Naf1(N) protein was emulsified in the same amount of Freund's complete adjuvant (Wako) for the first injection and in Freund's incomplete adjuvant for the following two booster injections. After the third injection, immune response was assessed by screening blood samples from the immunized mice with Western blot analysis of purified Naf1(N) protein. The mouse showing highest immune response was given a final intraperitoneal booster injection with 100 μL of antigen, 2-weeks after the third injection and 3 days before fusion with myeloma cells.

Hybridoma preparation

Three days after the last immunization, the mice were killed and the spleens removed aseptically. Spleen cells were mixed with SP 2/0 myeloma cells at a ratio of 5:1 and fused in the presence of polyethylene glycol (PEG 1500; Roche) at 37°C. The cells were then pelleted and resuspended in RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (FBS; Cansera International Inc), 100 U/mL penicillin, 100 g/mL streptomycin, and 2% HAT (100 μM hypoxanthine, 400 nM aminopterin, and 16 μM thymidine) for hybrid selection. Aliquots of the cell suspension were plated in 96-well plates and cultured at 37°C in 5% CO_2. After 24 h, 100 μL of HAT-selective medium was added, and every 2 or 3 days half of the medium from each well was replaced with fresh HAT medium. Between the 12th and 15th days, cell growth appeared in the majority of wells, and the supernatants were screened by immunofluorescence staining for the specific antibody-secreting clones. Cells from the positive hybridomas were transferred to a 24-well plate and cultured in 1 mL of 1% HT medium. As hybridoma cells grew well, aminopterin was omitted from the medium. At days 2 and 4, 0.5 mL of HT medium was added to each well, and supernatants were screened again by immunofluorescence staining. Among five positive hybridomas, one strongly positive hybridoma (Hybridoma-4) was selected and subcloned by limiting dilution in HT medium (GIBCO), followed by screening and selection of the strongest clone (C-3). Hybridoma clone (C-3) was cultured in large scale for inoculation into mice.

Purification and isotyping of MAb

Hybridoma cells (2×10^6) were inoculated into the peritoneal cavity of three BALB/c mice treated 1 week before with pristine (Sigma) to generate ascitic fluids containing anti-Naf1 MAb. After 10 days, ascitic fluid was collected and purified using HiTrap-Protein G Sepharose columns (Pharmacia Biotech). Immuno-globulin isotyping was performed using mouse MAb isotyping kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence staining

Immunofluorescence staining of Sf9 cells infected with recombinant baculovirus was done as described in the previous report.[33] In summary, cells were fixed in methanol for 5 min and incubated for 1 h at 37°C with anti-myc or anti-Naf1 MAb, followed by washing three times with PBS. Cells were then incubated with FITC-conjugated goat anti-mouse second antibody (American Qualex) for 1 h. As for control, the second antibody only was used. After thorough washings, cells were evaluated under fluorescence microscope (BX50F, Olympus Optical Co., Ltd.).

Silver staining and Western blot analysis

The extent of the purification of recombinant Naf1(N) protein was analyzed by silver staining and Western blot analysis. Aliquots of elusion, flow-through, and wash fractions were subjected to 12% SDS-PAGE, followed by silver staining.[34]

Cell lysates of 293 cells transfected with full-length Naf1 and Naf1(N) cDNA were subjected to Western blot analysis. Cell lysates were separated on a 12% SDS gel and blotted on an Immobilon-P (Millipore) transfer membrane. After incubation with anti-Naf1 MAb (1:1000 dilutions) and second anti-mouse antibody conjugated with horseradish peroxidase, antibody binding was detected by chemiluminescence reagent.

Epitope determination of anti-Naf1 MAb

For the epitope mapping of the anti-Naf1 MAb, truncated forms of Naf1(N) fusion proteins were expressed in *Escherichia coli*, as described previously.[35] cDNAs encoding 6×His and myc-tagged Naf1(N)-1,2,3 and Naf1(N)-A,B,C fusion protein fragments, ligated in pMAL-C2 vector after digestion with *Bam*H1 and *Hind*III were transformed into *E. coli* (XL 2-Blue). Single colonies of transformed *E. coli* were grown in Luria-Bertani (LB) medium with kanamycin (25 μg/mL). Protein expression was induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubated at 37°C for 3 h. The cells were then pelleted by centrifugation and resuspended in 100 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) followed by sonication. Cell lysates were applied to SDS-PAGE, followed by Coomassie Brilliant Blue staining or immunoblotting with anti-Naf1 MAb.

Immunofluorescence staining and confocal microscopy

Unstimulated peripheral blood lymphocytes (PBL), Jurkat, MT-4, and Molt-4 cells were washed with PBS and fixed in 2% paraformaldehyde for 20 min on ice. Cells were washed again with PBS containing 0.1% BSA, treated with 2% normal goat serum for 30 min to block non-specific binding, and then incubated with anti-Naf1 MAb (1:500 dilution) or control mouse IgG for 1 h at room temperature. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody for 1 h at room temperature. Cells were then washed and mounted with Fluorescent Mounting Medium (Dako). Fluorescent images of endogenous Naf1 were evaluated by confocal microscopy using a Zeiss (LSM-510, V-2.5) Axioplan-2, laser-imaging confocal microscope.

RESULTS

Expression and purification of recombinant Naf1(N) protein

To generate MAb against Naf1 cellular protein, we first constructed the recombinant baculovirus encoding Naf1(N) cDNA by homologous recombination of pMelBac A-Naf1(N) cDNA with the replication-deficient baculovirus DNA in Sf9 cells using Bac-N-Blue transfection kit. Recombinant virus encoding Naf1(N) was then used to infect High Five cells for large-scale

protein production. Expression of the recombinant Naf1(N) protein was examined by immunofluorescence staining of baculovirus-infected Sf9 cells with anti-myc MAb (Fig. 1A). After protein purification, the purity of recombinant Naf1(N) protein was analyzed by SDS-PAGE and Silver staining, showing recombinant Naf1(N) protein as a major band as shown in Figure 1B. Silver staining of purified Naf1(N) protein denoted the presence of minor contaminants; however, none of these bands reacted with purified MAb in Western blot analysis (Fig. 2B), indicating that the contaminants are not the components of recombinant Naf1(N) protein.

Production and characterization of anti-Naf1 MAb

Female Balb/c mice, subcutaneously immunized with purified recombinant Naf1(N) protein, revealed antibody responses in Western blot analysis using mice sera, and we selected a mouse with highest antibody response for hybridoma preparation. We obtained five positive hybridomas, and from them, one strongly positive hybridoma (Hybridoma number-4) was selected by immunofluorescent assay. We then subcloned the hybridoma by limiting dilution, and the strongest clone (C-3) was selected and cultured in large scale to be inoculated into the peritoneal cavity of BALB/c mice. Ascitic fluid, containing anti-Naf1 MAb, was collected and purified by using Protein G Sepharose columns. Immunoglobulin isotyping showed the isotype of the purified anti-Naf1 MAb as IgG1 with a lambda (λ) light chain. The specificity of MAb was examined by immunofluorescence staining of Sf9 cells infected with recombinant baculovirus (Fig. 2A) and Western blot analysis using purified anti-Naf1 MAb (Fig. 2B, C). Anti-Naf1 MAb recognized recombinant Naf1(N) protein, but not control BSA (Fig. 2B), as well as full-length Naf1 and Naf1(N) in transfected 293 cell lysates (Fig. 2C). These results clearly show that anti-Naf1 MAb is specific to Naf1 protein, not to histidine or myc, tagged to the recombinant Naf1(N) antigen. According to previous reports, it has already been shown that hexahistidine tagging takes advantage of a high-affinity to Ni-NTA resin in the purification procedure without interfering with the protein function.

Epitope analysis of MAb

To complete the characterization of MAb, we investigated the Naf1 epitope recognized by this MAb. Polyhistidine and myc-tagged Naf1(N) fusion protein fragments (1, 2, and 3) with

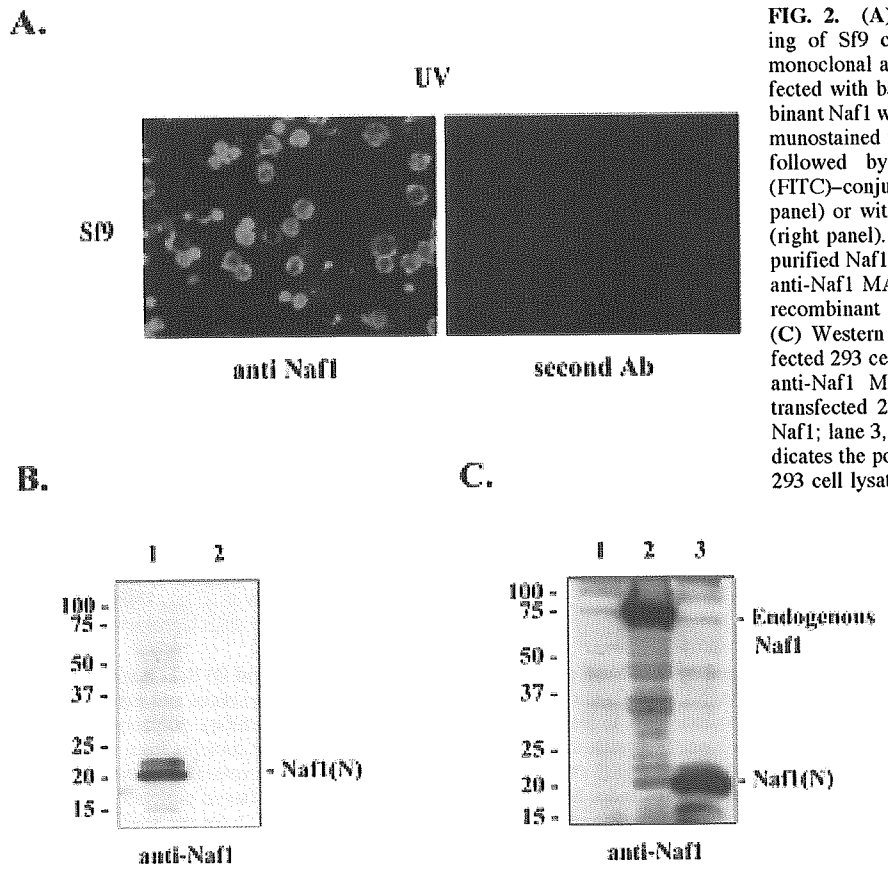


FIG. 2. (A) Immunofluorescence staining of Sf9 cells with purified anti-Naf1 monoclonal antibody (MAb). Sf9 cells infected with baculovirus expressing recombinant Naf1 were fixed in methanol and immunostained with purified anti-Naf1 MAb, followed by fluorescein isothiocyanate (FITC)–conjugated second antibody (left panel) or with second antibody (Ab) only (right panel). (B) Western blot analysis of purified Naf1(N), showing the reactivity of anti-Naf1 MAb. Lane 1, purified Naf1(N) recombinant protein; lane 2, control BSA. (C) Western blot analysis of Naf1-transfected 293 cell lysates for the specificity of anti-Naf1 MAb. Lane 1, control vector transfected 293 lysate; lane 2, full-length Naf1; lane 3, Naf1 (N). The arrowhead indicates the position of endogenous Naf1 in 293 cell lysate.

overlapping regions between amino acid residues 38 and 135, were generated (Fig. 3A). Protein expressions in induced *E. coli* were analyzed by SDS-PAGE and the Coomassie blue staining (Fig. 3B). Western blot analysis of Naf1(N) protein fragments using anti-myc antibody and the purified MAb shows that protein fragments were thoroughly produced in *E. coli*, and the epitope detected by the MAb was located at the overlapping region of protein fragments 1 and 2, between amino acid residues 64 and 88 (Fig. 3C).

We again constructed Naf1 fusion protein fragments (Fig. 3A–C) with overlapping regions between amino acid residues 64 and 88 (Fig. 3D). Expression of Naf1(N) protein fragments was ascertained by SDS-PAGE, followed by Coomassie blue staining (Fig. 3E) and Western blot analysis using anti-myc antibody (Fig. 3F). According to the result of Western blot analysis using anti-Naf1 MAb, as shown in Figure 3F, the MAb was found to have a specific binding ability to Naf1(N) fragment C. These results indicate that the epitope recognized by anti-Naf1 MAb was located at amino acid residues 81–88 region of Naf1.

