Table 1
Cell cycle arrest at G_1 by Vpr and C81

	24 h			48 h			72 h		
Phase/% Vpr-positive cells	Stop	Vpr	C81	Stop	Vpr	C81	Stop	Vpr	C81
G1	51.9	71.1	71.9	43.3	68.3	73.1	69.6	78.0	73.3
S	35.0	13.1	13.7	46.3	14.1	11.4	12.8	7.0	10.3
G2/M	13.1	15.6	14.4	10.4	17.5	15.6	17.7	15.0	16.5
Vpr-positive cells(%)	–	(39.8)	(34.2)	–	(12.1)	(6.3)	–	(5.7)	(1.1)

BHK cells were transfected with the Stop, Vpr and C81 expression plasmids. Cells were harvested at the indicated time, and cells were incubated with biotin-labeled Flag-specific antibody and then with streptavidin-FITC for detection of cells that expressed Vpr. Then they were stained with PI for analysis of DNA content and analyzed by flow cytometry. Cell cycle distributions were analyzed with ModFit LT software as noted in the text.

of the cell cycle. As shown in Fig. 1C, the majority of Rb in control cells was in a hyperphosphorylated form (upper major band). However, in cell extracts prepared from cells that expressed Vpr or C81, we observed a decrease in the level of the hyperphosphorylated form of Rb and an increase in the level of underphosphorylated form of Rb after 24 h and 48 h. Thus, the expression of Vpr resulted in the accumulation of underphosphorylated Rb, and the arrest of BHK cells in the G_1 phase of the cell cycle.

3.2. Vpr induces apoptosis in rodent cells

The expression of Vpr in BHK cells resulted in cell death, with rounding of cells and detachment from dishes. Therefore, we next examined whether Vpr could induce not only G_1 arrest but also apoptosis in rodent cells. We transfected rodent cells with Stop, Vpr and C81 plasmids, respectively, and prepared total cell extracts 24 h after transfections. Then we measured the activity of caspase-3, which plays a critical role in the induction of apoptosis. The activity of caspase-3 in cell extracts prepared from BHK cells that expressed Vpr or C81 was about three-fold higher than in the control cells that expressed Stop (Fig. 2A). Vpr also caused the activation of caspase-3 in extracts of NIH3T3 cells and L cells. Next, we counted the number of apoptotic cells by monitoring fluorescence after staining of cells with Hoechst 33258. As shown in Fig. 2B, the proportions of apoptotic cells with condensed chromatin in the cases of BHK cells that expressed Vpr and C81 were approximately 15.1% and 33.9%, respectively. These values were much higher in the case of cells that had been transfected with the control expression vector (2.5%). These results strongly suggest that Vpr is able to induce apoptosis in rodent cells independently of G_2/M arrest.

3.3. Vpr induces apoptosis predominantly in Vpr-expressing cells

Vpr is detectable in the serum of HIV-1 carriers and extracellular addition of Vpr to human cells in culture induces apoptosis. Thus, apoptosis in cells that do not express Vpr might be due to paracrine, as well as bystander, toxic effects. However, several studies have indicated that Vpr induces apoptosis in cells that express Vpr or have been infected with Vpr-positive HIV-1 isolates. To examine this issue at the single-cell level, we performed three-color flow cytometry. BHK cells were transfected with the three plasmids discussed above and harvested 24 h after transfection. Cells were immunostained for Flag-tag and the active form of caspase-3. Concurrently, they were stained with 7-amino-actinomycin D (7-AAD) for analysis of their DNA content. Gates for distinction between Vpr-expressing and Vpr-non-expressing cells were set on the basis of the control Stop-immunostain population (Fig. 3A). Samples were gated to exclude cells that were not stained by 7-AAD since they had already undergone apoptotic or necrotic death. As shown in Fig. 3B, cells transfected with Stop were not recognized by antibodies against active caspase-3. However, apoptosis of cells that has been transfected with Vpr or C81 was detected by this method. Notably, the majority of cells in which apoptosis was induced by Vpr were derived from the population of Vpr-expressing cells. By contrast, only a small population was apoptotic in the population of Vpr-non-expressing cells in the same culture (Fig. 3B). These results indicated that apoptosis occurred predominantly in Vpr-expressing cells.

3.4. Vpr induces apoptosis via disruption of mitochondrial function

In cell culture, Vpr targets mitochondria to induce apoptosis in human cells [25,26]. We examined whether endogenous Vpr targets mitochondria in rodent cells. First we measured the activities of caspase-8 and caspase-9 as well as caspase-3 24 h after transfection. Caspase-8 is activated by FasL and TNF-α. Cytochrome *c* is released from mitochondria in conjunction with the activation of caspase-9. As shown in Fig. 4A, Vpr doubled the activity of caspase-3 and caspase-9, as compared with controls. By contrast, caspase-8 was not activated upon expression of Vpr. Alternatively, Z-DEVD-FMK, Z-LETD-FMK and Z-LEHD-FMK as inhibitors of caspase-3, caspase-8 and caspase-9 were employed. These cell-permeable caspase inhibitors were added to the cultures of cells that had been transfected with Stop, Vpr and C81 plasmids at final concentration of 2 μM. Cells were incubated for 24 h in the presence of the inhibitors until apoptosis was assessed by determination of the activities of caspase-3, caspase-8 and caspase-9 (Fig. 4A). Apoptosis induced by Vpr and C81 was almost completely inhibited when inhibitors of caspase-3 and caspase-9 were added to both of cells that had

