Study on Regulation of Splicing by Human Immunodeficiency Virus Type1 (HIV-1) Vpr Protein

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Contents

Abbreviations

AIDS	:	acquired immunodeficiency syndrome
CA	:	capsid
CCR	:	CC chemokine receptor
CXCR	:	CXC chemokine receptor
Env	:	envelope
DNA	:	deoxyribonucleic acid
ER	:	endoplasmic reticulum
GR	:	glucocorticoid receptor
HIV	:	human immunodeficiency virus
HSV	:	Herpes simplex virus
hnRNP	:	heterogeneous nuclear ribonucleoprotein
IN	:	integrase
LTR	:	long terminal repeat
MA	:	matrix
MAb	:	monoclonal antibody
NE	:	nuclear extract
NC	:	nucleocapsid
NMR	:	nuclear magnetic resonance
PIC	:	preintegration complex
PR	:	protease
RNA	:	ribonucleic acid
RRM	:	RNA recognition motif
RTC	:	reverse transcriptase complex
RT	:	reverse transcriptase
RT-PCR	:	reverse transcription-polymerase chain reaction.
SAP	:	spliceosome-associated protein
SDS	:	sodium dodecyl sulfate
SF	:	splicing factor
SIV	:	simian immunodeficiency virus
snRNP	:	small nuclear ribonucleoprotein
SR protein	:	serine- and arginine-rich protein

TF	:	transcription factor
UDG	:	uracil DNA glycosylase

Abstract

Vpr, one of the accessory gene products of human immunodeficiency virus type 1 (HIV-1), affects both viral and cellular proliferation, being involved in long terminal repeat (LTR) activation, arrest of the cell cycle at the G2 phase, and apoptosis. Recently, it was found that Vpr interacted with spliceosome-associated protein (SAP) 145 in a yeast two-hybrid screen. Since SAP145 is an essential splicing factor, it was examined whether Vpr can regulate the splicing reaction and to discover a novel role for Vpr, which accumulates the cellular pre-mRNA *in vivo*. However, whether Vpr interacts with SAP145 in the host cells and inhibits splicing reaction directly, are unknown.

Here I addressed the mechanism of splicing inhibition by Vpr. First, it was found that Vpr interacted with SAP145 *in vitro* and *in vivo*. Coimmunoprecipitaion and colocalization showed that Vpr also interacts with other spliceosome factors and snRNPs. Next, using *in vitro* splicing assay, I clearly confirmed that Vpr inhibits splicing of *β-globin* pre-mRNA directly, and blocks spliceosome assembly at the early stages. Furthermore, by using mutant forms of Vpr with specific substitution in two domains on Vpr, I demonstrated that the interaction between Vpr and SAP145 is indispensable for splicing inhibition. Finally, coimmunoprecipitation and *in vitro* competitive binding assay indicated that Vpr associates with SAP145 and then suppresses the SAP145-SAP49 complex formation. These results suggest that cellular expression of Vpr may trigger spliceosome assembly suppression by interfering with the function of SAP145-SAP49 complex in the host cells.

Introduction

Acquired immunodeficiency syndrome (AIDS) was first reported in the United States in 1981 and has since become a major worldwide epidemic. AIDS is caused by the human immunodeficiency virus type 1 (HIV-1). By killing or damaging cells of the body's immune system, HIV-1 progressively destroys the body's ability to fight infections and certain cancers. People diagnosed with AIDS may get life-threatening diseases called opportunistic infections, which are caused by microbes such as viruses or bacteria that usually do not make healthy people sick.

In 1983, experimental data indicating an association between a retrovirus and AIDS were published by a research team in France led by Luc Montagnier (Barre-Sinoussi *et al.*, 1983). In 1984, the French group and researchers at the US National Institute of Health, led by Robert C. Gallo, published seminal papers that established, with viriological and epidemiological evidence, that the virus now known as HIV-1 was the cause of AIDS (Gallo, 2002; Montagnier, 2002). The virus was also isolated independently by Jay Levy in California from both individuals affected with AIDS and asymptomic individuals from groups at high risk for AIDS (Levy *et al.*, 1984).

All retrovirus contain three major open reading frames, including *gag* (generates the viral core after intravirion processing of a precursor polypeptide), *pol* [encodes the Reverse transcriptase (RT), Integrase (IN), and protease enzymes], *env* (directs the production of the transmembrane and surface glycoproteins). In addition, HIV-1, a member of the lentivirus subfamily of retrovirus contain genes for regulatory (*tat* and *rev*) and accessory (*vpr*, *vpu*, *vif*, and *nef*) proteins (Fig. 1). If HIV-1 life cycle is assumed to start at the binding to $CD4^+$ T cell (Dalgleish *et al.*, 1984) or macrophage,

the following stages are as follows. Following binding through interactions between HIV-1 envelope (Env) and CD4 and CC chemokine receptor (CCR) 5 (Alkhatib *et al.*, 1996) or CXC chemokine receptor (CXCR) 4, Env transmembrane region undergoes a conformational change that promotes virus-cell membrane fusion, thereby allowing entry of a viral core into the cell. The virion core is then uncoated to expose nucleoprotein complex where the genomic RNA is reverse transcribed by RT into viral genomic DNA. This complex is called preintegration complex (PIC) or reverse transcriptase complex (RTC). PIC goes through nucleapore complex to the nucleus (Jenkins *et al.*, 1998; Popov *et al.*, 1998; Fassati *et al.*, 2003). The nuclear import ability of the PIC, without a requirement for the breakdown of the nuclear envelope that occurs during cell division, is necessary for infection by HIV-1 of nondividing cells, such as quiescent T lymphocytes, terminally differentiated dendritic macrophages. Matrix (MA), IN and Vpr have been identified as possible mediators of the nuclear import of the PIC (Kamata *et al.*, 2005). Then, IN catalyzes integration of viral genomic DNA into host chromosome and the DNA is repaired. Viral transcripts are expressed from the promoter located in the 5' long terminal repeat (LTR), with Tat greatly enhancing the rate of transcription. A set of spliced (Purcell and Martin, 1993) and genomic-length RNAs are transported from the nucleus to the cytoplasm, where they can be translated or packaged. This step is regulated by Rev (Pollard and Malim, 1998). Viral mRNAs are translated in the cytoplasm, and the Gag and Gag-Pol polyproteins become localized to the cell membrane. The Env mRNA is translated at the endoplasmic reticulum (ER). The core particle is assembled from the Gag and Gag-Pol polyproteins [later processed to matrix, capsid (CA), nucleocapsid (NC), protease, RT and IN], Vif, Vpr, Nef, and the genomic RNA, and an immature virion begins to bud from the cell surface. As the particle buds

and is released from the cell surface coated with Env protein, the virion undergoes a morphologic change known as maturation. This step involves proteolytic processing of the Gag and Gag-Pol polyproteins by viral protease. The mature virion is then ready to infect the next cell (Frankel and Young, 1998).

The *vpr* gene, one of the accessory genes, encodes a 96-amino-acid protein (Fig. 2), was analyzed its three-dimensional structure by nuclear magnetic resonance (NMR) (Wecker *et al.*, 2003; Morellet *et al.*, 2003). Vpr is highly conserved among primate lentiviruses, including HIV-1, HIV-2, and simian immunodeficiency virus (SIV) (Planelles *et al.*, 1996) and associated with virus particles despite being a non-structural protein (Cohen *et al.*, 1990). In addition, the incorporation of Vpr has been shown to be specific involving a distinct domain, p6 region, in Gag (Lu *et al.*, 1995; Kondo and Gottlinger, 1996), by contrast other accessory proteins, Vif and Nef , were incorporated into virus particles by a non-specific mechanism (Azad, 2000). The experiments carried out using SIV in rhesus macaques indicated that pathogenesis in infected macaques was influenced by Vpr *in vivo* (Tungaturthi *et al.*, 2003). By contrast, it appears that Vpr is not absolutely required for viral replication because deletion of Vpr is not lethal to viral replication *in vitro*. However, it was reported that Vpr is present in significant amounts in the serum of HIV-1-infected patients and that it activates viral expression in latently infected cell lines and resting peripheral blood mononuclear cells (PBMC) (Levy *et al.*, 1993 and Levy *et al.*, 1994). These observations suggest that Vpr is very much involved in the life cycle of HIV-1 and controls both the replication and pathogenesis of this virus. Moreover, the fact that many copies of Vpr are packaged in progeny virions suggests that the protein might play a role early in the infectious process (Cohen *et al.*, 1990). Experimental support for an early functional role for Vpr comes from observations of a

contribution by Vpr to the nuclear import of proviral DNA in nondividing cells, such as macrophages (Heinzinger *et al.*, 1994). In addition, Vpr synthesized de novo plays an additional role in the replication of HIV-1 because Vpr that is supplied in trans does not fully complement the replication of Vpr-negative viruses in infected macrophages (Connor et al., 1995). It is possible that Vpr might upregulate the expression of viral genes. These reports indicated that Vpr could play important roles in HIV-1 infection (Connor *et al.*, 1995) and AIDS pathogenesis.

Moreover, Vpr has multiple activities in the host cells (Table 1.) such as nuclear localization (Heinzinger *et al.,* 1994; Kamata and Aida, 2000; Iijima *et al.*, 2004; Kamata *et al.*, 2005), regulation of transcription (Subbramanian *et al.*, 1998; Kino *et al.*, 2002), apoptosis (Ayyavoo *et al.*, 1997; Nishizawa *et al.*, 2000a, b; Azuma *et al.*, 2005), and cell cycle arrest at G2 phase (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Bartz *et al.*, 1996; Goh *et al*, 1998). These functions of Vpr must supported by its physical interactions with several identified cellular molecules (Kino and Pavlakis, 2004), such as nucleic acids (de Rocquigny *et al.*, 2000), Importin α (Popov *et al*, 1998; Kamata *et al.*, 2005) , Nucleoporin (Fouchier *et al.*, 1998), Transcription factor (TF) IIB (Agostini *et al.*, 1996), Proteasomal subunit mov34 (Mahalingam *et al.*, 1998), Glucocorticoid receptor (GR) type II (Kino *et al*, 2002), Sp1 (Wang *et al.*, 1995), Uracil DNA glycosylase (UDG) (Bouhamdan *et al.*, 1996), p300 (Felzien *et al.*, 1998) and 14-3-3 (Kino *et al.*, 2005) (Table 2), though the molecular mechanisms between function and Vpr associated-cellular molecule are still not clear.

