology, Inc.). The band on the membrane was then detected by ECL (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay (EMSA). Examination of AP-1 and NF-κB activation in IL-4-untreated and -treated cells was performed by EMSA, as described previously (14). The double-stranded oligonucleotides with the sequences of the binding sites for AP-1 (Promega) and NF-κB (Promega) were end-

labeled with [γ-^{32}P]ATP (Amersham Pharmacia) and T4 polynucleotide kinase (TaKaRa Shuzo Co., Ltd., Kyoto, Japan), then used as the probes. Nuclear extracts from CD38$^+$ and CD38$^-$ subsets treated by IL-4 were pre-incubated in 24 μl of a reaction mixture (EMSA mixture) containing 20 mM Hepes-KOH, pH 7.8, 168 mM KCl, 1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 8% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg of poly(dI-dC) for 15 min on ice and then the sample was mixed with 1 μl of ^{32}P-labeled probe (0.5 ng; ~50,000 cpm) and incubated for additional 30 min at room temperature. The DNA-protein complexes were separated on 6% polyacrylamide gels in the cold room, then the gels were dried and the radioactive bands were visualized with X-ray film.

The specificity of the binding was examined by specific competition with 1 μl (1.75 pmol/μl) unlabeled cold oligonucleotide and also by 1 μl (1.75 pmol/μl) unlabeled cold nonspecific competitor: for the AP-1 binding, we used the unlabeled NF-κB-binding sites and for the NF-κB binding, we used the unlabeled AP-1-binding sites. Meantime, the binding was blocked by 2 μg MAb to Jun B (C-11; Santa Cruz Biotechnology, Inc.) and polyclonal anti-NF-κB (American Research Products, Inc.) for the supershift assay.

Results

Effect of IL-4 on X4 HIV-1 Production in CD4$^+$ CD38$^+$ T-Cell Subsets

The CD4$^+$ CD38$^+$ and CD4$^+$ CD38$^-$ subsets at resting stage were prepared from 3 healthy donor-derived PBMCs. Their cell surface markers were as follows: 95% in CD4; <0.5% in CD8; <0.1% in CD25; <0.5% in CD19; and<0.5% in CD14. The CD38$^+$ and CD38$^-$ subsets from donors 1 to 3 were untreated and pretreated with IL-4 or PHA for 3 days, then infected with X4 HIV-1 (Fig. 1). Both PHA-pretreated CD38 subsets were susceptible to X4 HIV-1 infection and produced similar amounts of Gag p24 in their culture media. In contrast, the CD38$^+$ and CD38$^-$ subsets pretreated with IL-4 followed by X4 HIV-1 infection showed high and low susceptibilities to X4 HIV-1 infection, respectively. Both pretreatments of donor 1-derived CD38 subsets with PHA and IL-4 were not so effective for the HIV-1 susceptibility. However, higher susceptibility of the CD4$^+$ CD38$^+$ subset was observed by pretreatment with IL-4 as in donors 2 and 3. Untreated CD38 subsets from these donors were not effective for the HIV-1 susceptibility. The cell numbers of both subsets were similar during the course of infection in each treatment (data not shown). Thus, in contrast to PHA pretreatment, IL-4 pretreatment differentially affected the susceptibility of the CD38 subsets to X4 HIV-1. These results may suggest an IL-4-dependent activation of host factor(s) contributing to the virus production in the CD38$^+$ subset and such factor(s) could be different from that activated by PHA stimulation.

No Effect of IL-4 on Cell Activation and Proliferation Markers on the CD38 Subsets

Next, we examined the possible up- or down-regulation of T-cell activation marker CD25 and cell cycle-related molecule Ki-67 by flow cytometry. Both subsets were treated with IL-4 for 3 days. As a control, the same lot of CD38 subsets was untreated or treated with PHA for 3 days. The cells after these treatments showed different morphologies (data not shown). PHA treatment for 3 days greatly changed the cells to the blastic morphologies and they aggregated to form large clumps. Importantly, these changes were similarly observed in both CD38 subsets. In contrast, IL-4 treatment for 3 days did not significantly affect the morphology of CD38$^+$ subset, almost similar to the untreated one, although IL-4-treated CD38$^-$ subset had several small clumps. In fact, flow cytometric analyses revealed no apparent differences in the expression rates of CD25 and Ki-67 between both CD38 subsets (Fig. 2). The IL-4 treatment for 3 days induced only slight up-regulation of CD25 and Ki-67 in the subsets. However, PHA treatment induced significant up-regulation of both CD25 and Ki-67 molecules. Both subsets were almost similarly affected by such treatments. The cell percentages positive for CD25 and Ki-67 expression by means of triplicate experiments were as follows: 12.15 ± 3.86 in CD38$^+$ and 14.72 ± 5.41 in CD38$^-$ versus 88.36 ± 3.05 in CD38$^+$ and 74.08 ± 7.55 in CD38$^-$ for CD25; and 3.00 ± 2.92 in CD38$^+$ and 0.43 ± 0.14 in CD38$^-$ versus 40.82 ± 3.82 in CD38$^+$ and 22.97 ± 5.44 in CD38$^-$ for Ki-67. Thus, the susceptibility of primary CD4$^+$ T-cell subsets to X4 HIV-1 infection seems to be not correlated to the classically recognized cell activation stages and cell proliferation.

No Apparent Difference in the Integration Step between the CD38 Subsets

There were no apparent differences in the initial stage of HIV-1 interaction with host receptor CD4 and CXCR4 molecules as well as in the expression levels of IL-4 receptor between CD4$^+$ CD38$^+$ and CD4$^+$ CD38$^-$ subsets before and after IL-4 treatment (8). In fact, adsorption rates of X4 HIV-1 were similar in both subsets before and after stimulation with IL-4 (8). Therefore, we thought to compare the integration step between both subsets pretreated with IL-4 for 3 days, then infected with X4 HIV-1 (Fig. 3). The IL-4-treated

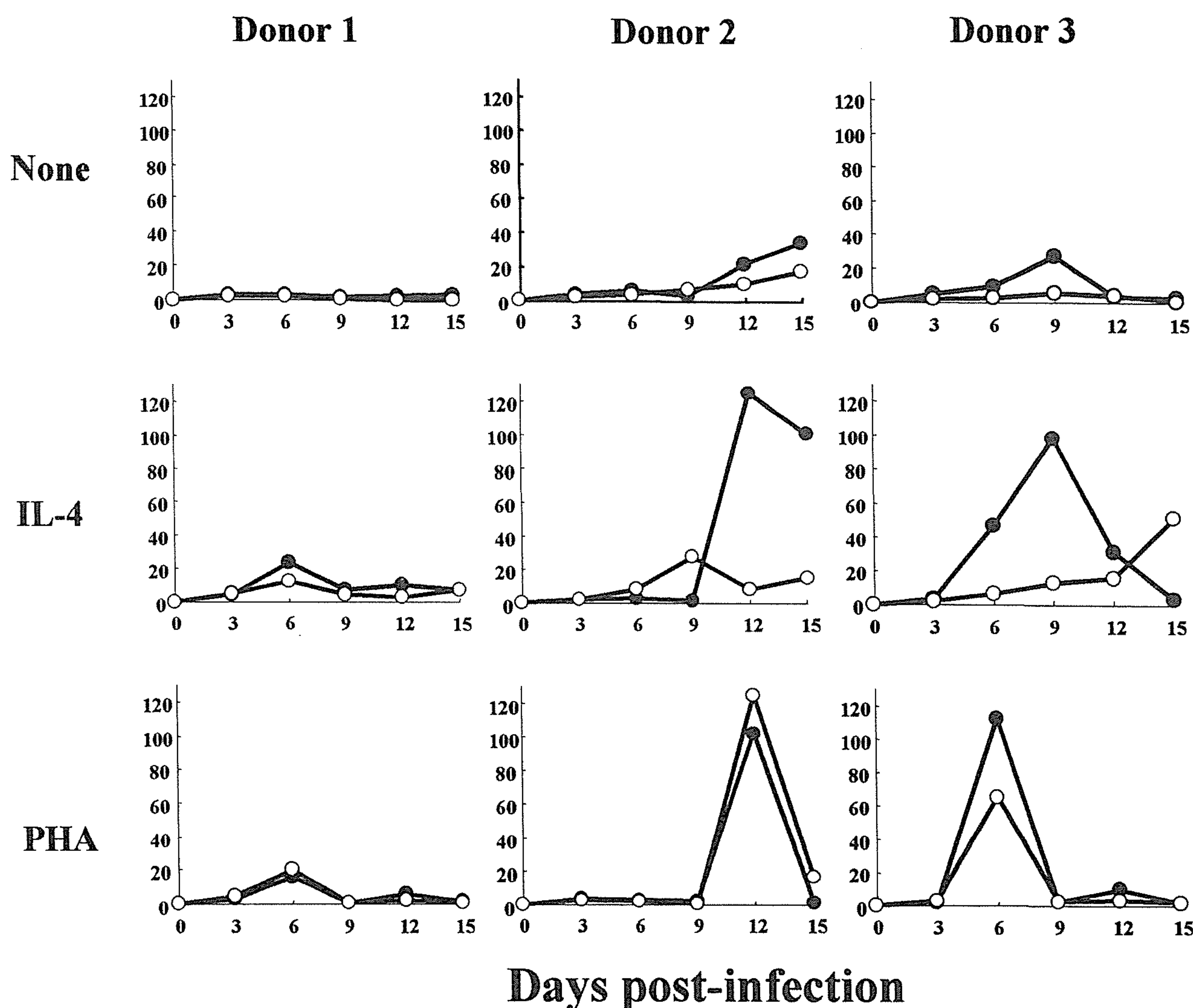

Fig. 1. Effect of IL-4 and PHA stimulations on the susceptibility of CD38 subsets to infection with X4 HIV-1. The $CD4^+$ $CD38^+$ (●) and $CD4^+$ $CD38^-$ (○) subsets prepared from donors 1 to 3 were unstimulated (None) or prestimulated with IL-4 or PHA for 3 days. These cells were infected with the same amount (30 ng/ml in Gag p24 antigen-capture ELISA) of HIV-1 NL4-3 for 1 hr at 37 C. The cell number was adjusted every 3 days in fresh complete medium. The virus production levels are shown by estimates of Gag p24 amounts in the culture media.