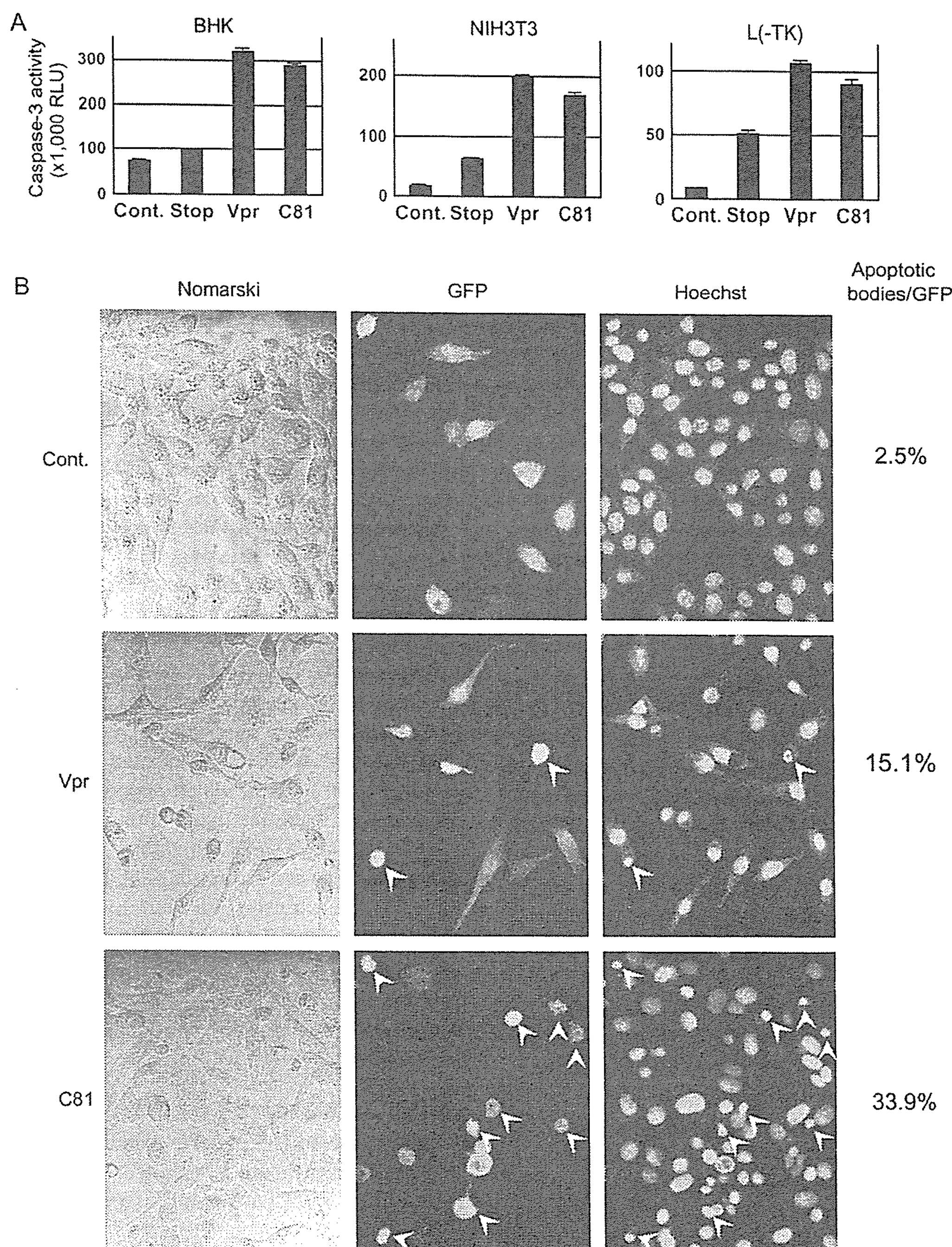

Fig. 2. The expression of Vpr induced apoptosis in rodent cells. (A) BHK, NIH3T3 and L cells were transfected with the Stop, Vpr or C81 expression plasmids. Then, 24 h after transfection, cells were lysed and the activity of caspase-3 was measured fluorometically as described in the text. Control extracts (Cont.) were derived from non-transfected cells. Each column and error bar represents the mean ± SD of results from three samples. (B) BHK cells were cotransfected with Stop, Vpr or C81 expression plasmids together with the GFP expression vector pEGFP-N1. GFP was used as the reporter molecule for discrimination between transfected and untransfected cells. Thirty-six hours after transfection, BHK cells were fixed in 1% formaldehyde and then in 70% ethanol and stained with Hoechst 33258 to monitor the morphology. Apoptotic bodies (arrowheads) were revealed by fluorescence microscopy. The percentage of apoptotic cells/GFP-positive cells is indicated in right of panel.

been transfected with Vpr and C81. By contrast, inhibitor of caspase-8 could not inhibit both Vpr- and C81-induced apoptosis. Thus, Vpr appears to induce apoptosis via the activation of caspase-9.

We monitored the release of cytochrome *c* from mitochondria into the cytosol by Western blotting with antibody against cytochrome *c*. A cytosolic fraction was prepared as described in Section 2 and analysis of cells that expressed Vpr indicated that the amount of cytochrome *c* released from mitochondria increased about three-fold by Vpr. However, in cells transfected with Stop, the release of cytochrome *c* was diminished (Fig. 4B). These results indicated that endogenously expressed Vpr disrupts mitochondrial function. We also analyzed the mitochondrial transmembrane potential with a cationic

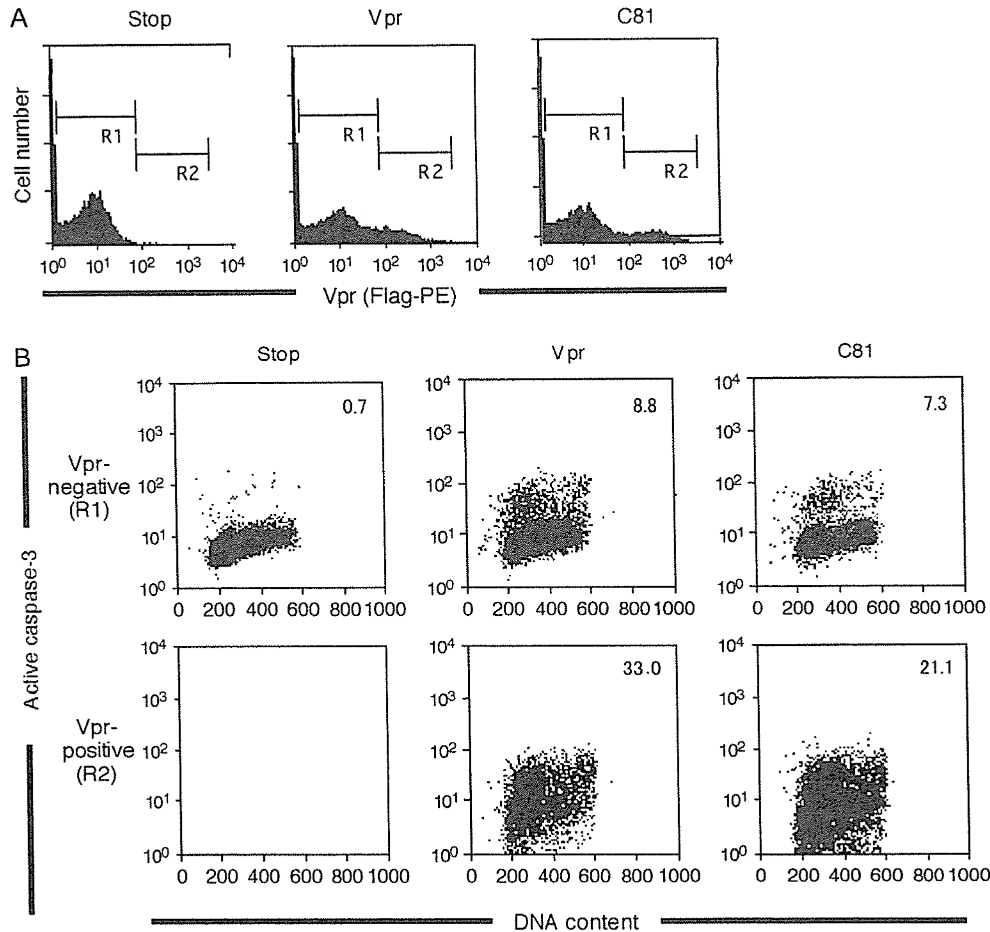

Fig. 3. Apoptosis occurred predominantly in Vpr-expressing cells. BHK cells were transfected with the Stop, Vpr or C81 expression plasmids and harvested 48 h after transfection. Cells were fixed and stained with FITC-labeled antibody against active caspase-3, which recognized only the active form of caspase-3, and biotin-labeled Flag-specific antibody. Then they were incubated with streptavidin-PE for detection of cells that expressed Vpr and stained with 7-amino-actinomycin D (7-AAD) for analysis of DNA content. Samples were analyzed by three-color flow cytometry with a FACSort system as described in the text. (A) Levels of expression of Vpr and of C81 were determined by flow cytometry. Gates were set for analysis of Region 1 (R1) to monitor the population of Vpr-non-expressing cells. Region 2 (R2) indicates the population of Vpr-expressing cells. (B) Cells were analyzed by flow cytometry for levels of active caspase-3 and for DNA content. DNA was stained with 7-AAD and cells that were not stained by 7-AAD were excluded. The numbers represent percent of cells recognized by antibodies against activated caspase-3.

mitochondrion-specific dye, MitoCapture, and flow cytometry. In healthy cells, MitoCapture aggregates in mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in mitochondria and no red fluorescence can be detected. Treatment of Camptothecin, which induces apoptosis via the activation of caspase-9, results in a significant change in the mitochondrial transmembrane potential. After transient transfections, we found that the expression of Vpr and that of C81 led to a change in the mitochondrial transmembrane potential, as compared with Stop (Fig. 4C).