Endogenous Naf1 expression and subcellular localization

The ability of the anti-Naf1 MAb to detect endogenous Naf1 in various cells was also investigated by Western blot analysis and the immunofluorescent staining of cells followed by confocal microscopy (Fig. 4A,B). Cell lysates of unstimulated human PBL, Jurkat, MT-4, Molt 4, 293, and U-937 cells were applied to 12% gel SDS-PAGE, and immunoblotting with Naf1 MAb showed endogenous Naf1 protein with the molecular weight of about 72 kDa (Fig. 4A). Immunofluorescent staining of human PBL, Jurkat, MT-4, and Molt-4 cells with anti-Naf1 MAb, followed by the assess-

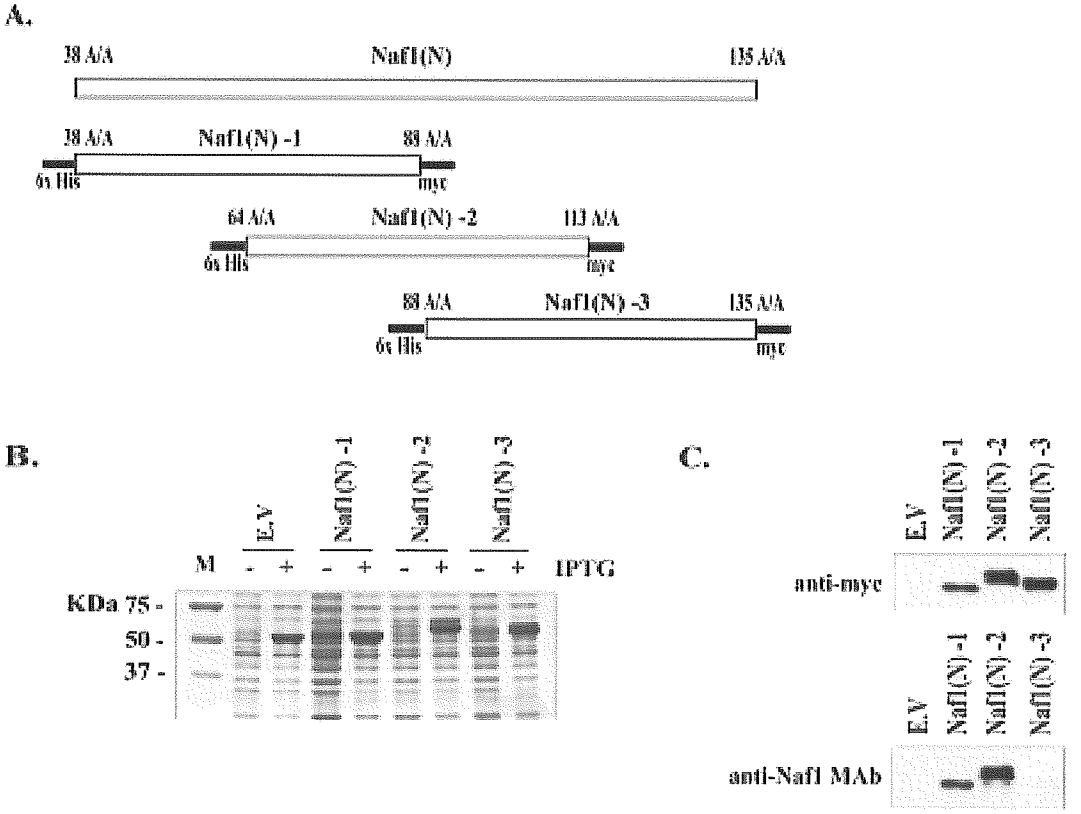


FIG. 3. Mapping of the Naf1 epitope recognized by anti-Naf1 monoclonal antibody (MAb). **(A, and D)** Schematic representation of N-terminally truncated Naf1 fusion proteins. The fusion proteins contain 6×His tag at N-terminus and myc tag at C-terminus. The numbers refer to the position of amino acids in Naf1 protein. **(B,E)** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining for the expression of Naf1 fusion proteins from un-induced (−) or isopropyl-D-thiogalactopyranoside (IPTG) induced (+) *E. coli*, transformed with control empty vector (EV) or with pMAL-6×His-Naf1-myc cDNAs. **(C,F)** Western blot analysis of induced bacterial lysates expressing Naf1 fusion proteins. Lysates of induced *E. coli* were electrophoresed in 12% acrylamide gels and immunoblotted with anti-Naf1 MAb for the specific epitope (upper panals) or anti-myc MAb for the protein input control (lower panals).

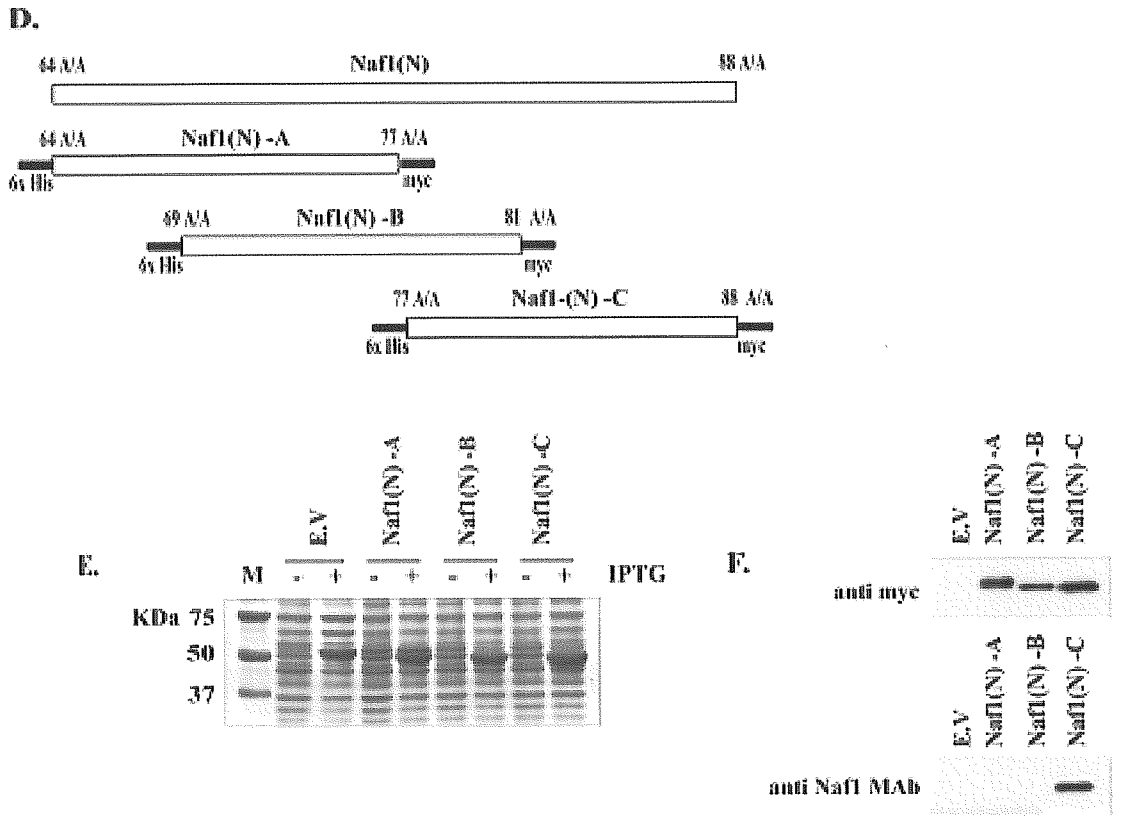


FIG. 3. *Continued.*

ment under confocal microscope, showed predominantly cytoplasmic localization of endogenous Naf1 (Fig. 4B).

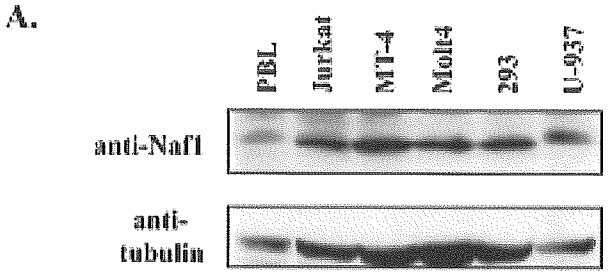
DISCUSSION

In this report, we present the production and characterization of an MAb specific for the cellular protein, Naf1/ABIN-1. We used the baculovirus expression system to synthesize recombinant Naf1(N) protein. High Five cells were described to achieve higher protein production when compared with Sf9 cells.[30] Previous reports showed that hexahistidine tagging takes advantage of a high affinity to Ni-NTA resin in the purification procedure and does not interfere with the protein function.[29]

The ability of anti-Naf1 MAb to recognize endogenous Naf1 protein in various cell lines was examined by Western blotting and immunostaining. We also investigated the expression and localization of endogenous Naf1 in human PBL and other cell lines showing its cytoplasmic localization. According to previous reports, Naf1 associates with HIV-1 viral proteins, Nef, and matrix in the yeast two-hybrid system and pull-down assays using Naf1-overexpressed cell lysates.[1,2] HIV-1 Nef contributes substantially to AIDS pathogenesis by augmenting virus replication and markedly perturbing the T cell functions. Host cell activation by viral protein Nef has been supposed to be a result of interaction between Nef with cellular protein Naf1, which is involved in the signal transduction of host cells. Naf1 has also been known to interact with A20 zinc finger protein, previously characterized as an inhibitor of NF-κB activation and apoptosis. Naf1 (ABIN-1) has been reported to inhibit NF-κB–dependent gene expression induced by TNF and IL-1. Naf1 blocks NF-κB activation by negative feedback regulation of NF-κB, and it has also been suggested that Naf1 has the potential to inhibit TNF-induced activation of NF-κB upon overexpression.[24,26] However, most of these earlier experiments were performed with Naf1-overexpressed cells because of the lack of specific MAb to the endogenous Naf1. To investigate the Naf1-interacting proteins and their molecular functions under physiological condition, anti-Naf1 MAb is required to access endogenous Naf1 in some specific examinations, such as immunoprecipitation and immunostaining assays. The improvement in understanding of the activation and regulation of NF-κB has opened the way for the development of new treatments in inflammatory diseases as well

as in HIV infection. From a therapeutic point of view, genetic engineering of specific human cells to express an NF-κB inhibitor such as Naf1 will offer a novel therapeutic tool in gene therapy.

In conclusion, anti-Naf1 MAb will be an indispensable tool in various immunoassay and functional investigations concerning the endogenous Naf1. We hope this MAb may lead to new findings and understanding of Naf1 in many aspects relating to HIV infection and various inflammatory diseases in human beings.



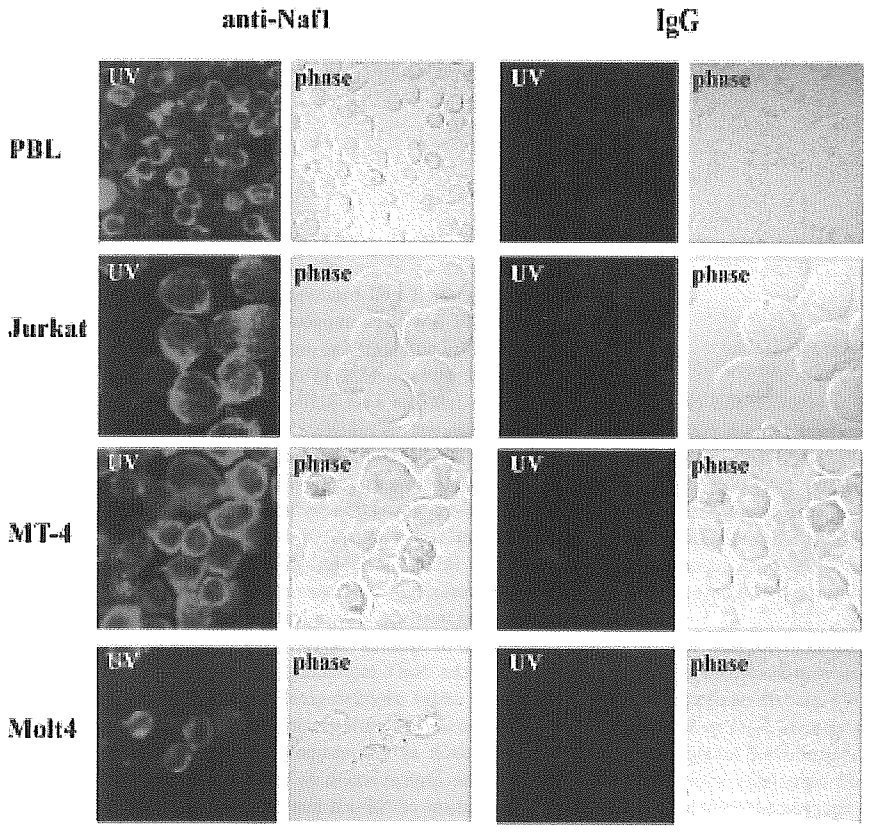


FIG. 4. Naf1 expression in human PBL, Jurkat, MT-4, Molt-4, 293, and U-937 cell lines. (**A**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of cell lysates for the expression of endogenous Naf1 using anti-Naf1 monoclonal antibody (MAb). (**B**) Subcellular localization of Naf1 in human PBL, Jurkat, MT-4, and Molt-4 cells by immunofluorescence staining with anti-Naf1 MAb or control IgG and assessment under confocal microscopy.

