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The Signal Peptide of a Simple Retrovirus Envelope Functions as a Posttranscriptional Regulator of Viral Gene Expression^{∇†}

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Retroviruses use different strategies to regulate transcription and translation and exploit the cellular machinery involved in these processes. This study shows that the signal peptide of the envelope glycoprotein (Env) of Jaagsiekte sheep retrovirus (JSRV) plays a major role in posttranscriptional viral gene expression. Expression of the JSRV Env *in trans* increases viral particle production by mechanisms dependent on (i) its leader sequence, (ii) an intact signal peptide cleavage site, (iii) a *cis*-acting RNA-responsive element located in the viral genome, (iv) Crm1, and (v) B23. The signal peptide of the JSRV Env (JSE-SP) is 80 amino acid residues in length and contains putative nuclear localization and export signals, in addition to an arginine-rich RNA binding motif. JSE-SP localizes both in the endoplasmic reticulum and in the nucleus, where it colocalizes with nucleolar markers. JSE-SP is a multifunctional protein, as it moderately enhances nuclear export of unspliced viral mRNA and considerably increases viral particle release by favoring a posttranslational step of the replication cycle.

The cell uses several strategies to regulate transcription and translation. For example, cellular mRNAs undergo various modifications in the nucleus (e.g., splicing and capping) before being exported to the cytoplasm, where translation occurs. Translation initiation and elongation are also subjected to various factors that can affect protein synthesis (42).

Retroviruses have evolved a variety of mechanisms to exploit the posttranscriptional gene expression machinery of the cell. For example, spleen necrosis virus, human T-cell leukemia virus (HTLV), and other retroviruses contain in their 5' untranslated regions (UTR) a *cis*-acting proximal posttranscriptional control element (7, 31) that interacts with the cellular RNA helicase A and modulates translation (30). Rous sarcoma virus uses *cis*-acting elements to regulate posttranscriptional events. The Rous sarcoma virus genome contains two direct repeat regions (47, 62), a negative regulator of splicing (75), and a stability element (73). These elements mediate, respectively, full-length mRNA nuclear export, stability, polyadenylation, and suppression of splicing.

Retroviruses, unlike the great majority of cellular genes, also need to export full-length unspliced RNA into the cytoplasm (in addition to single- or double-spliced mRNAs) (15–16, 21). The intron-containing full-length mRNA encodes the main structural and enzymatic viral proteins (i.e., Gag, Pro, and Pol) and also functions as the viral genome, which is then packaged into the newly formed virions (69). Some retroviruses synthesize regulatory proteins evolved specifically to facilitate posttranscriptional events (e.g., RNA nuclear export). These viruses are usually referred to as complex retroviruses in order to

differentiate them from the simple retroviruses, which possess only the canonical retroviral genes (*gag*, *pro*, *pol*, and *env*) (14). Human immunodeficiency virus (HIV), a complex retrovirus, encodes Rev, a multifunctional regulatory protein that facilitates export of full-length viral RNA (and the single-spliced *env* mRNA), Gag translation, and RNA encapsidation (5, 8, 17, 38). Rev interacts in *trans* with an RNA structure within the viral genome (the Rev-responsive element [RRE]) (38) and with the cellular karyopherin export factor Crm1 (chromosome region maintenance 1) (26, 45). The latter mediates RNA export by recruiting cellular adaptors and directing the RNA-protein complex to the nuclear pore (15). Other retroviruses, such as equine infectious anemia virus, feline immunodeficiency virus, HTLV, human endogenous retrovirus K (HERV-K), and mouse mammary tumor virus (MMTV), encode Rev-like proteins (32, 33, 43, 76). HTLV also encodes other accessory proteins, including posttranscriptional repressors (46, 77).

Mason-Pfizer monkey virus (MPMV), a simple retrovirus, does not use a viral protein to mediate RNA export but utilizes a structured *cis*-acting RNA element (the constitutive transport element [CTE]) (11, 79), which mediates RNA export by interacting with the cellular nuclear export factor Tap/NXF1 (10, 29, 54).

Here we investigated the posttranscriptional regulation of the exogenous and endogenous betaretroviruses of sheep (48, 50). JSRV is an exogenous and pathogenic virus that causes a transmissible lung adenocarcinoma in sheep (52). Interestingly, the sheep genome contains approximately 27 copies of JSRV-related endogenous retroviruses (enJSRVs), which play an essential role in the host reproductive biology and act as virus restriction factors (3, 4, 19, 44).

In this study we show that sheep betaretroviruses use a unique strategy to regulate posttranscriptional events of the viral replication cycle. More specifically, the signal peptide of the envelope glycoprotein of JSRV (JSE-SP) enhances nuclear export of full-length viral RNA and increases viral particle production by acting at a posttranslational step of the replication cycle.

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MATERIALS AND METHODS

Plasmids. Expression plasmids for the JSRV₂₁ infectious molecular clone (pCMV2JS21) and for the enJS56A1 (pCMV2enJS56A1) have been described (49, 52, 53). enJS56A1 is a defective provirus that is unable to exit transfected cells due to two critical amino acid substitutions in Gag at positions 21 and 98. We used the revertant enJS56A1 mutant enJS56-W21R-C98R (termed penJS-2 in this paper), which produces viral particles in transfected cells (44, 49). The expression plasmid for the JSRV Env (pCMV3JS21ΔGP) has also been described and is called pJSE in this paper for simplicity (37). pJSE34HAV5 was derived from JSE by the addition of a hemagglutinin (HA) tag after amino acid residue 34 of the JSRV Env and a V5 tag at the end of the cytoplasmic tail in the transmembrane (TM) domain. Plasmid pSARM4, containing the MPMV infectious molecular clone, was kindly provided by Eric Hunter (59). All mutant plasmids employed in this study were obtained by standard molecular biology techniques and/or site-directed mutagenesis using QuikChange XL (Stratagene) as recommended by the manufacturer. Deletion and tagged mutants of pJSE, pCMV2JS21, and pCMV2enJS56A1 are described in Results (see Fig. 1 and 3). Specific details of the cloning procedures for any plasmid employed in this study are available on request. The expression plasmids for B23 (pcDNA3.1 His-tagged NPM) and its dominant-negative version (pcDNA3.1 His-tagged NPMΔL) were kindly provided by Jason Weber (78). The expression plasmid pNup214/CAN (ΔCAN) expresses residues 1864 to 2090 of the human Nup214/CAN protein and functions as a dominant negative form of Crm1 (26). This plasmid was kindly provided by Gerard Grosveld. pcTapA17 is an expression plasmid for the dominant negative form of Tap and was a gift from Bryan Cullen (34).

Cell cultures. HEK293, HEK293T, COS, sheep choroid plexus (SCP), 293-enJS56A1, 208F.JSRV, and 208FJSRV₂₁ cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum at 37°C, 5% CO₂ and 95% humidity. SCP cells were kindly provided by David Griffiths. 293-enJS56A1 cells are HEK293 cells stably transfected with an expression plasmid for the enJS56A1 provirus (pCMV2enJS56A1) (49). 208F.JSRV (2) was derived from the 208F rat cell line (58), which had been transformed and which stably expressed the JSRV Env. Similarly, 208FJSRV₂₁ was derived from 208F cells which had been transformed with an expression plasmid of the entire JSRV₂₁ infectious molecular clone (pCMV2JS21) and which stably released viral particles in the supernatant.

Transfections, viral preparations, and Western blots. HEK293T cells were cultured in 10-cm-diameter petri dishes and were transfected with the appropriate plasmids (1 to 3 μg) using a Calphos mammalian transfection kit (Clontech) according to the manufacturer's instructions. Supernatants were collected at 24 to 48 h posttransfection, and virus particles were concentrated by ultracentrifugation as previously described (52, 53). For analysis of intracellular proteins, cells were lysed and proteins extracted by standard techniques as described previously (13). Gag expression was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting on concentrated viral particles from supernatants and protein extracts (50 μg) of lysates of transfected cells as described before, using a rabbit polyclonal antiserum against the JSRV p23 (matrix domain [MA]) or the capsid domain (CA) or monoclonal antibodies (MAbs) to HIV-1 p55/p24 (25, 52, 53). The JSRV CA antiserum cross-reacts with the MPMV CA and was used in experiments involving this virus, as indicated in Results. Western blots were quantified, when necessary, by chemifluorescence in a Molecular Dynamics Storm 840 imaging system using Image-Quant TL software (Molecular Dynamics). Each experiment was repeated independently at least three times.

Immunoprecipitations. In order to assess the association of the JSRV signal peptide with B23, we performed immunoprecipitation assays essentially as previously described (68). Briefly, cells were transfected with the appropriate plasmids as described above and processed for fractionation using a Proteoextract subcellular proteome extraction kit (Calbiochem). Subcellular fractions were immunoprecipitated with anti-FLAG M2 affinity gel antibody (Sigma) at 4°C overnight as recommended by the manufacturer. Proteins were eluted from the beads with a Flag peptide (200 ng/ml; Sigma) on ice and processed for SDS-PAGE and Western blotting. Antibodies used were rabbit polyclonal antibody against calnexin (an endoplasmic reticulum [ER] marker; Abcam), rabbit polyclonal antibody against histone H3 (a nuclear marker; Abcam), rabbit polyclonal antibody against GAPDH (a cytoplasmic marker; Abcam), rabbit polyclonal against B23 (Santa Cruz), and MAbs against FLAG (Sigma), as recommended by the manufacturers. When necessary, membranes were stripped using Restore Western blot stripping buffer (Thermo Scientific). V5-tagged Env proteins and, when indicated, HA-tagged proteins were detected by immunoprecipitation followed by SDS-PAGE and Western blotting of protein extracts of 293T cells

transfected with the appropriate plasmids using antibodies against V5 or HA (Sigma), as previously described (68).