3.5. Vpr also disrupts mitochondrial function in HeLa cells

The species-specific effects of Vpr might influence many of the functions of Vpr including its role in inducing G_2/M arrest [12–16,29]. Vpr induces G_2/M arrest in primate cell lines but not in rodent cell lines, such as BHK, NIH3T3 and L (TK-) cells (Fig. 1). To analyze whether Vpr exhibits species-specificity in the induction of apoptosis, we performed transient transfections of HeLa cells and measured the activities of caspase-3, caspase-8 and caspase-9. As shown in Fig. 5, the activities of caspase-3 and caspase-9 were doubled in cell extracts of cells transfected with Vpr, as compared with control cells transfected with Stop. However, the respective activities of caspase-8 were not different significantly. We also found that the expression of Vpr induced the release of cytochrome *c* from mitochondria and disruption of the mitochondrial transmembrane potential (data not shown). These results indicated that Vpr that was expressed endogenously in HeLa cells also induced apoptosis via disruption of mitochondrial function. Likewise, the activities of caspase-3 and caspase-9 increased in cell extracts of cells transfected with C81 expression vector (Fig. 5). Interestingly, the mutant C81 protein, which does not induce G_2/M arrest in HeLa cells, had stronger apoptosis-inducing effect than Vpr. These results suggest that Vpr might induce apoptosis through the activation of caspase-9 and that this pathway to apoptosis is independent of G_2/M arrest of the cell cycle in both primate and

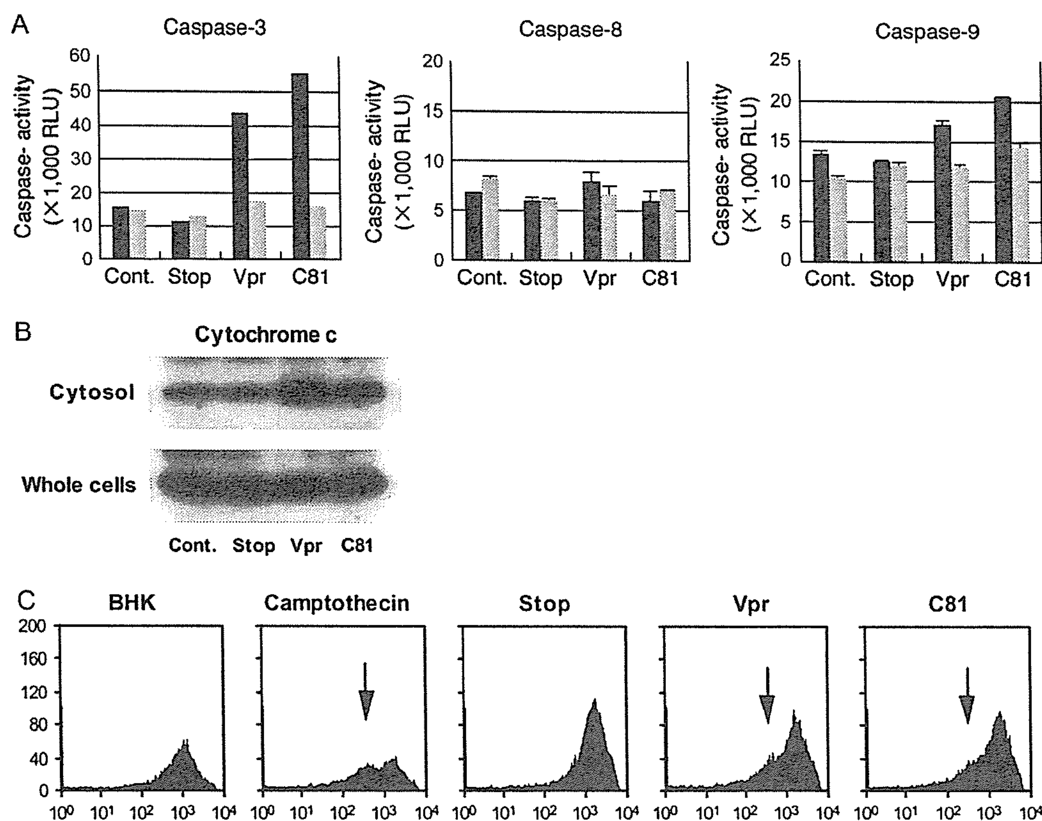

Fig. 4. Vpr induced apoptosis via disruption of mitochondrial function. (A) Effects on the activity of caspase-3, caspase-8 and caspase-9 of the expression of Vpr. BHK cells were transfected with the indicated plasmids and then treated with (gray bars) inhibitors of caspase-3, caspase-8 and caspase-9 or without (black bars). Twenty-four hours after transfection, cells were lysed and the activities of caspase-3, caspase-8 and caspase-9 were measured. Control extracts (Cont.) were derived from non-transfected cells. Each column and error bar represents the mean ± SD of results from three samples. (B) The release of cytochrome *c* from mitochondria into the cytosol was determined by Western blotting with cytochrome *c*-specific antibody. Twenty-four hours after transfection with 23.75 μg of a Vpr expression plasmid and 1.25 μg of pSV-β-galactosidase, cells were divided into two aliquots: one aliquot was subjected to an assay of β-galactosidase activity to monitor the efficiency of transfection and another aliquot was homogenized with cytosol extraction buffer. The cytosolic fraction was obtained as described in the text. Samples with equal β-gal activity were subjected to Western blotting. (C) The mitochondrial transmembrane potential was examined with the MitoCapture reagent. Cells were stained as described in the text. Arrows indicate the population of apoptotic cells. As a positive control, camptothecin was added to the growth medium at 1 μg/ml.

rodent cell lines. Furthermore, it appears that Vpr acts species-specifically in the induction of cell cycle arrest but not in the induction of apoptosis.

4. Discussion

We showed here that wild-type Vpr and C81 both induce G_1 arrest in rodent cells. In particular, the endogenous expression of Vpr inhibited the incorporation of BrdU, cells accumulated in the G_1 phase, and the phosphorylation of Rb was inhibited. In a previous study, we found that C81, a carboxy-terminally truncated form of Vpr, induced cell cycle arrest at the G_1 phase and apoptosis in human HeLa cells [31]. These findings together suggest that Vpr might be able to interfere with cell growth via two distinct pathways. Our results now suggest that Vpr acts via both pathways in primate cells but via only