ACKNOWLEDGMENTS

We thank Dr. Kazuo Terashima and Masami Koushi for their excellent technical assistance in immunostaining and confocal microscopy. This work was supported by grants from the Ministry of Education, Science and Culture, and the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Fukushi M, Dixon J, Kimura T, Tsurutani N, Michael Dixon M, and Yamamoto N: Identification and cloning of a novel cellular protein Naf1, Nef-associated factor 1, that increases cell surface CD4 expression. FEBS Lett 1999;442:83–88.
2. Gupta K, Ott D, Hope TJ, Siliciano RF, and Boeke JD: A Human nuclear shuttling protein that interacts with human immunodeficiency virus type 1 matrix is packed into virions. J Virol 2000;74: 11811–11824.
3. Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, and Desrosiers RC: Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 1991;65:651–662.
4. Miller MD, Warmerdam MT, Gaston I, Greene WC, and Feinberg MB: The human immunodeficiency virus–1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J Exp Med 1994;179:101–113.
5. Spina CA, Kwoh TJ, Chowers MY, Guatelli JC, and Richman DD: The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J Exp Med 1994;179:115–123.
6. Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, and Guatelli JC: Optimal infectivity *in vitro* of human immunodeficiency virus type 1 requires an intact nef gene. J Virol 1994;68:2906–2914.
7. Harris M: From negative factor to a critical role in virus pathogenesis: the changing fortunes of Nef J Gen Virol 1996;77:2379–2392.
8. Kawano Y, Tanaka Y, Misawa N, Tanaka R, Kira JI, Kimura T, Fukushi M, Sano K, Goto T, Nakai M, Kobayashi T, Yamamoto N, and Koyanagi Y: Mutational analysis of human immunodeficiency virus type 1 (HIV-1) accessory genes: requirement of a site in the nef gene for HIV-1 replication in activated $CD4^+$ T cells *in vitro* and *in vivo*. J Virol 1997;71:8456–8466.
9. Cullen BR: HIV-1 auxiliary proteins: making connections in a dying cell. Cell 1998;93:685–692.
10. Joseph AM, Kumar M, and Mitra D: Nef: "necessary and enforcing factor" in HIV infection. Curr HIV Res 2005;3:87–94.
11. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, and Desrosiers RC: Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med 1995; 332:228–232.
12. Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, and Trono D: Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes. EMBO J 1998; 17:2472–2481.
13. Kim YH, Chang SH, Kwon JH, and Rhee SS: HIV-1 Nef plays an essential role in two independent processes in CD4 down-regulation: dissociation of the CD4–$p56^{lck}$complex and targeting of CD4 to lysosomes. Virology 1999;257:208–219.
14. Swann SA, Williams M, Story CM, Bobbitt KR, Fleis R, and Collins KL: HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. Virology 2001;282:267–277.
15. Glushakova S, Munch J, Carl S, Greenough TC, Sullivan JL, Margolis L, and Kirchhoff F: CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with $CD4^+$ T-cell depletion in human lymphoid tissue *ex vivo*. J Virol 2001;75:10113–10117.
16. Lundquist CA, Tobiume M, Zhou J, Unutmaz D, and Aiken C: Nef-mediated downregulation of CD4 enhances human immunodeficiency virus type 1 replication in primary T lymphocytes. J Virol 2002;76:4625–4633.
17. Casartelli N, Di Matteo G, Potesta M, Rossi P, and Doria M: CD4 and major histocompatibility complex class I downregulation by the human immunodeficiency virus type 1 nef protein in pediatric AIDS progression. J Virol 2003;77:11536–11545.
18. Cortes MJ, Wong-Staal F, and Lama J: Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells. J Biol Chem 2002;277:1770–1779.
19. Christiane F, Stroka DM, Badrichani AZ, Cooper JT, Wrighton CJ, Soares M, Grey ST, and Bach FH: A20 inhibits NF-κB activation in endothelial cells without sensitizing to tumor necrosis factor–mediated apoptosis. Blood 1998;91:2249–2258.
20. Heyninck K, De Valck D, Vanden Berghe W, Van Criekinge W, Contreras R, Fiers W, Haegeman G, and Beyaert R: The zinc finger protein A20 inhibits TNF-induced NF-κB–dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-κB inhibiting protein ABIN. J Cell Biol 1999;145:1471–1482.
21. Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, and Ma A: Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. Science 2000;289:2350–2354.
22. Klinkenberg M, Van Huffel S, Heyninck K, and Beyaert R: Functional redundancy of the zinc fingers of A20 for inhibition of NF-κB activation and protein-protein interactions. FEBS Lett 2001; 498:93–97.
23. Yamamoto Y, and Gaynor RB: IκB kinases: key regulators of the NF-κB pathway. Trends Biochem Sci 2004;29:72–79.
24. Beyaert R, Heyninck K, and Van Huffel S: A20 and A20-binding proteins as cellular inhibitors of nuclear factor-κB-dependent gene expression and apoptosis. Biochem Pharmacol 2000;60: 1143–1151.
25. Heyninck K, Kreike MM, and Beyaert R: Structure-function analysis of the A20-binding inhibitor of NF-κB activation, ABIN-1. FEBS Lett 2003;536:135–140.
26. Gallagher J, Howlin J, McCarthy C, Murphy EP, Bresnihan B, FitzGerald O, Godson C, Brady HR, and Martin F: Identification of Naf1/ABIN-1 among TNF-α–induced expressed genes in human synoviocytes using oligonucleotide microarrays. FEBS Lett 2003;551:8–12.
27. El Bakkouri K, Wullaert A, Haegman M, Heyninck K, and Beyaert R: Adenoviral gene transfer of the NF-κB inhibitory protein ABIN-1 decreases allergic airway inflammation in a murine asthma model. J Biol Chem 2005;280:17938–17944.
28. Zhang S, Fukushi M, Hashimoto S, Gao C, Huang L, Fukuyo Y, Nakajima T, Amagasa T, Enomoto S, Koike K, Miura O, Yamamoto N, and Tsuchida N: A new ERK2 binding protein, Naf1, attenuates the EGF/ERK2 nuclear signaling. Biochem Biophys Res Commun 2002;297:17–23.
29. Massotte D, Baroche L, Simonin F, Yu L, Kieffer B, and Pattus F: Characterization of δ, κ, and μ human opioid receptors overexpressed in baculovirus-infected insect cells. J Biol Chem 1997;272: 19987–19992.
30. Kozasa T, and Gilman AG: Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of α_{12} and inhibition of adenylyl cyclase by α_z. J Biol Chem 1995;270:1734–1741.
31. Grennan Jones F, Wolstenholme A, Fowler S, Smith S, Ziemnicka K, Bradbury J, Furmaniak J, and Rees Smith B: High-level expression of recombinant immunoreactive thyroid peroxidase in the High Five insect cell line. J Mol Endocrinol 1996;17:165–174.

32. Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, and Murray PG: Monoclonal antibodies. Mol Pathol 2000;53: 111–117.
33. Horiuchi S, Ampofo W, Koyanagi Y, Yamashita A, Waki M, Matsumoto A, Yamamoto M, and Yamamoto N: High-level production of alternatively spliced soluble interleukin-6 receptor in serum of patients with adult T-cell leukaemia/HTLV-I–associated myelopathy. Immunology 1998;95:360–369.
34. Rabilloud T, Vuillard L, Gilly C, and Lawrence JJ: Silver-staining of proteins in polyacrylamide gels: a general overview. Cell Mol Biol 1994;40:57–75.
35. Na BK, Chung GT, and Song CY: Production, characterization, and epitope mapping of a monoclonal antibody against aspartic proteinase of *Candida albicans*. Clin Diagn Lab Immunol 1999;6: 429–433.

Address reprint requests to:
Naoki Yamamoto
Department of Molecular Virology
Bio-Response
Graduate School of Medicine
Tokyo Medical and Dental University
1-5-45, Yushima, Bunkyo-ku
Tokyo, 113-8519, Japan

E-mail: yamamoto.mmb@tmd.ac.jp

Received for publication June 17, 2005. Accepted for publication July 14, 2005.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Microbes and Infection 7 (2005) 1150–1160
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

A novel role for Vpr of human immunodeficiency virus type 1 as a regulator of the splicing of cellular pre-mRNA

Madoka Kuramitsu [a,b], Chieko Hashizume [a,b], Norio Yamamoto [c], Akihiko Azuma [a], Masakazu Kamata [a], Naoki Yamamoto [c], Yoshimasa Tanaka [b], Yoko Aida [a,b,*]

[a] *Retrovirus Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*
[b] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[c] *Department of Molecular Virology, Bio-Response, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8519, Japan*

Received 13 March 2005; accepted 21 March 2005

Available online 19 April 2005

Abstract

Vpr, one of the accessory gene products of human immunodeficiency virus type 1 (HIV-1), affects aspects of both viral and cellular proliferation, being involved in long terminal repeat (LTR) activation, arrest of the cell cycle at the G2 phase, and apoptosis. We have discovered a novel role for Vpr as a regulator of the splicing of pre-mRNA both in vivo and in vitro. We found, by RT-PCR and RNase protection analysis, that Vpr caused the accumulation of incompletely spliced forms of *α-globin 2* and *β-globin* pre-mRNAs in cells that had been transiently transfected with a Vpr expression vector. We postulated that this novel effect of Vpr might occur via a pathway that is distinct from arrest of the cell cycle at G2. By analyzing splicing reactions in vitro, we showed that Vpr inhibited the splicing of *β-globin* pre-mRNA in vitro. The splicing of intron 1 of *α-globin 2* pre-mRNA was modestly inhibited by Vpr but the splicing of intron 2 was unaffected. Interestingly, an experimental infection system which utilizes high-titered HIV-1/vesticular stomatitis virus G protein showed that Vpr expressed from an HIV-1 provirus was sufficient to accumulate endogenous *α-globin 2* pre-mRNA. Thus, it is likely that Vpr contributes to selective inhibition of the splicing of cellular pre-mRNA.

Keywords: Human immunodeficiency virus type 1; Vpr; Splicing; Pre-mRNA

1. Introduction

The genome of human immunodeficiency virus type 1 (HIV-1) contains both structural genes, such as *gag*, *pol*, and *env*, and accessory genes, such as *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef*. The *vpr* gene encodes a protein of 96 amino acids that is incorporated in significant quantities into virions [1,2]. Vpr is a nucleophilic protein with non-classical nuclear localization signals [3,4]. The presence of Vpr in the viral particle facilitates efficient infection of macrophages and other non-dividing cells [5–7] by mediating the active nuclear import of preintegration complexes (PIC) [8,9]. One of the important functions of Vpr is the promotion of growth arrest at the G2/M phase of the cell cycle [10–13]. Indeed, there are sufficient amounts of Vpr in incoming viral particles to induce G2 arrest of the cell cycle even prior to the initiation of the synthesis of viral proteins de novo [14,15]. In addition, G2 arrest enhances viral replication, in part by increasing the activity of the long terminal repeat (LTR) [16]. Other evidence also suggests that Vpr can regulate apoptosis both positively and negatively [17–22]. Vpr has also been reported to produce herniation and disruption of the nuclear envelope, which might be correlated with G2 arrest and suggests the possibility that Vpr might allow PICs to bypass the size restrictions of nuclear pore complexes [23].