HIV-derived plasmids and Gag enzyme-linked immunosorbent assay (ELISA). pNLgagSty330, a Rev-RRE and Tat-dependent HIV-1 Gag-Pol expression plasmid, and an expression plasmid for HIV-1 Rev (pCMV_sRev) were kindly provided by Barbara Felber (24, 41). In order to map the portions of the JSRV genome interacting with the JSRV signal peptide, we replaced the HIV RRE in pNLgagSty330 with various portions of the JSRV *env* and 3' UTR or with the MPMV CTE as an additional control. These plasmids are described in Results (see Fig. 4). The MPMV CTE was kindly provided by Marie-Louise Hammar-skjöld. The expression plasmid for HIV-1 Tat (pCMV-Tatflag) was a gift from Mauro Giacca (67). Plasmids (1 μg) were transfected into 10-cm dishes of 293T or SCP cells in the presence or absence of the JSRV signal peptide (or HIV Rev as a control) (1 μg) and Tat (0.2 μg). The medium was changed 24 and 40 h after transfection, and cell supernatants were assessed for the presence of HIV Gag 48 h after transfection using a Murex HIV antigen MAb kit (Abbot Murex) as recommended by the manufacturer. All experiments were repeated independently at least three times. Results are presented as means and standard deviations of the values obtained with the various constructs (see Fig. 4), normalized to the values obtained by the HIV Gag vector (pNLgagSty330) in the presence of Rev. The linear range of the test was predetermined.

Confocal microscopy. Experiments were performed on COS cells cultured on two-well chambered glass slides (Lab-Tek; Nalge Nunc International) and transfected with the appropriate plasmids using Lipofectamine (Invitrogen) according to the manufacturer's instructions. At 24 to 48 h posttransfection (or at earlier time points when indicated), cells were washed with phosphate-buffered saline and fixed with formaldehyde for 15 min. Cells were then processed as described previously (44). Primary antibodies used in confocal microscopy studies were mouse MAbs against protein disulfide isomerase (PDI) (Abcam), fibrillarin (Abcam), V5 (Invitrogen), and HA (Covance) or rabbit polyclonal antisera against HA (Abcam) and B23 (Sigma). Secondary antibodies used were anti-rabbit and anti-mouse immunoglobulin G conjugated with Alexa-488 and Alexa-594 (Molecular Probes), respectively. Slides were mounted with medium containing DAPI (4',6-diamidino-2-phenylindole [Vectashield]; Vector Laboratories), and images were analyzed with a Leica TCS SP2 confocal microscope. Time course experiments with cells transfected with JSE-34HAV5 were performed as follows. Cells were transfected with Lipofectamine (Invitrogen) and then fixed after either 5, 6, 7, or 24 h, when immunofluorescence was assayed as described above.

qRT-PCR and RT-PCR. RNA from the nuclear ($n = 28$) and cytoplasmic ($n = 38$) fractions of cells transfected with the plasmids described in Results were extracted 48 h after transfection using a Paris kit (Ambion) as recommended by the manufacturer. JSRV Gag cytoplasmic GAPDH and nuclear pre-GAPDH RNA were quantified by quantitative reverse transcriptase PCR (qRT-PCR) in an Mx30005 (Stratagene) thermocycler using a Brilliant II SYBR green qRT-PCR master mix one-step kit (Stratagene) according to the manufacturer's instructions. Contamination of the cytoplasmic RNA fraction with the nuclear fraction was ruled out by using the pre-GAPDH primer pairs in the RT-PCR. PCRs were carried out in a total volume of 25 μl. PCR conditions consisted of 10 min of *Taq* activation at 95°C, followed by 40 cycles of melting (95°C, 30 s), primer annealing at the temperature appropriate for each primer (57 to 59°C, 30 s), and extension (72°C, 20 to 30 s), ending with a melting curve analysis to validate the specificity of the PCR products. Primer pairs used for the Gag, GAPDH, and pre-GAPDH PCRs were the following: JSRVgagf (5'GTAGGA GAACAAATTCGGACGCA3') and JSRVgagr (5'TAGCAGCTTCTCGTCC AGTT), preGAPDHf (5'CCACCAACTGCTTAGCAC3') and preGAPDHr (5'CTCCCCACCTTGAAGGAAAT3'), and GAPDHf (5'TCTCCTGTACT TCAACAGCGAC3') and GAPDHr (5'CCCTGTTGCTGTAGCCAAATTC 3'). Primer pairs for GAPDH and pre-GAPDH were described previously (9, 20). Nuclear and cytosolic Gag RNA ("target") in the presence ("sample") or absence ("control") of JSESP-HA were normalized to the levels of nuclear pre-GAPDH and cytosolic GAPDH RNA ("reference") as previously described (9, 20). Amplification efficiencies (E) were as follows: Gag, 100%; GAPDH, 96%; and pre-GAPDH, 89%. The relative expression ratio of unspliced JSRV RNA in the presence or absence of JSE-SP was calculated using the following formula (55): $(E_{\text{target}})^{\Delta C_T}_{\text{target}}^{\text{(control-sample)}} / (E_{\text{reference}})^{\Delta C_T}_{\text{reference}}^{\text{(control-sample)}}$.

ΔC_T values were obtained by analyzing each individual RNA sample ($n = 66$) in triplicate. Statistical analysis of the data obtained was performed using the software REST (56), which allows the hypothesis test P value to be calculated for each sample group. P represents the probability that the difference between sample and control group is due only to chance and is calculated by performing 50,000 random reallocations of the data obtained. In order to identify JSRV mRNAs containing the *env* leader sequence, we performed RT-PCR on RNA of cells stably or transiently transfected with either pCMV2JS21, pCMV2enJS56A1, or

pJSE. RT-PCR using a SuperScript III kit (Invitrogen) according to the manufacturer's instructions. Primers and the different PCRs employed are described in the supplemental material.

Northern blotting. For Northern blotting analysis, cytoplasmic RNA was denatured, subjected to agarose gel electrophoresis, and blotted on nylon filters (Hybond; Amersham) as previously described (51). Hybridization was performed using a ^{32}P -labeled DNA probe corresponding to positions 7182 to 7455 of the JSRV₂₁ infectious molecular clone (overlapping *env* and U3) (52). In order to verify the integrity of the RNA samples, filters were stripped and rehybridized with a probe for the house-keeping gene for GAPDH. Hybridized probes were quantified by phosphorimaging using Image-Quant TL software (Molecular Dynamics). Values for each sample were calculated by the following formula: (arbitrary phosphorimager units of the JSRV probe - lane background)/(arbitrary phosphorimager units of the GAPDH probe - lane background). The values relative to full-length JSRV mRNA were set arbitrarily as 100%. Experiments were repeated independently seven times.

RESULTS

Optimal JSRV Gag expression and virus particle release are dependent on the N-terminal region of the Env glycoprotein functioning in *trans*. Our previous studies had shown that JSRV Gag expression and viral particle production are increased by the expression of the Env glycoprotein (12). In this study, we further investigated the determinants within Env that are important for JSRV Gag expression. To this end, we derived an expression plasmid for the JSRV₂₁ molecular clone modified by the addition of two premature termination codons at the beginning of *env* (pJSRVStop1-27) in order to block Env glycoprotein expression while maintaining the full-length viral genome (Fig. 1A). As expected, cells transfected with pJSRVStop1-27 expressed smaller amounts of intracellular Gag and released fewer viral particles than cells transfected with pCMV2JS21 (an expression plasmid for wild-type JSRV) (Fig. 1B, lanes 7 and 8). Cotransfection of pJSRVStop1-27 with an expression plasmid for the JSRV Env (pJSE) substantially increased Gag expression and viral particle release from the transfected cells (Fig. 1B, lane 6). In order to map the determinants within Env critical for viral particle production, we cotransfected pJSRVStop1-27 with a series of pJSE mutants containing premature termination codons or large deletions (Fig. 1A). As illustrated in Fig. 1B, the introduction of a premature termination codon at the beginning of the surface (SU) domain (amino acid residue 96; pJSEStop96), just before the TM domain (amino acid residue 374; pJSEStop374), or within the cytoplasmic tail of the TM domain (amino acid residue 590; pJSEStop590) did not abrogate the ability of Env to increase Gag expression and viral particle production (Fig. 1B, lanes 3 to 5). Also, large deletions within the SU (pJSE Δ 103-352) and TM (pJSE Δ 378-574) domains did not alter the capacity of the resulting expression plasmids to facilitate viral particle production of JSRVStop1-27 (Fig. 1B, lanes 1 and 2). Collectively, the data obtained indicated that in most cases, the N-terminal region of the JSRV Env influenced Gag expression and especially viral particle release in the supernatant of transfected cells. Next, we cotransfected pJSRVStop1-27 with truncated mutants containing the N-terminal 318 (pJSE Δ Env-Flag), 98 (pJSE Δ 98-615HA), 85 (pJSE Δ 85-615HA), 80 (pJSESP-Flag), or 67 (pJSE Δ 67-615HA) amino acid residues of the JSRV Env tagged with a carboxy-terminal Flag or HA epitope. All the truncation mutants, with the exception of the one containing only the Env N-terminal 67 amino acid residues (Fig. 1C, lane 1), maintained the ability to enhance Gag ex-

pression and viral particle release (Fig. 1C and D). Thus, the shortest truncation mutant influencing Gag expression possessed the N-terminal 80 amino acid residues of Env, corresponding exactly to the signal peptide of this glycoprotein (pJSESP-Flag). Indeed, a pJSE mutant containing a heterologous signal peptide (derived from the preprotrypsin protein; pJSEHSP-Flag) failed to enhance Gag expression of pJSRVStop1-27 in cotransfection assays (Fig. 1D, lane 2), although it efficiently mediated viral entry and transformation of rodent fibroblasts analogously to wild-type JSRV Env (data not shown).

Similar results were obtained using plasmid penJS-2 mutants derived from enJS56A1, a JSRV-related endogenous betaretrovirus of sheep, as reporters (44, 49) (data not shown).