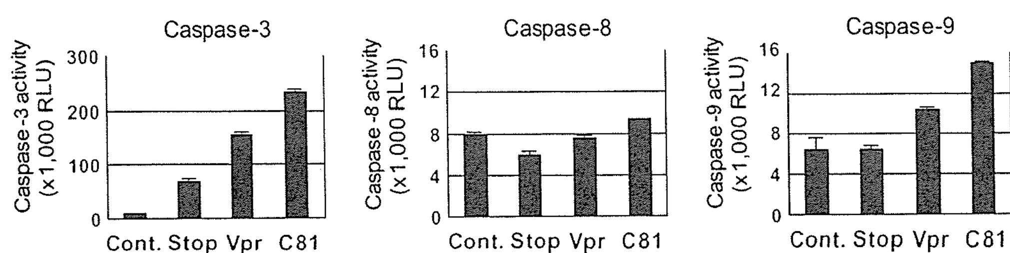

Fig. 5. Vpr also induced apoptosis in human cells via disruption of mitochondrial functions and independently of G_2/M arrest. HeLa cells were transfected with the Stop, Vpr or C81 expression plasmids. C81, a carboxy-terminally truncated form of Vpr, is unable to induce G_2/M arrest in human cells. Cells were lysed 24 h after transfection, and the activities of caspase-3, caspase-8 and caspase-9 were measured. Control extracts (Cont.) were derived from non-transfected cells. Each column and error bar represents the mean ± SD of results from three samples.

one pathway, which is independent of G_2/M arrest, in rodent cells. We were unable to observe G_1 arrest 72 h after transfection and it seems likely that growth inhibition by Vpr might induce apoptosis within a relatively short time, while surviving cells, in which apoptosis is not induced, might grow slowly. In fact, NIH3T3 cells transfected with a plasmid that encoded Vpr were able to form colonies [29].

Apoptosis occurred in all lines of rodent cells tested in this study. However, our results differ from those of Chang et al. [33], who reported that, in transient transfection experiments, HIV-1 Vpr induced apoptosis in human cells but not in rodent cells. The discrepancy between our data and those of Chang et al. might be related to the expression level of Vpr since we demonstrated previously, by time course experiments and analysis of a mutant form of Vpr [10], that overexpression of Vpr is able to induce apoptosis independently of G_2/M arrest. Furthermore, the stable expression of Vpr at a low level has been reported to have a strong anti-apoptotic effect [9,38]. Thus, Vpr might influence the regulation of apoptosis both positively and negatively depending on its level of expression.

We showed, in the present study, that Vpr induces apoptosis through the activation of caspase-9 and disruption of mitochondrial function. Jacotot et al. reported that soluble Vpr might bind directly to adenine nucleotide translocator and induce apoptosis [24,25], whereas soluble Vpr has been reported to induce apoptosis via the activation of caspase-8 in human neural cells [26]. Thus, apoptosis induced by soluble Vpr might be specific to cell type. It is also possible that endogenous expression of Vpr and soluble Vpr might induce apoptosis via different pathways. Muthumani et al. reported that adenovirus that encoded HIV-1 Vpr induced apoptosis via the activation of caspase-9 in human cells [39,40]. Furthermore, they proposed that HIV-1 itself induces apoptosis through an identical pathway. We confirmed that Vpr induces apoptosis via the activation of caspase-9 in rodent cells, as well as in human cells. These results indicate that the pathway for the induction of apoptosis by Vpr is similar in primate and rodent cells. Rodent cells do not support the replication of HIV-1 because entry into cells by the virus is blocked, as are expression of provirus and virion assembly [41]. Thus, for example, Tat transactivation of the HIV-1 LTR is inefficient in rodent cells. The defect in Tat function can be complemented by human cyclin T_1 [42]. The amino acid sequences of mouse cyclin T_1 and human cyclin T_1 differ at several positions, which might explain the inefficient Tat transactivation of the HIV-1 LTR in rodent cells. Vpr might display species specificity in terms of induction of G_2/M arrest but not in terms of induction of apoptosis. Thus, the targets of Vpr in the induction of G_2/M arrest and apoptosis might be different.

The activity of caspase-3 induced by C81 was about three times higher than that induced by wild-type Vpr in HeLa cells. By contrast, the activity of caspase-3 induced by C81 and by Vpr was similar in rodent cells, which did not undergo G_2/M arrest in response to Vpr and to C81. Thus, our data in rodent cells suggest that induction of G_2/M arrest might inhibit Vpr-induced and C81-induced apoptosis [10]. Therefore, rodent cells might be useful tools for elucidation of the mechanism of induction of apoptosis by Vpr in the absence of the effect of Vpr on G_2/M arrest. It has been reported that Vpr induces apoptosis after G_2/M arrest [27]. However, our data suggest that Vpr might also induce apoptosis via a G_1 cell cycle arrest, independently of G_2/M arrest. Further studies are required to determine whether Vpr can induce apoptosis that is related to G_1 arrest or G_2/M arrest. Most viruses have evolved a number of strategies both for the inhibition and for the activation of apoptosis. Therefore, additional studies are needed to determine how apoptosis and G_2/M arrest are regulated during the life cycle of HIV and during the pathogenesis of AIDS.

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Original article

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

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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/vesicular 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.
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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)

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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′-GCTCTAGATTAGTGATACTTGTGGGC-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-