CD38 subsets on 2, 5, and 15 days post-infection with X4 HIV-1, similarly prepared as for Fig. 1, were used for DNA extraction. Semi-quantitative Alu-PCR analysis using serial dilutions of individual DNAs revealed that similar levels of integrated proviral genome were present in DNAs from infected $CD4^+$ $CD38^+$ and $CD4^+$ $CD38^-$ subsets. The provirus amounts were significantly increased on day 5 from that on day 2 (Fig. 3), and subsequently steady levels were maintained till day 15 (data not shown). Such slow integration process in both subsets was consistent with the previous observation that the RT step is slow in the resting T cells (28). However, these levels of the provirus were almost the same in both CD38 subsets. Thus, difference in the production rates of X4 HIV-1 in the two subsets seemed to be due to a post-integration event.

A Significant Difference in the Transcription Step of X4 HIV-1 between the CD38 Subsets

The RNA fractions extracted from $CD4^+$ $CD38^+$ and $CD4^+$ $CD38^-$ subsets that were treated with IL-4 for 3 days then infected with NL4-3 and cultured for 12 days, as for Fig. 1, were subjected to northern blot analysis with probe at the *nef* region. The viral mRNA level in infected $CD38^+$ subset was significantly high and this was greatly in contrast to that in infected $CD38^-$ subset (Fig. 4). Therefore, next to confirm that the different susceptibility of IL-4-treated CD38 subsets to X4 HIV-1 infection is derived from varied levels at the transcription step during HIV-1 life cycle, we prepared VSV-G

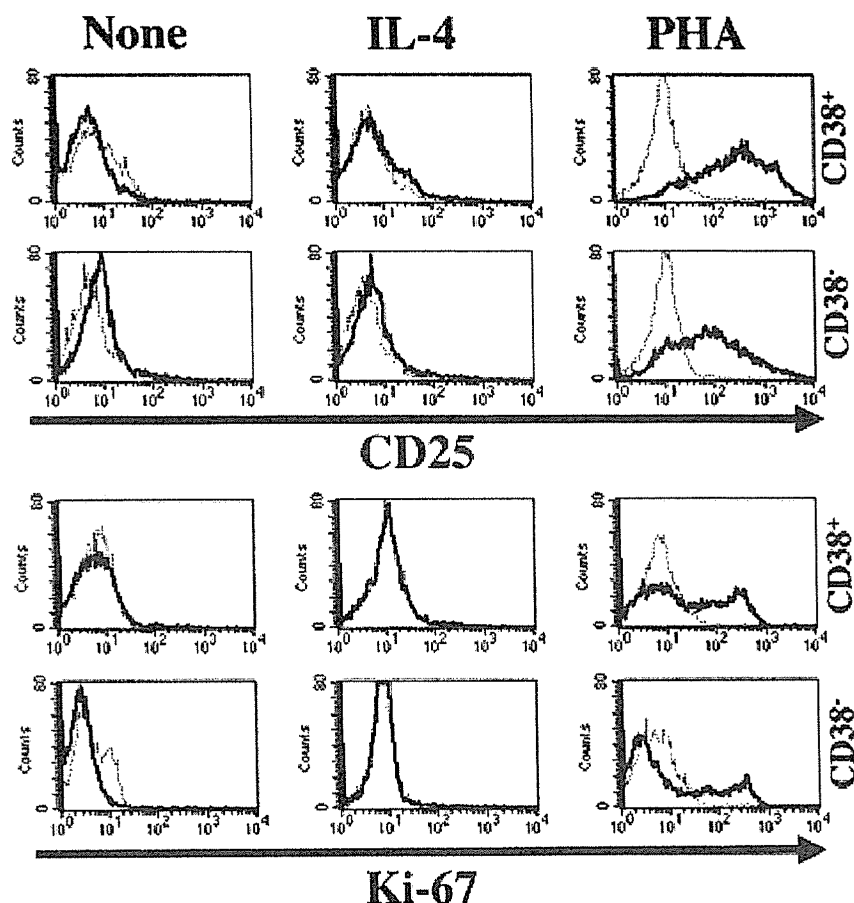

Fig. 2. Expression of cell activation and proliferation markers on the CD38 subsets after IL-4 or PHA treatment. The CD38 subsets similarly untreated (None), treated with IL-4 or PHA, as for Fig. 1, were subjected to flow cytometry for the expression of CD25 and Ki-67 (——). As a control antibody, normal mouse-derived IgG was used (----).

pseudovirus particles from 293T cells co-transfected with pNL-ΔEnvΔNef/Luc and pHIT-G. After adsorption for 1 hr with the pseudovirus, the cells were treated with IL-4 or PHA for 3 days. The expression rates of HIV-1 in these CD38 subsets were determined by luciferase activity assay. The results clearly showed that $CD38^+$ subset gave significantly higher activity than $CD38^-$ subset after IL-4 treatment (Fig. 5). Similarly, PHA treatment also gave slightly higher activity in $CD38^+$ than $CD38^-$ subsets (Fig. 5). Additional four independent experiments using $CD38^+$ and $CD38^-$ subsets from different donors showed similar results (data not shown), although the luciferase activities were variable among individual subset samples. Thus, the significant difference in the susceptibility of primary CD38 subsets to X4 HIV-1 apparently occurs at the transcription level. These data strongly suggest that a transcription factor(s) could be induced in the $CD38^+$ subset by IL-4 treatment. Therefore, we next examined the possible difference in the activation levels of several transcription factors between both subsets.

Difference in the Activation Level of Transcription Factors between the CD38 Subsets

The $CD38^+$ and $CD38^-$ subsets similarly prepared above were stimulated with IL-4 for 3 days. Since the high susceptibility of the $CD38^+$ subset to X4 HIV-1 was tightly dependent on IL-4 treatment, the phosphorylated STAT-6 levels were first compared between the CD38 subsets. However, the result showed that the $CD38^+$ and $CD38^-$ subsets contained similar amounts of the activated STAT-6 after IL-4 treatment for 3 days and such STAT-6 was not detected in both subsets without IL-4 treatment (data not shown).

Next, the levels of the AP-1 and NF-κB transcription factors were determined by EMSA using the nuclear fractions from both CD38 subsets treated with IL-4 for 3 days. As shown in Fig. 6, a significantly higher level of AP-1 activation detected in the $CD38^+$ than the $CD38^-$ subset. The specificity of the AP-1 activation in the $CD38^+$ subset was confirmed by competition with unlabeled oligonucleotides containing AP-1-binding site, but not with unlabeled NF-κB-binding site. Also, the specificity of this AP-1 activation in this $CD38^+$ subset