Pre-mRNA splicing, the removal of non-coding introns from mRNA precursors, is a pre-requisite for the expression of most eukaryotic genes. This essential reaction was carried out in the nucleus. The pre-mRNA splicing machinery consists of five small

nuclear ribonucleoproteins particles (snRNPs) such as U1, U2, U4, U5 and U6 and more than fifty proteins (Hastings and Krainer, 2001). During splicing, successive recognition of splice sites occurs as spliceosomal complex E, A, B, and C assemble on pre-mRNA in a stepwise manner (Fig. 3). In the commitment complex E, U1 snRNP is bound to the 5' splice site and splicing factors U2AF and SF1 are attached to the polypyrimidine tract upstream of the 3' splice site and the branch site, respectively. As U2 snRNP binds to the branch site, complex E is converted to presplicing complex A (Gozani *et al.*, 1996), a short helix forms between a single-stranded region in U2 snRNA and the intron branch site which requires both the 12S snRNP and splicing factors (SF) 3a and SF3b, which contain subunits of SF3a60, SF3a66, and SF3a120, or SF3b10, SF3b14b, p14, spliceosome-associated protein (SAP) 49, SAP130, SAP145 and SAP155, respectively (Wells *et al.*, 1996; Das *et al.*, 1999; Will *et al.*, 2001). Next step is binding of the U4/U5/U6 tri-snRNP at the 5' splice site to form the B complex. Just prior to splicing, conformational changes within complex B destabilize the association of U1 and U4 snRNPs and transform complex B into complex C (Fig. 3). SAP145, an essential component of the SF3b subunit in U2 snRNP, can bind to pre-mRNA (Staknis and Reed, 1994) and is implicated in the tethering of U2 snRNP to the branch point site that is required for complex A assembly (Champion-Arnaud and Reed, 1994). SAP49 has two RNA recognition motifs (RRMs) that are located on the surface of SF3b (Golas *et al.*, 2003), and interacts directly and highly specifically with both SAP145 and pre-mRNA. Thus, the observation that SAP145 and SAP49 interact directly with both U2 snRNP and the pre-mRNA suggests that this protein complex plays an important role in tethering U2 snRNP to the branch site (Gozani *et al.*, 1996).

Several viral proteins have also been shown to regulate the splicing of cellular

pre-mRNAs. For example, the Herpes Simplex Virus type 1 (HSV-1) ICP27 protein has also been reported to inhibit splicing through its association with SAP145 and to interact with SRPK1 resulting in hypophosphorylation of serine- and arginine-rich (SR) proteins, which impairs their roles in spliceosome assembly (Bryant *et al.*, 2001; Lindberg *et al.*, 2002; Sciabica *et al.*, 2003; Smith *et al.*, 2005). NS1, an influenza virus non-structural protein, inhibits the splicing of the major class of mammalian pre-mRNAs (GU-AU introns) by binding to a specific stem-bulge in U6 snRNA, thereby blocking the formation of U4/U6 and U2/U6 complexes (Fortes *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995). HIV-1 proteins Tat, which acts as a transactivator of viral and cellular gene, and Rev, which is essential for nuclear export of incompletely spliced viral mRNAs, have also been shown to inhibit HIV-1 splicing by the interaction with p32, a cofactor of ASF/SF2 (Berro *et al.*, 2006; Powell *et al.*, 1997), however, whether HIV-1 protein regulates splicing of cellular pre-mRNA, but not viral pre-mRNA, are unknown.

Recently, it was found that Vpr interacted with SAP145 in a yeast two-hybrid screen (Zhang *et al.*, personal communication). Since SAP145 is an essential splicing factor, it was examined whether Vpr can regulate the splicing reaction and discovered a novel role for Vpr, which accumulates *α-globin 2* and *β-globin* pre-mRNA *in vivo* (Kuramitsu *et al.*, 2005). It was also indicated strong evidence that Vpr expressed from an HIV-1 provirus was sufficient to accumulate for *α-globin2* pre-mRNA (Kuramitsu *et al.*, 2005).

The main aim of this thesis is to demonstrate that regulatory and molecular mechanisms of splicing inhibition of the cellular pre-mRNA by Vpr. Firstly, I confirmed that Vpr interacted with SAP145 in a pull-down assay, coimmunoprecipitation and

colocalization. Vpr also colocalized with SC35, a well-known spliceosome marker. These results showed that Vpr interacts with SAP145 and exists in the spliceosome. Next, to confirm that whether Vpr directly acts on splicing inhibition, I examined splicing assay *in vitro*. Although my previous study showed that Vpr accumulates the cellular pre-mRNA *in vivo* (Kuramitsu *et al.*, 2005), it was possible that the expression of Vpr, which act as a regulator of transcription (Agostini *et al.*, 1996; Felzien *et al.*, 1998; Subbramanian *et al.*; Kino *et al.*, 2002), may affect the transcription of several genes such as SR protein and heterogeneous nuclear ribonucleoprotein (hnRNP) which are essential for the splicing regulation. Therefore, by using *in vitro* splicing assay, it was clearly shown that Vpr inhibits *β-globin* pre-mRNA splicing. By native gel electrophoresis analysis for *in vitro* splicing assay, it was unable to find the formation of complex B and C of spliceosome when Vpr is present. These results indicated that Vpr inhibits splicing reaction directly by blocking the early stages of spliceosome assembly. Furthermore, by using mutant forms of Vpr with specific substitution in third α-helical domain and arginine-rich region, I demonstrated that the interaction between Vpr and SAP145 was indispensable for splicing inhibition by Vpr. Finally, coimmunoprecipitation and *in vitro* competitive binding assay showed that Vpr associates with SAP145 and subsequently suppresses the SAP145-SAP49 complex formation. The evidence suggests that the function of SAP145-SAP49 complex might be inhibited by Vpr when Vpr localizes in the U2 snRNP, and then the splicing reaction is inhibited. My results reveal an understanding of the molecular mechanisms of splicing regulation by Vpr and a novel mechanism of Vpr at the early stage of the splicing assembly.

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Materials and Methods

Cells, transfection and extraction of RNA and DNA. Human cervical HeLa-S3 cells were grown in minimum essential medium eagle (S-MEM) (Sigma, St. Louis, MO.) supplemented with 5% heat-inactivated calf serum (Invitrogen, Carlsbad, CA.). Human cervical HeLa cells were grown in Dulbecco's modified Eagle's medium (Sigma) that contained 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

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.

Genomic DNA was extracted from HeLa cells with a Wizard^{TM} genomic DNA purification kit (Promega, Madison, WI.). Total RNA was extracted from HeLa cells using TRIzol^{TM} reagent (Invitrogen).

Construction of plasmids. The derivative of the expression vector pME18Neo that encodes Flag-tagged wild-type Vpr, namely, pME18Neo-FVpr, has been described previously (Nishino *et al.*, 1997; Nishizawa *et al.*, 1999). To generate the cloning plasmid for the *β-globin* gene, including three exons and two introns, by PCR with genomic DNA from HeLa cells as template and primers βG-1-Eco RV-5' (5'-GGCGATATCCATGGTGCACCTGACTCCT-3') and βG-end-Xba I-3' (5'-GCTCTAGATTAGTGATACTTGTGGGC-3'). Then I cloned the amplified fragment between the *Eco*RV and *Xba*I sites of pBluescript II (SK$^+$) (pSK) (Stratagene, La Jolla, CA.). The resulting construct was designated pSK-*β-globin*. The DNA fragment encoding U1 snRNA or U2 snRNA was obtained by PCR using total genomic DNA from HeLa cells as template as template and the primers for U1 snRNA , U1-1-5': 5'- ATACTTACCTGGCAGGGG-3' and U1-end-3':

5'-CAGGGGAAAGCGCGAACG-3', and for U2 snRNA , U2-1-5': 5'-ATCGCTTCTCGGCCTTTT-3', and U2-end-3': 5'-GGGTGCACCGTTCCTGGA-3' were used, and was inserted into pSK, designated pSK-U1 or pSK-U2 respectively. To generate the cloning plasmid pSK/SAP145, containing human SAP145 cDNA was amplified by PCR with HeLa cDNA as template and the primers, 5'-Bgl II/SAP145: 5'-CAGGAGATCTGTCTGATGAG-3' and SAP145/5'-stop-3': 5'-CAAGTTTTAGGTCCCCTCACACTAGC-3'. The product, C terminal side of SAP145, was cloned between *Bgl* II and *Sma* I sites of the pSK. N terminal side of human SAP145 cDNA was amplified by PCR with HeLa cDNA as template and the primers, 5'-SAP145 (EcoRV): 5'-GGCGATATCCGCGCCTGGGGCTGCCC-3', and SAP145/3'-Bgl II: 5'-CTTAGCTCATCAGACAGATCTCCTGG-3'. The product was inserted between *Eco* RV and *Bgl* II sites of the pSK/C terminal side of SAP145. The cloned plasmid named pSK/SAP145NC. The 5' end of human SAP145 cDNA was amplified by PCR with HeLa cDNA as template and the primers: SAP145-AKM-5' GCGATATCCGCTAAGATGGCGACGGAG and SAP145-XhoI-3': CTACTCGAGGACCCAGAGG. The product was cloned *Eco* RV site of the pSK+. pSK/SAP145NC was digested with *Not* I followed by blunting and was digested *Xho* I. The fragment subcloned into pSK/5' end of SAP145, was digested with *Kpn* I followed by blunting and was digested *Xho* I. To generate the cloning plasmid pSK/SAP49, containing human SAP49 cDNA was amplified by PCR with HeLa cDNA as template and the primers: SAP49-2-EcoRV: 5'-GAGATATCTGCTGCCGGGCCGATCTCC-3' and SAP49NotI: 5'-GAGCGGCCGCTTACTGAGGGAGAGGGCC-3'. The product was cloned *Eco* RV and *Not* I site of the pSK+. To generate the Vpr substitution mutants

designated R80A, R88A, L67P/R80A and L67P/R88A, I introduced site-specific mutations into pSK-Fvpr (Nishizawa *et al.*, 1999) by ExSite PCR-based site-directed mutagenesis using a kit from Stratagene. Arg was changed to Ala codon by accurate PCR with pSK-Fvpr as the template for R80A and R88A, pSK-L67P/Vpr as the template for L67P/R80A and L67P/R88A and the following primers: 5'-AATAGGCGTTACTCGACAGA-3' and 5'-GCGCTATGTCGACACCCAAT-3' for R80A; 5'-AGCAAGAAATGGAGCCAGTA-3' and 5'-GCCCTCTGTCGAGTAACGCC-3' for R88A. Each *Xho* I-*Not* I fragment, including the site-mutated *vpr* gene and Flag sequences, in pSK-Fvpr was excised and subcloned into pME18Neo. pGEX-5X3-Vpr was a kind gift from Dr. K. Fujinaga (Hokkaido University, Japan). All constructs described above were verified by nucleotide sequencing.

***In vitro* splicing assay.** The pSK-*β-globin* plasmid was linearized with *Bam*H I, and then transcribed by the Riboprobe system (Promega) with T7 RNA polymerase, 7mG(ppp)G RNA Cap Structure Analog (New England Biolabs, Ipswich, MA.) and 50 μCi [α-^{32}P] CTP (PerkinElmer, Wellesley, MA.). Splicing reactions were carried out as described previously (Mayeda and Krainer, 1999). 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 (Dignam *et al.*, 1983; Lee and Green, 1990). Transcripts were separated on a 7% polyacrylamide-7 M urea denaturing gel which was exposed to an imaging plate.

Immunoprecipitation and Western blotting. Following transfection, cells were

lysed at 4°C in CHAPS lysis buffer as described previously (Kamata and Aida, 2000) and subjected to immunoprecipitations with anti-FLAG mouse M2 MAb (Sigma) or anti-SAP145 serum, which was raised against the SAP145 peptide sequence, CPSVGPKIPQALEKILQLKE. The immunoprecipitates were applied to SDS–PAGE (8 or 15% polyacrylamide). The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA.) for analysis by Western blotting. Each membrane was blocked for 1 h in a solution of 5% (w/v) skim milk powder in PBS prior to incubation with FLAG-specific monoclonal antibody (M2, Sigma), anti-SAP145 serum or anti-SAP49 donkey polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA.). Then the membrane was incubated with horseradish peroxidase-linked sheep antibodies against mouse, rabbit or donkey, IgG (Amersham Biosciences, Uppsala, Sweden). Bands of immunoreactive proteins were detected with the SuperSignal™ West Pico chemiluminescent substrate (Pierce, Rockford, Il.).