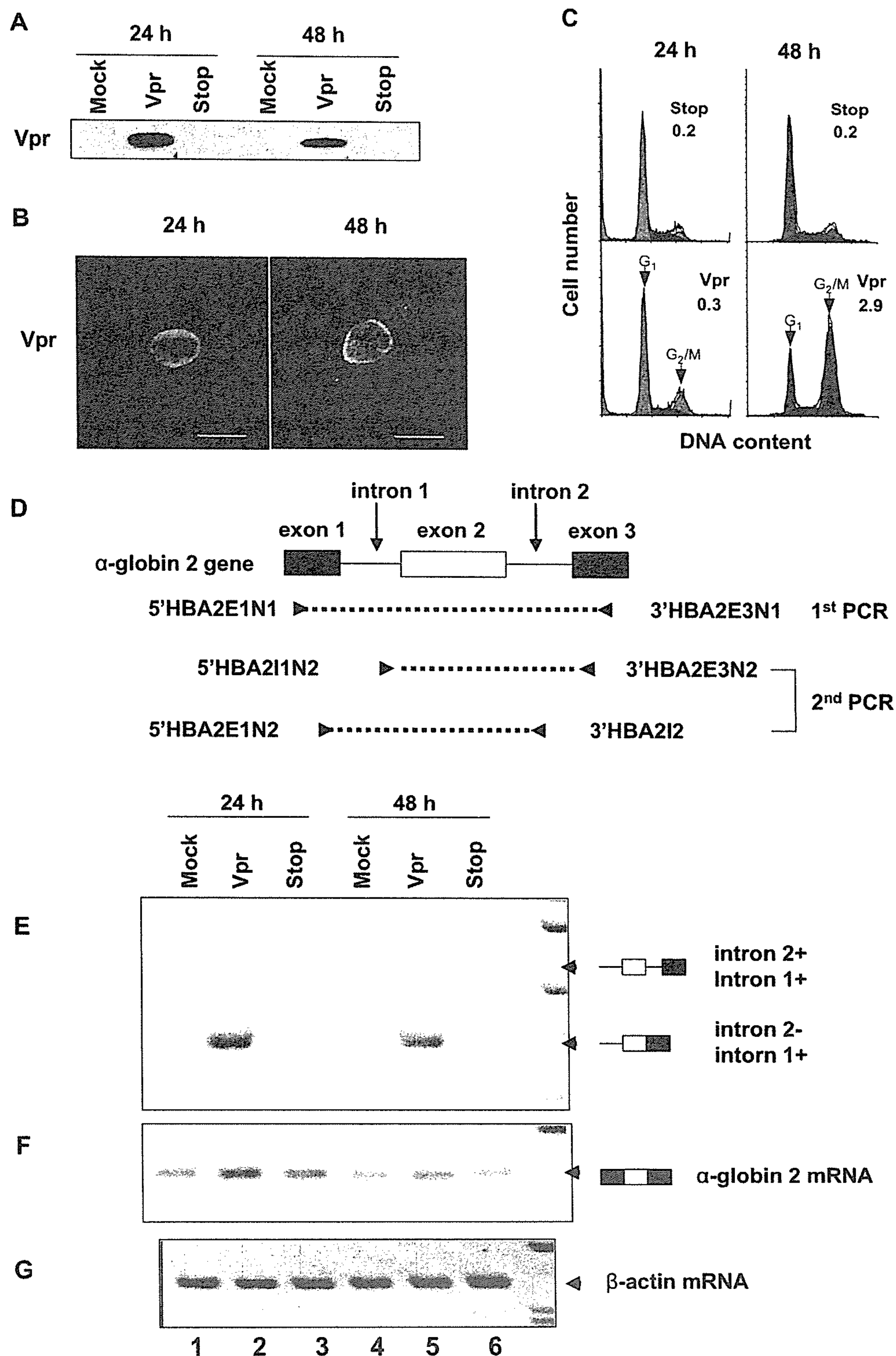

Fig. 1. Vpr prevented splicing of endogenous intron 1 of *α-globin* 2 pre-mRNA in HeLa cells. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or with 30 μg of control pME18Neo-Stop (Stop), or they were mock-transfected. Then, 24 and 48 h after transfection, 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.

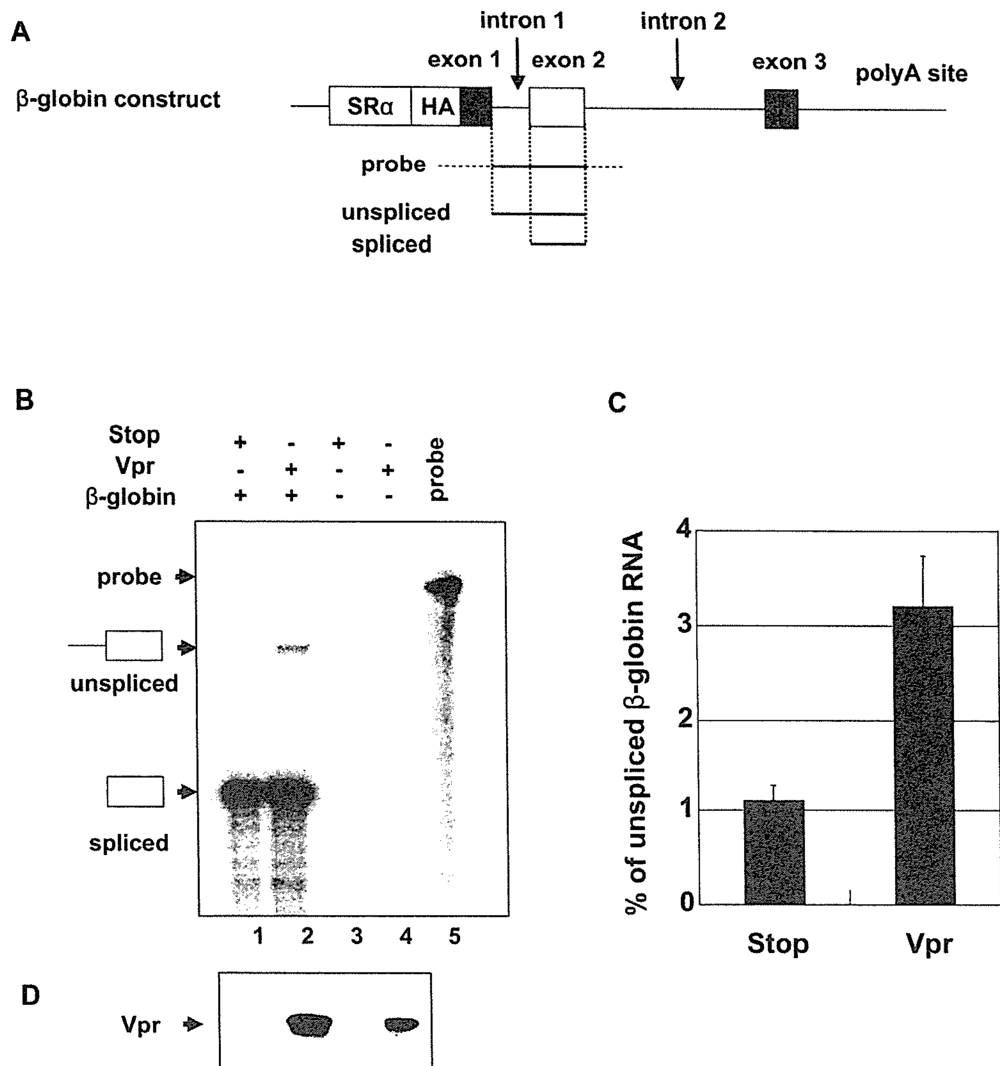

Fig. 2. Vpr inhibited the splicing of exogenous *β-globin* pre-mRNA in HeLa cells that had been transfected with a vector that encoded Vpr and a vector that encoded *β-globin* pre-mRNA. (A) The pME18Neo construct, showing the *β-globin* gene that contained three exons and two introns under the control 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

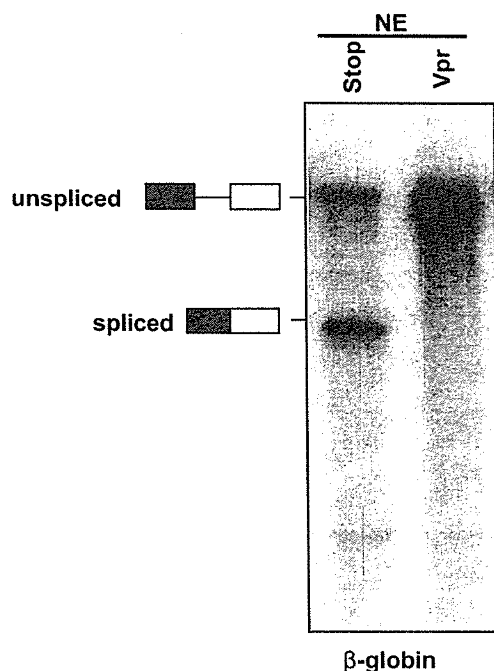

Fig. 3. Vpr inhibited splicing in vitro. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or 30 μg of control pME18Neo-Stop (Stop). Then, 24 h after transfection, nuclear extracts (NE) were prepared from cells and subjected to in vitro splicing assay with ^{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