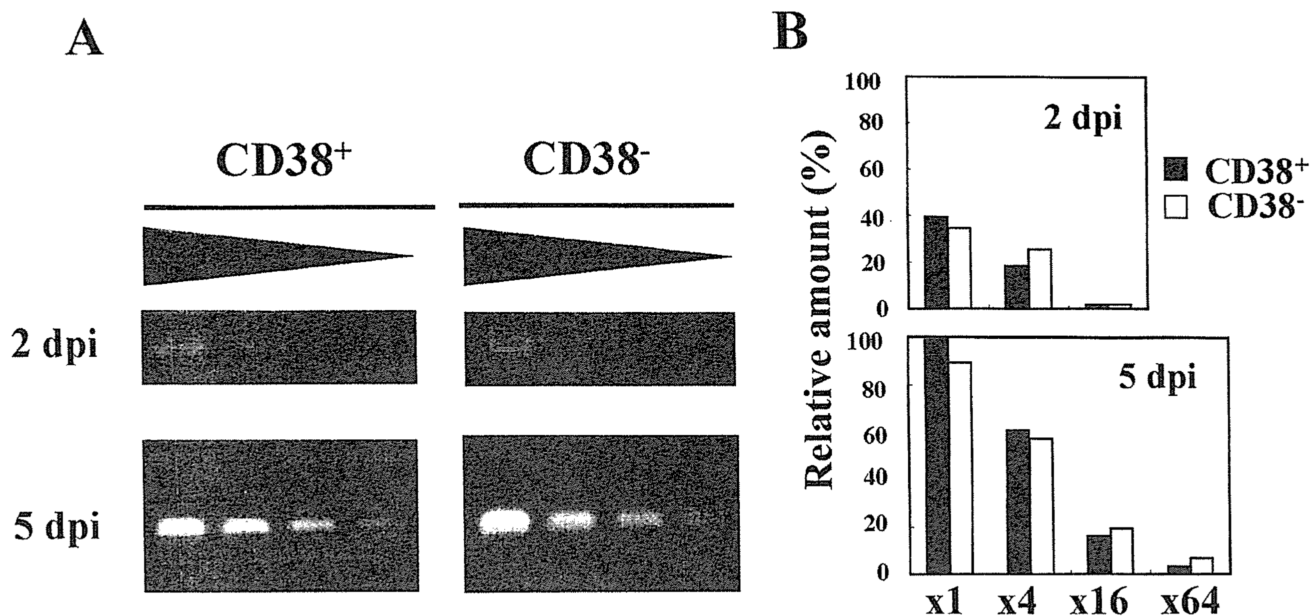

Fig. 3. Alu-PCR comparison of the integration levels in the CD38 subsets infected with X4 HIV-1. The CD38 subsets were treated with IL-4 for 3 days, then infected with NL4-3, as in Fig. 1. The DNA purified from the CD38 subsets 2 and 5 days post-infection (dpi) with NL4-3 were subjected to Alu-PCR to compare the amounts of integrated provirus in both subsets. Serial dilutions of the DNA (1-, 4-, and 16-fold dilutions of 500 ng/μl on day 2; and 1-, 4-, 16-, and 64-fold dilutions of 200 ng/μl on day 5) were used (A). The densitometric quantification of the integrated provirus shown in left figure was calculated by using the NIH Image software (B).

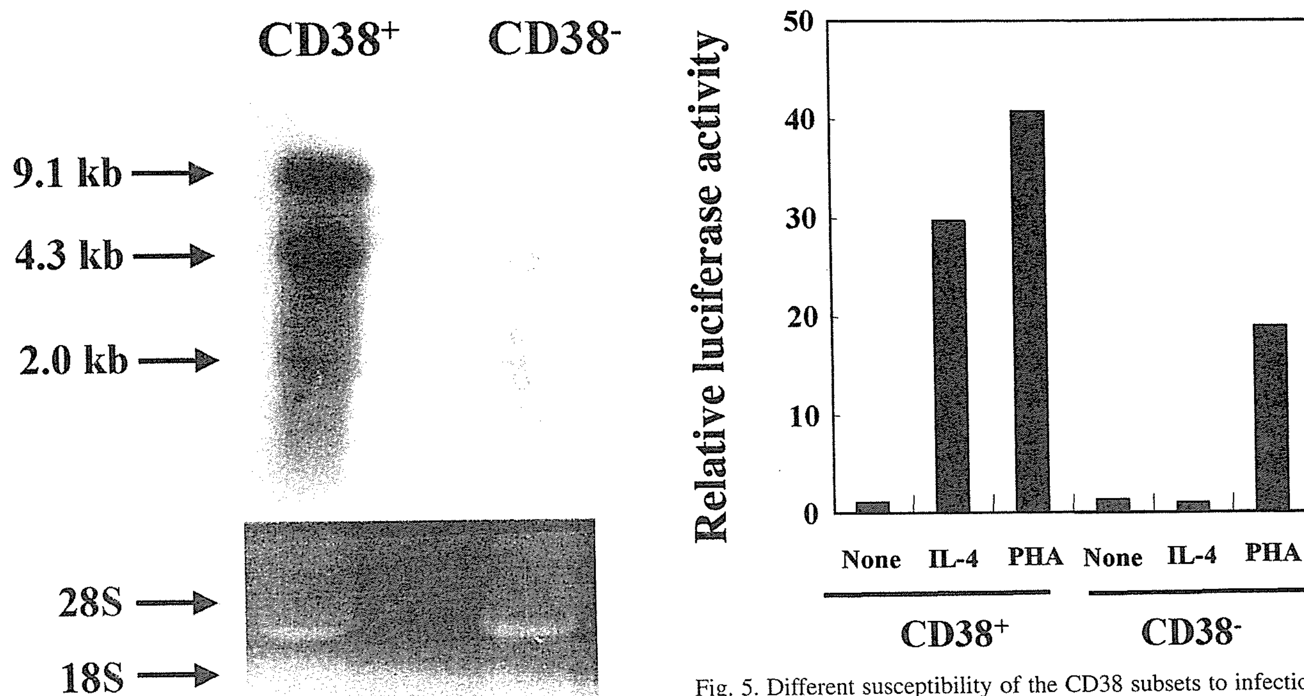

Fig. 4. Northern blot analysis for the comparison of the viral mRNA in the CD38 subsets infected with X4 HIV-1. The same CD38 subset cultures from donor 2 on day 12 after infection with NL4-3, as in Fig. 1, were used for northern blotting with the probe at *nef* region. As a control, stained ribosomal RNAs in the gel are shown in the lower panel.

Fig. 5. Different susceptibility of the CD38 subsets to infection with VSV-G pseudovirus with NL4-3 background. The CD38 subsets were infected with the same amount of VSV-G pseudovirus (adjusted by HIV-1 Gag p24 antigen-capture ELISA). After adsorption for 1 hr, the cells were untreated (None), or treated with IL-4 or PHA for 3 days. The cells were subjected to the luciferase activity assay.

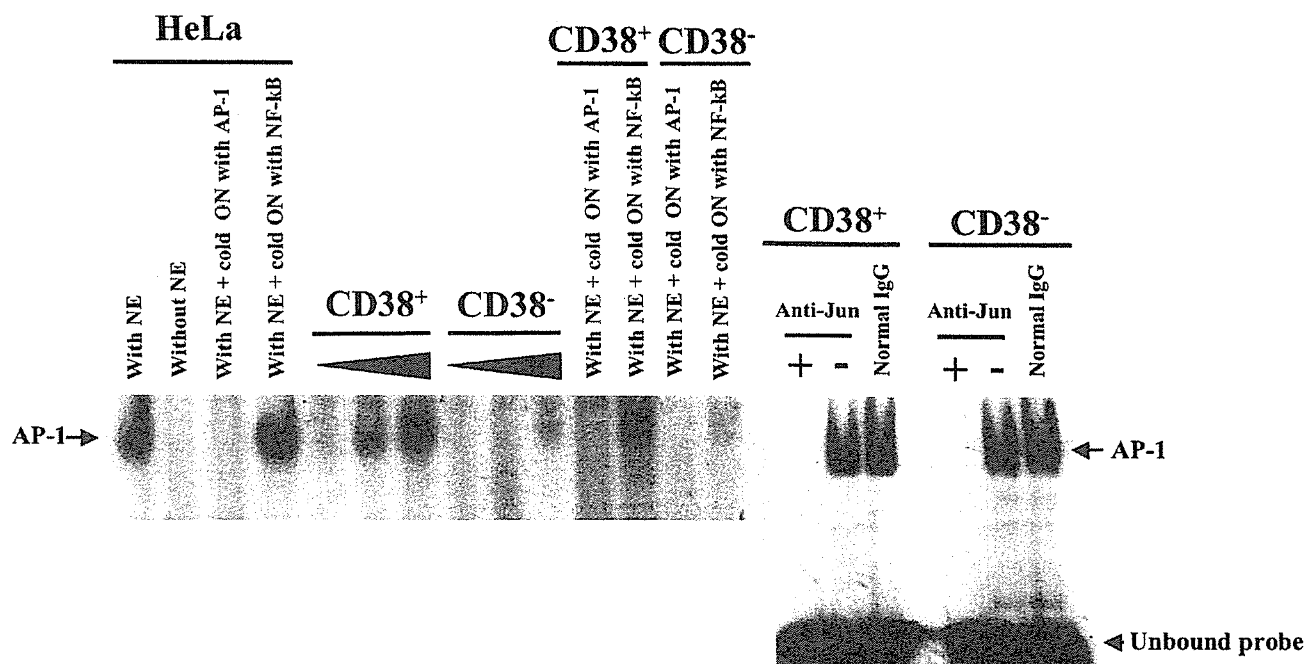

Fig. 6. Significant difference in the activation of the AP-1 between IL-4-treated CD38 subsets. The CD38 subsets were treated with IL-4 for 3 days. The nuclear extracts prepared from the treated CD38$^+$ and CD38$^-$ subset cells were solubilized in lysis buffer. The nuclear extracts were serially diluted (1-, 3-, and 9-fold dilutions of 10 μg/lane). The diluted samples were incubated with ^{32}P-labeled oligonucleotides (ON) containing AP-1-binding site in the HIV-1 LTR. After incubation for 1 hr, the samples were separated on 6% polyacrylamide gel. As a positive control, HeLa cell-derived nuclear extract (NE) supplied with the Kit were used: with NE, without NE, with NE with cold ON containing AP-1-binding site, and NE with cold ON containing NF-κB-binding site. Also, specific competition with unlabeled cold ON containing AP-1-binding site and unspecific unlabeled cold ON containing NF-κB-binding site were done for both subsets. In addition, EMSA was performed with NE from CD38$^+$ subset and CD38$^-$ subset in the absence (−) or presence (+) of anti-Jun B antibodies or normal IgG.

was confirmed by EMSA in the presence of anti-Jun B antibodies. Western blotting also revealed a significantly higher amount of Jun B, but not C-Jun, in the CD38$^+$ than the CD38$^-$ subset after IL-4 treatment (data not shown). Thus, AP-1 activation induced in the CD4$^+$ CD38$^+$ subset by IL-4 stimulation could be involved in the higher susceptibility of this subset to X4 HIV-1. In contrast, using the same nuclear fractions from both CD38 subsets treated with IL-4 for 3 days, NF-κB was detected in both subsets at similar levels (data not shown).