JSE-SP increases Gag expression and virus particle release. Collectively, the data described above show that JSE-SP influences JSRV (and enJSRV) Gag expression and especially viral particle release. In order to determine whether the cleavage and release of JSE-SP from Env were necessary for its effect on Gag expression, we mutated the amino acid residues around the cleavage site of the signal peptidase (Env amino acid residues 78 to 82, GAAAA to WPPVP) in both the expression plasmid for the full-length JSRV₂₁ infectious molecular clone (pJSRV Δ SPC) and pJSE (Fig. 2A). Transfection of 293T cells with pJSRV Δ SPC resulted in a dramatic reduction of viral particle release in the supernatant compared to particle release by cells transfected with the expression plasmid for JSRV (Fig. 2B). The levels of viral particles in cells transfected with pJSRV Δ SPC were restored to the levels of wild-type JSRV when JSE-SP was provided in *trans* by cotransfecting an expression plasmid for JSE-SP tagged with an HA epitope (pJSESP-HA) (Fig. 2B, lane 5). A JSRV Env mutant bearing the same mutations in the signal peptide cleavage site as pJSRV Δ SPC (WPPVP, Env amino acid residues 78 to 82) did not show increased Gag expression or viral particle production of JSRVStop1-27 in *trans* (data not shown; see below). On the other hand, a mutated signal peptide expression plasmid terminating with the mutated amino acid residues (position 78 to 82, WPPVP) before the HA tag was able to increase JSRVStop1-27 Gag expression and viral particle production in *trans* (data not shown). Thus, expression of JSE-SP on its own (i.e., not in the context of the JSRV Env) is not influenced by the mutated residues within the signal peptidase cleavage site. In order to test whether the mutations introduced in the JSE-SP cleavage site indeed blocked the signal peptidase cleavage site, we derived the Env expression plasmids pJSE-V5, pJSE-34HAV5, and pJSE-34HA Δ SPCV5. These plasmids contain a V5 epitope tag fused to the carboxy-terminal end of the TM domain. In addition, pJSE-34HAV5 and pJSE-34HA Δ SPCV5 contain an HA epitope in the leader peptide region (between amino acid residues 34 and 35). pJSE-V5 and pJSE-34HAV5 were able to enhance viral particle release in cells transfected with penJS-2Stop1-27 while, as expected, pJSE-34HA Δ SPCV5 was not able to do so (Fig. 2C). Immunoprecipitation analysis of cells transfected with the plasmids described above suggests that pJSE-34HA Δ SPCV5 (containing a mutated signal peptide cleavage site like pJSRV Δ SPC) expresses only a polypeptide corresponding to the unprocessed pre-Env glycoprotein (corresponding to the full-length Env plus its signal peptide) (Fig. 2D).

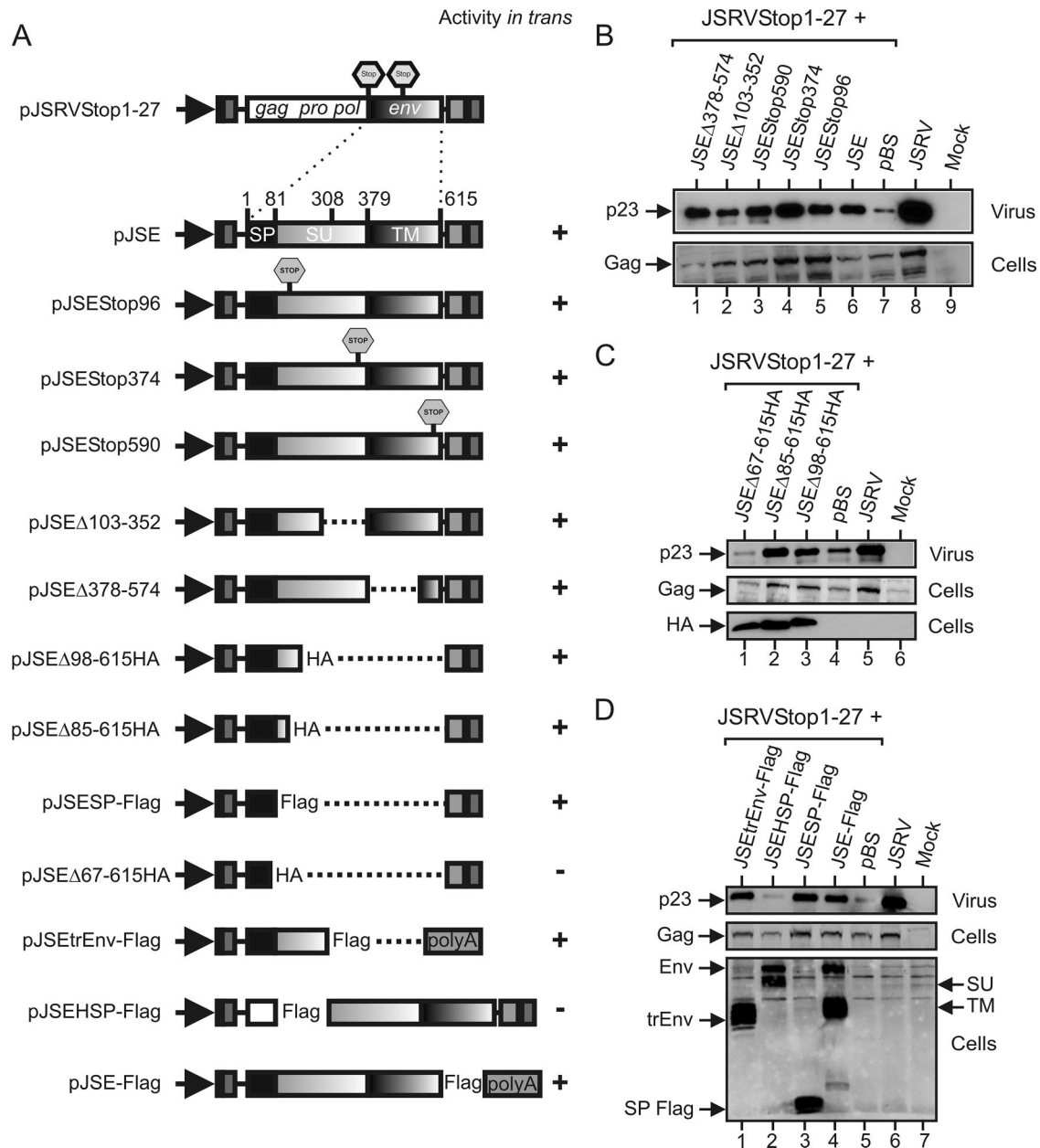


FIG. 1. The N-terminal region of Env facilitates Gag expression and virus particle production *in trans*. (A) Schematic representation of Env expression plasmids used in this study. Mutants are derived from a JSRV Env (pJSE) expression plasmid. Numbers refer to amino acid residues of the JSRV₂₁ Env (52). Premature termination codons and deletions are indicated with stop signs and dashed lines, respectively. In pJSEHSP-Flag, the signal peptide of the JSRV Env has been replaced by the signal peptide of the human preprotrypsin. The influence of each Env mutant on Gag expression of a JSRV provirus containing two premature termination codons in Env (pJSRVStop1-27) is indicated with a plus or minus sign. (B) Representative Western blots of virus pellets and cell protein extracts of 293T cells cotransfected with pJSRVStop1-27 and various JSE mutants (or the empty plasmid pBS). Cells were analyzed 48 h posttransfection by SDS-PAGE and Western blotting employing an antiserum against the JSRV p23 (matrix). Gag expression of JSRVStop1-27 is increased by all the mutants employed in the experiment. The lack of a suitable antiserum does not allow detection of the truncated JSRV Env proteins, although the ability of each expression plasmid used in this panel to enhance viral particle release of JSRVStop1-27 is indirect evidence of protein expression. Lane JSRV represents virus pellets and protein extracts of cells transfected with the expression plasmid pCMV2JS21 (wild-type JSRV). (C) Representative Western blots, as described for panel B, of virus pellets and protein extracts of cells transfected with pCMV2JS21 (JSRV) or cotransfected with pJSRVStop1-27 and various pJSE truncation mutants (tagged with an HA epitope) or the empty plasmid (pBS). (D) Representative Western blots, as described for panel B, showing that JSESP-Flag (and not JSEHSP-Flag) increases Gag expression of JSRVStop1-27. The Flag epitope in JSEHSP-Flag remains fused to the SU domain of Env.

JSE-SP function depends on an RNA-responsive element located at the 3' end of *env* and in the 3' UTR of the viral genome. Here, we tested whether JSE-SP function depends on *cis*-acting regions present in the JSRV (or enJSRVs) *env* and 3'

UTR. We constructed within the JSRV and enJSRV expression plasmids a series of *env* 3' UTR deletion mutants and used them in cotransfection assays with pJSESP-HA (Fig. 3A). As expected, all the deletion mutants used in these experiments