Splicing of pre-mRNA is not only a nearly ubiquitous and essential step in gene expression but it is also an important mechanism for the generation of protein diversity and the regulation of gene expression. The splicing reaction is performed by the spliceosome, which consists of five small nuclear ribonucleoprotein (snRNP) complexes, namely, U1, U2, U4, U5, and U6, and a large number of non-snRNPs, which include members of the serine- and arginine-rich (SR)

* Corresponding author. Tel.: +81 48 462 4408; fax: +81 48 462 4399.
E-mail address: aida@riken.jp (Y. Aida).

1286-4579/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.micinf.2005.03.022

family of proteins. The spliceosome acts, through a multitude of RNA–RNA, RNA–protein and protein–protein interactions, to excise each intron precisely and to join exons in the correct order [24]. In mammalian cells, SR proteins and other splicing factors are present in regions of the nucleus known splicing factor compartments, nuclear speckles, or interchromatin granules, and they appear to function at several steps in gene expression [25]. Several viral proteins have also been shown to regulate the splicing of pre-mRNAs. For example, NS1 of influenza virus [26,27] and ICP27 of herpes simplex virus (HSV) [28–31] were reported to interfere with splicing of cellular pre-mRNA as part of the mechanism for blockage of host protein synthesis. Moreover, it has been proposed that production of unspliced or partially spliced transcripts of HIV-1 and other lentiviruses might be mediated by the action of virus-encoded Rev, which binds to a specific RNA sequence (the Rev-responsive element or RRE). Rev binds viral RNAs that contain an RRE [32,33] and it exports HIV-1 RNA to the cytoplasm in a CRM1-dependent manner [34–36].

In the present study, we discovered a novel role for Vpr of HIV-1. We demonstrated that Vpr inhibits the splicing of cellular pre-mRNA both in vivo and in vitro. In analyses by RT-PCR and RNase protection assays, we showed that Vpr-induced the accumulation of unspliced forms of *α-globin 2* and *β-globin* pre-mRNA. Furthermore, we confirmed that Vpr inhibited pre-mRNA splicing in an in vitro splicing assay using *β-globin* pre-mRNA as the substrate. We also present strong evidence that Vpr contributes to selective inhibition during the splicing of cellular pre-mRNA. In addition, we demonstrated that HIV-1 infection was sufficient to inhibit splicing of *α-globin 2* pre-mRNA using vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viruses. Our results reveal a novel function of Vpr and contribute to an enhanced understanding of splicing mechanisms and the life cycle of HIV-1.

2. Materials and methods

2.1. Cells, transfection and extraction of RNA and DNA

Human cervical HeLa cells and human 293T cells were grown in Dulbecco's modified Eagle's medium that contained 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The Jurkat line of human T-lymphoid cells was grown in similarly supplemented RPMI 1640 medium.

Transfections were performed by electroporation in a 4-mm-diameter cuvette using a Gene Pulser (Bio-Rad, Richmond, CA.) at 300 V and 975 μF for HeLa cells and at 260 V and 975 μF for Jurkat cells.

Genomic DNA was extracted from HeLa cells with a Wizard™ genomic DNA purification kit (Promega, Madison, WI.). Total RNA was extracted from HeLa cells and Jurkat cells using TRIzol™ reagent (Invitrogen, Carlsbad, CA.).

2.2. Construction of plasmids

The derivative of the expression vector pME18neo that encodes Flag-tagged wild-type Vpr, namely, pME18Neo-Fvpr, has been described previously [37,38]. To generate the control vector pME18Neo-Stop, in which a stop codon was inserted at the amino terminus of the *vpr* sequences, we performed PCR using pME18Neo-Fvpr as the template and primers VprSTOP (5′-ATCCGAATAAGCCCCAGAAGACC-3′) and PMER2 (5′-GGGGAGGTGTGGGAGGTTTT-3′). Then we subcloned the mutated *vpr* gene between the *Eco*RV and *Not*I sites of pME18Neo-Fvpr. To generate the expression vector for the *β-globin* gene, we amplified the *β-globin* gene, including three exons and two introns, by PCR with genomic DNA from HeLa cells as template and primers βG-1-EcoRV-5′ (5′-GGCGATATCCATGGTGCACCTGACTCCT-3′) and βG-end-XbaI-3′ (5′-GCTCTAGATTAGTGA-TACTTGTGGGC-3′). Then we cloned the amplified fragment between the *Eco*RV and *Xba*I sites of pBluescript II (SK$^+$) (Stratagene, La Jolla, CA.). The resulting construct was designated pSK-β-globin. The *Eco*RV and *Xba*I fragment of pSK-β-globin was excised and subcloned into pME18Neo-HA and encoded the following amino acid sequence: M-A-Y-P-Y-D-V-P-D-Y-A-COOH. To generate the expression vector for the *α-globin 2* gene, we amplified the *α-globin 2* gene, including three exons and two introns, by PCR with genomic DNA from HeLa cells as template and primers HBA2EcoRV (5′-ATCCATGGTGCTGTCTCCTGCC-3′) and HBANotI (5′-CAGCGGCCGCTTAACGGTATTTGGAGG-3′). Then we cloned the amplified fragment between the *Eco*RV and *Not*I sites of pME18Neo-HA.

Infectious molecular clone HIV-1 pNF462 was a kind gift from A. Adachi, Tokushima University, Japan [39]. To generate *env*-negative mutant designating pNF462Δ*env*, frame sift was introduced at *env* region of pNF462 as described below. Parental clone pNF462 was digested using *Bst*EII (TOYOBO, Osaka, Japan) and blunt-ended by KOD (TOYOBO). Then the fragment was self-ligated to introduce 5-base frame sift. To generate *vpr*-negative mutant designating pNF462Δ*env*Δ*vpr*, *Nde*I-*Sal*I fragment of pNL432Δ*vpr* were inserted at *Nde*I-*Sal*I site of pNF462Δ*env*. The generation of pNL432Δ*vpr* was described previously [40]. The expression plasmid of VSV-G, designated pCMV-G, has been previously described [41].

2.3. Reverse transcription-PCR

Samples of RNA were treated with RNase-free DNase I (Invitrogen) for 30 min at room temperature to remove genomic and plasmid DNA. Then 4 μg of total RNA were reverse-transcribed in the presence of oligo(dT) by SuperScript™ II Reverse Transcriptase (Invitrogen) in a total volume of 20 μl.

We amplified intron 1 of endogenous *α-globin 2* pre-mRNA by PCR using primers 5′HBA2E1N1 (5′-TTCTGGTCCCCACAGACTCA-3′) and 3′HBA2E3N1 (5′-

TTATTCAAAGACCAGGAAGGGC-3′) for the first PCR (15 cycles; see below for details of cycles), primers and 5′HBA2I1N2 (5′-GACCCACAGGCCACCCTCAA-3′) and 3′HBA2E3N2 (5′-GTGCTCACAGAAGCCAGGAACTTG-3′) for the second nested PCR or 5′HBA2E1N2 (5′-CCCACCATGGTGCTGTCTCC-3′) and 3′HBA2I2 (5′-CAGTGGCTTAGGAGCTGTGCAG-3′) for 35 cycles, as shown in Fig. 1D. We amplified endogenous *α-globin 2* mRNA by PCR (34 cycles) using primers 5′HBA2E1N1 (5′-TTCTGGTCCCCACAGACTCA-3′) and 3′HBA2E3N1 (5′-TTATTCAAAGACCAGGAAGGGC-3′). Introns 1 and 2 of unspliced *α-globin 2* pre-mRNA and spliced *α-globin 2* mRNA were detected by PCR with 0.5 μl of cDNA as template and the primers shown in Fig. 1D. To detect intron 1 of exogenous *α-globin 2* pre-mRNA, we performed PCR for 32 cycles with primers 5′HBA2I1 (5′-GACCCACAGGCCACCCTCAA-3′) and 3′HBA2E3 (5′-TAACGGTATTTGGAGGTCAGCACG-3′). To detect intron 2 of exogenous *α-globin 2* pre-mRNA, we performed PCR for 34 cycles with primers 5′HBA2E1 (5′-CGAGTATGGTGCGGAGGC-3′) and 3′HBA2I2. To detect exogenous *α-globin 2* mRNA, we performed PCR for 23 cycles with primers 5′HBA2E1 and 3′HBA2E3. We amplified *β-actin* mRNA by PCR (16 cycles) using primers 5′β-act (5′-CGTCGCCCTGGACTTCGAGCA-3′) and 3′β-act (5′-GCTGGAAGGTGGACAGCGAGGCCAGGA-3′). The details of each cycles of PCR were as follows: 2 min at 94 °C; then the indicated number of cycles of incubation at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 45 s; with a final 2-min extension at 72 °C. Reaction products were subjected to electrophoresis on a 2% agarose gel.

All products of PCR were cloned into pBluescript II (SK^+) and their identities were confirmed by nucleotide sequencing by the dideoxy chain-termination method with a CEQ™ 2000 DNA-analysis system (Beckman-Coulter, Fullerton, CA.).

2.4. Quantitative PCR

The real-time quantitative PCR of exogenous *α-globin 2* pre-mRNA was performed on LightCycler system (Roche Diagnostics, Mannheim, Germany) in the presence of LightCycler-FirstStart DNA Master SYBR Green I (Roche Diagnostics) using the following primers AG/1stF (5′-TTCTGGTCCCCACAGACTCA-3′) and AG/1stR (5′-TTATTCAAAGACCAGGAAGGGC-3′) for the first PCR, and AG/2nd/IN1F (5′-AGGCCACCCTCAACCGT-3′) and AGExon2R (5′-CTTGAAGTTGACCGGGTC-3′) for the second PCR. For normalization, quantitation of total *α-globin 2* RNA was performed using primers AG/1stF and AG/1stR for the first PCR, and AGExon2F (5′-GATGTTCCTGTCCTTCCC -3′) and AGExon2R for the second PCR. The first PCR product was treated with ExoSAP-IT™ (containing exonuclease I and shrimp alkaline phosphatase) to remove the first PCR primers according to the manufacturer's instructions (Amersham Bioscience, Uppsara, Sweden) and subjected to the second PCR.

2.5. RNase protection assay

RNase protection assays were performed with an RPA III kit (Ambion, Austin, TX.) according to the instructions in the manual from the manufacture. In brief, 10 μg of total RNA were allowed to hybridize with 8×10^4 cpm ^{32}P-labeled probe overnight at 46 °C. Unprotected single strand RNA was digested with RNases A and T1 and protected fragments were fractionated on a 5% polyacrylamide–7 M urea denaturing gel that was then exposed to an imaging plate (Fuji film, Tokyo, Japan).

For the generation of the probe for detection of splicing of *β-globin* pre-mRNA, we amplified full-length intron 1-exon 2 of *β-globin* by PCR using KOD plus DNA polymerase (TOYOBO) with genomic DNA from HeLa cells as template. We cloned the product of PCR into pBluescript II (SK^+) at the *Eco*RV site. The construct was verified by nucleotide sequencing. After linearization with *Not*I, an antisense probe was generated using a Riboprobe System (Promega) according to the manufacturer's standard protocol, 50 μCi [^{32}P]CTP (PerkinElmer, Boston, MA.) and T7 RNA polymerase. At the end of the reaction, 20 units of RNase-free DNase I (Promega) were added and incubation was continued at 37 °C for 15 min. The probe was then purified by gel filtration on a Sephadex G-50 spin column (Amersham Biosciences) to remove unincorporated nucleotides.

2.6. In vitro splicing assay

The pSK-β-globin plasmid including three exons and two introns, was linearized with *Bam*HI and then transcribed by the Riboprobe system (Promega) with T7 RNA polymerase and 50 μCi [^{32}P]CTP (PerkinElmer). Splicing reactions were carried out as described previously [42]. In brief, approximately 25 fmol of RNA transcript were incubated for 2 h at 30 °C with 60% (v/v) nuclear extract in Dignam's buffer D with 20 mM creatine phosphate, 3 mM $MgCl_2$, 0.8 mM ATP, and 2.6% (w/v) polyvinyl alcohol. HeLa nuclear extracts were prepared basically as described previously [43]. Transcripts were separated on a 7% polyacrylamide–7 M urea denaturing gel which was exposed to an imaging plate.