Discussion

In this study, IL-4 was shown to play a significant role on the higher susceptibility of the primary CD4$^+$ CD38$^+$ than CD4$^+$ CD38$^-$ T-lymphocyte subset to X4 HIV-1. This effect of IL-4 on the CD38$^+$ subset occurred at the transcription step in X4 HIV-1 replication cycle. The activation of the transcription factor AP-1 detected in the CD38$^+$ subset after IL-4 stimulation could be involved in the higher susceptibility of this subset to X4 HIV-1.

IL-4 expression was found to be up-regulated in some HIV-1-infected individuals at advanced stages of the disease (4). In the *in vitro* studies, both enhancing (15, 26, 43) and suppressive (26) effects of IL-4 on HIV-1 production from the cultures of monocytes, macrophages, CD4$^+$ T cells, and/or microglia cells have been reported. It was also reported that IL-4 favored X4 HIV-1 replication, while down-modulated that of R5 HIV-1 (39). These effects have been explained in most cases by IL-4-induced up-regulation of CXCR4 on the T lymphocytes (34, 39). Moreover, transcriptional enhancement of X4 HIV-1 in T lymphocytes was also previously reported to occur as a consequence of IL-4 stimulation, however, the mechanism of which was not yet clarified (39).

IL-4 was secreted from the CD4$^+$ CD38$^+$ subset derived from all examined donors at higher levels than CD4$^+$ CD38$^-$ subset on stimulation, although the IL-4 levels were greatly variable among individual samples (8). Thus, the CD38$^+$ cells even in the resting stage comprise a specific cell population that can preferentially secrete the Th2 cytokine on stimulation. Interestingly, although PHA pretreatment resulted in similar

susceptibility of both subsets to X4 HIV-1 infection, IL-4 treatment enhanced this susceptibility only in the CD38$^+$ subset. Flow cytometric analyses revealed that in contrast to PHA treatment, IL-4 treatment did not affect cell activation and proliferation, suggesting that the activation and proliferation properties of host CD4$^+$ T lymphocytes are not significantly important for the replication and particle production after the entry event of X4 HIV-1. In fact, it was previously reported that IL-4 inhibits several IL-2-induced effects including proliferation of human PBMCs (23, 42). This finding in the CD38 subsets is consistent with recent reports (17, 31, 44). Together with our results in this study, these data are apparently in contrast to other reports demonstrating that virus expression is tightly associated with the appearance of markers of classical T-cell activation after treatment of the cells with mitogens (44), progression out of G_0/G_1 (18), as well as cytokine secretion (38). Consequently, undetectable cellular stimulation by means of the classic T-cell activation seems to be sufficient for the virus replication.

Our result in this study is in agreement with that several host cellular transcription factors could regulate gene expression of HIV-1. The clear difference in the X4 HIV-1 gene expression between the CD38$^+$ and CD38$^-$ subsets was dependent on IL-4 stimulation. The activation levels of the NF-κB, the typical transcription factor involved in the HIV-1 transcription through binding to elements in the LTR (5), did not differ in both subsets. On the contrary, active form of AP-1 was significantly detected in this study in the IL-4-treated CD38$^+$, but not in the IL-4-treated CD38$^-$ subset, the untreated CD38$^+$ subset as well. The AP-1-binding sites were identified in the X4 HIV-1 genome, where they play a critical role in transactivation of the viral LTR via a Rev-independent but Tat-dependent mechanism (40). Further, synergistic role of AP-1 was also demonstrated on the transactivation of the X4 HIV-1 LTR through the NF-κB sites (45). Thus, studies using X4 HIV-1-infected primary CD4$^+$ T-lymphocyte subsets strongly suggest a significance of the AP-1 transcription factor that is activated after IL-4 stimulation for the up-regulated production of X4 HIV-1 in the CD38$^+$ compared with the CD38$^-$ subset. Further study is necessary to clarify the mechanism for the AP-1 function for the up-regulation of X4 HIV-1 transcription in the CD38$^+$ subset.

We thank Fuyoko Sasao for technical contribution. This study was supported in part by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

References

1) Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M.A. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**: 284–291.
2) Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A., and Mackay, C.R. 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. **94**: 1925–1930.
3) Calcaterra, S., Cappiello, G., Di Caro, A., Garbuglia, A.R., and Benedetto, A. 2001. Comparative analysis of total and integrated HIV-1 DNA in peripheral CD4 lymphocytes and monocytes after long treatment with HAART. J. Infect. **43**: 239–245.
4) Clerici, M., Hakim, F.T., Venzon, D.J., Blatt, S., Hendrix, C.W., Wynn, T.A., and Shearer, G.M. 1993. Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. J. Clin. Invest. **91**: 759–765.
5) Cullen, B.R. 1991. Regulation of HIV-1 gene expression. FASEB J. **5**: 2361–2368.
6) Eckstein, D.A., Penn, M.L., Korin, Y.D., Scripture-Adams, D.D., Zack, J.A., Kreisberg, J.F., Roederer, M., Sherman, M.P., Chin, P.S., and Goldsmith, M.A. 2001. HIV-1 actively replicates in naïve CD4$^+$ T cells residing within human lymphoid tissues. Immunity **15**: 671–682.
7) Hofmann, B., Nishanian, P., Fahey, J.L., Esmail, I., Jackson, A.L., Detels, R., and Cumberland, W. 1991. Serum increases and lymphoid cell surface losses of IL-2 receptor CD25 in HIV infection: distinctive parameters of HIV-induced change. Clin. Immunol. Immunopathol. **61**: 212–224.
8) Horikoshi, K., Kinomoto, M., Kurosu, T., Komoto, S., Shiraga, M., Otake, T., Mukai, T., and Ikuta, K. 2002. Resting CD4$^+$ T cells with CD38$^+$ CD62L$^+$ produce interleukin-4 which contributes to enhanced replication of T-tropic human immunodeficiency virus type 1. Virology **293**: 94–102.
9) Horikoshi, H., Kinomoto, M., Sasao, F., Mukai, T., Luftig, R.B., and Ikuta, K. 2001. Differential susceptibility of resting CD4$^+$ T lymphocytes to a T-tropic and a macrophage (M)-tropic human immunodeficiency virus type 1 is associated with their surface expression of CD38 molecules. Virus Res. **73**: 1–16.
10) Ibegbu, C., Spira, T.J., Nesheim, S., Mendez, H., Lee, F., Polliotti, B., Caba, J., and Nahmias, A. 1994. Subpopulations of T and B cells in perinatally HIV-infected and non-infected age-matched children compared with those in adults. Clin. Immunol. Immunopathol. **71**: 27–32.
11) Ibrahim, M.S., Watanabe, M., Palacios, J.A., Kamitani, W., Komoto, S., Kobayashi, T., Tomonaga, K., and Ikuta, K. 2002. Varied persistent life cycles of Borna disease virus in a human oligodendroglioma cell line. J. Virol. **76**: 3873–3880.
12) Janossy, G., Borthwick, N., and Lomnitzer, R. 1992. Proliferative defects of CD4 and CD8 lymphocytes in HIV-1