Immunofluorescence assay. HeLa cells, growing on coverslips, were examined 48 h after transfection by an immunofluoresence assay, as described previously (Kamata and Aida, 2000).

GST pull-down assay. GST pull down assays were performed by combining glutathione-Sepharose 4B beads-bound GST or GST-Vpr proteins with 10 μl ^{35}S-labeled SAP145 proteins from *in vitro* translation using the TNT system (Promega), as described previously (Iijima *et al.*, 2004; Kamata *et al.*, 2005).

Northern blot analysis. After incubation at 30°C for 10 min, the splicing reaction was added to 2.5 nmol of GST or GST-Vpr and this mixture was added Glutathione-Sepharose 4FF beads, as described previously (Lu *et al.*, 1994). RNA were

extracted with ISOGEN (Nippon gene, Toyama, Japan) from the RNA-protein complexes, remained binding to the beads, and then separated on 10 % polyacrylamide-7 M urea denaturing gels and transferred on a Hybond N+ (Amersham biosciences). The membrane was hybridized with digoxigenin (DIG)-containing riboprobes specific for the U1 or U2 snRNAs, synthesized from pSK-U1 or U2. U snRNAs on the membrene were detected by DIG Nothern Starter Kit (Roche Diagnotics, Indianapolis, IN.).

Results

Vpr interacts with the essential splicing factor SAP145

Recently, it was found that Vpr interacted with SAP145 in a yeast two-hybrid screen (Zhang *et al.*, personal communication). To confirm the interaction between Vpr and SAP145 *in vitro*, I performed GST-pull down assay. As shown in Fig. 4A, GST-Vpr (lanes 3-5), but not GST alone (lane 2), bound to *in vitro* translated ^{35}S-labeled SAP145 on a dose dependent manner, indicating that Vpr directly interacts with SAP145. To confirm whether Vpr associates with SAP145 *in vivo*, cellular extracts from HeLa cells transfected with pME18Neo-FVpr or without plasmid (Mock) were subjected to immunoprecipitation with anti-Flag M2 MAb and anti-SAP145 serum. The immunoprecipitated proteins were analyzed by Western blotting using an anti-Flag M2 MAb (Fig. 4B). The Western blots demonstrated that Vpr coimmunoprecipitated with SAP145, in consistent with the *in vitro* binding results. By contrast, no specific band was observed in the analysis using extracts from HeLa cells transfected without plasmid (Fig. 4B). To examine whether Vpr colocalizes with SAP145 in the nucleus, a Flag-tagged Vpr expression plasmid was transfected into HeLa cells, and subsequently immunostained with anti-Flag M2 MAb (Fig. 5, left panel) and anti-SAP145 antibody (Fig. 5, middle panel). Vpr was localized predominantly in the nucleus and nuclear envelope. By contrast, endogenous SAP145 was localized in the nucleus and showed a speckled distribution. Interestingly, it is clearly shown that a part of Vpr colocalizes with endogenous SAP145 in a specked distribution (Fig. 5, right panel). These results confirmed that Vpr interacts with SAP145 in the nucleus.

Vpr localizes in the spliceosome

To examine the interaction between Vpr and functional spliceosomal complexes, I examined whether Vpr were coimmunoprecipitated with SAP49, which is a component of U2 snRNP and binds to SAP145 directly (Champion-Arnaud and Reed, 1994), U1-70K, which is a component of U1 snRNP and U2B", which is a component of U2 snRNP. As indicated in Fig. 6, Western blotting of the immunoprecipitates with anti-U1-70K and U2B" clearly showed that they interacted with Vpr as well as SAP145. However, SAP49 did not interact with Vpr.

Next, to confirm whether Vpr was associated with the spliceosomes that were formed, a splicing reaction containing the GST-Vpr protein was incubated for 10 min at 30°C, and the reaction mixture was then subjected to GST pull down. If the GST-Vpr became associated with the spliceosomes, the affinity selection should yield the snRNAs that participated in splicing. The precipitated RNAs were extracted and analyzed by Northern blotting using digoxigenin-labeled riboprobes directed against U1 or U2 snRNA (Fig. 7). Although only the background amounts of the U1 and U2 snRNAs were found in the reactions containing any of the GST alone, bands of GST-Vpr were greater observed than that in GST alone, indicating that GST-Vpr was associated with U1 and U2 snRNP. Moreover, I determined whether Vpr colocalized with SC35, which was a non-snRNP splicing factor of the SR family of proteins that was commonly used to define splicing nuclear speckles (Lamond and Spector, 2003). Same as Fig. 5, in the merge panel (Fig. 8, right panel) showed that Vpr was colocalized with endogenous SC35, suggesting that Vpr localizes in the spliceosome (Fig. 8).

Collectively, my results strongly suggested that Vpr is associated with SAP145 in the spliceosome.

Vpr directly inhibits splicing reactions

In the previous study, it was appeared 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 (Kuramitsu *et al.*, 2005). To determine whether Vpr directly inhibits splicing reaction, I firstly prepared the splicing-competent nuclear extracts (NE) from HeLa-S3 cells, mixed them with NE prepared from HeLa cells transfected with pME18Neo-FVpr and immediately analyzed the splicing activity of NE on *β-globin* pre-mRNA substrate. As shown in Fig. 9, *β-globin* pre-mRNA was efficiently spliced in NE from HeLa-S3 by addition of ATP/creatine phosphate (lane 2). Interestingly, after the addition of Vpr-containing NE, the splicing was decreased by approximately 80% compared to that achieved by NE from HeLa-S3 only (lane 8). The inhibition was showed on a dose-dependent manner (lanes 6-8). This *in vitro* splicing assay demonstrated that Vpr targets splicing directly and inhibits it.

Vpr inhibits splicing at the early stages of spliceosome assembly

Assembly of a catalytically active metazoan spliceosome sequentially proceeds through a series of intermediate complexes termed heterogeneous (H), E, A, B, and C (Fig. 3), which can be distinguished *in vitro* by separating splicing reactions using native gel electrophoresis. As shown in Fig. 10, when *β-globin* pre-mRNA used as substrate, complexes H+E, A and B+C were detected in addition of nuclear extracts from Mock (lanes 1-3). E complex is hidden within the nonspecific H complex and can therefore not be distinguished using this assay. In contrast, while E and A prespliceosome complexes were evident in addition of Vpr-containing NE, higher

complexes B and C representing functional spliceosomes were not seen (lanes 4-6). These results indicate that when Vpr was present, splicing is inhibited because spliceosome assembly is interrupted before the complexes B and C formed.

Vpr inhibits splicing of *β-globin* pre-mRNA via interaction with SAP145

In the previous study (Kuramitsu *et al.*, personal communication), the domain(s) involved in splicing inhibition of Vpr, was identified by producing pEGFP-N1 that encoded chimeric FLAG-GFP fusion proteins that consisted of three putative structural regions on the basis of the amino acid sequence and analysis of three dimentional structure of the Vpr utilizing nuclear magnetic resonance (Wecker *et al.*, 2003): i) the first α-helical region, extending from aa 17 to aa 33 (α-H1); ii) the third α-helical region, extending from aa 56 to aa 77 (αH3); and iii) the arginine-rich carboxy-terminal domain from aa 77 to aa 96 (77-96) (Fig. 11). This study showed that both αH3 and 77-96 are indispensable for the accumulation of *α-globin2* pre-mRNA by Vpr.

To verify that the interaction with SAP145 is necessary for splicing inhibition by Vpr, I created mutant forms of Vpr. To modify the structure and interaction ability of Vpr, I took particular note of L67, R80 and R88 residues of Vpr because the previous study suggested that αH3 and arginine rich region of Vpr are necessary for the splicing inhibition by Vpr (Fig. 11). Thus, it was designated point mutations of Vpr as L67P, R80A, R88A, L67P/R80A and L67P/R88A (Fig. 12). Although three mutants, L67P, L67P/R80A and L67P/R88A were remarkably impaired the abilities to interact with SAP145 (Fig. 13). In particular, double point mutants, L67P/R80A and L67P/R88A, were more severely impaired that compared with single point mutant L67P. These mutants partially restored the splicing reaction by *in vitro* splicing assay with substrate

of *β-globin* pre-mRNA (Fig. 14). By contrast, the R80A and R88A had kept activities to interact with SAP145 and inhibited the splicing reaction. These results indicated that the interaction between Vpr and SAP145 is required for splicing inhibition of *β-globin* pre-mRNA by Vpr.

Vpr alters the interaction between SAP145 and SAP49

Splicing factor SAP49 interacts directly and highly specifically with both SAP145 and pre-mRNA and the SAP49-SAP145 complex directly interact with both U2 snRNP and the pre-mRNA (Champion-Arnaud and Reed, 1994). To firstly verify the effect of Vpr on SAP145-SAP49 complex formation, cellular extracts from HeLa cells transfected with pME18Neo-FVpr or with control vector pME18Neo were subjected to immunoprecipitation with anti-FLAG M2 MAb or anti-SAP145 serum, and the resulting immunoprecipitates were subjected to the Western blot with anti-SAP49 serum (Fig. 15A). Same as Fig. 6, in the immunoprecipitation analysis with anti-FLAG M2 MAb, the interaction between SAP49 and Vpr was not detected. SAP145 was coimmunoprecipitated with SAP49 in the control extracts, indicating that both proteins form in a tight complex. In contrast, the expression of Vpr was inhibited the complex formation.

To further define how the Vpr interaction with SAP145 inhibits SAP145-SAP49 complex formation, I examined to see if mutants L67P, L67P/R80A and L67P/R88A, which show reduced interaction with SAP145, were proficient for inhibiting SAP145-SAP49 complex formation (Fig. 15B) compared to the mutants R80A and R88A. In the presence of Vpr wild type, R80A and R88A, I observed less coimmunoprecipitation of SAP145 and SAP49, but it appeared that the intensity of the

band of SAP49 in presence of wild type Vpr and the R80A mutant. In contrast, SAP145 and SAP49 interacted efficiently together in the presence of L67P, L67P/R80A or L67P/R88A. These results suggest that Vpr associates with SAP145 and then suppresses the SAP145-SAP49 complex formation.

To next determine whether Vpr competitively inhibits the interaction between SAP145 and SAP49, ^{35}S-labeled *in vitro* translated SAP49, non-labeled SAP145 and GST-Vpr protein were mixed and immunoprecipitated with anti-SAP145 serum and protein A-Sepharose. Immunoprecipitated proteins were analyzed by SDS-PAGE. As shown in Fig. 16, the amount of SAP49 that bound to SAP145 decreased as a function of the amount of GST-Vpr. This was not the case for the control using increasing amounts of GST alone, suggesting that Vpr competes for the interaction between SAP145 and SAP49.

Collectively, these results strongly indicate that Vpr competes with SAP49 for interaction with SAP145.

Discussion

At the beginning of this study, I expected that SAP145 was one of the candidates for Vpr-interacting protein, since it has found that Vpr interacted with SAP145 in a yeast two-hybrid screen (Zhang *et al.*, personal communication). In my study, I firstly confirmed that Vpr interacts with SAP145 *in vitro* and *in vivo* (Fig. 4), and Vpr was colocalized with SAP145 in the nucleus, indicating the SAP145 is a Vpr-interacting protein in the host cells. Moreover, I found that Vpr was associated with U2B", a component of U2 snRNP, U1-70K, a component of U1 snRNP (Fig. 6), and U1 and U2 snRNP (Fig. 7), and colocalized with SC35, a well-known spliceosome marker, suggesting Vpr interacts with SAP145 in the spliceosome.