2.7. Western blotting

Cells were lyzed with a 1% solution of SDS 24 or 48 h after transfection. Lysates were suspended in Laemmli's [44] buffer and equal amounts of total protein were fractionated by SDS-PAGE (15% polyacrylamide). The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA.) for analysis by immunoblotting. Each membrane was blocked for 1 h in a 5% (w/v) solution of skim milk powder in PBS prior to incubation with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO.). Then the membrane was incubated with horseradish peroxidase-linked sheep antibodies against mouse IgG (Amersham Biosciences). Bands of immunoreac-

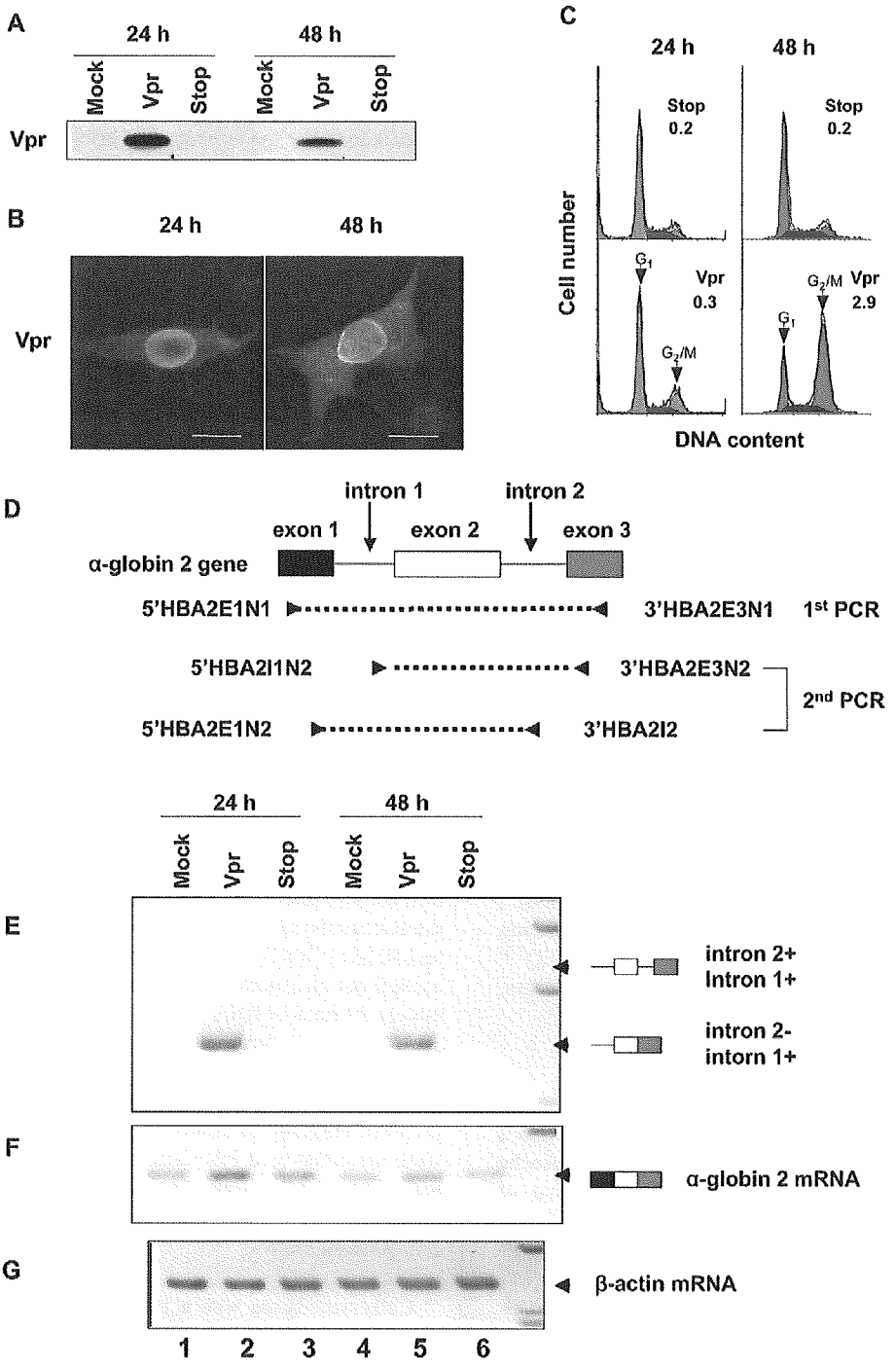


Fig. 1. Vpr prevented splicing of endogenous intron 1 of *α-globin 2* pre-mRNA in HeLa cells. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or with 30 μg of control pME18Neo-Stop (Stop), or they were mock-transfected. Then, 24 and 48 h after transfection, cells were subjected to Western blotting (A), immunofluorescence (B), analysis of the cell cycle (C) and RT-PCR (D–G). (A) Western blotting of Vpr. Transfected cells were lyzed and then lysates containing 25 μg of protein were subjected to Western blotting analysis with the Flag-specific MAb M2. (B) Subcellular localization of Vpr. Transfected cells were subjected to immunofluorescence staining with Flag-specific MAb M2 and Alexa 488-conjugated goat antibodies against mouse IgG and analyzed by confocal laser scanning microscopy. Bar, 20 μm. (C) The DNA content of HeLa cells that expressed Vpr. Transfected cells were treated with the Flag-specific monoclonal antibody M2 and propidium iodide (PI). Cells that bound M2 were quantitated by flow cytometry. Arrowheads indicate peaks of cells at the G1 and G2/M phase. The ratio of cells at G2/M to those at G1 is indicated in the upper right of each graph. (D) Schematic representation of the human *α-globin 2* gene and the positions of primers used for nested RT-PCR. (E) Result of nested RT-PCR for examination of endogenous *α-globin 2* pre-mRNA. Total RNA was extracted from cells, as indicated, and subjected to the first RT-PCR with primers specific for exon 1 (5'HBA2E1N1) and

tive proteins were detected with the SuperSignal™ West Pico chemiluminescent substrate (Pierce, Rockford, IL.).

2.8. Immunofluorescence assay

HeLa cells, growing on coverslips, were examined 24 or 48 h after transfection by an immunofluoresence assay, as described previously [4].

2.9. Analysis of the cell cycle

HeLa cells were harvested 24 or 48 h after transfection and analyzed by flow cytometry for DNA content, as described previously [45].

2.10. Virus infection assay

To generate VSV-G pseudotyped virus, 293T cells (1×10^6 cells) were transfected with 5 μg of VSV-G expression vector together with 10 μg of pNF462Δ*env* or pNF462Δ*env*Δ*vpr*. Supernatants of transfected cells were harvested 24 h after transfection, filtered through 0.45-μm-pore-size filters and treated with DNase I (250 U, Sigma). The amount of HIV-1 p24 antigen was quantified with Lumipulse (Fujirebio, Tokyo Japan). HeLa cells were infected with VSV-G pseudotyped virus containing 100 ng of p24 antigen per 5×10^5 cells.

3. Results

3.1. Accumulation of the incompletely spliced form of endogenous α-globin 2 pre-mRNA

Vpr is involved in the import of the PIC of HIV-1 into the nuclei of non-dividing cells, in cellular differentiation, in the induction of cell cycle arrest at the G2/M phase, in immune suppression, and in enhancement of the replication of the HIV-1 itself [10–14,16,46]. However, although Vpr is a nucleophilic protein with non-classical nuclear localization signals [3,4], the function of Vpr in the nucleus remains to be clarified. To examine whether Vpr might affect splicing by the spliceosome, which is an important event in the nucleus, we transfected HeLa cells with pME18Neo-Fvpr that encoded Flag-tagged Vpr and with the control vector pME18Neo-Stop, in which a stop codon was located at the amino terminus of the *vpr* sequence. We extracted total cellular RNA and examined levels of unspliced endogenous human *α-globin 2* pre-mRNA and of spliced mRNA by nested RT-PCR (Fig. 1). We selected the human gene for *α-globin 2* as the cellular target gene, because it is a simple construct with only three exons and two introns and the length of the coding region, including two introns, is only 685 bps. Vpr was expressed at detectable levels within 24 h after transfection (Fig. 1A) and was localized predominantly in the nucleus and nuclear envelope (Fig. 1B). However, it was minimally effective in inducing G2 arrest (Fig. 1C). To our surprise *α-globin 2* pre-mRNA containing intron 1 but not intron 2 was clearly detectable in HeLa cells that had been transiently transfected with the Vpr-coding vector after the second PCR with forward primer 5′HBA2I1N2, which is located in intron 1, and reverse primer 3′HBA2E3N2, which is located in exon 3. We sequenced the fragment obtained by PCR and confirmed that the fragment was *α-globin 2* pre-mRNA that retained intron 1 but lacked intron 2 (data not shown). By contrast, pre-mRNA that contained intron 2 did not accumulate in cells that had been transfected with the Vpr-coding vector, or in cells transfected with the control vector pME18Neo-Stop and in mock-transfected cells. In addition, the second PCR with forward primer 5′HBA2E1N2, which spans exon 1, and reverse primer 3′HBA2I2, which spans intron 2, yielded neither of two possible types of pre-mRNA that contained intron 2 (data not shown). By contrast, in all cells transfected with the control vector pME18Neo-Stop and all mock-transfected cells, essentially all of the *α-globin 2* pre-mRNA had been spliced and spliced mRNA was produced (Figs. 1E, F). The levels of expression of *β-actin* mRNA were the same in all samples examined (Fig. 1G), as were levels of mRNAs that encoded GAPDH and L13a (data not shown). Our results indicated that splicing of *α-globin 2* pre-mRNA had been partially inhibited by overexpression of Vpr, allowing incompletely spliced pre-mRNA to accumulate. Moreover, we obtained similar results later in 48 h period after transfection, namely, during the time when Vpr is able to induced significant G2 arrest, as shown in Fig. 1C. These observations suggest that the Vpr-induced inhibition of splicing and G2 arrest are independent phenomena.

3.2. Vpr inhibits splicing of exogenous β-globin pre-mRNAs

To confirm that Vpr affects splicing of pre-mRNA in vivo, we selected the human gene for *β-globin*, which is also a comparatively simple construct. We produced a derivative of pME18Neo that included the *β-globin* gene, with three exons and two introns, under the control of the SRα promoter, as shown in Fig. 2A. Then, we transiently transfected HeLa cells with two pME18Neo expression vectors, namely, one that encoded Vpr and one that encoded *β-globin* pre-mRNA. We examined the splicing of *β-globin* pre-mRNA by RNase protection analysis using a ^{32}P-labeled antisense probe specific for *β-globin* pre-mRNA (Fig. 2). This probe was able to dif-

exon 3 (3′HBA2E3N1), and then the second nested RT-PCR with primers specific for intron 1 (5′HBA2I1N2) and exon 3 (3′HBA2E3N2), or with specific primers of exon 1 (5′HBA2E1N2) and intron 2 (3′HBA2I2). (F) Result of RT-PCR for examination of *α-globin 2* mRNA with primers specific for exon 1 (5′HBA2E1N1) and exon 3 (3′HBA2E3N1). (G) Result of RT-PCR for examination of *β-actin* mRNA. The products of PCR were subjected to electrophoresis on a 2% agarose gel. The data are representative of five independent experiments that gave similar results.