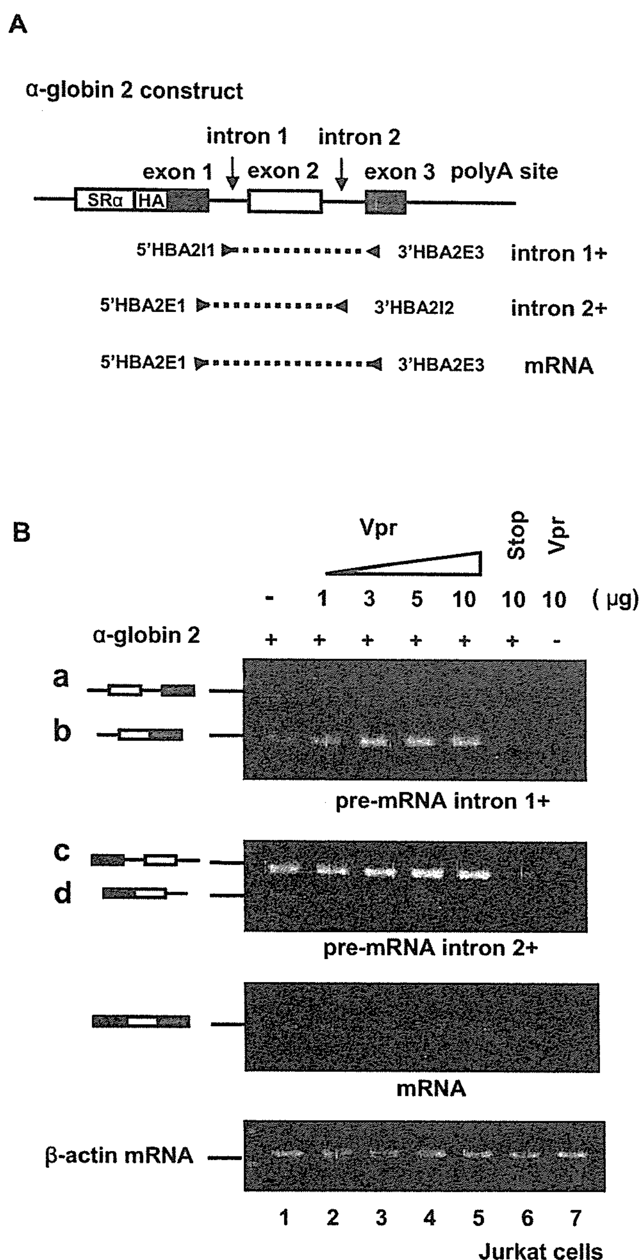

Fig. 4. Analysis by RT-PCR of exogenous *α-globin 2* pre-mRNA in Jurkat cells that had been transfected with a vector that encoded Vpr and a vector that encoded *α-globin 2* pre-mRNA. (A) The pME18Neo construct including *α-globin 2* gene that contained three exons and two introns under the control of 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.

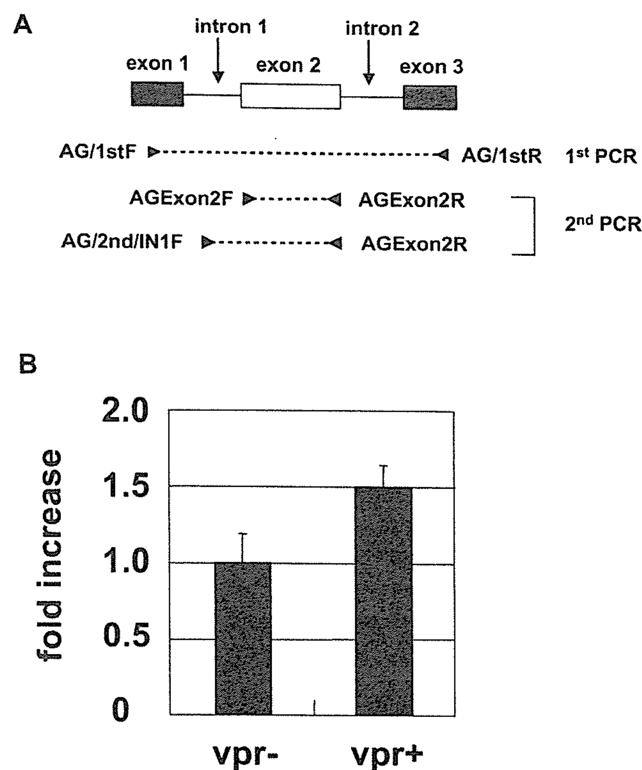

Fig. 5. Vpr expressed from an HIV-1 provirus accumulates endogenous α-globin2 pre-mRNA. Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viruses were harvested from 293T cell cultures 24 h after cotransfection with VSV-G expression vector together with pNF462Δ*env* or pNF462Δ*env*Δ*vpr*. 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.

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, suggesting 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.

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Importin-α Promotes Passage through the Nuclear Pore Complex of Human Immunodeficiency Virus Type 1 Vpr

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Viral protein R (Vpr) of human immunodeficiency virus type 1 has potent karyophilic properties, but details of the mechanism by which it enters the nucleus remain to be clarified. We reported previously that two regions, located between residues 17 and 34 (αH1) and between residues 46 and 74 (αH2), are indispensable for the nuclear localization of Vpr. Here, we reveal that a chimeric protein composed of the nuclear localization signal of Vpr, glutathione *S*-transferase, and green fluorescent protein was localized at the nuclear envelope and then entered the nucleus upon addition of importin-α. An in vitro transport assay using a series of derivatives of importin-α demonstrated that the carboxyl terminus was required for this nuclear import process. We also showed that Vpr interacts with importin-α through αH1 and αH2; only the interaction via αH1 is indispensable for the nuclear entry of Vpr. These observations indicate that importin-α functions as a mediator for the nuclear entry of Vpr.

The nuclear import of proteins occurs through nuclear pore complexes (NPCs) and typically requires specific signals: the nuclear localization signals (NLSs). Soluble factors involved in the nuclear import of proteins include importin-α (10, 11, 25, 31, 42, 51, 56), importin-β (9, 13, 21, 26), small GTPase Ran/TC4 (20, 36, 37, 39, 40), and NTF2 (39, 46). Importin-α functions as an adaptor molecule, binding importin-β via its amino-terminally located importin-β-binding (IBB) domain and binding an NLS-bearing protein via two NLS-binding sites in the central region of importin-α (23, 30). Importin-β is the transport receptor that carries the importin-α–NLS complex from the cytoplasm to the nuclear side of the NPC. Once the heterotrimer consisting of importin-α, importin-β, and the NLS-bearing protein reaches the nuclear face of the NPC, the GTP-bound form of Ran binds directly to importin-β, with resultant release of importin-α and the NLS-bearing protein into the nucleoplasm. Ran, which is found in the GDP-bound form in the cytoplasm and in the GTP-bound form in the nucleus, is a major determinant of the directionality of transport across the nuclear membrane.

Primate lentiviruses have the unusual ability to infect and replicate in nondividing cells. This property of lentiviruses and, in particular, of human immunodeficiency virus type 1 (HIV-1), depends on the active transport of the viral genome into the nucleus of the infected cell, without a requirement for the breakdown of the nuclear envelope that occurs during cell division (8, 32). Transport of the genome into the nucleus requires that the preintegration complex (PIC) of HIV-1 should be actively imported into the nucleus of the host cell. It has been suggested that the targeting of the PIC to the nucleus is accomplished by the cooperative actions of several dozen NLSs that are located on various proteins in the PIC (16, 57), including matrix antigen (MA), integrase (IN), and viral protein R (Vpr) (7, 18, 22, 44, 55) Both MA and IN have a functional NLS, and both utilize the classical nuclear import pathway that includes interactions with importin-α/β heterodimer (18, 19). In contrast, the mechanism responsible for nuclear import of Vpr remains poorly understood. It has been reported that a small region of HIV-1 DNA, known as the central DNA flap, acts as a *cis* determinant of the nuclear import of PIC (59). Moreover, Fassati et al. (14) clearly showed that importin 7, an import receptor for ribosomal proteins and histone H1, is involved in nuclear import of PIC in a transport assay in vitro.