Since SAP145 is an essential splicing factor, it has been examined whether Vpr can regulate the splicing reaction and discovered a novel role for Vpr, which accumulates *α-globin 2* and *β-globin* pre-mRNA *in vivo* (Kuramitsu *et al.*, 2005). Moreover, it has been also indicated strong evidence that Vpr expressed from an HIV-1 provirus was sufficient to accumulate for *α-globin2* pre-mRNA. However, it is possible that the expression of Vpr, which acts as a regulator of transcription (Agostini *et al.*, 1996; Felzien *et al.*, 1998; Subbramanian *et al.*, 1998; Kino *et al.*, 2002), may affect the transcription of several genes such as SR protein and hnRNP which are essential for the splicing regulation in the transfected cells. Thus, *β-globin* pre-mRNA substrate was incubated under *in vitro* splicing conditions in NE prepared from Vpr-expressing cells (Fig. 9). By *in vitro* splicing assays, I confirmed that Vpr targets splicing directly and inhibits it. Moreover, native gel electrophoresis analysis demonstrated that Vpr inhibits splicing at the early stages of spliceosome assembly (Fig. 10). This result indicates that splicing factors that are involved in the initial stages of the splicing process are

hampered in the presence of Vpr.

The three-dimentional NMR structure of the full-length Vpr showed the intact Vpr molecule also contains three helices, which hold around of a hydrophobic core constituted of Leu, Ile, Val and aromatic residues (Morellet *et al.*, 2003). This could explain some of the binding properties of Vpr with different targets, and the modification of the biological activity induced by specific changes of amino acids achieved by site-directed mutagenesis. Indeed, replacement of Leu residue by Pro at position 67 within third α-helical region αH3 of Vpr remarkably impaired the splicing inhibition, the interaction with SAP145 and the inhibition of SAP49 and SAP145 complex formation (Figs. 13-15). Thus, my present data indicates that the third α-helical region and in particular the Leu residues at position 67, is indispensable for the splicing inhibition via an interaction between Vpr and SAP145. By contrast, although the arginine-rich carboxy-terminal domain 77-96 appeared to be involved in splicing inhibition, substitutions of Arg for Ala at positions 80 and 88 within this domain have no effect on the splicing inhibition and interaction with SAP145 by Vpr. This clearly indicated that Arg residues at positions 80 and 88 are dispensable for the splicing inhibition via an interaction between Vpr and SAP145. The NMR structure also showed that basic carboxy-terminal domain of Vpr is flexible and stabilized when Vpr interacts with its partner (Morellet *et al.*, 2003). In fact, R80A mutant interacted with SAP145 and completely inhibited the splicing reaction, but to some extent could not inhibit the SAP49 and SAP145 complex formation. By contrast, R88A as well as wild type Vpr impaired the SAP49 and SAP145 complex formation. These results suggest that R80A may be stabilized its structure by interacting with SAP145 and SAP49

complex. Additionally, *in vitro* competitive binding assay confirmed clearly that Vpr competed with SAP49 against SAP145 (Fig. 16), indicating that Vpr and SAP49 do not bind to each other, nor compete with each other to binding site on SAP145. In the presence of Vpr, the loss-of-interaction between SAP145 and SAP49 were able to induce splicing inhibition result in similar phenotypes, it has been predicted that the interaction between Vpr and the SAP145-SAP49 complex positively and negatively may relate with a splicing function of Vpr. Just during my preparation of this manuscript, it has been suggested that Vpr induces cell cycle arrest, which is one of functions of Vpr, by binding of SAP145 and by interfering with the interaction between SAP145 and SAP49 (Terada and Yasuda, 2006). Thus, it is likely that the expression of Vpr inhibits the interaction between SAP145 and SAP49. My *in vitro* competitive binding assay (Fig. 16) also suggested that the splicing inhibition might be carried out that Vpr competed with SAP49 against SAP145.

It is unknown why Vpr inhibits the function of SAP145-SAP49 by interfering with the interaction between SAP145-SAP49 in the host cells. Although little is known about detail function of SAP145-SAP49 complex, there is one possible hypothesis with regard to its function. Recently, the three-dimensional structures of SF3b (Fig. 17, Golas *et. al*, 2003) was determined by single-particle electron cryomicroscopy at a resolution of less than 10 angstroms and then it appeared that SAP49 has two RRMs which are located on the surface of SF3b (Fig. 17). Moreover, Champion-Arnaud and Reed (1994) had demonstrated that SAP49 UV crosslinks to pre-mRNA immediately upstream of the branchpoint sequence in the pre-spliceosomal complex A, and, in addition, SAP49 participates in direct protein-protein interactions with SAP145, suggesting that SAP49 and SAP145 interact in a U2 snRNP associated complex that functions to tether U2

snRNP to the branch site. Therefore, it is possible that Vpr interacts with SAP145 and thereby interfering formation of the SAP145-SAP49 complex that directly interacts with both U2 snRNP and the pre-mRNA. Thus, it is likely that this protein complex plays a role in stable tethering U2 snRNP to the branch site. Indeed, my *in vitro* splicing assays (Fig. 9) and native gel electrophoresis (Fig. 10) indicate that authentic B and C complexes were not formed when Vpr is present, and that complexes A and B transition was very inefficient in nuclear extract from that transiently expressed Vpr. Likewise, HSV-1 protein ICP27 has been reported to interact with SAP145 and inhibit splicing at the early stage of the splicing process (Bryant *et al.*, 2001, Smith *et al.*, 2005). Thus, my present results indicate that the direct binding between SAP145 and SAP49, and in consequence, stable tethering U2 snRNP to the branch site requires for B complex spliceosome assembly on pre-mRNA and the expression of Vpr inhibit its assembly. Therefore I propose a putative model for splicing inhibition by Vpr (Fig. 18). This also is the first report showing that an HIV-1 accessory protein, Vpr, regulates directly the cellular pre-mRNA splicing. My results reveal an understanding of the splicing regulatory and molecular mechanism by Vpr and a novel role of the viral protein in the splicing reactions.

In addition to splicing inhibition, it has been well known that Vpr has many functions in both viral and cellular proliferation, such as cell cycle G2 arrest (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995), apoptosis (Ayyavoo *et al.*, 1997; Nishizawa *et al.*, 2000a, b, Azuma *et al.*, 2005) and regulation of transcription (Agostini *et al.*, 1996; Felzien *et al.*, 1998; Subbramanian *et al.*, 1998; Kino *et al.*, 2002). On the other hand, several studies reported links between splicing and cell signaling such as cell cycle and apoptosis (Shin and Manley, 2004; Schwerk and Schulze-Osthoff, 2005). For

example, the yeast *PRP17* gene, encodes a splicing factor, has been identical to the cell division cycle *CDC40* gene, and it was identified the homologue of human gene (Ben Yehuda *et al.*, 1998). TIA-1, an apoptosis-promoting protein, regulates alternative pre-mRNA splicing of *the apoptotic gene Fas* (Forch *et al.*, 2000). Therefore, further study is required to define clearly the relation between Vpr splicing regulation and the other functions.

Recently, the strong evidence was reported that 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 (Kuramitsu *et al.*, 2005) and an NL43-2-drived mutant virus lacking *vpr* produced low level of *de novo* synthesized *env* mRNA and Env protein, and correspondingly showed lower viral infectivity (Zhang *et al.*, personal communication). These reports suggest that Vpr may regulate both cellular and viral pre-mRNA splicing, and play important roles in HIV-1 infection and replication. While the regulation both cellular and HIV-1 pre-mRNA splicing remain to be fully elucidated, in this study demonstrated a novel role for Vpr in the splicing reaction. My results provide important insight for the design and selection of novel targets for anti-HIV-1 drug development.

Collectively, I propose that the interaction between Vpr and SAP145 and the splicing regulation by Vpr can be novel targets for anti-HIV-1 drug development.

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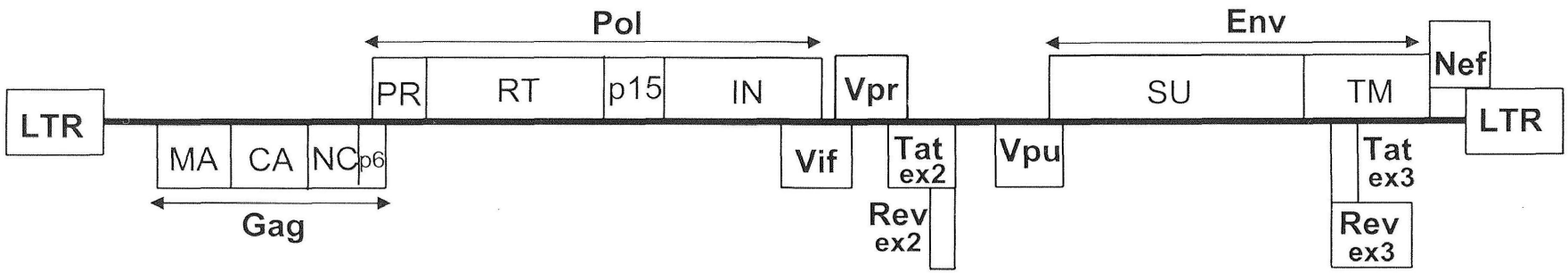
Figure 1. **The structure of HIV-1 genome.** All retrovirus contain three major open reading frames, including *gag* (generates the viral core after intravirion processing of the $p55^{gag}$ precursor polypeptide), *pol* (encodes the RT, IN, and protease enzymes), and *env* (directs the production of the transmembrane and surface glycoprotein). In addition, HIV-1 contains genes for regulatory (*tat* and *rev*) and accessory (*vpr*, *vpu*, *vif*, and *nef*) proteins.



Gag : Group specific antigen

Pol : Polymerase

Env : Envelope protein

Rev : Regulation of virion protein expression

Tat : *trans*-Activator

Vif : Virion infectivity factor

Vpr : Viral protein R

Vpu : Viral protein U

Nef : Negative factor

LTR: Long terminal repeat

RT: Reverse transcriptase

IN : Integrase

MA: Matirix

CA: Capsid

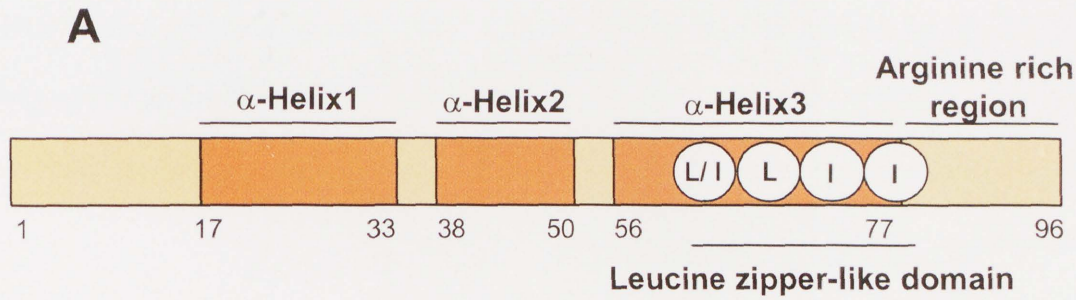
NC: Nucleocapsid

SU: Surface

TM: Transmembrane

Figure 1.