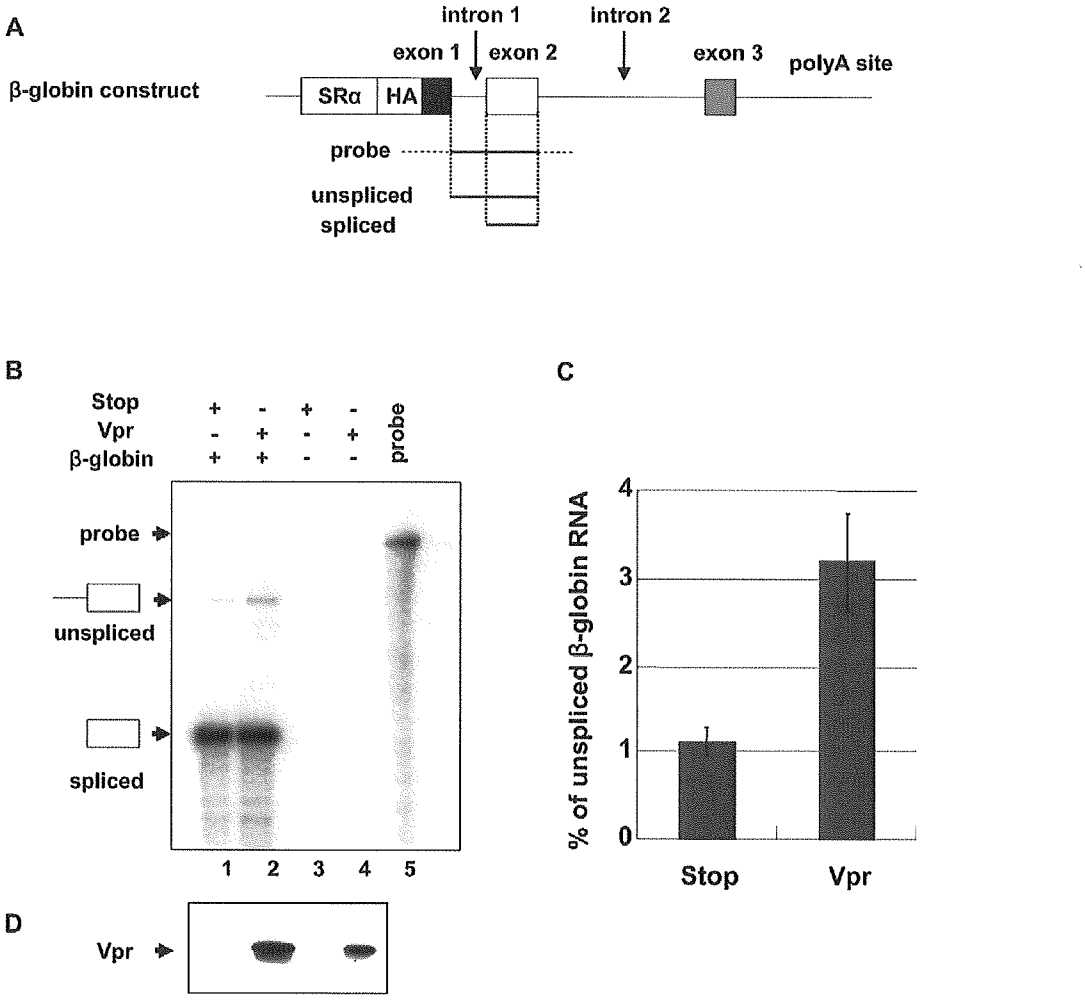


Fig. 2. Vpr inhibited the splicing of exogenous *β-globin* pre-mRNA in HeLa cells that had been transfected with a vector that encoded Vpr and a vector that encoded *β-globin* pre-mRNA. (A) The pME18Neo construct, showing the *β-globin* gene that contained three exons and two introns under the control of the SRα promoter. Bar shows the location of the antisense probe specific for *β-globin* pre-mRNA. In the RNase protection assay, this probe allowed discrimination between unspliced pre-mRNA and spliced mRNA, as indicated. B-D, HeLa cells were cotransfected with 25 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (lanes 2 and 4) or control pME18Neo-Stop (lanes 1 and 3) together 5 μg of pME18Neo that encoded *β-globin* pre-mRNA (lanes 1 and 2). HeLa cells were harvested 24 h after transfection and divided into two portions. (B) Some cells were subjected to an RNase protection assay. Total RNA was extracted from cells and subjected to an RNase protection assay with the ^{32}P-labeled *β-globin* antisense probe. After treatment with RNases A and T1, the protected fragments were separated by electrophoresis on a 5% polyacrylamide–7 M urea denaturing gel. Arrows indicate positions of the intact probe, of unspliced *β-globin* RNA (intron 1+), and of spliced *β-globin* RNA (intron 1-). (C) Intensities of the unspliced and spliced β-globin pre-mRNA signals were quantitated using BAS2500 (Fujifilm Co., Tokyo, Japan) and percentage of the intensity of unspliced *β-globin* RNA against that of unspliced plus spliced *β-globin* RNA was calculated in each transfection. Each column and error bar represent the mean ± S.D. of results from three independent experiments. (D) The remaining cells were subjected to Western blotting with the Flag-specific monoclonal antibody M2 to determine levels of expression of Vpr. The data are representative of three independent experiments that gave similar results.

ferentiate unspliced *β-globin* pre-mRNA that contained intron 1 from spliced mRNA, as shown in Fig. 2A. Protected fragments were fractionated on a 5% acrylamide–7 M urea denaturing gel. We found that, 24 h after transfection, pre-mRNA that contained intron 1 modestly accumulated in cells that had been transfected with pME18Neo-Fvpr plus the *β-globin* expression vector, as compared to levels in HeLa cells that had been transfected with pME18Neo-Stop plus the *β-globin* expression vector (Fig. 2B, lanes 1 and 2). The percentage of unspliced *β-globin* RNA against total *β-globin* RNA in HeLa cells that had been transfected with pME18Neo-Fvpr was approximately threefold higher than that in HeLa cells that had been transfected with control vector pME18Neo-Stop (Fig. 2C). Thus, it appeared that Vpr had modestly inhibited splicing of exogenous *β-globin* pre-mRNA in vivo. Furthermore, *β-globin* pre-mRNA was spliced and spliced *β-globin* mRNA also accumulated. By contrast, no bands of unspliced pre-mRNA and spliced mRNA were detected in the analysis of RNA from HeLa cells had not been transfected with the *β-globin* expression vector (Fig. 2B, lanes 3 and 4), indicating the absence of endogenous *β-globin* pre-mRNA and spliced mRNA in this system. Moreover, we obtained similar

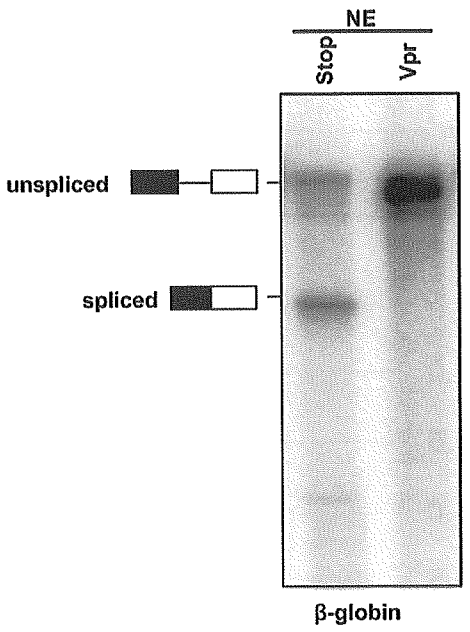


Fig. 3. Vpr inhibited splicing in vitro. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or 30 μg of control pME18Neo-Stop (Stop). Then, 24 h after transfection, nuclear extracts (NE) were prepared from cells and subjected to in vitro splicing assay with ^{32}P-labeled *β-globin* pre-mRNA as substrate. The products were separated on a 7% polyacrylamide–7 M urea denaturing gel.

results in RNase protection analysis with a derivative of pME18Neo that included the *α-globin 2* gene with three exons and two introns under the control of the SRα promoter (data not shown). Since our findings tended to confirm the results of amplification by nested RT-PCR of the endogenous pre-mRNA, it appeared that Vpr might partially inhibit the splicing of *β-globin* and *α-globin 2* pre-mRNAs in vivo.

3.3. Vpr inhibits pre-mRNA splicing in vitro

To obtain definitive evidence that Vpr acts on splicing, we included this protein in in vitro splicing assays with a nuclear extract (NE) of HeLa cells that had been transiently transfected with either pME18Neo-Fvpr or pME18Neo-Stop (Fig. 3). As a substrate we used ^{32}P-labeled *β-globin* pre-mRNA with a G capped at the 5′ end. As expected, the splicing of *β-globin* was dramatically suppressed by the nuclear extract from HeLa cells that expressed Vpr. By contrast, splicing activity of a nuclear extract from HeLa cells that had been transfected with pME18Neo-Stop was sufficient to splice *β-globin* pre-mRNA. This in vitro splicing assay demonstrated that Vpr regulated the splicing of *β-globin* pre-mRNA.

3.4. Vpr inhibits splicing of exogenous α-globin 2 pre-mRNA

As shown in Fig. 1, amplification of endogenous *α-globin 2* pre-mRNA by nested RT-PCR demonstrated that pre-mRNA that contained only intron 1 and not intron 2 accumulated in the presence of Vpr. Thus, inhibition of splicing upon expression of Vpr might be sequence-specific. Therefore, we examined the effects of the expression of Vpr on cellular splicing by RT-PCR using total RNA from Jurkat cells that had been transfected with pME18Neo-Fvpr or pME18Neo-Stop and a derivative of pME18Neo that included the *α-globin 2* gene (Fig. 4). In this experiment, we used Jurkat cells, a line of human T-lymphoid cells that is permissive with respect to infection by HIV-1 and from which it is not possible to amplify the endogenous *α-globin 2* pre-mRNA by nested RT-PCR. In our analysis, 24 h after transfection, we detected three products amplification by RT-PCR of pre-mRNA. They contained intron 1 but not intron 2 (Fig. 4Bb), intron1 and intron 2 (Fig. 4Ba, Bc), and intron 2 but not intron 1 (Fig. 4Bd) both in the absence and in the presence of Vpr. The levels of *β-actin* mRNA were similar in all samples examined (Fig. 4B). These results indicate that splicing of intron 1 might be partially inhibited by Vpr, while splicing of intron 2 was unaffected. We obtained similar results in HeLa cells (data not shown). Collectively, our results indicate that Vpr of HIV-1 is involved in selective inhibition during the splicing of cellular pre-mRNAs.

3.5. Vpr expressed from an HIV-1 provirus accumulates endogenous α-globin 2 pre-mRNA

To monitor the potential of Vpr to inhibit the splicing of cellular pre-mRNAs, we assessed whether HIV-1 infection accumulated endogenous *α-globin 2* pre-mRNA. HeLa cells were infected with 100 ng equivalent of p24 of VSV-G pseudotyped HIV-1 *vpr*$^+$ or *vpr*$^-$ virus. Then 24 h after infection, total RNA was isolated from HeLa cells and real-time quantitative RT-PCR were carried out to quantitate the level of endogenous *α-globin 2* pre-mRNA. The level of total *α-globin 2* RNA was measured for normalization and fold-production of the level of *α-globin 2* pre-mRNA that contains intron 1 was quantified (Fig. 5B). Interestingly, a higher level of *α-globin 2* pre-mRNA that contains intron 1 was detected in HeLa cells infected with *vpr*$^+$ virus than that in HeLa cells infected with *vpr*$^-$ virus. This result indicated that Vpr produced from an HIV-1 provirus was sufficient to accumulate endogenous *α-globin 2* pre-mRNA in an infection system as well as in a transient transfection system.