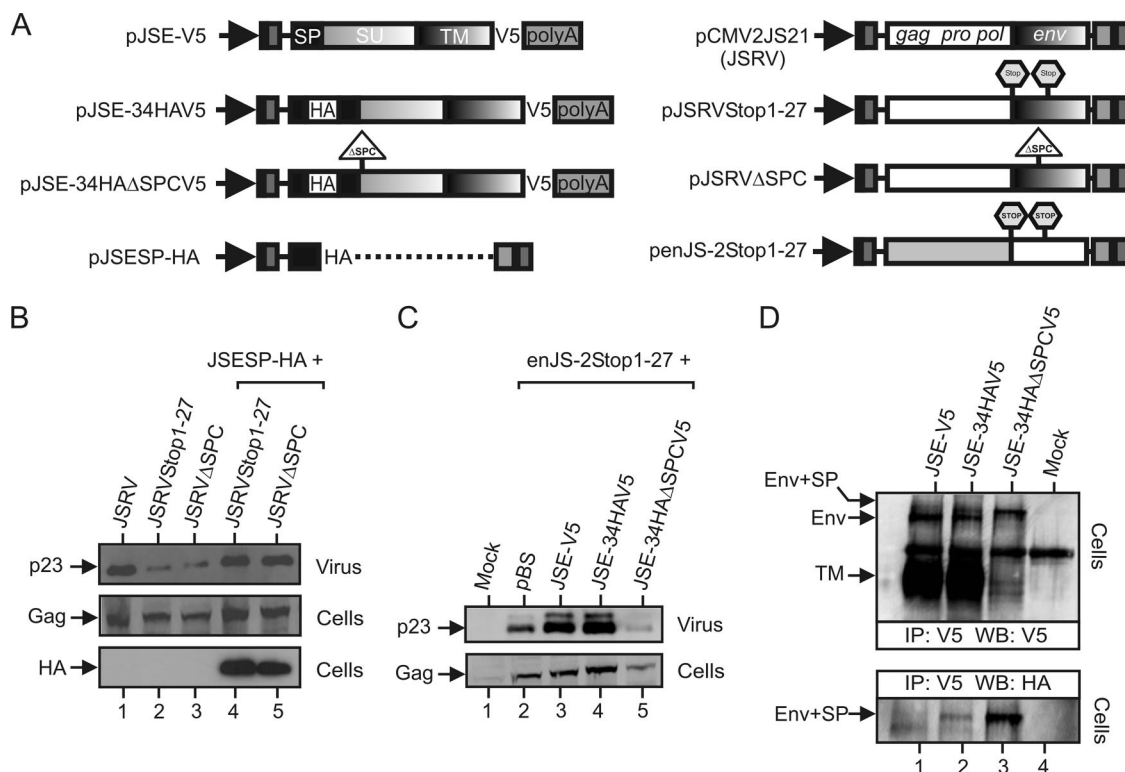


FIG. 2. The signal peptide cleavage site of the JSRV Env is necessary for optimal Gag expression. (A) Schematic representation of the plasmids used in the experiments shown in this figure. Premature termination codons and a mutated signal peptide cleavage site of the JSRV Env are indicated, as are the epitope tags HA and V5. (B) Representative Western blots of viral pellets and protein extracts of 293T cells transfected with the plasmids indicated in each lane. Samples were analyzed 48 h posttransfection employing an antiserum against the JSRV p23 (matrix) or HA to confirm expression of JSESP-HA. (C) Western blotting of virus pellets and protein extracts of 293T cells transfected with the plasmids indicated above each lane. (D) Immunoprecipitation and Western blotting of protein extracts from 293T cells transfected with the indicated plasmids using V5 and HA antisera. Cell lysates are from the experiments reflected in panel C. In cells transfected with pJSE-34HAV5 or pJSE-V5, both the full-length Env and the TM domain are visible. However, in cells transfected with JSE-34HAV5ΔSPC, only the full-length Env (including the uncleaved signal peptide) is visible.

induced barely detectable levels of viral particles in transfected cells in the absence of JSESP-HA. Levels of viral particles released by cells transfected with the deletion mutants pJSRVΔSN and pJSRVΔAN were substantially enhanced by cotransfection of the JSESP-HA expression plasmid (Fig. 3B, lanes 5 and 8). pJSRVΔAN has a large deletion in most of *env*, with the exceptions of 131 bp at the 5' extremity and 160 bp at the 3' end. Interestingly, JSRV mutants with the 5' extremity of *env* deleted (pJSRVΔBS, pJSRVΔBB, and pJSRVΔBN) were unable to cause transfected cells to produce viral particles in the presence or absence of JSESP-HA (Fig. 3B, lanes 4, 7, and 9). Similar results have been obtained with the corresponding enJSRV-based plasmids (data not shown). Thus, the data obtained so far suggested that a *cis*-acting region(s) necessary for JSE-SP function is located at the extremities of the *env* 3' UTR.

To refine the data shown above, we used a Rev-RRE-dependent HIV Gag-Pol expression vector (pNLgagSty330) (23, 24, 41). We replaced the RRE region of pNLgagSty330 with the entire or portions of JSRV *env* or the 3' UTR (Fig. 4A). In constructs containing the entire JSRV *env* and 3' UTR, we introduced either two premature termination codons in the N-terminal region (pH-JSStop1-27) or a mutated SP cleavage site (pH-JSΔSPC) to avoid spurious expression of JSE-SP.

Cells were transfected with pH-JSStop1-27 or pH-JSΔSPC, in the presence or absence of pJSESP-HA, and HIV Gag released in the supernatants was quantified by ELISA. Controls included HIV Gag-Pol maintaining the HIV RRE (pNLgagSty330) and used in cotransfection assays with HIV Rev or pNLgagSty330, where the RRE was replaced by the CTE of MPMV (pH-MPMV-CTE). Release of HIV Gag in the supernatant of cells transfected with pH-JSStop1-27 or pH-JSΔSPC increased severalfold (8 to 22 times) when JSESP-HA was provided in *trans*. In these cases, the levels of HIV Gag in the supernatants of transfected cells was comparable to the levels present in cells transfected with the controls pNLgagSty330 (in the presence of Rev) or pH-MPMV-CTE (Fig. 4B). Cotransfection of pJSESP-HA with any of the pNLgagSty330-derived constructs containing only the 5' extremity (pH-JSE5'A, pH-JSE5'B, or pH-JSE5'C) or the 3' extremity of the JSRV *env* and 3' UTR (pH-JSE3'A) did not result in an increased level of HIV Gag in the supernatant of transfected cells. However, the presence of the JSESP-HA in *trans* increased the levels of Gag in the supernatant of cells transfected with constructs containing both the 5' and 3' extremities of the JSRV *env* 3' UTR (pH-JSE5'3'A) or a large portion of the *env* UTR with the exception of the 5' extremity (pH-JSE3'B). Construct pH-

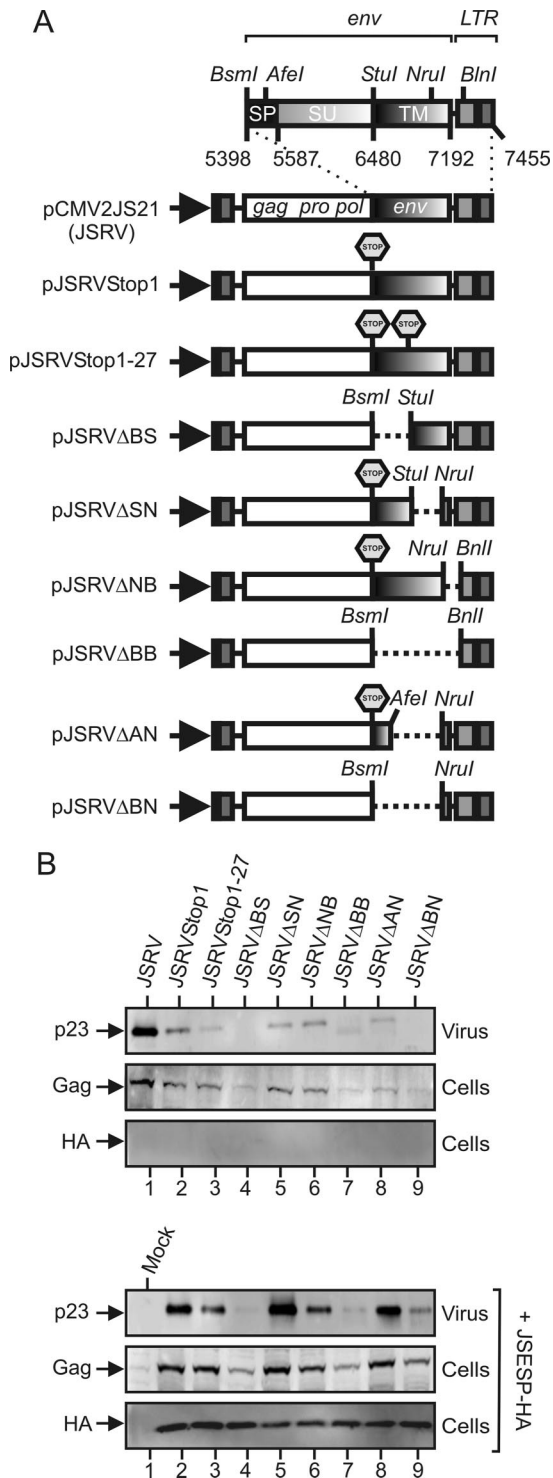


FIG. 3. JSE-SP function depends on a *cis*-acting region located in the JSRV genome. (A) Schematic representation of the deletion mutants of pCMV2JS21 used in the experiments reflected in panel B. Premature termination codons and deletions are indicated with stop signs and dashed lines. (B) Representative Western blots of virus pellets and protein extracts of 293T cells transfected with the plasmids indicated in each lane in the absence or presence of JSESP-HA. Samples were analyzed 48 h posttransfection by SDS-PAGE and Western blotting employing an antiserum against the JSRV p23 (matrix) or HA.

JSE5'3'A is the equivalent of the JSRV-derived deletion mutant pJSRV Δ AN (Fig. 3A) described above.

These data supported our model that the *env* and 3' UTR contained a *cis*-acting region(s) that is necessary for JSE-SP activity. Mfold analysis of the JSRV *env* 3' UTR contained in pH-JSE5'3'A (and pJSRV Δ AN) identified a predicted RNA secondary structure in the 3' end of the genome including the last 50 nucleotides (nt) of the *env* gene and 114 proximal nt of the U3 (note that there are 21 nt overlapping between *env* and U3) (Fig. 4C and D). Cotransfection of pJSESP-HA with pH-JSSPRE (the HIV Gag-Pol vector containing this predicted RNA secondary structure) was sufficient to increase Gag release in the supernatant of transfected cells by a factor of approximately 5, to levels comparable to those found in cells transfected with pNLgagSty330 in the presence of Rev or pH-MPMV-CTE. Our results suggest that this region, which we termed the signal peptide-responsive element (SPRE), is the minimal region necessary for JSE-SP function.