Figure 2. **Schematic representation of the Structure of Vpr identified by amino acid sequence analysis (A) and the three-dimensional structure using NMR (B).** The structure of the protein was characterized by the defined first α-helical domain extending from aa 17 to aa 33, the second α-helical domain extending from aa 38 to aa 50, the third α-helical domain extending from aa 56 to aa 77, the arginine rich region extending from aa 77 to 96, and leucine zipper-like domain extending from aa 60 to aa 81.


A
α-Helix1
α-Helix2
α-Helix3
Arginine rich region
L/I
L
I
I
1
17
33
38
50
56
77
96
Leucine zipper-like domain

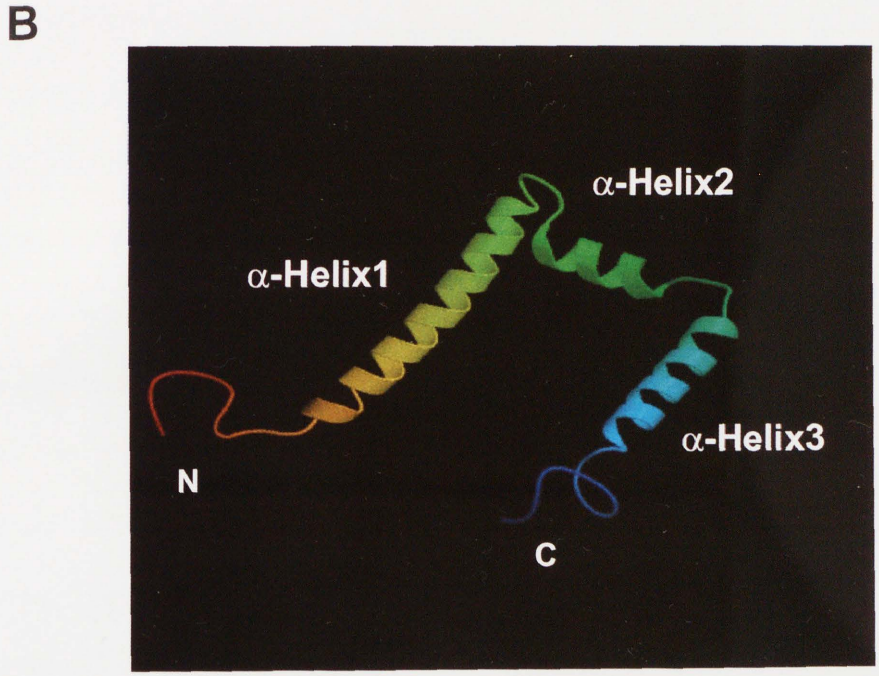

B
α-Helix2
α-Helix1
α-Helix3
N
C

Figure 2

Figure 3. **Schematic representation of pre-mRNA splicing and the spliceosome assembly pathway.** Splicing is an essential step of gene expression in which introns are removed from pre-mRNA to generate mature mRNA that can be translated by the ribosome. This reaction is catalyzed by a large and dynamic macromolecular RNP complex called the spliceosome. The spliceosome is formed by the stepwise integration of five snRNPs composed of U1, U2, U4, U5, and U6 snRNAs and more than 50 proteins binding sequentially to pre-mRNA.

In the early step, U1 snRNP (yellow circle) binds to 5' splice site of pre-mRNA, yielding complex E. Spliceosome formation initially involves the interaction of U1 and U2 (blue circle) snRNPs with the 5'splice site and the branch site (red), respectively, yielding the pre-spliceosome or complex A. The U4/5/6 tri-snRNP (gray circles), in which U4 and U6 snRNAs are base-pared, is then stably bound to give complex B. which, however, still has no catalytic center. For catalytic activation of the spliceosome, complex B undergoes a dramatic structural change that involves the dissociation of the intermolecular U4-U6 RNA helices and the formation of an intricate network of interactions between the U6, U2, and pre-mRNA molecules, which together constitute part of the catalytic core of the spliceosome. Prior to this RNA rearrangement, U1 snRNP has to dissociate from the 5' splice site. The activated complex B undergoes the first catalytic step of splicing, which generates complex C. Complex C undergoes the second catalytic step after which the post-spliceosomal intron-containing complex is dismantled and mRNA product is released (Stark and Luhrmann, 2006). SAP145, SAP49 and U2B" are components of SF3b in U2 snRNP. U1-70K is a component of U1 snRNP. U2AF65 and U2AF/35 are splicing factors.

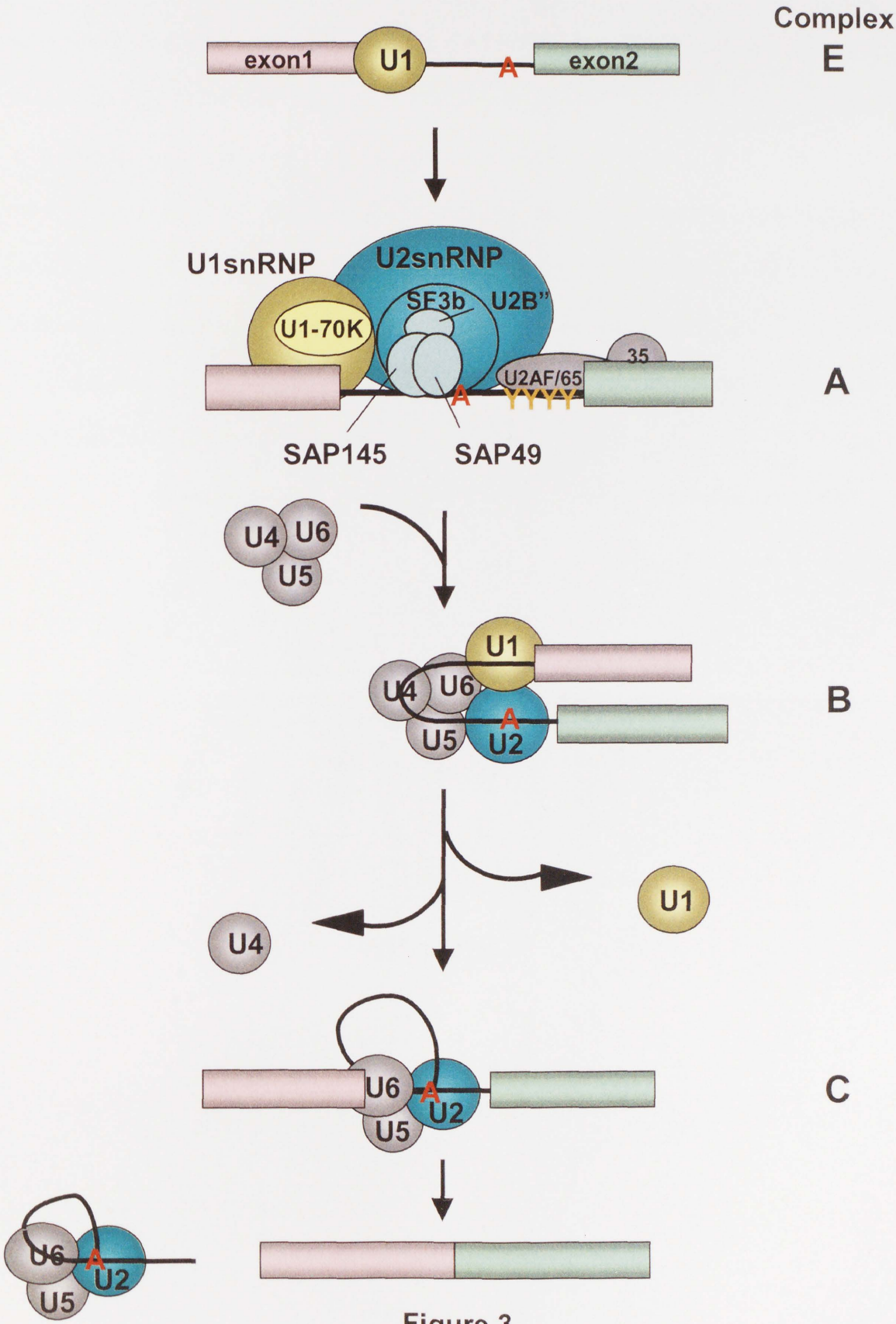

Complex
E
exon1
U1
A
exon2
U1snRNP
U2snRNP
SF3b
U2B''
U1-70K
35
U2AF/65
YYYY
A
SAP145
SAP49
A
U4
U6
U5
U1
U4
U6
A
U5
U2
B
U1
U4
U6
A
U2
U5
C
U6
A
U2
U5

Figure 3

Figure 4. **Vpr interacted with SAP145 *in vitro* and *in vivo*.** (A) GST pull-downs were performed with ^{35}S-labeled, *in vitro* translated SAP145 and 100 pmol of GST (lane 2) or 10, 30, 100 pmol of GST-Vpr (lanes 3, 4 and 5) respectively. Glutathione-Sepharose-precipitated proteins or 2% of input SAP145 (lane 1) were analyzed by 6% SDS-PAGE gels and visualized by autoradiography. (B) Extracts from HeLa cells transfected either without (Mock) or with pME18Neo encoding Flag-tagged Vpr (pME18Neo-FVpr) transiently and immunoprecipitated with mouse preimmune IgG, anti-FLAG M2 MAb, or anti-SAP145 antibody. Bound proteins and 2.5% of the extract (Input) were subjected to Western blot analysis with anti-FLAG M2 MAb.

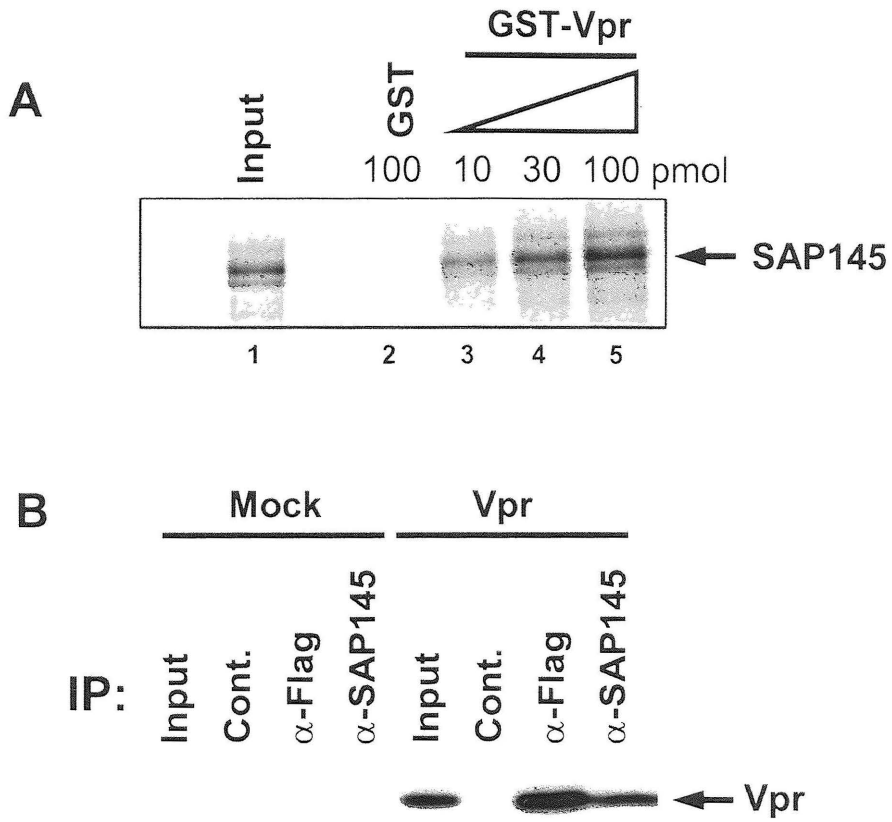

A
GST-Vpr
GST
Input
100 10 30 100 pmol
SAP145
1 2 3 4 5
B
Mock
Vpr
IP:
Input
Cont.
α-Flag
α-SAP145
Input
Cont.
α-Flag
α-SAP145
Vpr
WB: α-Flag

Figure 4

Figure 5. **Vpr colocalized with SAP145 in the nucleus.** HeLa cells that were transiently transfected with pME18Neo-FVpr were stained with anti-FLAG M2 MAb (left panel) together with anti-SAP145 serum (middle panel), and then cells were analyzed by confocal laser scanning microscopy. Bar, 10 μm. The fields were merged (right panel) to show colocalization (arrow heads) in yellow.