infection, p. 96–114. *In* Janossy, G., Autran, B., and Miedema, F. (eds), Immunodeficiency in HIV infection and AIDS, Karger, Basel.
13) Janossy, G., Jiang, J.D., and Roboz, J.P. 1992. Activated CD45RO, CD8$^+$ T lymphocytes in HIV infection, p. 195–210. *In* Janossy, G., Autran, B., and Miedema, F. (eds), Immunodeficiency in HIV infection and AIDS, Karger, Basel.
14) Kameoka, M., Ota, K., Tetsuka, T., Tanaka, Y., Itaya, A., Okamoto, T., and Yoshihara, K. 2000. Evidence for regulation of NF-κB by poly(ADP-ribose) polymerase. Biochem. J. **346**: 641–649.
15) Kazazi, F., Mathijs, J.M., Chang, J., Malafiej, P., Lopez, A., Dowton, D., Sorrell, T.C., Vadas, M.A., and Cunningham, A.L. 1992. Recombinant interleukin 4 stimulates human immunodeficiency virus production by infected monocytes and macrophages. J. Gen. Virol. **73**: 941–949.
16) Kestens, L., Vanham, G., Vereecken, C., Vandenbruaene, M., Vercauteren, G., Colebunders, R.L., and Gigase, P.L. 1994. Selective increase of activation antigens HLA-DR and CD38 on CD4$^+$ CD45RO$^+$ T lymphocytes during HIV-1 infection. Clin. Exp. Immunol. **95**: 436–441.
17) Kinter, A.L, Umscheid, C.A., Arthos, J., Cicala, C., Lin, Y., Jackson, R., Donoghue, E., Ehler, L., Adelsberger, J., Rabin, R.L., and Fauci, A.S. 2003. HIV envelope induces virus expression from resting CD4$^+$ T cells isolated from HIV-infected individuals in the absence of markers of cellular activation or apoptosis. J. Immunol. **170**: 2449–2455.
18) Korin, Y.D., and Zack, J.A. 1999. Nonproductive human immunodeficiency virus type 1 infection in nucleoside-treated G0 lymphocytes. J. Virol. **73**: 6526–6532.
19) Lee, B., Sharron, M., Montaner, L.J, Weissman, D., and Doms, R.W. 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc. Natl. Acad. Sci. U.S.A. **96**: 5215–5220.
20) Liu, Z., Cumberland, W.G., Hultin, L.E., Prince, H.E., Detels, R., and Giorgi, J.V. 1997. Elevated CD38 antigen expression on CD38$^+$ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4$^+$ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **16**: 83–92.
21) Lund, F.E., Cockayne, D.A., Randall, T.D., Solvason, N., Schuber, F., and Howard, M.C. 1998. CD38: a new paradigm in lymphocyte activation and signal transduction. Immunol. Rev. **161**: 79–93.
22) Mahalingam, M., Pozniak, A., McManus, T.J., Vergani, D., and Peakman, M. 1995. Cell cycling in HIV infection: analysis of *in vivo* activated lymphocytes. Clin. Exp. Immunol. **102**: 481–486.
23) Martinez, O.M., Gibbons, R.S., Garovoy, M.R., and Aronson, F.R. 1990. IL-4 inhibits IL-2 receptor expression and IL-2-dependent proliferation of human T cells. J. Immunol. **144**: 2211–2215.
24) Michael, N.L., Mo, T., Mersouki, A., O'Shaughnessy, M., Oster, C., Burke, D.S., Redfield, R.R., Birx, D.L., and Cassol, S.A. 1995. Human immunodeficiency virus type 1 cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. J. Virol. **69**: 1868–1877.
25) Mocroft, A., Bofill, M., Lipman, M., Medina, E., Borthwick, N., Timms, A., Batista, L., Winter, M., Sabin, C.A., Johnson, M., Lee, C.A., Phillips, A., and Janossy, G. 1997. CD8$^+$, CD38$^+$ lymphocyte percent: a useful immunological marker for monitoring HIV-1-infected patients. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14**: 158–162.
26) Naif, H.M., Li, S., Ho-Shon, M., Mathijs, J.M., Williamson, P., and Cunningham, A.L. 1997. The state of maturation of monocytes into macrophages determines the effects of IL-4 and IL-13 on HIV replication. J. Immunol. **158**: 501–511.
27) Phillips, A.N., Sabin, C.A., Elford, J., Bofill, M., Lee, C.A., and Janossy, G. 1993. CD8 lymphocyte counts and serum immunoglobulin A levels early in HIV infection as predictors of CD4 lymphocyte depletion during 8 years of follow-up. AIDS **7**: 975–980.
28) Pierson, T.C., Zhou, Y., Kieffer, T.L., Ruff, C.T., Buck, C., and Siliciano, R.F. 2002. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. J. Virol. **76**: 8518–8531.
29) Resino, S., Navarro, J., Bellon, J.M., Gurbindo, D., Leon, J.A., and Munoz-Fernandez, M.A. 2001. Naïve and memory CD4$^+$ T cells and T cell activation markers in HIV-1 infected children on HAART. Clin. Exp. Immunol. **125**: 266–273.
30) Roederer, M., Raju, P.A., Mitra, D.K., Herzenberger, L.A., and Herzenberg, L.A. 1997. HIV does not replicate in naïve CD4 T cells stimulated with CD3/CD28. J. Clin. Invest. **99**: 1555–1564.
31) Scales, D., Ni, H., Shaheen, F., Capodici, J., Cannon, G., and Weissman, D. 2001. Nonproliferating bystander CD4$^+$ T cells lacking activation markers support HIV replication during immune activation. J. Immunol. **166**: 6437–6443.
32) Schnittman, S.M., Lane, H.C., Greenhouse, J., Justement, J.S., Baseler, M., and Fauci, A.S. 1990. Preferential infection of CD4$^+$ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. Proc. Natl. Acad. Sci. U.S.A. **87**: 6058–6062.
33) Sousa, A.E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z., and Victorino, R.M. 2002. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J. Immunol. **169**: 3400–3406.
34) Suzuki, Y., Koyanagi, Y., Tanaka, Y., Murakami, T., Misawa, N., Maeda, N., Kimura, T., Shida, H., Hoxie, J.A., O'Brien, W.A., and Yamamoto, N. 1999. Determination in human immunodeficiency virus type 1 for efficient replication under cytokine-induced CD4$^+$ T-helper 1 (Th1)- and Th2-type conditions. J. Virol. **73**: 316–324.
35) Tedder, T.F., Crain, M.J., Kubagawa, H., Clement, L.T., and Cooper, M.D. 1985. Evaluation of lymphocyte differentiation in primary and secondary immunodeficiency diseases. J. Immunol. **135**: 1786–1791.
36) Tobiume, M., Fujinaga, K., Kameoka, M., Kimura, T., Nakaya, T., Yamada, T., and Ikuta, K. 1998. Dependence on

host cell cycle for activation of human immunodeficiency virus type 1 gene expression from latency. J. Gen. Virol. **79**: 1363–1371.

37) Tokunaga, K., Greenberg, M.L., Morse, M.A., Cumming, R.L., Lyerly, H.K., and Cullen, B.R. 2001. Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. J. Virol. **75**: 6776–6785.

38) Unutmaz, D., KewalRamani, V.N., Marmon, S., and Littman, D.R. 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. **189**: 1735–1746.

39) Valentin, A., Lu, W., Rosati, M., Schneider, R., Albert, J., Karlsson, A., and Pavlakis, G.N. 1998. Dual effect of interleukin 4 on HIV-1 expression: implications for viral phenotypic switch and disease progression. Proc. Natl. Acad. Sci. U.S.A. **95**: 8886–8891.

40) Van Lint, C., Amella, C.A., Emiliani, S., John, M., Jie, T., and Verdin, E. 1997. Transcription factor binding sites downstream of the human immunodeficiency virus type 1 transcription start site are important for virus infectivity. J. Virol. **71**: 6113–6127.

41) van Rij, R.P., Blaak, H., Visser, J.A., Brouwer, M., Rientsma, R., Broersen, S., de Roda Husman, A.M., and Schuitemaker, H. 2000. Differential coreceptor expression allows for independent evolution of non-syncytium-inducing and syncytium-inducing HIV-1. J. Clin. Invest. **106**: 1039–1052.

42) Wagner, F., Fischer, N., Lersch, C., Hart, R., and Dancygier, H. 1989. Interleukin 4 inhibits the interleukin 2-induced production of its functional antagonist, interferon γ. Immunol. Lett. **21**: 237–241.

43) Wang, J., Harada, A., Matsushita, S., Matsumi, S., Zhang, Y., Shioda, T., Nagai, Y., and Matsushima, K. 1998. IL-4 and a glucocorticoid up-regulate CXCR4 expression on human $CD4^+$ T lymphocytes and enhance HIV-1 replication. J. Leukoc. Biol. **64**: 642–649.

44) Wong, J.K., Hezareh, M., Gunthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A., and Richman, D.D. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278**: 1291–1295.

45) Yang, X., Chen, Y., and Gabuzda, D. 1999. ERK MAP kinase links cytokine signals to activation of latent HIV-1 infection by stimulating a cooperative interaction of AP-1 and NF-κB. J. Biol. Chem. **274**: 27981–27988.

46) Zaunders, J., Carr, A., McNally, L., Penny, R., and Cooper, D.A. 1995. Effects of primary HIV-1 infection on subsets of $CD4^+$ and $CD38^+$ T lymphocytes. AIDS **9**: 561–566.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 18, pp. 12495–12505, May 5, 2006
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Transcriptional Repression of Human Immunodeficiency Virus Type 1 by AP-4*

Received for publication, November 1, 2005, and in revised form, March 14, 2006 Published, JBC Papers in Press, March 15, 2006, DOI 10.1074/jbc.M511773200

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Elucidation of the mechanism of transcriptional silencing of human immunodeficiency virus type 1 (HIV-1) provirus in latently infected cells is crucial to understand the pathophysiology of HIV-1 infection and to develop novel therapies. Here we demonstrate that AP-4 is responsible for the transcriptional repression of HIV-1. We found that AP-4 site within the viral long terminal repeat (LTR) is well conserved in the majority of HIV-1 subtypes and that AP-4 represses HIV-1 gene expression by recruiting histone deacetylase (HDAC) 1 as well as by masking TATA-binding protein to TATA box. AP-4-mediated transcriptional repression was inhibited by an HDAC inhibitor, tricostatin A, and could be exerted even at distant locations from the TATA box. In addition, AP-4 interacted with HDAC1 both *in vivo* and *in vitro*. Moreover, chromatin immunoprecipitation assays have revealed that AP-4 and HDAC1 are present in the HIV-1 LTR promoter in latently infected ACH2 and U1 cells, and they are dissociated from the promoter concomitantly with the association of acetylated histone H3, TBP, and RNA polymerase II upon TNF-α stimulation of HIV-1 replication. Furthermore, when AP-4 is knocked down by siRNA, HIV-1 production was greatly augmented in cells transfected with a full-length HIV-1 clone. These results suggest that AP-4 may be responsible for transcriptional quiescence of latent HIV-1 provirus and give a molecular basis to the reported efficacy of combination therapy of conventional anti-HIV drugs with an HDAC inhibitor in accelerating the clearance of HIV-1 from individuals infected with the virus.