JSESP-HA was also able to substantially increase H-JSSPRE Gag expression in SCP cells, suggesting that this phenomenon was not restricted to a specific cell line (data not shown). In addition, by inserting into pNLgagSty330 portions of the enJS56A1 *env* and 3' UTR, we identified also the minimal SPRE (203 nt) of enJSRVs (data not shown). JSRV and enJSRVs SPREs are located in the same region of the viral genome and possess similar predicted RNA secondary structures (Fig. 4C and D).

JSE-SP localizes in the ER and in the nucleoli. JSE-SP possesses a predicted nuclear localization signal (NLS) and a nuclear export signal (NES). In addition, JSE-SP contains a putative arginine-rich RNA binding motif (ARM) overlapping the NLS (Fig. 5A) (66). By confocal microscopy, we observed that JSE-SP localizes both in the cytoplasm and in the nucleus of transfected cells (Fig. 5 and 6). Within the nucleus, JSE-SP staining appeared as punctate foci or as ring-like structures colocalizing with nucleolar markers, such as B23 and fibrillarin (60).

In cotransfected cells, the SP mutant with deletions of the NLS (pJSESP Δ NLS-HA) or the NES (pJSESP Δ NES-HA) were not able to increase Gag expression and viral particle release of enJS2Stop1-27 or JSRVStop1-27 (Fig. 5C and data not shown). These data suggest that both NLS and NES are essential for JSE-SP function, although we cannot exclude the possibility that the deletions per se may alter the structure and function of JSE-SP. By confocal microscopy, we observed that JSESP Δ NLS displayed a diffuse staining pattern in the cytoplasm and in the nucleus, with no punctate foci or ring-like structures surrounding the nucleoli. It is not surprising that JSESP Δ NLS-HA is still able to diffuse in the nucleus, considering that JSE-SP is only 80 amino acid residues in length and can therefore passively diffuse through the pores of the nuclear membrane. On the other hand, cells transfected with JSESP Δ NES-HA presented the typical staining pattern of JSESP-HA. We also mutated in the context of JSESP-HA the arginine residue at position 9 of the signal peptide to a tryptophan residue in order to alter the function of the putative ARM. The resulting mutant, JSESPR9W-HA, was expressed in transfected cells at levels comparable to those of JSESP-HA and localized in the nucleus and nucleoli (data not shown) but was not able to increase viral particle release of JSRVStop1-27 or enJS-2Stop1-27 (Fig. 5C and data not shown). These data

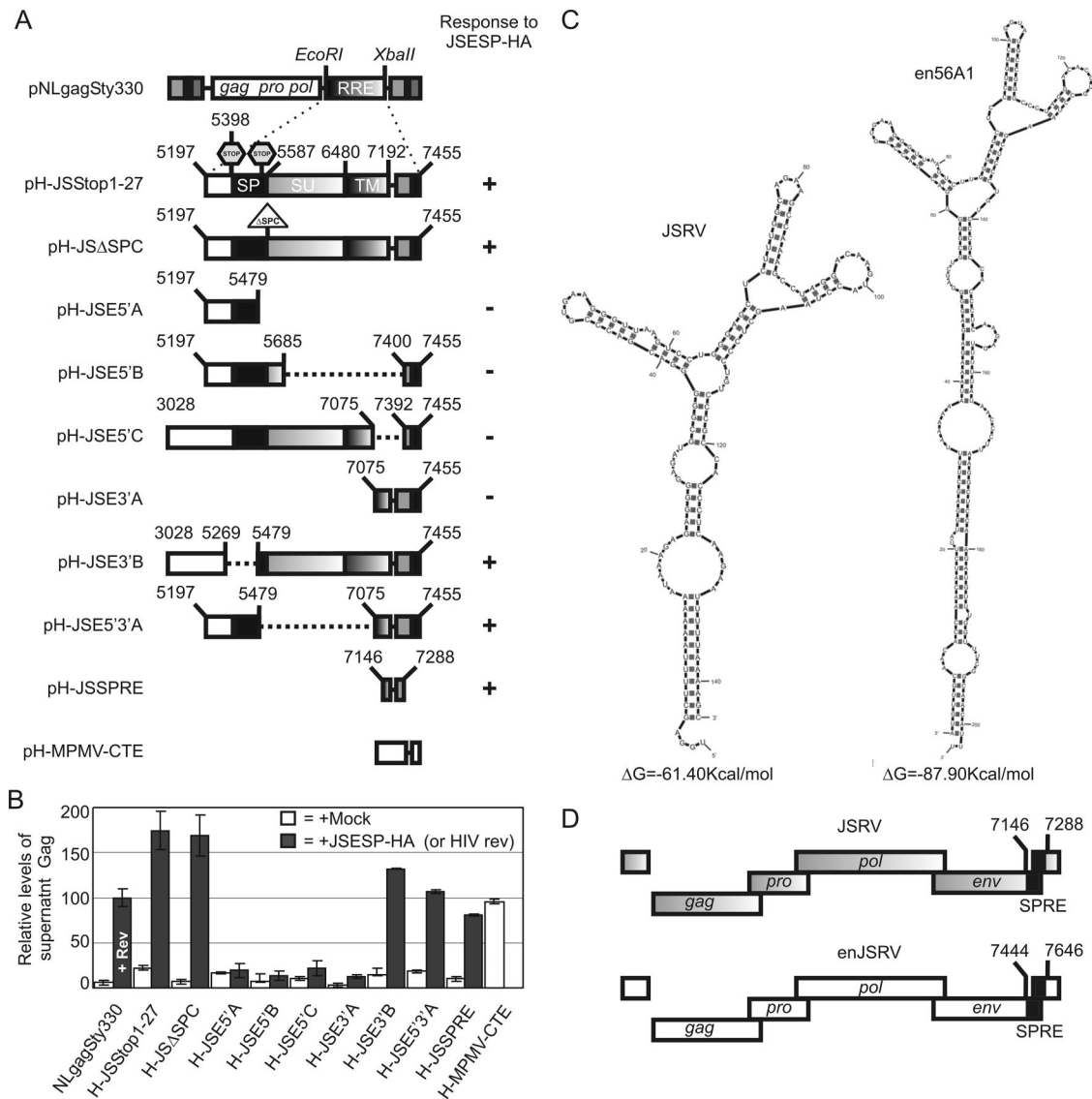


FIG. 4. Identification of the SPRE. (A) Schematic representation of the HIV Gag-Pol expression plasmid (pNLgagSty330) and derived constructs. In these plasmids the HIV RRE was replaced by various portions of the JSRV *env* 3' UTR or by the MPMV CTE. Dashed lines represent deletions, while numbers refer to the nucleotide sequence of the infectious molecular clone JSRV₂₁ (52). (B) Results of the HIV Gag ELISA performed as described in Materials and Methods. Each plasmid (with the exception of pNLgagSty330) was transfected with pJSESP-HA or with an empty plasmid. pNLgagSty330 was cotransfected with an expression plasmid for the HIV Rev or with an empty plasmid, as described above. Results are averages and standard deviations from at least three independent experiments and were normalized to the values obtained with pNLgagSty330 in the presence of the HIV Rev ($414 \pm 42.9 \text{ pg/ml}$), which were arbitrarily set at 100%. The minimal region responding to the JSESP-HA is 146 nt in length and is located in the JSRV *env* 3' UTR. Plasmid pH-JSSPRE contains the minimal SPRE. (C) The RNA secondary structure of the JSRV and enJS56A1 SPREs as predicted by the Mfold program (version 3.3). Predicated ΔG values are indicated (80). (D) Genomic organization of JSRV and enJS56A1. The relative positions of their respective SPREs are indicated. Numbers refer to nucleotide residues of the JSRV₂₁ and enJS56A1 molecular clones (49, 52).

suggest that the ARM may play a role in JSE-SP function, but more experiments are needed to test this hypothesis.

Next, we wanted to determine whether JSE-SP was able to localize in the nucleus in the context of the full-length envelope glycoprotein. In order to perform this experiment, we used pJSE-34HAV5 as described above (Fig. 6A). The resulting plasmid was transfected into COS cells, and confocal microscopy was performed using antibodies against nucleolar markers (fibrillarin) or PDI (a marker for the ER) (74). Given that JSE-SP is expected to shuttle between the cytoplasm and the

nucleus relatively quickly, cells were fixed and stained as described above at 5, 6, 7, and 24 h posttransfection. Cells were also scored for the presence of JSE-SP either exclusively in the cytoplasm or in both the nucleus and cytoplasm. HA staining (indicating the presence of both uncleaved Env and cleaved JSE-SP) was detected, as expected, both in the cytoplasm (co-localizing with ER markers) and in the nucleus (colocalizing with nucleolar markers). V5 staining (indicating full-length Env and the TM subunit) was instead detected only in the cytoplasm. Time point experiments revealed a progressive in-