4. Discussion

Our present results lead to three major conclusions. First, the present study reveals that Vpr, when expressed transiently in cells, can regulate the splicing reaction of cellular pre-mRNA both in vivo and in vitro. Using RT-PCR and RNase protection assays, we showed that Vpr induced the accumulation of unspliced forms of *α-globin 2* and *β-globin* pre-mRNAs in HeLa cells that expressed Vpr. We confirmed the modulation of splicing by Vpr in in vitro splicing assays

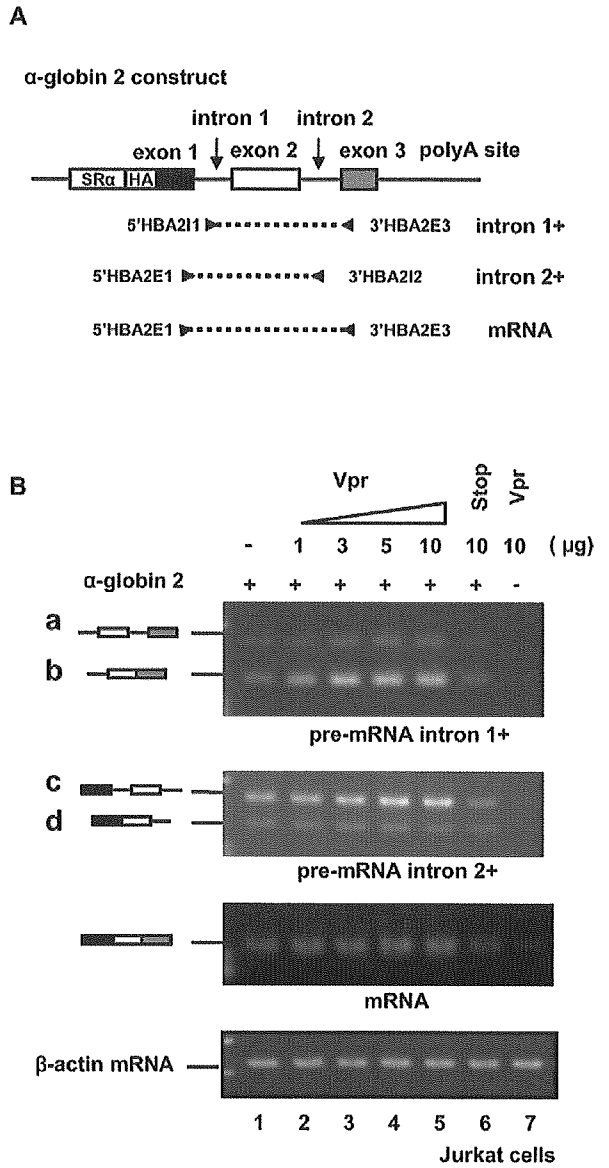


Fig. 4. Analysis by RT-PCR of exogenous *α-globin 2* pre-mRNA in Jurkat cells that had been transfected with a vector that encoded Vpr and a vector that encoded *α-globin 2* pre-mRNA. (A) The pME18Neo construct including *α-globin 2* gene that contained three exons and two introns under the control of the SRα promoter and the position of each primer used for RT-PCR. (B) Jurkat cells were transfected with 1, 3, 5 and 10 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (lanes 2–5), 10 μg of control pME18Neo-Stop (lane 6) or none of this plasmid (lane 1), and 1 μg of pME18Neo that encoded *α-globin 2* pre-mRNA (lanes 1 and 2). Then, 24 h after transfection, RT-PCR was performed with a pair of primers specific for *α-globin 2* pre-mRNA, and products of PCR were subjected to electrophoresis on a 2% agarose gel. RT-PCR to amplify cellular *β-actin* mRNA was performed as a control. Total amounts of DNA were equalized by addition of control pME18neo. The data are representative of results of three independent experiments.

as follows. The splicing of *β-globin* pre-mRNA was dramatically suppressed when a nuclear extract from HeLa cells that expressed Vpr was added to an in vitro splicing system, sug-

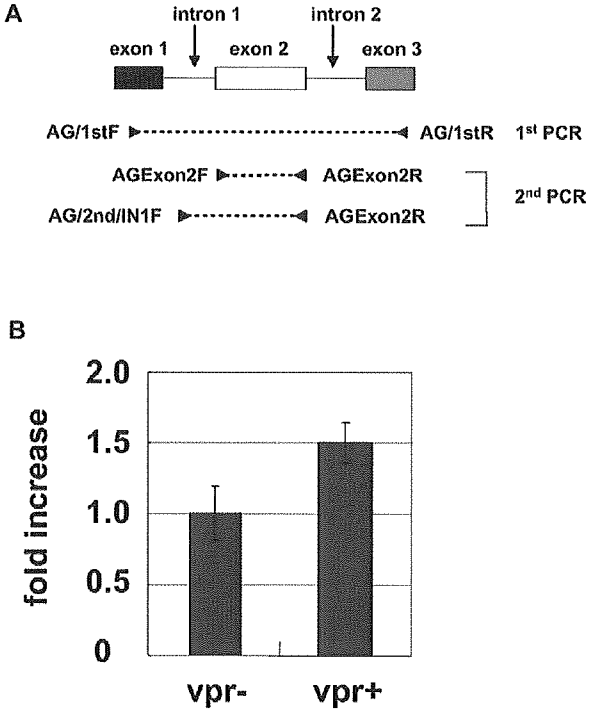


Fig. 5. Vpr expressed from an HIV-1 provirus accumulates endogenous α-globin2 pre-mRNA. Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viruses were harvested from 293T cell cultures 24 h after cotransfection with VSV-G expression vector together with pNF462Δ*env* or pNF462Δ*env*Δ*vpr*. HeLa cells (5×10^5) were infected with 100 ng of p24 antigen equivalent of pseudotyped viruses. Two hours after infection, cells were washed with serum-free medium twice and cultured with fresh medium containing 10% fetal bovine serum. Then, 24 h after infection, quantitative RT-PCR was performed using specific primers for total *α-globin 2* RNA or *α-globin 2* pre-mRNA that contains intron 1. (A) Schematic representation of *α-globin 2* gene and the position of primers used for quantitative RT-PCR. (B) Fold increase of the level of *α-globin 2* pre-mRNA that contains intron 1. Signals of amplification products were normalized by those of total *α-globin 2* RNA. Each column and error bar represent the mean ± S.D. of results from three independent experiments.

gesting that Vpr had an effect on the splicing machinery. The level of inhibition of splicing by Vpr was modest in vivo but high in vitro. The reason why such difference arose between in vivo and in vitro was not clear, but there was a possibility that some factors that could alleviate the inhibition of splicing by Vpr in vivo existed at low levels or lacked in HeLa nuclear extract that used in the in vitro splicing assay. Thus, the mechanism by which Vpr inhibits pre-mRNA splicing appears to be novel. Second, although the inhibition of splicing induced by Vpr was not as strong as that shown in a transient transfection system, we also indicated strong evidence that Vpr expressed from an HIV-1 provirus was sufficient to accumulate endogenous α-globin 2 pre-mRNA in an infection system using VSV-G pseudotyped HIV-1 vpr^+ or vpr^- virus. Third, our results also indicate the potential sequence-specific nature of the Vpr-induced modulation of splicing. Amplification of endogenous *α-globin 2* pre-mRNA by nested RT-PCR demonstrated that pre-mRNA that contained only intron 1 and not intron 2 accumulated in presence of Vpr.

Moreover, RT-PCR with total RNA from Jurkat cells that had been transfected with a pME18Neo-Fvpr plus a derivative of a pME18Neo that included the *α-globin 2* gene revealed that Vpr-induced an increase in the accumulation of all products of pre-mRNA that contained intron 1. Further studies are required to clarify why Vpr inhibit splicing of the particular intron.

The NS1 protein of influenza virus [26,27,47] and the ICP27 protein of HSV [29–31,48] have been reported to inhibit the splicing of cellular pre-mRNA, perhaps as a part of the mechanism for shutting down the synthesis of host proteins. The NS1 protein binds to U6 small nuclear RNA, inhibiting the formation of U4/U6 and U2/U6 complex [47], while ICP27 inhibits splicing by interfering with assembly of spliceosomes [31]. In addition, it has been proposed that ICP27 interacts with SRPK1 and inhibits splicing by altering the phosphorylation of SR protein [48]. Moreover, ICP27 also interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step [30]. It has been demonstrated that Vpr binds to ribonucleic acid via a process that requires the carboxy-terminal basic domain of the protein (in particular the helical 70–80 domain) [49], which suggests the possibility of a functional association with pre-mRNA. Indeed, we have preliminary evidence (Kuramitsu and Aida, personal communication) that a carboxy-terminal domain of Vpr is essential for the inhibition of splicing. Therefore, our present results and the previous demonstration that NS1 and ICP27 can associate with spliceosomes that contained splicing intermediates suggest that Vpr might interact with spliceosomes, which inhibit splicing at the stage when the splicing complex is formed. It is also possible that Vpr inhibits splicing by preventing pre-mRNA from association with spliceosomes or stabilizing pre-mRNA through the association with pre-mRNA. These issues will be further elucidated.

The correlations between the novel ability of Vpr to inhibit splicing of cellular pre-mRNA and the previously characterized functions of Vpr in phenomena such as LTR activation [16], apoptosis [50] and G2 arrest [10–13], suggest some intriguing possibilities. Splicing and transcription are tightly coupled [51]. For example, spliceosomal UsnRNP forms a complex with elongation factor TAT-SF1, which associates with RNA polymerase II (pol II) via the carboxy-terminal domain (CTD) kinase PTEFb. This UsnRNP-TAT-SF1 complex stimulates both transcription and splicing in vitro [52]. Kino and Pavlakis [53] have reported that hsRBP7, a subunit of pol II, bind Vpr in a yeast two-hybrid screening assay. This observation strongly suggests that Vpr might regulate transcription via an interaction with hsRBP7 and might then coincidentally, participate in the splicing reaction. Moreover, apoptosis and splicing influence one another. For example, the cellular apoptosis-promoting factor TIA-1 is a regulator of the splicing of pre-mRNA [54,55]. However, it remains unclear whether Vpr can inhibit splicing via these two processes. By contrast, Vpr-induced G2 arrest might be a key event related to the inhibition of splicing by Vpr. Roshal et al. [56] showed that treatment with LY294002, an inhibitor of phosphatidylinositol 3-kinase, alleviated Vpr-induced G2 arrest in HeLa cells. Moreover, recent reports indicated that RNA processing is also the target of several signal-transduction pathways, including phosphatidylinositol 3-kinase pathway [57,58]. However, in the present study, we detected the accumulation of pre-mRNA during the 24-h period after transfection, namely, during the time when Vpr is unable to induce G2 arrest. This result clearly indicates that the inhibition of splicing by Vpr is not the result of G2 arrest. Further studies are required to determine whether inhibition of the splicing of pre-mRNA influences the induction of G2 arrest by Vpr.

The generation of the 2 or 4-kb form of HIV-1 RNA from unspliced 9-kb genomic RNA by splicing is orchestrated by *cis* elements like exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing silencers, and the viral protein Rev [59–64]. It has been suggested that, Rev-mediated nuclear export of incompletely spliced HIV-1 RNA regulates splicing of HIV-1 RNA [63]. However, splicing of HIV-1 RNA is also controlled by many cellular splicing factors, such as SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). For example, the generation of tat mRNA is regulated via splicing acceptor site A3. Splicing at the A3 site is regulated by ESS2p [62], ESE2 [60] and ESS2 [61]. The ribonucleoprotein designated hnRNP H binds to the ESS2p element to repress activity at splice site A3. SC35 binds to the ESE2 to activate splicing, whereas hnRNP A1 binds ESS2 to repress splicing. ESE2 and ESS2 overlap and binding of hnRNP A1 to ESS2 masks binding site for SC35 and inhibits splicing at the A3 site. By contrast, it has been suggested that Vpr acts multifunctionally via interactions with numerous cellular partner molecules, such as the 14-3-3, the p300/CREB-binding protein, and the importin α [53]. Therefore, it is possible that Vpr might be associated not only with host splicing but also with the alternative splicing of HIV-1 RNA. However, we do not know whether Vpr interacts with regulators of splicing that control splicing of HIV-1 RNA. Thus, while our present results suggest that Vpr inhibits splicing of cellular pre-mRNA, it remains to be determined whether Vpr regulates splicing of the HIV-1 genome. Our understanding of the life cycle of HIV-1 and the progression of AIDS pathogenesis will be enhanced as we improve our understanding of the roles of Vpr both in cellular splicing and in the life cycle of HIV-1.

Acknowledgments

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 Hearth, 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.

We thank Dr. K. Nagata, Mrs. K. Sugiyama and K. Murano (University of Tsukuba, Ibaraki, Japan) for helpful discussions and technical advice and to Mrs. K. Muneta and Mrs. K. Kimata (Retrovirus Research Unit, RIKEN) for their skilled technical assistance.

References

[1] X. Yuan, Z. Matsuda, M. Matsuda, M. Essex, T.H. Lee, Human immunodeficiency virus vpr gene encodes a virion-associated protein, AIDS Res. Hum. Retroviruses 6 (1990) 1265–1271.

[2] E.A. Cohen, G. Dehni, J.G. Sodroski, W.A. Haseltine, Human immunodeficiency virus vpr product is a virion-associated regulatory protein, J. Virol. 64 (1990) 3097–3099.

[3] Y. Jenkins, M. McEntee, K. Weis, W.C. Greene, Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways, J. Cell Biol. 143 (1998) 875–885.