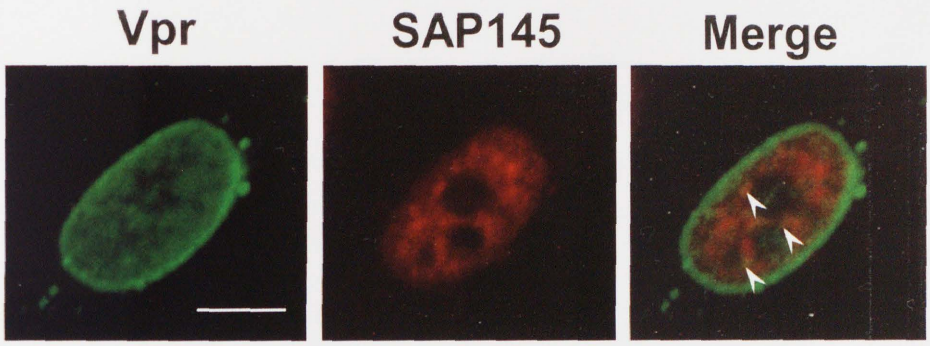


Figure 5

Figure 6. **Vpr was associated with other spliceosomal proteins.** Extract from HeLa cells transiently transfected with pME18Neo-FVpr (Vpr) or with the control vector pME18Neo (Cont.). At 24 h after transfection, cells were subjected to immunoprecipitation with anti-FLAG M2 MAb, anti-SAP145, anti-SAP49, anti-U1-70K serum or anti-U2B'' MAb. The blot was probed with anti-FLAG M2 MAb.

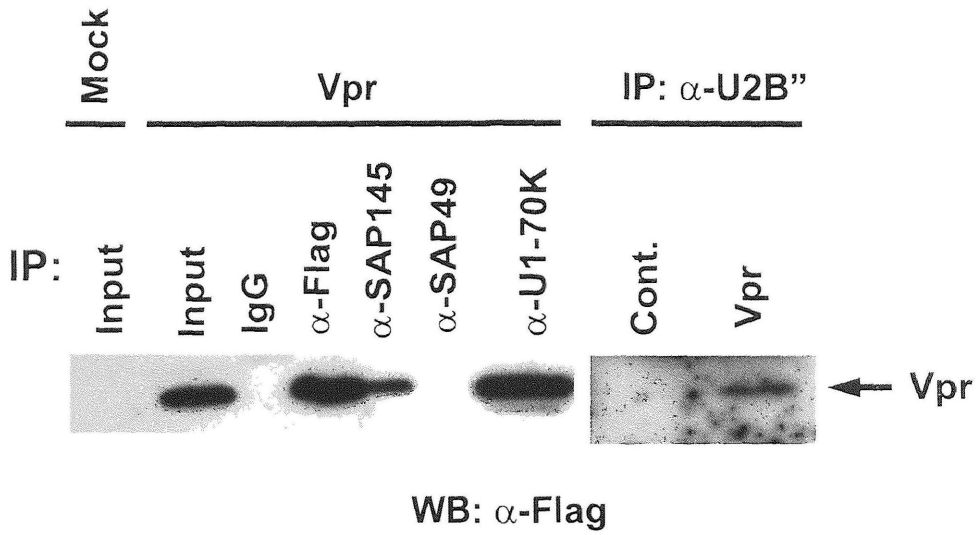

Mock
Vpr
IP: α-U2B"
IP:
Input
Input
IgG
α-Flag
α-SAP145
α-SAP49
α-U1-70K
Cont.
Vpr
Vpr
WB: α-Flag

Figure 6

Figure 7. **Vpr was associated with U1 and U2snRNPs.** GST pull-downs were performed with nuclear extract (NE) from HeLa cells under splicing conditions in a final volume of 50 μl in the presence of 2.5 nmol of the GST or GST-Vpr. Glutathione-Sepharose-precipitated RNA and 2% of input NE (Input) were analyzed by denaturing polyacrylamide gels. The blot was probed with the U1 or U2 snRNA riboprobe.

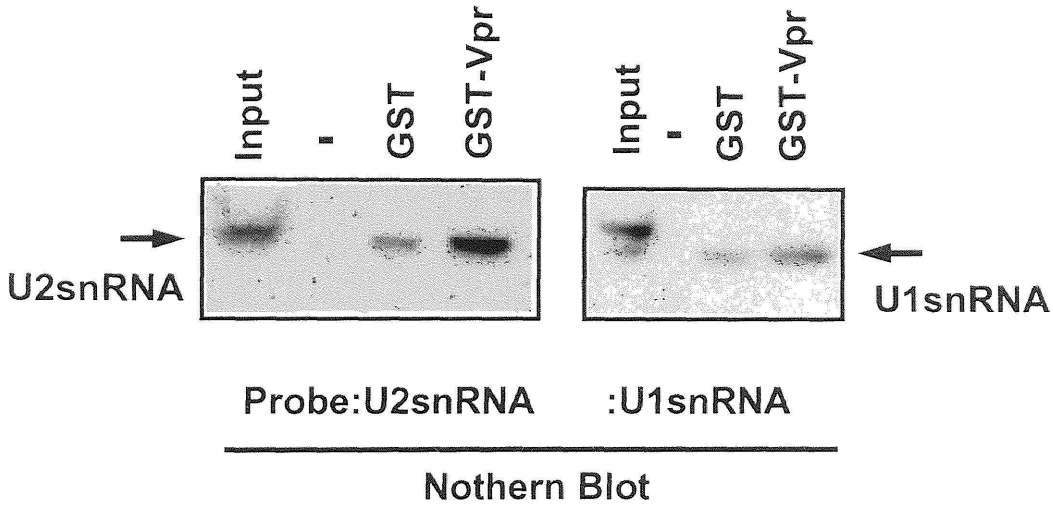

Input
-
GST
GST-Vpr
Input
-
GST
GST-Vpr
U2snRNA
U1snRNA
Probe:U2snRNA
:U1snRNA
Nothern Blot

Figure 7

Figure 8. **Vpr existed in the spliceosome.** HeLa cells that were transiently transfected with pME18Neo-FVpr were stained with anti-FLAG M2 MAb (left panel) together with anti-SC35 antibody (middle panel), and then cells were analyzed by confocal laser scanning microscopy. Bar, 10 μm. The fields were merged (right panel) to show colocalization (arrow heads) in yellow.

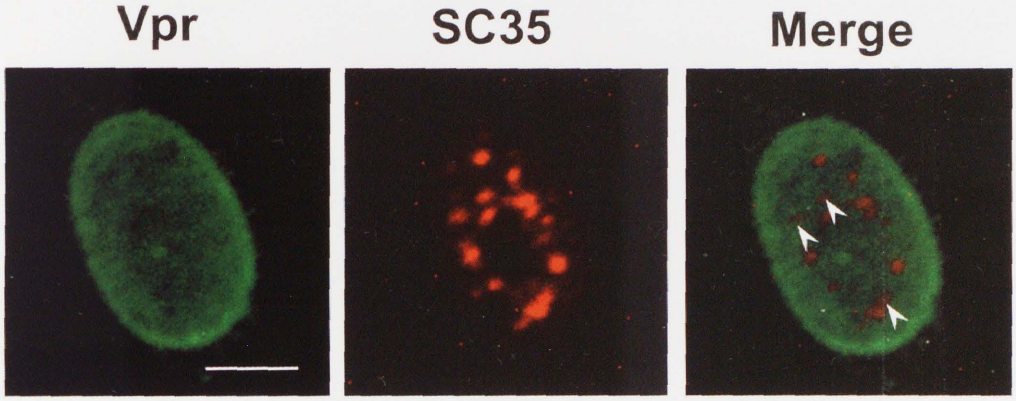


Figure 8

Figure 9. **Vpr inhibited β-globin splicing *in vitro*.** NE was prepared from transfected HeLa cells with either without (Mock) or with pME18Neo-FVpr (Vpr) transiently. ^{32}P-Labeled *β-globin* pre-mRNA was incubated for 2 h at 30 °C with NE from HeLa-S3 cells with (lane 2) or without energy (ATP/CP) (lane 1), and in the indicated amounts of Mock NE (lanes 3, 4 and 5; 4, 12 and 20 μg respectively) or Vpr-containing NE (lanes 6, 7 and 8; 4, 12 and 20 μg respectively) were added. The RNA products were separated on a 7% polyacrylamide-7 M urea denaturing gel and the bands were visualized by autoradiography. Splicing efficiencies of *β-globin* pre-mRNAs were quantified by using a BAS2500. Splicing activity was taken to be the product of mRNA/(pre-mRNA+mRNA) X 100, and relative splicing was taken to be the product of each splicing activity/control splicing activity X 100. All experiments were done at least three times. Average splicing efficiencies and standard deviations are shown.

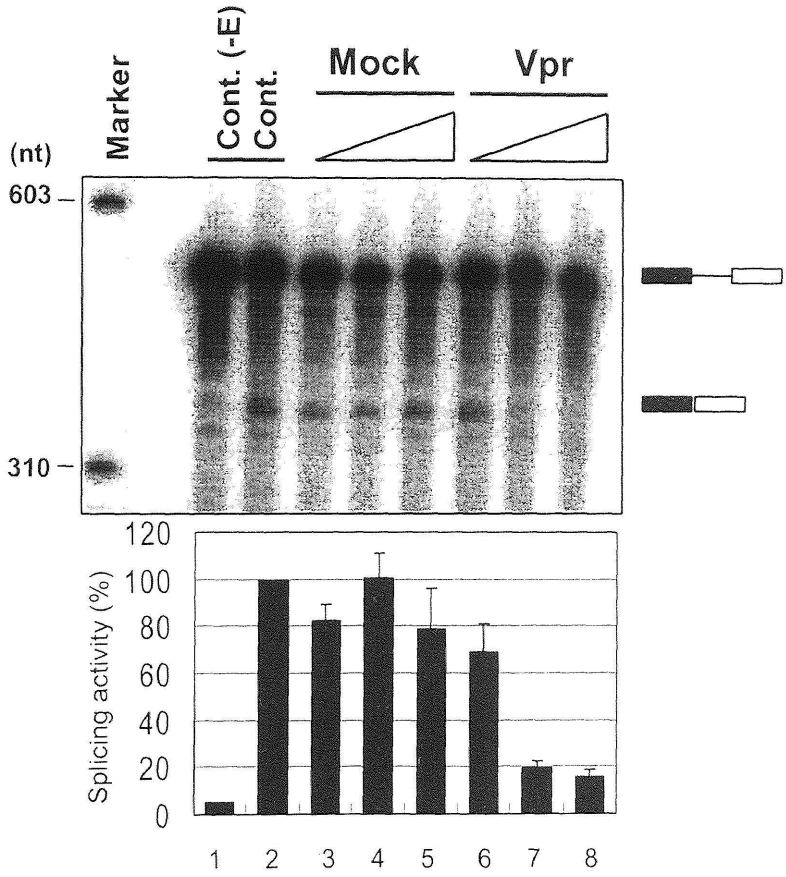

Marker
Cont. (-E)
Cont.
Mock
Vpr
(nt)
603
310
Splicing activity (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8

Figure 9

Figure 10. **Analysis of spliceosome formation in Vpr-containing extracts.** *In vitro* splicing reactions were performed in Mock NE (Mock) or Vpr-containing NE (Vpr) using ^{32}P-labeled *β-globin* pre-mRNA as substrate. At indicated time points (0, 30 and 60 min) samples were withdrawn and directly analyzed for spliceosome formation using native 4.2% polyacrylamide gel electrophoresis and visualized by autoradiography.