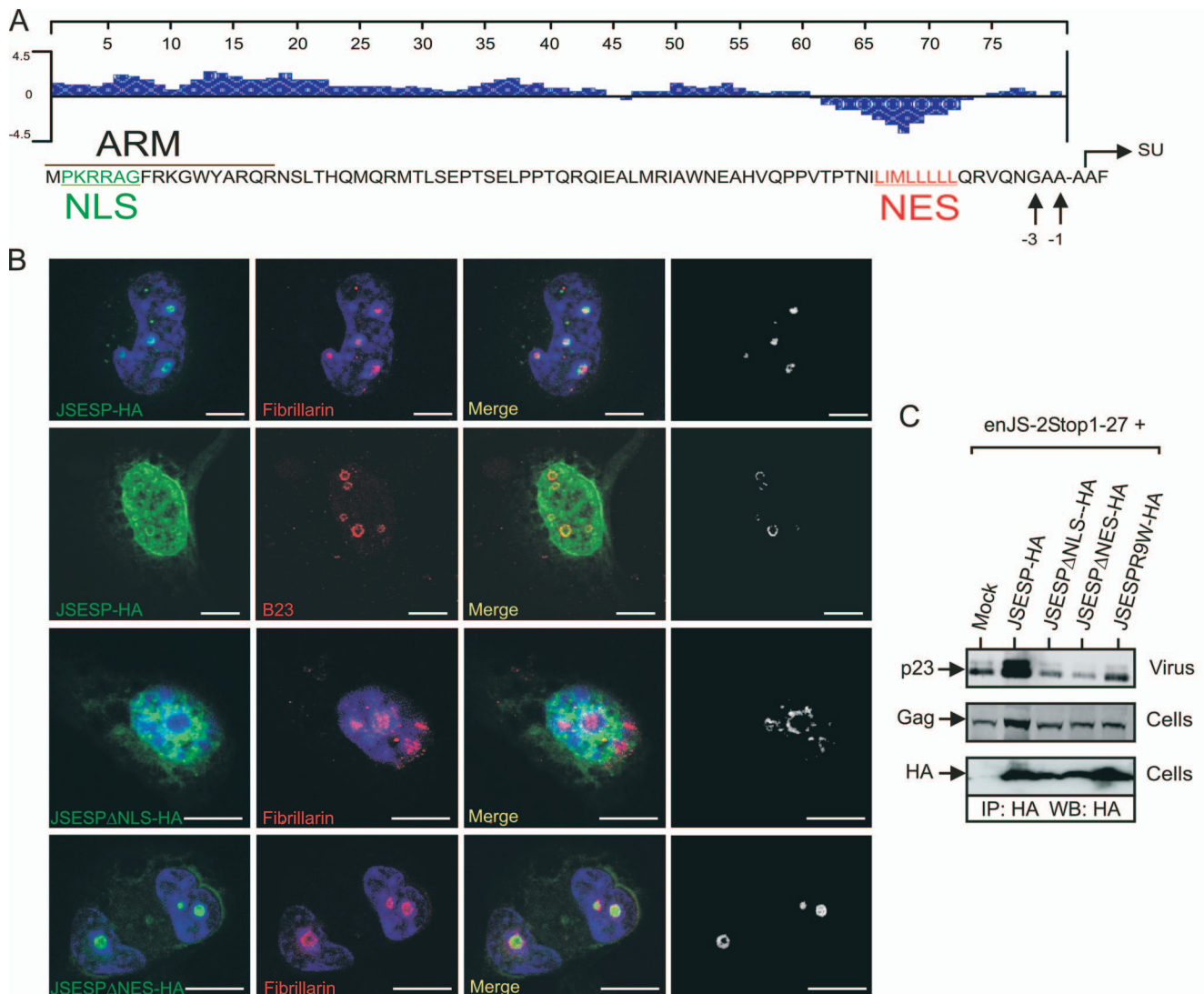


FIG. 5. The signal peptide of the JSRV envelope colocalizes with nucleolar markers. (A) Schematic representation of JSE-SP. Kyte-Doolittle hydrophobicity plot of JSE-SP. JSE-SP also shows a predicted NLS, an NES, and an ARM. (B) Confocal microscopy of COS cells transfected with pJSESP-HA or with the deletion mutants pJSESP Δ NLS-HA and pJSESP Δ NES-HA. At 24 h posttransfection, cells were fixed and analyzed by confocal microscopy using antibodies against HA and the nucleolar markers fibrillarin and B23 with the appropriate conjugates, as described in Materials and Methods. Note the nucleolar staining pattern of JSESP-HA. Cells transfected with pJSESP Δ NLS-HA still display nuclear staining although this is diffused and does not localize in the nucleoli. Panels in black and white show colocalization using the plug-in of the Image J software (<http://rsbweb.nih.gov/ij/>). Bars, 10 μ m. (C) Representative Western blots of virus pellets and protein extracts of 293T cells transfected with the plasmids indicated in each lane. Samples were analyzed 48 h posttransfection by SDS-PAGE and Western blotting employing an antiserum against the JSRV p23 (matrix) or HA. None of the JSESP-HA mutants substantially increase virus particle release of enJS-2Stop1-27.

crease of JSE-SP staining in the nuclei of transfected cells as early as 6 h posttransfection. Thus, the JSE-SP NLS appears to be important for targeting the signal peptide to the nucleus and the nucleoli after cleavage from Env, while the latter is directed to the cell membrane via the ER compartment.

JSE-SP facilitates cytoplasmic accumulation of unspliced genome-length JSRV RNA. The data presented so far indicate that JSE-SP functions as a posttranscriptional regulator of viral gene expression. The activity of JSE-SP depends on the presence of an RNA-responsive element, similarly to other retroviral regulatory proteins, such as Rev. As mentioned before, Rev regulates various steps of the HIV replication cycle, but it

was originally identified as a protein facilitating nuclear export of unspliced HIV RNA (57). In order to test whether JSE-SP facilitates cytoplasmic accumulation of unspliced viral RNA, we performed qRT-PCR on the nuclear and cytoplasmic RNA fractions of cells transfected with pJSRV Δ SPC (nuclear fraction, $n = 8$; cytoplasmic fraction, $n = 12$), pJSRV-SPRE (a deletion mutant of the JSRV provirus with most of *env* deleted but retaining the SPRE region; nuclear fraction, $n = 14$; cytoplasmic fraction, $n = 14$), and pCMV2JS21 (a JSRV infectious molecular clone; nuclear fraction, $n = 6$; cytoplasmic fraction, $n = 12$) in the presence or absence of JSESP-HA (Fig. 7A and B). GAPDH and pre-GAPDH RNAs were used to normalize

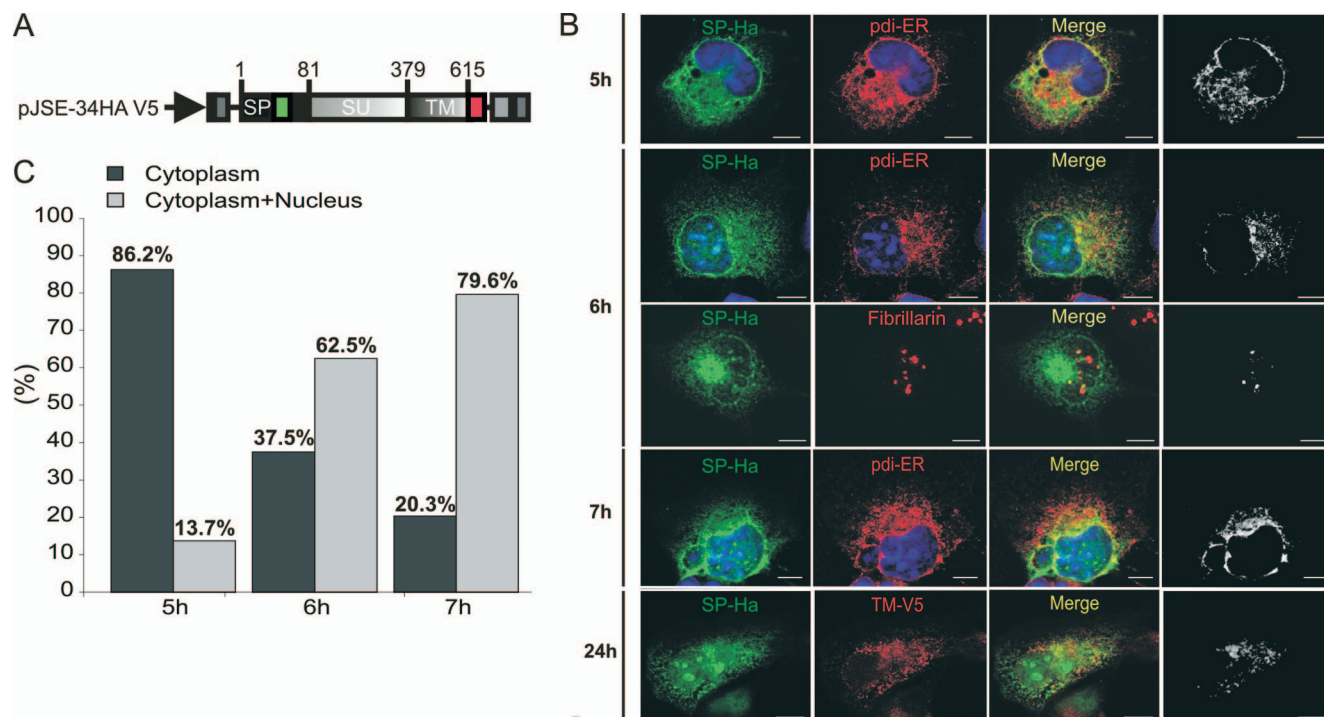


FIG. 6. Localization kinetics of JSE-SP. (A) Schematic representation of the JSRV Env expression plasmid pJSE-34HAV5, which bears an HA tag within the signal peptide and a V5 tag at the carboxy-terminal end of the TM domain. (B) COS cells were transfected with pJSE-34HAV5 and fixed at various times posttransfection as indicated on the left. Cells were analyzed by confocal microscopy using antibodies toward HA (green), V5 (red), PDI (an ER marker, shown in red), and fibrillarlin (a nucleolar marker, shown in red), and the appropriate conjugates. HA nuclear staining starts to appear as early as 6 h posttransfection. As expected, HA colocalizes with both nucleolar and ER markers, while V5 colocalizes only with ER markers. Panels in black and white show colocalization using the plug-in of the Image J program as in Fig. 5. Bars, 10 μ m. (C) Quantification of JSE-SP staining patterns in confocal microscopy of COS cells expressing JSE-34HAV5 at various times posttransfection. Cells were scored as having either exclusively cytoplasmic staining or both cytoplasmic and nuclear staining. Approximately 100 cells were evaluated at each time point. Values are averages from two independent experiments.

the data in the cytoplasmic and nuclear fractions, respectively. Pre-GAPDH RNA RT-PCR was also used to rule out nuclear RNA contaminations in the cytoplasmic fraction (data not shown). The levels of cytoplasmic unspliced JSRV RNA in the presence of JSE-SP were 3.5- to 3.7-fold higher in cells transfected with either pJSRVSPRE, pJSRV Δ SPC, or pCMV2JS21. On the other hand, the presence of JSE-SP did not alter the levels of unspliced viral RNA in the nuclei of any of the samples tested.