[4] M. Kamata, Y. Aida, Two putative alpha-helical domains of human immunodeficiency virus type 1 Vpr mediate nuclear localization by at least two mechanisms, J. Virol. 74 (2000) 7179–7186.

[5] N.K. Heinzinger, M.I. Bukinsky, S.A. Haggerty, A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson, M. Emerman, 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 (1994) 7311–7315.

[6] R.I. Connor, B.K. Chen, S. Choe, N.R. Landau, Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes, Virology 206 (1995) 935–944.

[7] P. Gallay, V. Stitt, C. Mundy, M. Oettinger, D. Trono, Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import, J. Virol. 70 (1996) 1027–1032.

[8] R.A. Fouchier, B.E. Meyer, J.H. Simon, U. Fischer, A.V. Albright, F. Gonzalez-Scarano, M.H. Malim, Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex, J. Virol. 72 (1998) 6004–6013.

[9] S. Popov, M. Rexach, L. Ratner, G. Blobel, M. Bukrinsky, Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex, J. Biol. Chem. 273 (1998) 13347–13352.

[10] J. He, S. Choe, R. Walker, P. Di Marzio, D.O. Morgan, N.R. Landau, Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity, J. Virol. 69 (1995) 6705–6711.

[11] J.B. Jowett, V. Planelles, B. Poon, N.P. Shah, M.L. Chen, I.S. Chen, The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle, J. Virol. 69 (1995) 6304–6313.

[12] F. Re, D. Braaten, E.K. Franke, J. Luban, Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B, J. Virol. 69 (1995) 6859–6864.

[13] M.E. Rogel, L.I. Wu, M. Emerman, The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection, J. Virol. 69 (1995) 882–888.

[14] B. Poon, K. Grovit-Ferbas, S.A. Stewart, I.S. Chen, Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents, Science 281 (1998) 266–269.

[15] M. Hrimech, X.J. Yao, F. Bachand, N. Rougeau, E.A. Cohen, Human immunodeficiency virus type 1 (HIV-1) Vpr functions as an immediate-early protein during HIV-1 infection, J. Virol. 73 (1999) 4101–4109.

[16] W.C. Goh, M.E. Rogel, C.M. Kinsey, S.F. Michael, P.N. Fultz, M.A. Nowak, B.H. Hahn, M. Emerman, HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo, Nat. Med. 4 (1998) 65–71.

[17] V. Ayyavoo, A. Mahboubi, S. Mahalingam, R. Ramalingam, S. Kudchodkar, W.V. Williams, B.H. Hahn, M. Emerman, HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B, Nat. Med. 3 (1997) 1117–1123.

[18] M. Nishizawa, M. Kamata, T. Mojin, Y. Nakai, Y. Aida, Induction of apoptosis by the Vpr protein of human immunodeficiency virus type 1 occurs independently of G(2) arrest of the cell cycle, Virology 276 (2000) 16–26.

[19] E. Jacotot, L. Ravagnan, M. Loeffler, K.F. Ferri, H.L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J.P. Briand, T. Irinopoulou, E. Daugas, S.A. Susin, D. Cointe, Z.H. Xie, J.C. Reed, B.P. Roques, G. Kroemer, The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore, J. Exp. Med. 191 (2000) 33–46.

[20] S.A. Stewart, B. Poon, J.Y. Song, I.S. Chen, Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation, J. Virol. 74 (2000) 3105–3111.

[21] K. Muthumani, D.S. Hwang, B.M. Desai, D. Zhang, N. Dayes, D.R. Green, D.B. Weiner, HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells, J. Biol. Chem. 277 (2002) 37820–37831.

[22] T. Roumier, H.L. Vieira, M. Castedo, K.F. Ferri, P. Boya, K. Andreau, S. Druillennec, N. Joza, J.M. Penninger, B. Roques, G. Kroemer, The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway, Cell Death Differ. 9 (2002) 1212–1219.

[23] C.M. de Noronha, M.P. Sherman, H.W. Lin, M.V. Cavrois, R.D. Moir, R.D. Goldman, W.C. Greene, Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr, Science 294 (2001) 1105–1108.

[24] J.F. Caceres, A.R. Kornblihtt, Alternative splicing: multiple control mechanisms and involvement in human disease, Trends Genet. 18 (2002) 186–193.

[25] A.I. Lamond, D.L. Spector, Nuclear speckles: a model for nuclear organelles, Nat. Rev. Mol. Cell Biol. 4 (2003) 605–612.

[26] Y. Lu, X.Y. Qian, R.M. Krug, The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing, Genes Dev. 8 (1994) 1817–1828.

[27] P. Fortes, A. Beloso, J. Ortin, Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport, EMBO J. 13 (1994) 704–712.

[28] M.A. Hardwicke, R.M. Sandri-Goldin, The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection, J. Virol. 68 (1994) 4797–4810.

[29] W.R. Hardy, R.M. Sandri-Goldin, Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect, J. Virol. 68 (1994) 7790–7799.

[30] H.E. Bryant, S.E. Wadd, A.I. Lamond, S.J. Silverstein, J.B. Clements, Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step, J. Virol. 75 (2001) 4376–4385.

[31] A. Lindberg, J.P. Kreivi, Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27, Virology 294 (2002) 189–198.

[32] M.H. Malim, J. Hauber, S.Y. Le, J.V. Maizel, B.R. Cullen, The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA, Nature 338 (1989) 254–257.

[33] U. Fischer, J. Huber, W.C. Boelens, I.W. Mattaj, R. Luhrmann, The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs, Cell 82 (1995) 475–483.

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

[35] M. Neville, F. Stutz, L. Lee, L.I. Davis, M. Rosbash, The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export, Curr. Biol. 7 (1997) 767–775.

[36] S. Nakielny, G. Dreyfuss, Transport of proteins and RNAs in and out of the nucleus, Cell 99 (1999) 677–690.
[37] Y. Nishino, T. Myojin, M. Kamata, Y. Aida, Human immunodeficiency virus type 1 Vpr gene product prevents cell proliferation on mouse NIH3T3 cells without the G2 arrest of the cell cycle, Biochem. Biophys. Res. Commun. 232 (1997) 550–554.
[38] M. Nishizawa, T. Myojin, Y. Nishino, Y. Nakai, M. Kamata, Y. Aida, A carboxy-terminally truncated form of the Vpr protein of human immunodeficiency virus type 1 retards cell proliferation independently of G(2) arrest of the cell cycle, Virology 263 (1999) 313–322.
[39] M. Kawamura, T. Ishizaki, A. Ishimoto, T. Shioda, T. Kitamura, A. Adachi, Growth ability of human immunodeficiency virus type 1 auxiliary gene mutants in primary blood macrophage cultures, J. Gen. Virol. 75 (Pt. 9) (1994) 2427–2431.
[40] S. Iijima, Y. Nitahara-Kasahara, K. Kimata, W. Zhong Zhuang, M. Kamata, M. Isogai, M. Miwa, Y. Tsunetsugu-Yokota, Y. Aida, Nuclear localization of Vpr is crucial for the efficient replication of HIV-1 in primary CD4+ T cells, Virology 327 (2004) 249–261.
[41] J.K. Yee, A. Miyanohara, P. LaPorte, K. Bouic, J.C. Burns, T. Friedmann, A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes, Proc. Natl. Acad. Sci. USA 91 (1994) 9564–9568.
[42] A. Mayeda, A.R. Krainer, Mammalian in vitro splicing assays, Methods Mol. Biol. 118 (1999) 315–321.
[43] K.A. Lee, M.R. Green, Small-scale preparation of extracts from radiolabeled cells efficient in pre-mRNA splicing, Methods Enzymol. 181 (1990) 20–30.
[44] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[45] M. Nishizawa, M. Kamata, R. Katsumata, Y. Aida, A carboxy-terminally truncated form of the human immunodeficiency virus type 1 Vpr protein induces apoptosis via G(1) cell cycle arrest, J. Virol. 74 (2000) 6058–6067.
[46] D.N. Levy, L.S. Fernandes, W.V. Williams, D.B. Weiner, Induction of cell differentiation by human immunodeficiency virus 1 vpr, Cell 72 (1993) 541–550.
[47] Y. Qiu, M. Nemeroff, R.M. Krug, The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing, RNA 1 (1995) 304–316.
[48] K.S. Sciabica, Q.J. Dai, R.M. Sandri-Goldin, ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation, EMBO J. 22 (2003) 1608–1619.
[49] H. de Rocquigny, A. Caneparo, T. Delaunay, J. Bischerour, J.F. Mouscadet, B.P. Roques, Interactions of the C-terminus of viral protein R with nucleic acids are modulated by its N-terminus, Eur. J. Biochem. 267 (2000) 3654–3660.
[50] K. Muthumani, A.Y. Choo, D.S. Hwang, M.A. Chattergoon, N.N. Dayes, D. Zhang, M.D. Lee, U. Duvvuri, D.B. Weiner, Mechanism of HIV-1 viral protein R-induced apoptosis, Biochem. Biophys. Res. Commun. 304 (2003) 583–592.
[51] D. Bentley, The mRNA assembly line: transcription and processing machines in the same factory, Curr. Opin. Cell Biol. 14 (2002) 336–342.
[52] Y.W. Fong, Q. Zhou, Stimulatory effect of splicing factors on transcriptional elongation, Nature 414 (2001) 929–933.
[53] T. Kino, G.N. Pavlakis, Partner molecules of accessory protein Vpr of the human immunodeficiency virus type 1, DNA Cell Biol. 23 (2004) 193–205.
[54] F. Del Gatto-Konczak, C.F. Bourgeois, C. Le Guiner, L. Kister, M.C. Gesnel, J. Stevenin, R. Breathnach, The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5' splice site, Mol. Cell. Biol. 20 (2000) 6287–6299.
[55] P. Forch, O. Puig, N. Kedersha, C. Martinez, S. Granneman, B. Seraphin, P. Anderson, J. Valcarcel, The apoptosis-promoting factor TIA-1 is a regulator of alternative pre-mRNA splicing, Mol. Cell 6 (2000) 1089–1098.
[56] M. Roshal, B. Kim, Y. Zhu, P. Nghiem, V. Planelles, Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R, J. Biol. Chem. 278 (2003) 25879–25886.
[57] X. Liu, A. Mayeda, M. Tao, Z.M. Zheng, Exonic splicing enhancer-dependent selection of the bovine papillomavirus type 1 nucleotide 3225 3' splice site can be rescued in a cell lacking splicing factor ASF/SF2 through activation of the phosphatidylinositol 3-kinase/Akt pathway, J. Virol. 77 (2003) 2105–2115.
[58] N.A. Patel, C.E. Chalfant, J.E. Watson, J.R. Wyatt, N.M. Dean, D.C. Eichler, D.R. Cooper, Insulin regulates alternative splicing of protein kinase C beta II through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells, J. Biol. Chem. 276 (2001) 22648–22654.
[59] T.O. Tange, C.K. Damgaard, S. Guth, J. Valcarcel, J. Kjems, The hnRNP A1 protein regulates HIV-1 tat splicing via a novel intron silencer element, EMBO J. 20 (2001) 5748–5758.
[60] A.M. Zahler, C.K. Damgaard, J. Kjems, M. Caputi, SC35 and heterogeneous nuclear ribonucleoprotein A/B proteins bind to a juxtaposed exonic splicing enhancer/exonic splicing silencer element to regulate HIV-1 tat exon 2 splicing, J. Biol. Chem. 279 (2004) 10077–10084.
[61] B.A. Amendt, D. Hesslein, L.J. Chang, C.M. Stoltzfus, Presence of negative and positive cis-acting RNA splicing elements within and flanking the first tat coding exon of human immunodeficiency virus type 1, Mol. Cell. Biol. 14 (1994) 3960–3970.
[62] S. Jacquenet, A. Mereau, P.S. Bilodeau, L. Damier, C.M. Stoltzfus, C. Branlant, A second exon splicing silencer within human immunodeficiency virus type 1 tat exon 2 represses splicing of Tat mRNA and binds protein hnRNP H, J. Biol. Chem. 276 (2001) 40464–40475.
[63] B.R. Cullen, Nuclear mRNA export: insights from virology, Trends Biochem. Sci. 28 (2003) 419–424.
[64] M. Caputi, A. Mayeda, A.R. Krainer, A.M. Zahler, hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing, EMBO J. 18 (1999) 4060–4067.