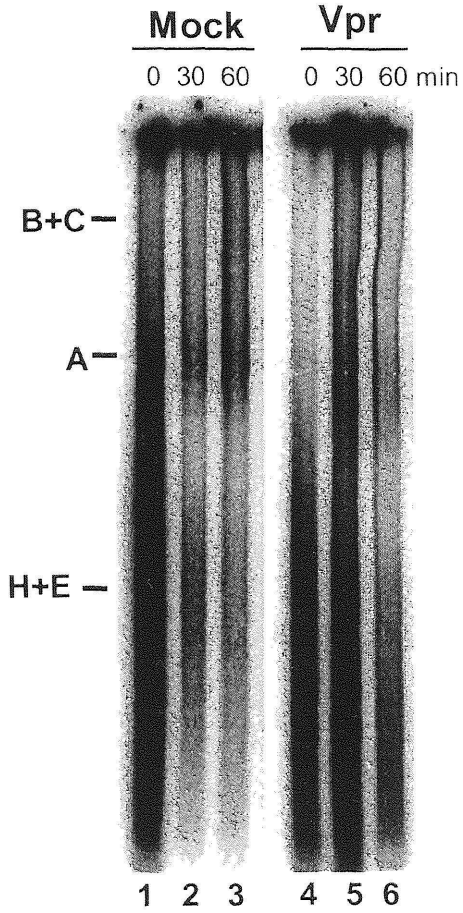


Figure 10

Figure 11. **Vpr mutants accumulated *a-globin 2* pre-mRNA.** Schematic presentation of the predicted first α-helical domain (α-Helix1), second α-helical domain (α-Helix2), the third α-helical domain (α-Helix3) as well as the arginine rich region of Vpr protein are indicated. Grey areas represent the FLAG-tag and GFP-tag. The results of *a-globin 2* pre-mRNA accumulation by Vpr mutants are summarized (right panel).

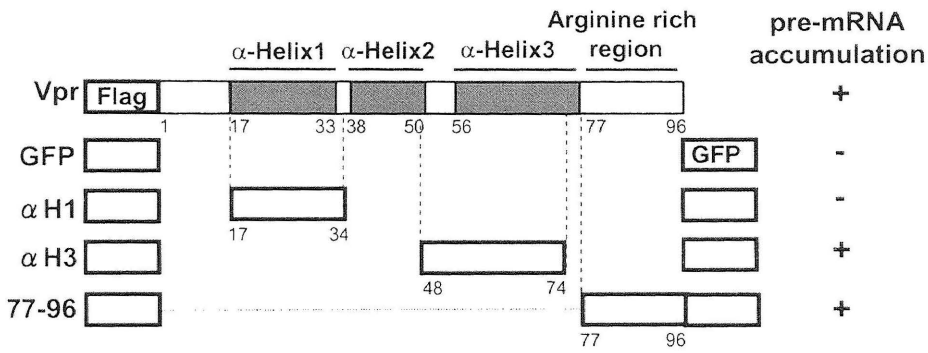

α-Helix1
α-Helix2
α-Helix3
Arginine rich region
pre-mRNA accumulation
Vpr
Flag
1
17
33
38
50
56
77
96
+
GFP
GFP
-
α H1
17
34
-
α H3
48
74
+
77-96
77
96
+

Figure 11

Figure 12. **Construction of point mutant(s) Vpr proteins.** The mutations introduced within the third α-helical domain (α-Helix3) and Arginine rich region of Vpr protein. The diagram shows schematic representations of the various proteins and putative domains. Grey areas show the FLAG-tag. It was named replacing that the Leucine at position 67 by Proline, L67P, the Arginine at position 80 or 88 by Alanine, R80A or R88A, respectively.

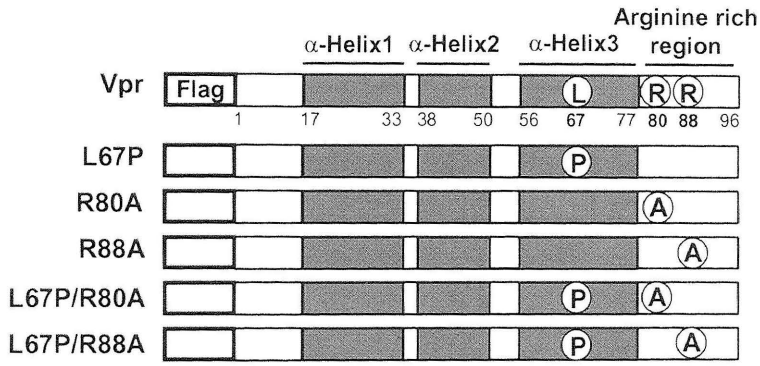

α-Helix1
α-Helix2
α-Helix3
Arginine rich region
Vpr
Flag
L
R
R
1
17
33
38
50
56
67
77
80
88
96
L67P
P
R80A
A
R88A
A
L67P/R80A
P
A
L67P/R88A
P
A

Figure 12

Figure 13. **Several Vpr mutants are decreased in binding to SAP145.** Extracts from HeLa cells were transfected transiently with pME18Neo-FVpr, Vpr/L67P, Vpr/R80A, Vpr/R88A, Vpr/L67P/R80A or Vpr/L67P/R88A, or with the control vector pME18Neo. At 24 h after transfection, cells were subjected to immunoprecipitation with anti-SAP145 serum. Bound proteins (upper panel) and 5% of the extract (Input, lower panel) were subjected to Western blot analysis with anti-FLAG M2 MAb.

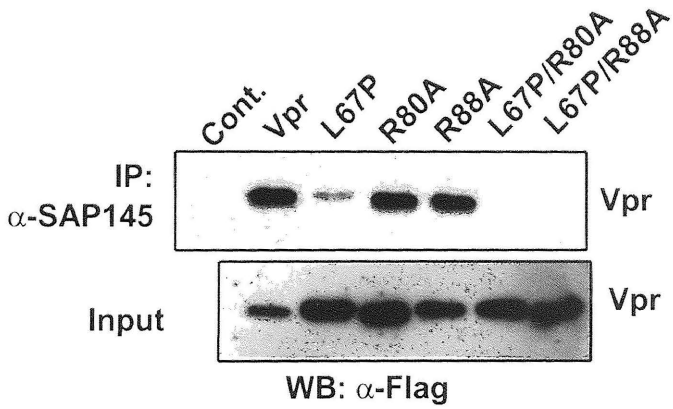

Cont.
Vpr
L67P
R80A
R88A
L67P/R80A
L67P/R88A
IP:
α-SAP145
Vpr
Input
Vpr
WB: α-Flag

Figure 13

Figure 14. **Vpr mutants with decreased binding to SAP145 were restored *in vitro* splicing activity.** *In vitro* splicing reactions were performed in NE from HeLa cells with the control vector pME18Neo (Cont.) or NE of transfected cells with pME18Neo-FVpr Vpr/L67P, Vpr/R80A, Vpr/R88A, Vpr/L67P/R80A or Vpr/L67P/R88A using ^{32}P-labeled *β-globin* pre-mRNA, as substrate. The RNA products were separated on a 7% polyacrylamide-7 M urea denaturing polyacrylamide gel electrophoresis and visualized by autoradiography (upper panel). The NE used for *in vitro* splicing was subsequently subjected to Western blot analysis with anti-FLAG M2 MAb (Input, lower panel).

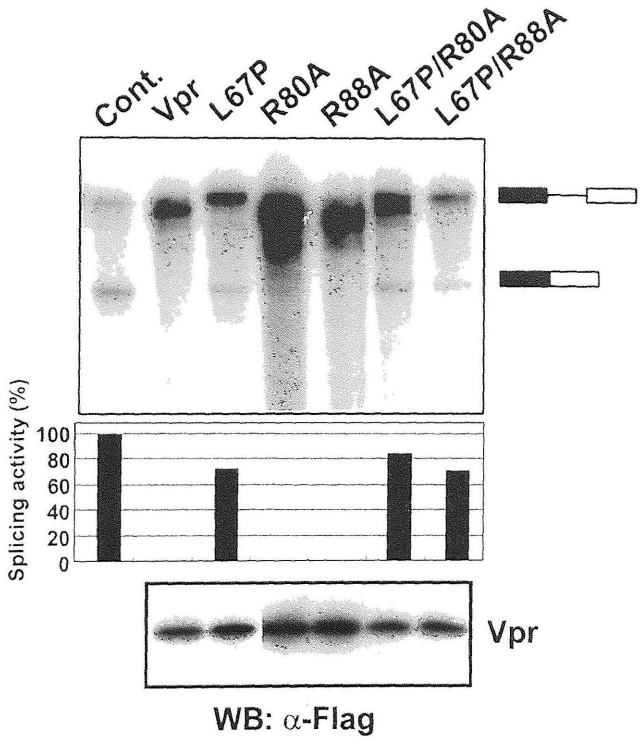

Cont.
Vpr
L67P
R80A
R88A
L67P/R80A
L67P/R88A
Splicing activity (%)
100
80
60
40
20
0
Vpr
WB: α-Flag

Figure 14

Figure 15. **Vpr inhibits the interaction between SAP145 and SAP49.** (A) Extracts from HeLa cells transiently with pME18Neo-FVpr (+) or with the control vector pME18Neo (-), were immunoprecipitated with anti-FLAG M2 MAb or anti-SAP145 antibody. The bound proteins and 5% of extract (Input) were subjected to Western blot analysis with anti-SAP49 antiserum. (B) Extracts from HeLa cells transfected with control vector pME18Neo (Cont.) or with pME18Neo-FVpr, Vpr/L67P, Vpr/R80A, Vpr/R88A, Vpr/L67P/R80A or Vpr/L67P/R88A were immunoprecipitated with anti-SAP145 antibody and analyzed by 8% SDS-PAGE gels. The bound proteins (upper panel) and 5% of the extract (Input, middle panel) were analyzed with anti-SAP49 antibody or anti-FLAG M2 MAb (Input, lower panel).