Samples from the cytoplasmic RNA fractions described above were also analyzed by Northern blotting in which the levels of unspliced viral RNA were quantified by phosphorimaging (Fig. 7C). Results are expressed as mean arbitrary phosphorimaging units for each sample (after subtracting lane background levels) normalized to the GAPDH RNA levels. The level of JSRV full-length mRNA was set arbitrarily as 100%. The levels of unspliced cytoplasmic genomic-length mRNA (expected size, \sim 7.5 kb) in pJSRV Δ SPC- and pCMV2JS21-transfected cells increased by a factor of approximately 3 in the presence of JSE-SP-HA. Levels of unspliced JSRV-SPRE RNA (expected size, \sim 5.8 kb due to the large deletion in *env*) in the cytoplasmic fraction of transfected cells were barely above background levels, while they increased substantially when JSE-SP-HA was provided in *trans* by co-transfection.

Thus, the data presented above suggest that JSE-SP facilitates cytoplasmic accumulation of unspliced viral RNA. Reassuringly, the estimated effect of JSE-SP on cytoplasmic accumulation of unspliced viral RNA was approximately threefold as determined by both qRT-PCR and Northern blotting. The only difference noted is that the levels of cytoplasmic accumulation of unspliced JSRVSPRE RNA was estimated to be about 40-fold higher in the presence of JSE-SP by Northern blotting, as opposed to only 3-fold by qRT-PCR. However, the levels of JSRV-SPRE RNA in the absence of JSE-SP were around the detection limits of the Northern blotting analysis, and this may have resulted in an overestimation of the influence of JSE-SP on the cytoplasmic RNA accumulation of this particular construct. These data also suggest that the *env* region may contain stability factors analogous to those found in the genomes of other retroviruses, such as Rous sarcoma virus (73).

In the same experimental set, we also quantified the levels of JSRV Gag present in cell lysates and supernatants of transfected cells by Western blotting and chemifluorescence (Fig. 7D). The influence of JSE-SP on steady-state Gag levels in lysates of cells transfected with the plasmids described above mirrored the results obtained by Northern blotting. JSE-SP-HA increased Gag expression of the various JSRV constructs by a factor between 2 and 4 when it was provided in *trans*. On the

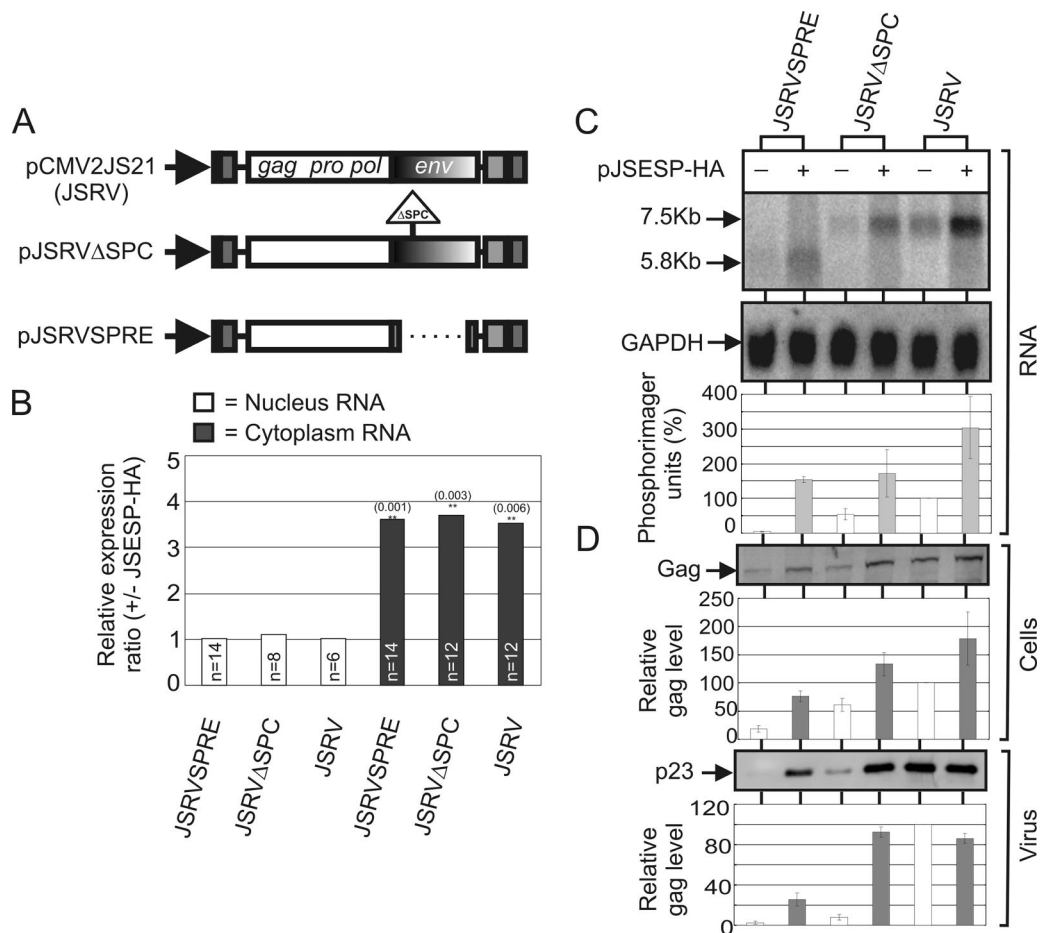


FIG. 7. JSE-SP facilitates cytoplasmic accumulation of unspliced viral RNA. (A) Schematic representation of the plasmids used in these experiments. pJSRVSPRE contains a large deletion within *env* but maintains the minimal SPRE identified in Fig. 4. The dashed line in pJSRVSPRE indicates a large deletion in *env*. (B) qRT-PCR of nuclear and cytoplasmic RNA fractions of cells transfected with the plasmids shown in panel A. Samples were analyzed as described in Materials and Methods. The number of samples analyzed is indicated within each bar, and results are expressed as the relative expression ratio of Gag RNA in the presence or absence of JSESP-HA. Each sample was analyzed in triplicate, and values were normalized to pre-GAPDH (nuclear fraction) or GAPDH (cytoplasmic fraction) values, taking into consideration the efficiency of each PCR using the formula indicated in Materials and Methods. Statistical analysis of the data was performed using the software REST (56). *P* values indicating a statistically significant difference between samples in the presence or absence of JSE-SP are indicated above each bar. (C) Northern blotting (top) using a JSRV *env* U3 probe showing the unspliced full-length RNAs for JSRVSPRE, JSRVΔSPC, and JSRV. The expected length of full-length unspliced RNA for JSRV and JSRVΔSPC is 7.5 kb, while that for JSRVSPRE is 5.8 kb. Positive signals were quantified by phosphorimaging (bottom) and normalized with signals obtained with a probe for GAPDH after subtracting the background of each individual lane. Values are expressed as percentages of the value obtained by the cytoplasmic fraction of the full-length JSRV RNA, which was arbitrarily assigned a value of 100%. Molecular sizes of the JSRV RNAs are given. (D) Protein extracts and virus pellets from culture supernatants from the same cells were analyzed by Western blotting employing an antiserum against JSRV p23 (matrix). Positive signals were quantified by phosphorimaging and reported as percentages of the arbitrary chemifluorescence units of JSRV Gag, which was arbitrarily assigned a value of 100%.

other hand, the levels of Gag associated with viral particles in the supernatants of cells transfected with pJSRVΔSPC and pJSRVSPRE were 10-fold higher in the presence of JSESP-HA. Thus, JSE-SP likely also facilitates a posttranslational step(s) of the viral replication cycle, such as assembly or viral particle trafficking (see Discussion).

JSE-SP function depends on Crm1 and not Tap/Nxf1. We next investigated whether JSE-SP function was dependent on the Crm1 pathway, similar to the HIV-1 Rev regulatory protein. Rev recruits the nucleoporin Nup214/CAN, among other cellular factors (64). Here we found that overexpression of a defective form of Nup214/Can (Δ Can), previously shown to inhibit the Crm1 pathway (6), also inhibited JSRV exit. In

these experiments, we cotransfected the expression plasmid for JSRV, MPMV, or HIV-1 Gag-Pol (pNLgagSty330) with increasing amounts of Δ Can and quantified the viral particles released in the supernatant of transfected cells by Western blotting. Δ Can inhibited both JSRV and HIV-1 exit very efficiently in a dose-dependent manner, while MPMV exit was minimally affected (Fig. 8A). We used MPMV as a negative control, considering that it utilizes a Tap/NXF1-dependent and not a Crm-1 dependent pathway (34). Furthermore, we established that a dominant negative mutant of Tap/NXF1, TapA17 (34), has only a relatively minor effect on the JSRV SPRE that is not dose dependent and is similar to its effects on the HIV RRE (while it inhibits the MPMV CTE function as expected).