Human immunodeficiency virus type 1 (HIV-1)[2] is a cytopathic retrovirus and the primary etiological agent of acquired immunodeficiency syndrome (AIDS) and related disorders. Among the various steps of viral life cycle, the step of transcription from HIV-1 provirus is conceived to be crucial for viral replication since amplification of the viral genetic information is attainable only through transcription. HIV-1 transcription is directed by the promoter located in the 5′ long terminal repeat (LTR) of the integrated provirus and is controlled by cellular factors that bind to the multiple *cis*-regulatory elements located in the LTR as well as the virally encoded Tat protein (reviewed in Refs. 1 and 2). In cells chronically infected with HIV-1, activation of nuclear factor-κB (NF-κB) by external stimuli such as tumor necrosis factor (TNF)-α and its binding to LTR triggers the initiation of transcription of viral genes including Tat, which results in explosive HIV-1 replication (reviewed in Refs. 3 and 4). However, little is known how transcription from HIV-1 provirus remains silent during the viral latency.

There are multiple mechanisms known to be involved in the negative regulation of HIV transcription including elimination of transcriptional activator TATA-binding protein (TBP) transcriptional factor IID (TFIID) and the initiator protein complex by leader-binding protein (LBP)-1 (5) and YY-1 (6) that recruits histone deacetylase (HDAC) (7, 8), and actions of transcription factors that interact with the negative regulatory element (NRE) located from −340 to −184 of HIV-1 LTR (2, 9). Regarding the action of NRE, the mechanism by which NRE exerts its negative effect on transcription remains unknown because most of the transcription factors that interact with NRE are transcriptional activators. In addition, an *in vitro* study has revealed a potential role of activator protein (AP)-4 in blocking the TBP binding to TATA box (10). However, biological significance of this finding has not been clarified although the sequence comparison has revealed conservation of AP-4 sites in the majority of HIV-1 isolates (Fig. 1*A*) (11–13).

The HIV-1 LTR TATA box is located at −27 to −23 relative to the transcription initiation site (2, 11–14). TFIID interacts with TATA box and is crucial for HIV-1 gene expression (9, 14–16). TFIID contains the 38-kDa TBP as the major component and induces transcriptional initiation by interacting with other general transcription factors and recruiting RNA polymerase II (RNAPII) (17). TBP (TFIID) also serves as the target of DNA-binding factors binding to the *cis*-regulatory elements within HIV-1 LTR in both positive and negative fashions and thus determines its promoter activity (reviewed in Refs. 16, 18, and 19).

AP-4 is a ubiquitously expressed transcription factor of the basic helix-loop-helix leucine-zipper (bHLH-Zip) subgroup of bHLH proteins and binds to the symmetrical DNA sequence 5′-CAGCTG-3′ (20, 21). AP-4 site is found adjacent (−21/−16) to the HIV-1 TATA box (−27/−23) (10). Although AP-4 was initially identified as a cellular protein that binds to the simian virus 40 (SV40) enhancer and activates the viral late gene transcription (21), transcriptional repression by AP-4 was reported in a number of other genes including angiotensinogen (21) and E7 oncoprotein of human papillomavirus type 16 (23). In addition, we recently found that AP-4 negatively regulates transcription of 8-oxo guanine DNA glycosylase 1 (*OGG1*) gene (24). However, because AP-4 sites are not located adjacent to the TATA box in these promoters, the molecular mechanism of its repressive action is yet to be clarified.

In this study we investigated the role of AP-4 in HIV-1 gene expression. Here we show that AP-4 represses HIV-1 transcription by recruiting HDAC1 as well as by masking the TBP to the HIV-1 TATA box. Biological and therapeutic implications are discussed.

* This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; TBP, TATA-binding protein; HDAC, histone deacetylase; NRE, negative regulatory element; aa, amino acids; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay; TNF, tumor necrosis factor; TSA, tricostatin A; ELISA, enzyme-linked immunosorbent assay.

EXPERIMENTAL PROCEDURES

Cell Culture—CEM, HL-60, Jurkat, ACH2, and U1 cells were maintained at 37 °C in RPMI 1640 (Sigma) with 10% fetal bovine serum (Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml). To maintain the latency of the HIV-1 in ACH2 and U1, 20 μM AZT was added in the culture medium and was excluded prior to experiments. Human embryonic kidney 293 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Sigma) with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin.

Plasmids—Construction of mammalian expression plasmids pMyc-AP-4, containing the full-length AP-4 cDNA, pCMV-Tat, and pNL4-3 were described previously (24, 25). pCMV-TBP was a generous gift from T. Tamura (Chiba University). To generate pcDNA-AP-4 (full), pcDNA-ΔN100 AP-4 (100–355 aa), pcDNA-ΔN143 AP-4 (143–355 aa), pcDNA-ΔN180 AP-4 (180–355 aa), pcDNA-ΔC179 AP-4 (1–179 aa), and pcDNA-ΔC130 AP-4 (1–130 aa), each containing a FLAG epitope tag in the N terminus and a V5 epitope tag in the C terminus, the various portions of AP-4 cDNA were amplified by PCR using pMyc-AP-4 as a template with 5′ and 3′ oligonucleotide primers. These products were subcloned into pcDNA 3.1 TOPO V5 vector (Invitrogen). Construction of HIV-1 LTR-based luciferase expression plasmid: CD12-luc (containing the HIV-1 LTR U3 and R) was previously described (25). The mutant HIV-1 LTR luciferase reporter constructs lacking AP-4 binding were generated using a QuikChange site-directed mutagenesis kit (Stratagene). The mutant sequences (sense strand) utilized were the following: CD12-luc-m1, GAT CCT GCA TAT AAG tcg cga CTT TTT GCC TGT AC; CD12-luc-m2, GCA TAT AAG CAG CTc CTT TTT GCC TGT AC; CD12-luc-m3, GCA TAT AAG CAG Cgc TTA AGA TAC AGC; CD12-luc-m4, CCT GCA TAT AAG CAG tcG CTT TTT GCC TGT AC (consensus AP-4 binding sites are underlined, and the mutated sequences are in small letters). The mutant HIV-1 LTR-directed reporter constructs, in which the authentic AP-4 site is mutated and an AP-4 site is aberrantly inserted into various positions of the CD12-luc-m2 reporter plasmid, were constructed by PCR using CD12-luc-m2 DNA as a template and site-directed mutagenesis kit with the following mutagenesis oligonucleotide primer pairs: CD12-luc-m2(+55), forward (5′-GCT AGC TAG GGA ACA GCT GCC CAC TGC TTA AG-3′) and reverse (5′-CTT AAG CAG TGG GCA GCT GTT CCC TAG CTA GC-3′); CD12-luc-m2(−79), forward (5′-CTG GGG ACT TTC CAC AGC TGG GGA GGC GTG GCC-3′) and reverse (5′-GGC CAC GCC TCC CCA GCT GTG GAA AGT CCC CAG-3′); CD12-luc-m2(-150), forward (5′-GTG GCC CGA GAG CTC AGC TGG CAT CCG GAG TAC-3′) and reverse (5′-GTA CTC CGG ATG CCA GCT CAG CTC TCG GGC CAC-3′); CD12-luc-m2(−400), forward (5′-GAT CTG TGG ATC TCA GCT GAC CAC ACA CAA GG-3′) and reverse (5′-CCT TGT GTG TGG TCA GCT GAG ATC CAC AGA TC-3′). The mutant pNL4-3 containing mutation in AP-4 binding was generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene) (24) with oligonucleotide primer pairs: forward, 5′-CAT ATA AGC AGC TcC TTT TTG CCT GTA C-3′ and reverse 5′-GTA CAG GCA AAA AGg AGC TGC TTA TAT G-3′ (mutated AP-4 binding sites are underlined and the mutated nucleotides are in lowercase letters). We first constructed the 5′-LTR AP-4 site mutant in the background of pNL4-3 by site-directed mutagenesis and additional AP-4 site mutation in the 3′-LTR was subsequently introduced into this mutant by site-directed mutagenesis. All constructs were confirmed by dideoxynucleotide sequencing using ABI PRISM™ dye terminator cycle sequencing ready kit (PerkinElmer Life Sciences) on an Applied Biosystems 313 automated DNA sequencer.