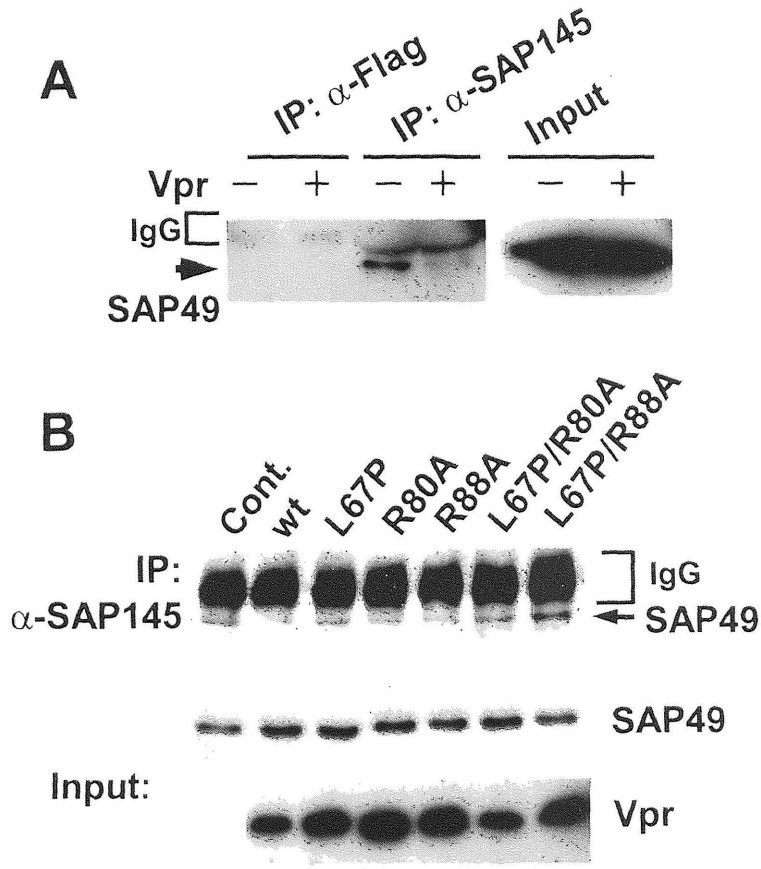

A
IP: α-Flag
IP: α-SAP145
Input
Vpr
IgG
SAP49
B
Cont.
wt
L67P
R80A
R88A
L67P/R80A
L67P/R88A
IP:
α-SAP145
IgG
SAP49
SAP49
Input:
Vpr

Figure 15

Figure 16. **Vpr inhibits with SAP49 for formation of SAP49-SAP145 complex.** ^{35}S-labeled *in vitro* translated SAP49, non-labeled *in vitro* translated SAP145 and GST (10 and 100 pmol, respectively) or GST-Vpr protein (10 and 100 pmol, respectively) were mixed and immunoprecipitated with anti-SAP145 antiserum and protein A-Sepharose. Immunoprecipitated proteins or 2% of input SAP49 (Input) were analyzed by 8% SDS-PAGE gels and visualized by autoradiography (upper panel). Immunoprecipitation without GST and GST-Vpr was performed as a control. The band signal intensity was quantified by using a BAS2500 and the intensities of bands were normalized with that of the control (lower panel).

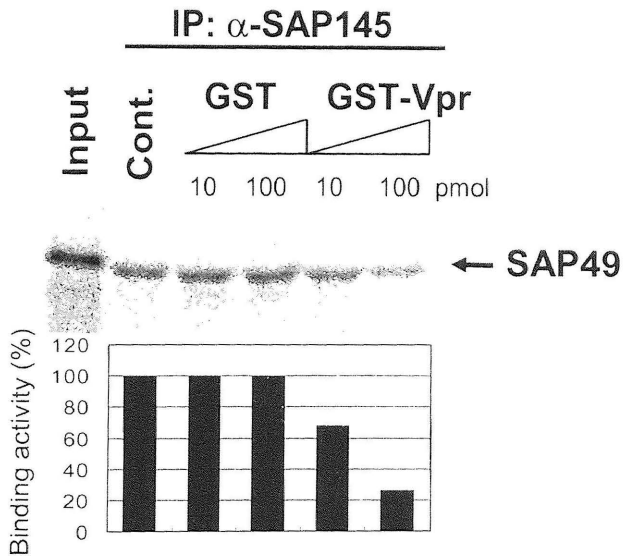

IP: α-SAP145
Input
Cont.
GST
GST-Vpr
10 100 10 100 pmol
SAP49
Binding activity (%)
120
100
80
60
40
20
0

Figure 16

Figure 17. **The Three-dimensional structure of SF3b.** The Three-dimensional reconstruction of the multiple protein complexes SF3b at ~10 Å resolution by single-particle electron cryomicroscopy (Golas *et al.*, 2003). Two RRMs belonging to the SAP49 could be identified clearly (green).

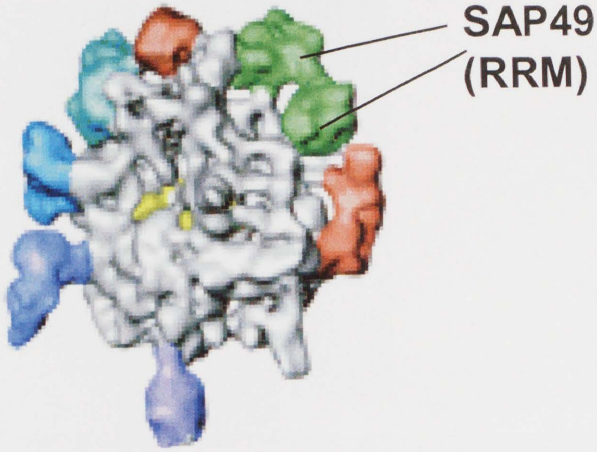


Figure 17

Figure 18. **A model for inhibitory mechanism of splicing reaction by Vpr in the *β-globin* pre-mRNA.** Vpr interacts with SAP145 resulting in the suppression of direct interaction between SAP145 and SAP49. Since SAP49 interacts directly and highly specifically with both SAP145 and pre-mRNA in essential splicing factor SF3b, binding of the U2 snRNP to pre-mRNA might be reduced. At the early stages of spliceosome assembly, the reduction, decrease of SAP145-SAP49 complex may suppress that complex A changes to complex B (Complex A, right side). Thus, splicing reaction is inhibited by Vpr.

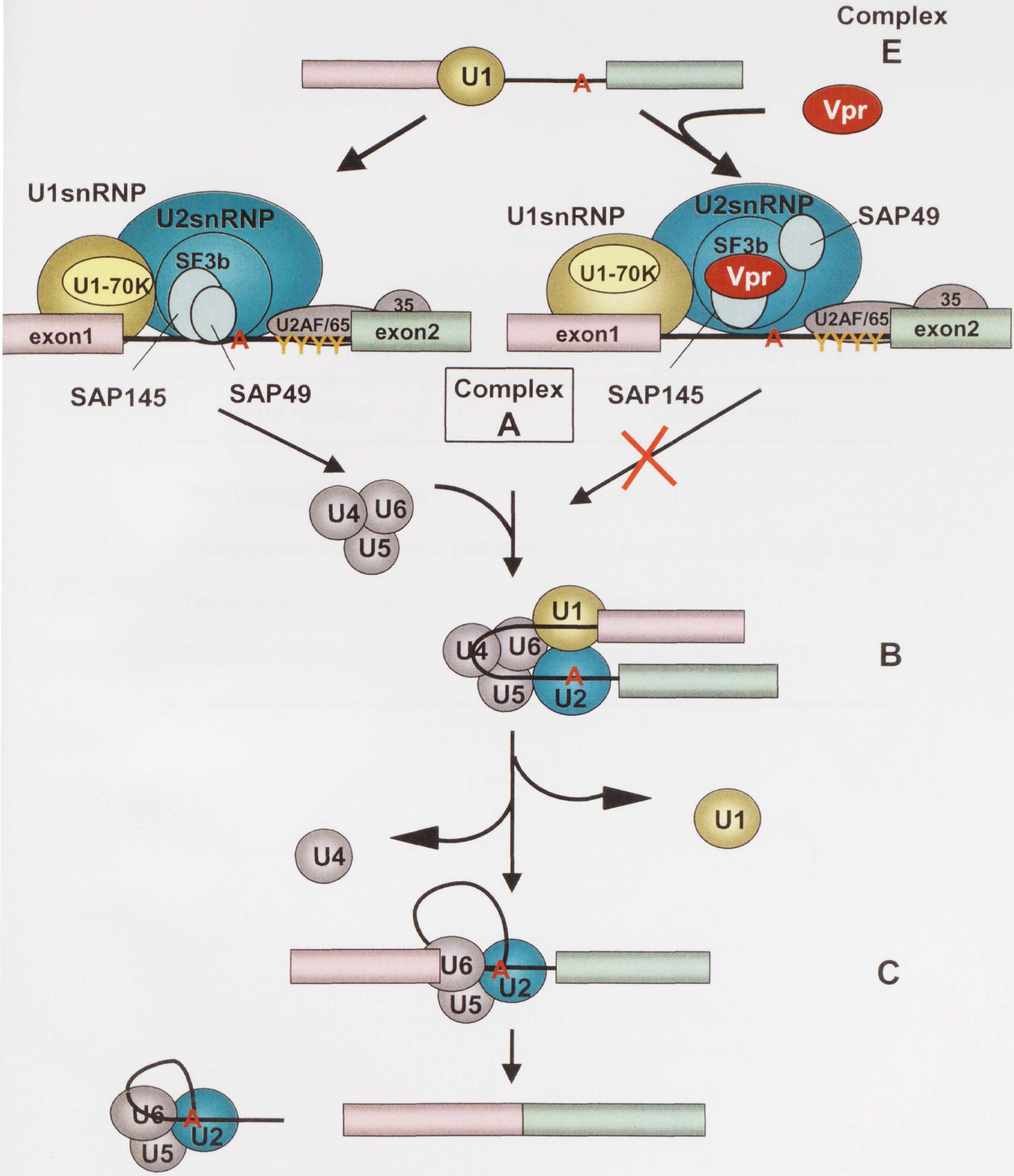

Complex
E
U1
A
Vpr
U1snRNP
U2snRNP
SF3b
U1-70K
exon1
A
U2AF/65
35
exon2
SAP145
SAP49
U1snRNP
U2snRNP
SAP49
SF3b
Vpr
U1-70K
exon1
A
U2AF/65
35
exon2
SAP145
Complex
A
U4
U6
U5
U1
U4
U6
A
U5
U2
B
U1
U4
U6
A
U5
U2
C
U6
A
U5
U2

Figure 18

Table 1. Characteristics of Vpr

Virus related	*Cellular level*
Virion-associated protein	Localizes in the nucleus
Incorporation into the virus particles	Cell cycle arrest at the G2 stage
Transcriptional activator	Apoptosis
Effect on virus replication	Oligomerization

Table 2. Interacting Partners and characteristics of Vpr

Cellular proteins	*Biological significance of the interaction*
Importin α	Nuclear translocation of the HIV-1 PIC and nuclear localization of Vpr
Pom 121	Localizes in the nucleus
14-3-3	Cell cycle arrest at the G2 stage
ANT	Apoptosis
CyclinT1	Transactivation
HHR23A	Cell cycle arrest at the G2 stage and apoptosis (?)
P34mov	Cell cycle arrest at the G2 stage (?)
Lysine tRNA synthetase	HIV-1 reverse transcription
TFIIB	Transactivation of promoter
Sp1	Transactivation of the HIV-1 LTR
GRII	Transactivation of the glucocorticoid-Responsive promoter

? = has been controversial evidence.