Vpr, one of the possible mediators of the nuclear localization of PIC, is a small (14-kDa) nuclear protein of 96 amino acids that plays various roles in viral infection and cellular functions (6, 12). This protein is localized predominantly in nuclei and at the nuclear envelope (29, 54), but it lacks any identifiable classical import signal (35). Consistent with the absence of such a classical import signal, the nuclear import of Vpr is unaffected by the addition of an excess of the NLS peptide of the large T antigen of SV40 or of a peptide that corresponds to the IBB domain of importin-α or the M9 signal sequence (18, 27, 28) that is located in hnRNP A1 and is related to the nuclear import, which is mediated by transportin (3, 47). Furthermore, a dominant-negative mutant form of Ran, RanQ69L, which is a potent suppressor of nuclear import (5, 43), has no inhibitory effects on the nuclear import of PIC that is mediated by Vpr (28). It has also been suggested that Vpr binds importin-α (2, 48, 49, 54), in addition to phenylalanine-glycine repeat (FG repeat)-containing nucleoporins (17, 48, 50, 54). However, the significance of these interactions is still unknown.

In a previous study, we showed that the region between residues 17 and 74 of Vpr is associated with the distinctive localization of wild-type Vpr and two smaller regions, between residues 17 and 34 (αH1) and between residues 46 and 74

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(αH2) of Vpr, are indispensable for the karyophilic nature (29). However, the exact function of each residue and the mechanism of nuclear import, which included responsible cellular factors, have been unknown. In this report, we demonstrate first that the region between residues 17 and 74 is a bona fide NLS of Vpr, using microinjection and in vitro transport assays. Furthermore, we demonstrate that Vpr traverses the NPC in an importin-α-dependent manner. We also show that Vpr interacts with importin-α through the αH1 and αH2 regions and that the interaction via αH1 is indispensable for entry into the nucleus.

MATERIALS AND METHODS

Culture and transfection of cells. HeLa cells and Madin-Darby bovine kidney (MDBK) cells were grown, respectively, in RPMI 1640 medium and Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Transfections were performed as described previously (45).

Plasmids and constructs. The construction of glutathione *S*-transferase (GST)-tagged N17C74-green fluorescent protein (GFP) and $L67P_{N17C74}$-GFP was described previously (24). For construction of GST-tagged αLA_{N17C74}-GFP, a fragment was amplified with the primers 5′-GCGGATATCCGAATGGACA GCCGA-3′ and 5′-CGCGGATCCCCAATTCTGAAA-3′, using pSK-FαLA (29) as a template. For construction of GST-tagged NLS_{SV40}-GFP, the fragment of interest was amplified by PCR with primers 5′-GCGCGAGATCTATCCCA AAAAAGAAG-3′ and 5′-CTAGAGTCGCGGCCGCTTTACT-3′, using pGFP-SV40 NLS (29) as template. These fragments were then subcloned into pGEX-6P3 at the BamHI and NotI sites. For construction of GST-tagged GFP, the XhoI and NotI fragment of pEGFP-N1 (Clontech Laboratories, Inc.) was subcloned into pGEX-5T3 (Amersham Pharmacia Biotech) that had been digested with SalI and NotI. The deletion mutants of importin-α were prepared as follow: DNA fragments were generated by PCR with appropriate oligonucleotides and cloned into a maltose-binding protein (MBP) epitope tag expression vector, pMAL-c2X (New England Biolabs). All constructions were sequenced with a BigDye Terminator Cycle Sequencing kit and a Genetic Analyzer (ABI PRISM 310; PE Applied Biosystems).

Preparation of recombinant proteins. We expressed GST-tagged mutant forms of Vpr in *Escherichia coli* strain NovaBlue (Novagen) or BL21 CodonPlus (DE3)-RIL (Stratagene), respectively. After overnight culture at 16°C, cells were collected, lysed by sonication, and GST-tagged proteins in the supernatant were allowed to absorb to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as described elsewhere (24). The proteins were eluted with 16 mM glutathione and were dialyzed against transport buffer (20 mM HEPES-KOH [pH 7.3], 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 2 mM EGTA, 2 mM dithiothreitol [DTT]) and then concentrated in a Vivaspin centrifugal concentrator (Sartorius AG).

The following proteins were expressed in *E. coli* BL21 CodonPlus (DE3)-RIL and purified as described elsewhere: GST-tagged importin-α (mouse importin-α1/PTAC58) (26), importin-β (mouse importin-β1/PTAC97) (25), NTF2 (53), and Ran/TC4 (36). The MBP-tagged proteins were prepared according to the instructions. For preparation of GST-free proteins, GST-fused proteins bound to glutathione-Sepharose 4B were digested with thrombin (Amersham Pharmacia Biotech) or with PreScission protease (Amersham Pharmacia Biotech). The cleaved products were separated on a HiTrap Q FF column (Amersham Pharmacia Biotech), and peak fractions containing each protein were pooled and dialyzed against transport buffer. The purity of each recombinant protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were stored at −80°C.

In vitro transport assay. The basic assay for examination of nuclear import was performed as described elsewhere (1). HeLa cells were permeabilized by treatment with 50 μg of digitonin (Fluka AG) per ml in transport buffer on ice for 5 min. In some case, the cells were further treated with 10 U of apyrase per ml (Sigma) in transport buffer at 30°C for 5 min to deplete the pool of nucleotide triphosphates. To clarify the involvement of NPC in nuclear import of each protein, the digitonin-permeabilized cells were incubated with 200 μg of wheat germ agglutinin (WGA; E.Y. Laboratories) per ml. Twenty-five microliters of test solution usually contained a final concentration of 1 μM chimeric GFP in transport buffer. Where indicated, recombinant importin-α, importin-β, RanGDP, transportin, NTF2, or an energy source (a mixture of 1 mM ATP, 5 mM creatine phosphate, and 20-U/ml creatine kinase) was included in the 25 μl of test solution mentioned above. The import reaction was allowed to proceed for 30 min at 30°C or on ice, and the cells were then washed twice with ice-cold transport buffer and fixed with 1% formaldehyde in transport buffer for 30 min on ice. Specimens were examined with a confocal laser-scanning microscope with a ×63 (NA 1.4) objective (LSM 510; Carl Zeiss).

Microinjection. MDBK cells were grown on coverslips, and GST-tagged N17C74-GFP (2 mg/ml) was injected into the cytoplasm of cells with or without 1 mg of WGA per ml. After injection and incubation for 30 min at 37°C or on ice, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). The injected fluorescent proteins were detected by fluorescence microscopy with a ×63 (NA 1.4) objective (Axiophot; Carl Zeiss).

Preparation of an extract of HeLa cells. To prepare an extract of whole cells, we suspended 10^7 HeLa cells in 500 μl of ice-cold PBSMT (PBS plus 3 mM KCl, 2.5 mM $MgCl_2$, and 0.5% Triton X-100) (54) supplemented with a cocktail of protease inhibitors, and then DNA in the sample was sheared by five passages through a 27-gauge needle. Lysates were cleared by centrifugation for 15 min in a microcentrifuge (15,000 × *g*) at 4°C.

Binding assays using a cell extract or MBP-tagged proteins. An extract of HeLa cells (100 μg of protein) or a purified MBP-tagged derivative of importin-α (12.5 pmol) was incubated with GST or GST-tagged chimeric proteins that had been preadsorbed to 12.5 μl of a preparation of glutathione-Sepharose 4FF beads (Amersham Pharmacia Biotech) at 4°C for 1 h in the presence of bovine serum albumin (10 mg/ml) in PBSMT. The beads were washed extensively with PBSMT, and bound proteins were eluted by incubation with sample buffer for SDS-PAGE at 60°C for 15 min. Eluted proteins were fractionated by SDS-PAGE and detected by Western blotting with antibody specific for the Flag tag (M2; Sigma-Aldrich) or MBP (New England Biolabs).