Recombinant Protein and Purification—pGEX expression vector (Amersham Biosciences) was utilized to express glutathione *S*-transferase (GST) fusion proteins in bacteria. To generate pGEX-AP-4-expressing GST-AP-4, the AP-4 cDNA was amplified by PCR using pMyc-AP-4 as a template with oligonucleotide primer pairs: forward, 5′-CGG GAT CCC GGA GTA TTT CAT GGT GCC CAC TCA G-3′, containing an BamHI site; reverse, 5′-GGA ATT CCT CAG GGA AGC TCC CCG TCC CCC G-3′, containing an EcoRI site. This product was digested with BamHI and EcoRI, and subcloned in-frame into pGEX-5X-3 vector at the BamHI/EcoRI sites. pGEX-AP-4 was transformed in *Escherichia coli* strain DH5 and expression of recombinant GST-AP-4 protein was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 6 h. Recombinant GST proteins were purified by affinity chromatography on glutathione-agarose beads as described previously (26).

Electrophoretic Mobility Shift Assay (EMSA)—The experimental procedure was carried out as described previously (24). Purified recombinant TBP and GST-TBP proteins were purchased from Promega and Santa Cruz Biotechnology, respectively. The double-stranded DNA oligonucleotides corresponding to −42/+4 of HIV-1 LTR (CD12) containing the binding sites of TBP and AP-4 and their mutants were synthesized. The wild-type and mutant oligonucleotide sequences (sense strand) were the following: wild-type (5′-CCC TCA GAT CCT GCA TAT AAG CAG CTG CTT TTT GCC TGT A-3′) and mutants (the underlined AP-4 site has been changed to TGACGG (m1), TAGCTC (m2), CAGCGC (m3), and CAGTCG (m4)) (Fig. 2*B*). These oligonucleotides were labeled using the 5′-end-labeling kit (Takara, Otsu, Shiga, Japan) in the presence of [γ-^{32}P]dATP (Amersham Biosciences). DNA binding reactions were performed at 30 °C for 30 min for TBP and room temperature for 20 min for AP-4. Analysis of protein-DNA complexes was performed by electrophoresis in 6% native polyacrylamide gels with 0.5× Tris borate-EDTA buffer at a constant voltage of 125 V at 4 °C, followed by autoradiography. The specificity of DNA binding was assessed by preincubating with purified GST-AP-4, GST-TBP, or control GST proteins with specific antibodies or competitors for 20 min prior to the addition of the probe.

Anti-AP-4 Antibody—Anti-AP-4 antibody was obtained by immunizing rabbits with GST-AP-4 fusion protein as no immunoprecipitable anti-AP-4 antibody was currently available from any commercial source. The immunized rabbit anti-AP-4 sera were affinity-purified by passing through affinity columns, and the lack of immunoreactivity with GST and other *E. coli* components was confirmed.

Immunoprecipitation and Immunoblot Assays—The experimental procedures for immunoprecipitation and immunoblotting were performed as described (24, 27). Briefly, cells were harvested with lysis buffer (25 mM HEPES-NaOH, pH 7.9, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation, and the supernatants were incubated with anti-AP-4 antibody overnight at 4 °C. Immune complexes were washed three times with 1 ml of lysis buffer and antibody-bound proteins were dissolved by boiling in 2× Laemmli sample buffer. After centrifugation, the supernatant proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C, Amersham Biosciences). The membrane was probed with anti-AP-4 antibody, and immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce). To evaluate the level of AP-4 protein, cells were similarly treated with the lysis buffer, and the cell lysates were analyzed by immunoblotting using anti-AP-4 or V5 antibody (Invitrogen).

Transfection and Luciferase Assay—293 cells cultured in 12-well plates were transfected using Fugene-6 transfection reagent (Roche Applied Science) as described previously (26, 27). CEM and HL60 cells were transiently transfected by electroporation as reported (24). Briefly,

2×10^7 cells/ml were electroporated with 2 μg of CD12-luc together with 2 μg of pCMV-Tat and indicated amounts of Myc-AP-4 in 400 μl of serum-free RPMI using the Electro Cell Manipulator 600 (BTX Electroporation System) apparatus at 260 V/1050 μfarads. For the internal control, we employed pRL-TK, expressing *Renilla* luciferase under the control of the thymidine kinase promoter not containing the AP-4 site. The transfected cells were harvested, and the extracts were subjected to the luciferase assay using the Luciferase Assay System™ (Promega). All the experiments were carried out in triplicates, and the data were presented as the fold increase in luciferase activities (means ± S.D.) relative to the control for three independent transfections.

RNA Interference—The siRNAs with two thymidine residues (dTdT) at the 3′-end of the sequence were synthesized by Takara. The target sequences were as follows: AP-4-1 (5′-GUG CCC UCU UUG CAA CAU U-3′), AP-4-2 (5′-GGU CAU CAA CUC UGU UUC C-3′), and GFP (5′-GGC UAC GUC CAG GAG CGC ACC-3′). Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) reagents.

In Vitro Binding Assay—An *in vitro* protein-protein interaction assay was carried out as described previously (26). Briefly, AP-4 and luciferase proteins were labeled with [^{35}S]methionine *in vitro* transcription/translation using the TNT wheat germ extract-coupled system (Promega) according to the manufacturer's protocol. Approximately 20 μg of GST fusion proteins were immobilized on 20 μl of glutathione-Sepharose beads and washed two times with 1 ml of modified HEMNK buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After the final wash, 0.6 ml of beads suspension was incubated with radiolabeled proteins for 12 h at 4 °C. The beads were then washed two times with 1 ml of HENMK buffer and two times with HENMK buffer containing 150 mM KCl. Bound radiolabeled proteins were eluted with 30 μl of Laemmli sample buffer, boiled for 3 min, and resolved by 10% SDS-PAGE.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed according to the provider's protocol (Upstate Biotechnology) with some modifications as previously described (24). Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold phosphate-buffered saline, and lysed for 10 min at 2×10^6 cells in 200 μl of SDS lysis buffer. The cross-linked chromatin was sheared by sonication 13 times for 10 s at one-third of the maximum power of microson XL sonicator (Wakenyaku, Co., LTD., Kyoto, Japan) with 20 s of cooling on ice between each pulse. Cross-linked and released chromatin fractions were precleared with salmon sperm DNA and protein A-agarose beads for 1 h, followed by immunoprecipitation with the desired antibodies overnight at 4 °C. The immunoprecipitates were sequentially washed once with lysis buffer, twice with high salt buffer, twice with low salt buffer, and twice with TE buffer. After the wash, immune complexes were collected with salmon sperm DNA and protein A-agarose beads at room temperature for 1 h and extracted with 1% SDS, 0.1 M $NaHCO_3$. The eluted samples were reverse cross-linked by proteinase K at 45 °C for 1 h and treated with RNase at 37 °C for 1 h. DNA was recovered by phenol/chloroform and chloroform extractions, and ethanol precipitation. Finally, DNA was dissolved in 30 μl of TE buffer and subjected to PCR. The primer sequences used for PCR were the following: HIV-1 LTR (−109 to +79): forward (5′TAC AAG GGA CTT TCC GCT GG-3′) and reverse (5′-TTG AGG CTT AAG CAG TGG G-3′); β-actin promoter (−980 to −915) (as a control): forward (5′-TGC ACT GTG CGG CGA AGC-3′) and reverse (5′-TCG AGC CAT AAA AGG CAA-3′). The number of PCR cycles was as the following: 33 PCR cycles for all the ChIP experiments and 24 PCR cycles for the input samples, in which PCR amplification was obtained under the linear range of AP-4 binding to the HIV-1 LTR DNA. For each reaction, 10% of cross-linked released chromatin was saved and reversed by proteinase K digestion at 45 °C for 1 h followed by DNA extraction, and the recovered DNA was used as input control.

Antiviral Assay and Measurement of Viral p24 Antigen—Antiviral activity of AP-4 was evaluated based on the extent of inhibition of viral antigen expression in the culture supernatants of Jurkat or 293 cells transfected with a full-length HIV-1 molecular clone (pNL4-3) or mutant pNL4-3, in which AP-4 site is mutated. 293 cells were transfected with 0.1 μg of pNL4-3, together with various amounts of plasmids encoding wild-type AP-4 or AP-4 mutants with Fugene-6 transfection reagent. For siRNA studies, 100 nM siRNAs were introduced with 0.1 μg of pNL4-3 using Lipofectamine 2000 reagent. Jurkat cells were transfected by Nucleofector™ kit V for Jurkat cell (Amaxa Biosystems) according to the manufacturer's protocol. Briefly, 3×10^6 cells were mixed with 0.2 μg of wild-type or mutant pNL4-3 together with indicated amounts of FLAG-AP-4 in 100 μl of Nucleofector™ solution V. These samples were transferred into a transfection cuvette and subjected to electroporation using program T-14. The transfected cells were incubated in culture flasks with a complete media for 36 h. Then, cells were incubated for an additional 24 h in the presence or absence of TNF-α (3 ng/ml). The p24 antigen level in the cell culture supernatant was measured by p24 antigen capture ELISA assay using a commercial kit (RETRO-TEK HIV-1 p24 Antigen ELISA kit; Zepto Metrix Corp., Buffalo, NY) as described previously (25).

RESULTS

AP-4 Competes with TBP for Binding to the HIV-1 TATA Element—The HIV-1 TATA is located at nucleotide position from −27 to −23 relative to the transcription initiation site. The consensus AP-4 site, CAGCTG, is located −21 to −16 nucleotides immediately downstream of the TATA box. The AP-4 binding site in the HIV-1 LTR appears to be conserved in the majority of HIV-1 isolates (11–13). As shown in Fig. 1*A*, a majority of HIV-1 clones contain typical AP-4 binding sequence, CAGCTG, whereas it is mutated to CAGCCG in HIV-1 subtypes F1-F2, G, O, and 01-AE.

Because the AP-4 binding site is located close to the TATA box, we first examined the effect of AP-4 on the binding activity of TBP to the TATA box *in vitro*. To address this issue, recombinant AP-4 protein was produced and purified (Fig. 1, *B* and *C*). As shown in Fig. 1*D* (*left panel*), EMSA analysis using a DNA probe (−42/+4) containing both the TATA box and AP-4 site, showed that AP-4 blocked the TBP binding to TATA box in a dose-dependent manner (*lanes 6* and *7*). The control GST proteins did not alter the DNA binding activity of TBP (Fig. 1*D*, *right panel*). These results were consistent with a previous study by Ou *et al.* (10).

Repression of HIV-1 LTR Gene Expression by AP-4—Because AP-4 masks the TBP binding to the HIV-1 TATA box *in vitro*, we examined the effect of AP-4 on transcription from HIV-1 LTR. The luciferase reporter plasmid containing the HIV-LTR (CD12-luc) was cotransfected with an AP-4 expression vector (pMyc-AP-4) into CEM, HL-60, and 293 cell lines. As shown in Fig. 2*A*, the basal transcriptional level from HIV-1 LTR was inhibited by AP-4 in a dose-dependent manner in all the cell lines tested. Upon stimulation of HIV-1 promoter by TNF-α, a physiological inducer of NF-κB, AP-4 could similarly exert its negative effect. In addition, AP-4 also inhibited the Tat-induced HIV-1 gene expression in these cells.

To address whether the inhibitory effect of AP-4 depends on the presence of AP-4 site, we have created HIV-1 LTR mutants where the AP-4 binding site was mutated (Fig. 2*B*). As shown in Fig. 2*C*, EMSA confirmed that these mutants lost AP-4 binding. Although the inhibitory effect of AP-4 on wild-type LTR was clearly observed (Fig. 2*A*), it was abolished when basal, TNF-α-stimulated, and Tat-stimulated gene expression were

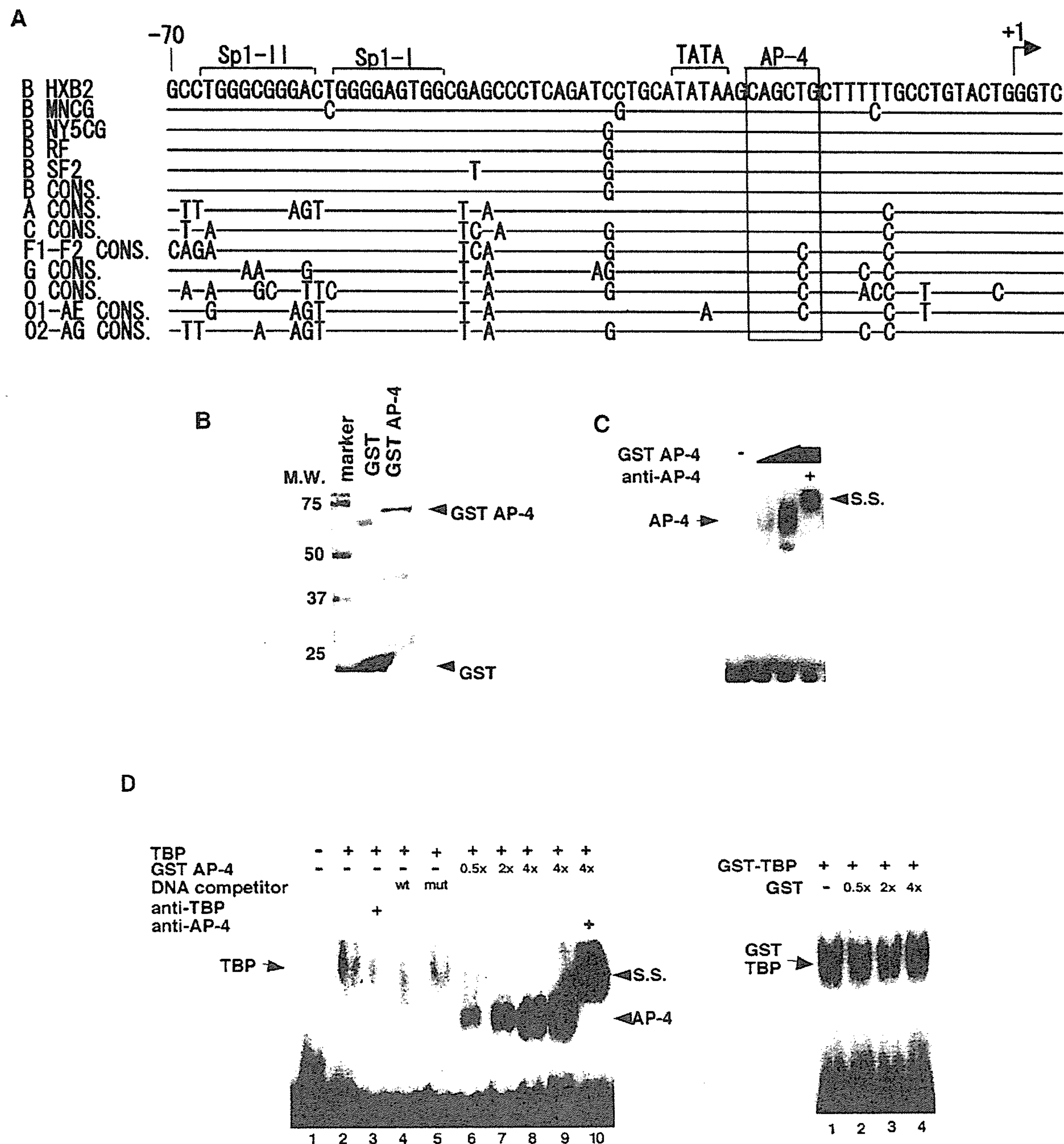

FIGURE 1. **Presence of AP-4 binding site in the vicinity of TATA box of HIV-1 LTR.** *A*, alignment of DNA sequences near the TATA box of various HIV-1 subtypes. The portions of LTR sequences encompassing two Sp1 sites, TATA box, and AP-4 site are shown. AP-4 sites are indicated by a *bracket*. *CONS*, consensus sequences of each HIV-1 subtype. *B*, recombinant GST-AP-4 and GST proteins. The procedures of synthesis and purification are described under "Experimental Procedures." Proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining. *Arrows* indicate the positions of GST-AP-4 and GST. *C*, DNA binding activity of recombinant GST-AP-4. The DNA binding activity of purified recombinant AP-4 protein was analyzed by EMSA with the ^{32}P-labeled double-stranded oligonucleotide probe containing the TATA box and AP-4 site. To verify the AP-4 and DNA complex, the reaction mixtures were incubated with AP-4 antibody. *D*, effect of AP-4 on the DNA binding activity of TBP. The TBP-DNA binding was analyzed by EMSA using the same probe as *C*. TBP was preincubated with various amounts of either GST-AP-4 at the molar ratios indicated (*left panel*). To verify the TBP-DNA and AP-4-DNA complexes, the reaction mixtures were incubated with the TBP and AP-4 antibodies, respectively. For the cold DNA competition experiment, unlabeled double-stranded competitor oligonucleotides (containing wild-type (*wt*) or mutated (*mut*) TATA sequences) were added to the reaction mixtures at 30-fold excess relative to the DNA probe. To confirm that the GST moiety does not affect the TBP-DNA binding, GST-TBP was preincubated with GST prior to the DNA binding reaction (*right panel*). Experiments were carried out at least three times, and reproducible results were obtained. The representative results are shown. Positions of specific DNA-protein complex and supershifted complex (*S.S.*) are indicated by *arrows*.

assessed with mutant HIV-1 LTR reporter constructs (Fig. 2*D*). To further address whether the inhibitory effect of AP-4 depends on the presence of AP-4 site, we repeated similar experiments using other luciferase reporter plasmids including 5× κB-TATA-luc and 4× CRE-TATA-luc (26), in which no AP-4 site is present. AP-4 did not inhibit gene expression from 5× κB-TATA-luc nor 4× CRE-TATA-luc (data not shown). These results demonstrated for the first time that AP-4 exhibits repressive action on HIV-1 gene expression in cultured cells *in vivo*.

Overexpression of TBP Overcomes the Inhibitory Effect of AP-4—To confirm that the repressive effect of AP-4 is through masking the TBP binding to the HIV-1 TATA box, we examined the effect of TBP overexpression on the action of AP-4. As shown in Fig. 3*A*, the inhibitory effect of AP-4 on basal gene expression was abrogated by TBP overexpression in a dose-dependent manner. Similarly, AP-4-mediated repression of the TNF-α-stimulated HIV-1 gene expression was abolished by TBP overexpression (Fig. 3*B*).