RESULTS

The region between residues 17 and 74 is a bona fide NLS of Vpr. As reported previously, the region between residues 17 and 74 is indispensable for the nuclear translocation of wild-type Vpr (29). To confirm that this region is sufficient for nuclear localization of Vpr, we constructed a chimeric protein that consisted of the region between residues 17 and 74, designated N17C74, fused at its amino-terminal end to GST and at its carboxy-terminal end to GFP. We examined the subcellular localization of this protein in vivo after microinjection into MDBK cells. The chimeric protein (~62 kDa) was adequately large to preclude passive diffusion into the nucleus. As shown in Fig. 1A, the chimeric protein was localized in the nucleus, and the localization was dependent on temperature. The entry into the nucleus was completely inhibited in the presence of WGA, indicating that the nuclear translocation involved the NPC (15, 58). A chimeric protein that consisted of GST plus GFP (GST-GFP), which served as a negative control, failed to enter the nucleus.

We confirmed the subcellular localization of the chimeric protein in vitro, using digitonin-permeabilized HeLa cells (Fig. 1B). To detect chimeric proteins, we stained cells with a GFP-specific monoclonal antibody (MAb) and Cy3-conjugated antibody against mouse immunoglobulin G (IgG) after the import reaction. In the absence of soluble factors, the N17C74 chimeric protein was localized predominantly in the perinuclear region and a certain amount was present inside the nucleus. The amount of chimeric protein in the nucleus increased in the presence of a mixture of soluble factors that included importin-α, importin-β, RanGDP, NTF2, and an energy-regenerating system, as in the case for the GST- and GFP-tagged NLS of the large T antigen of SV40 (SV40 NLS), which is a positive control for in vitro transport assays. GST-GFP failed to enter the nucleus even in the presence of the above-described import mixture. The results indicated that the region

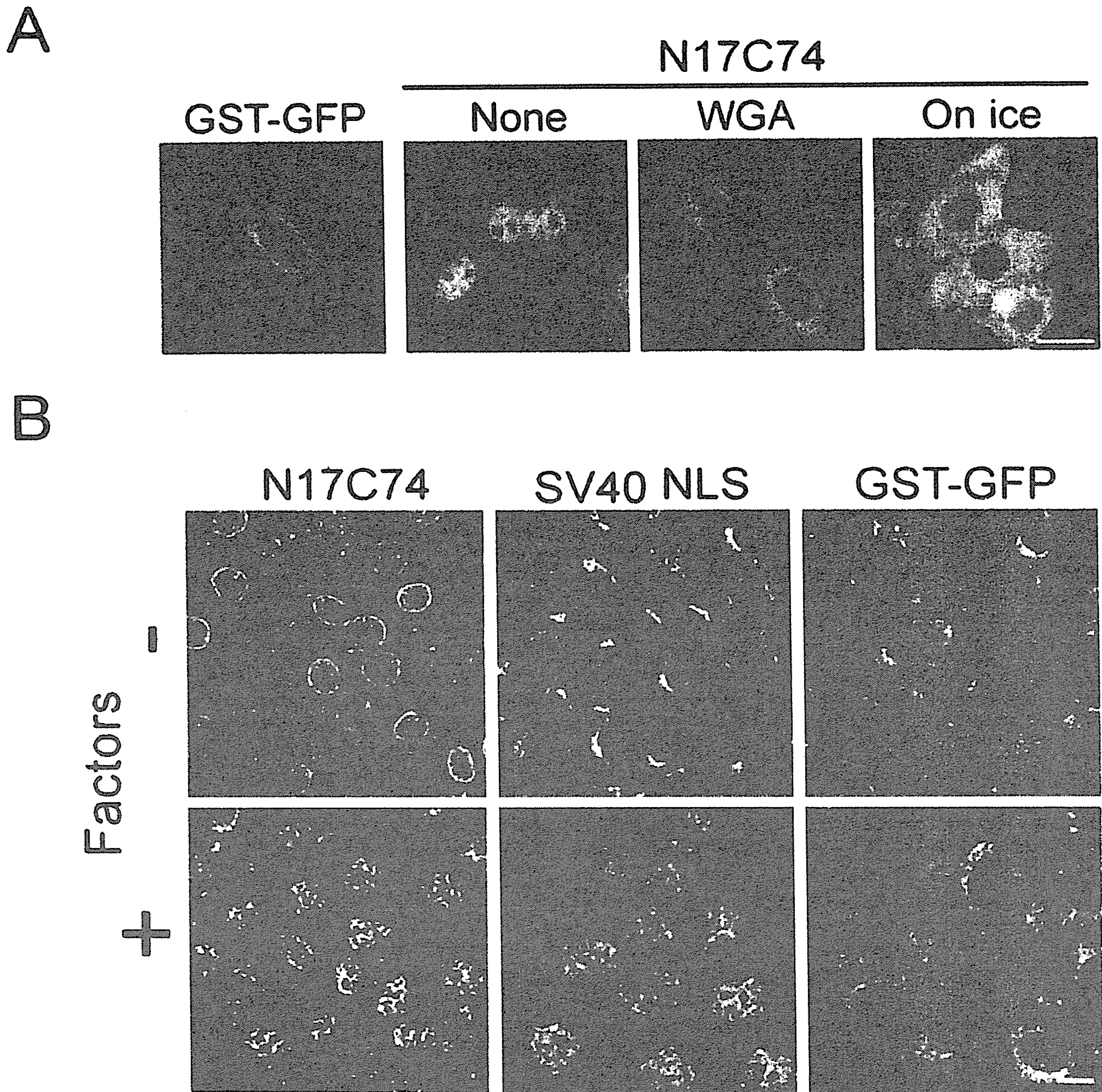

FIG. 1. Characterization of the putative NLS of Vpr. (A) MDBK cells were grown on coverslips, and GST- and GFP-tagged N17C74 (N17C74) or GST-tagged GFP (GST-GFP; 2 mg/ml) was injected into the cytoplasm with (WGA) or without (None and On ice) 1-mg/ml WGA. After incubation at 37°C or on ice, cells were observed by fluorescence microscopy. Bar = 20 μm. (B) Digitonin-permeabilized HeLa cells were incubated with N17C74, GST- and GFP-tagged NLS of SV40 (SV40 NLS), and GST-GFP, as indicated, in the absence (−) or presence (+) of soluble factors. After fixation and permeabilization with 0.2% Triton X-100 in PBS, cells were stained with GFP-specific MAb and Cy3-conjugated antibody against mouse IgG and analyzed by confocal laser-scanning microscopy. The preparation of soluble factors contained the following factors at the indicated concentrations: importin-α, 1 μM; importin-β, 1 μM; RanGDP, 2 μM; NTF2, 0.21 μM; and an energy source, namely, 1 mM ATP, 20 U/ml creatine phosphokinase, and 10 mM creatine phosphate. Bar = 20 μm.

between residues 17 and 74 is a bona fide NLS of Vpr and that soluble factors are required for entry of Vpr into the nucleus.

Entry into the nucleus of the N17C74 chimeric protein through the NPC requires importin-α. We next attempted to identify the factors necessary for the nuclear entry of N17C74, using an in vitro transport assay (Fig. 2). To be clear about the participation of energy in the nuclear import, the cells were pretreated with apyrase to deplete the pool of nucleotide triphosphates before incubation with the import mixture. Upon addition of importin-α, most of the chimeric protein had entered the nucleus. Similar results were obtained when we added an energy source to the import mixture. Addition of