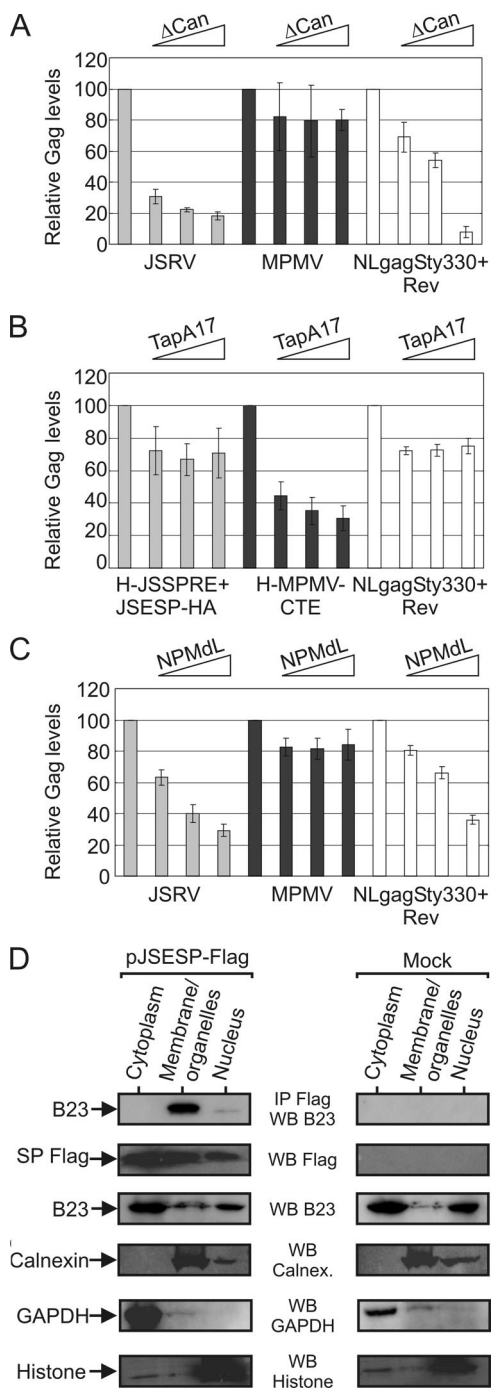


FIG. 8. JSE-SP function is dependent on Crm1 and B23. (A) COS cells were cotransfected with 1 μ g of the expression plasmid for JSRV, MPMV, or HIV (the latter including a Rev expression plasmid) and increasing amounts of dominant negative forms of Crm1 (0.5 to 5 μ g). Virus pellets from supernatants of transfected cells were analyzed 48 h posttransfection by Western blotting employing an antiserum against the JSRV CA or HIV p55/p24. Results were quantified by phosphorimaging and are shown as arbitrary chemifluorescence units relative to those for JSRV, MPMV, or HIV (which were arbitrarily assigned a value of 100%). Values are averages and standard deviations from three independent experiments. (B) 293T cells were transfected with pH-JSSPRE plus pJSESP-HA, with pH-MPMV-CTE, or with pNLgagSty330 plus pCMVsRev. In each transfection set, cells were cotransfected with an expression plasmid of the dominant negative Tap/NXF1 (0.5 to 5 μ g) or with the empty expression plasmid as a control.

In order to quantify the effects of Tap/NXF1 on JSRV exit, we used pNLgagSty330 containing the JSRV SPRE (pH-JSSPRE) (in the presence of JSESP-HA) or the MPMV CTE (pH-MPMV-CTE) as a positive control (Fig. 8B). In our hands, H-MPMV-CTE was inhibited by TapA17 in 293T cells much more efficiently than MPMV and thus represented a better experimental system (data not shown). Viral particle production in cells transfected by the JSRV expression plasmid pCMV2JS21 was not inhibited at all by TapA17 (data not shown).

In addition, a dominant negative form of the multifunctional protein B23 (NPMdL) substantially downregulated JSRV and HIV Gag particle release in a dose-dependent manner, while it had a relatively minor effect on MPMV (Fig. 8C). B23 shuttles in the nucleolus and its nuclear export is dependent on the Crm1 pathway (61, 78). B23 has been found to be associated with both the nuclear export protein Rev and Rex of HIV-1 and HTLV, respectively (1, 22, 65). By coimmunoprecipitation, we determined that JSE-SP and B23 associate in the membrane/organelle fraction (including ER, membranes, and Golgi), despite being more abundant in the nuclear and cytoplasmic fractions (Fig. 8D).

Transcripts encoding JSE-SP. In the context of wild-type JSRV, JSE-SP could be derived from cleavage of Env following translation of the single-spliced *env* mRNA or from the translation of other viral mRNAs that include the *env* leader sequence. In a previous study, we showed that in transfected 293T cells JSRV encodes, beside the spliced Env mRNA, a truncated Env (tr-Env) derived from premature polyadenylation of the *env* mRNA (37, 51). Tr-Env uses the splice donor and splice acceptor of the *env* mRNA and includes the leader sequence and a portion of the SU domain. This messenger is prematurely polyadenylated due to the presence of a non-canonical poly(A) signal (ATTAAA) within the SU. However, the signal peptide within tr-Env is not necessary (although it may contribute in 293T cells) to the positive influence of the JSRV Env on Gag expression, as expression plasmids for both the predicted tr-Env and the JSRV Env with the noncanonical poly(A) signal deleted maintained JSE-SP activity (data not shown). In addition, the enJS5F16 Env (which does not express

At 48 h posttransfection, Gag was measured in the supernatant of transfected cells by ELISA, as described in Materials and Methods. Results are expressed relative to the values obtained with pH-JSSPRE, pH-MPMV-CTE, or pNLgagSty330 plus pCMVsRev cotransfected with the empty expression plasmid in three independent experiments. (C) COS cells were cotransfected with 1 μ g of the expression plasmids for JSRV (pCMV2JS21) or MPMV (pSARM4) and increasing amounts of dominant negative forms of B23 (0.5 to 5 μ g). Virus pellets from supernatants of transfected cells were analyzed as described for panel A. (D) 293T cells were transfected with JSESP-Flag or an empty plasmid (Mock), and at 48 h posttransfection, proteins were extracted from different subcellular compartments (cytosol, nucleus, and membranes and organelles). Protein extracts were coimmunoprecipitated with a Flag antibody and analyzed by Western blotting using a B23 antibody. Controls included Western blots of the same protein lysates using antibodies against Flag, B23, calnexin, GAPDH, and histone H3. B23 coimmunoprecipitates with JSESP-Flag, especially in the membrane/organelle fraction, although it is clearly less abundant in this fraction than in the other two fractions.

tr-Env) was able to enhance JSRV^{Stop1-27} Gag expression and particle production in *trans* (data not shown).

We also investigated whether JSRV had any additional mRNAs that could encode an accessory protein formed in part by JSE-SP. In order to verify the possible presence of mRNAs that are formed at least in part by the JSRV/enJSRVs *env* region, we extracted RNA from a variety of transiently and stably transfected cells expressing either JSRV, enJS56A1, or the JSRV Env and performed RT-PCR (see the supplemental material). Only the single-spliced transcript encoding Env was detected as a prominent band consistently in every sample (see the supplemental material). Faint PCR bands were detected in some of the reactions, and 26 different transcripts containing *env* sequences were cloned and sequenced. Thus, available data suggest that Env is sufficient to provide JSE-SP function. Alternative transcripts, including tr-Env, are not absolutely required for JSE-SP activity, although the possibility that they may play a redundant function cannot be excluded.

DISCUSSION

In this study, we showed that the signal peptide of the JSRV envelope acts as a posttranscriptional regulator of viral gene expression. JSE-SP is a multifunctional protein that functions in *trans* and requires an RNA-responsive element located in the 3' portion of the viral genome (end of *env* and 3' UTR). Signal sequences are N-terminal extensions of nascent membrane and secretory proteins that mediate targeting and translocation to the ER (39, 71). Signal sequences usually include 15 to 25 amino acid residues that are normally formed by a central hydrophobic core (7 to 10 amino acid residues) flanked by a positively charged N region and a neutral, polar C region. The latter contains the cleavage site for the signal peptidase that is responsible for releasing the signal peptide (70) from the rest of the protein. Signal peptides are thought to be subsequently degraded or further processed by an intramembrane protease (signal peptide peptidase). Few exceptions are represented by the signal peptide of prolactin (72) and the internal signal sequences of the hepatitis C virus polyprotein, which are processed by signal peptide peptidase and liberated into the cytosol, where they act as functional proteins (40).

The signal peptides of JSRV and related enJSRVs are unusually long (80 amino acid residues) compared to those of other retroviruses, which are around 20 to 30 amino acid residues in length. Notable exceptions are the signal peptides of the envelope (or overlapping accessory proteins) of MMTV, HERV-K, and foamy viruses. These long signal peptides, by themselves or as part of accessory proteins, are involved in some biological processes critical for the viral replication cycle, besides targeting their respective Env to the ER (35, 63). For example, the signal peptides of both MMTV and HERV-K (betaretroviruses like JSRV) Envs are part of regulatory proteins named, respectively, Rem and Rec (32, 36, 43, 76). Both Rem and Rec are derived from double-spliced transcripts that include the envelope leader sequence, and both proteins function as RNA export proteins (32, 36, 43, 76). Interestingly, it was recently shown that the signal peptide of MMTV Rem is released from the ER and accumulates in the nucleoli (18). Thus, it is now increasingly clear that the signal peptides of the envelopes of betaretroviruses have evolved by acquiring bio-

logical functions besides ER protein targeting. MPMV has a short signal peptide, unlike other betaretroviruses, but its envelope region (in contrast to the products of *gag* and *pol*) is phylogenetically more similar to that of gammaretroviruses than betaretroviruses.

Similar to Rev, JSE-SP is a multifunctional protein acting at different levels of the viral replication cycle. Under our experimental conditions, JSE-SP favored cytoplasmic accumulation of full-length viral mRNA (taken as an indirect measure of nuclear export) approximately threefold, as assessed by qRT-PCR and Northern blotting analysis. These data may underestimate the true effects of JSE-SP on mRNA export due to the fact that our assays have been performed on cells transiently transfected with expression plasmids under the control of the strong cytomegalovirus promoter. The effect of JSE-SP on steady-state intracellular Gag levels is similar to its influence on cytoplasmic RNA accumulation. On the other hand, we found JSE-SP to have a more pronounced effect on viral particle release. One of the technical issues to consider is that both cytoplasmic RNA and protein levels are measured in cell lysates and extracts and therefore are influenced by the half-life of the respective molecules and by their "loss" due to assembly and release in the supernatant of newly formed viral particles. Gag associated with viral particles accumulates over time in the cell supernatant and has a different half-life than intracellular Gag. Therefore, there may not necessarily be a linear relationship between intracellular Gag expression and viral particle release and accumulation, and more experiments will be needed to address this point. However, JSE-SP appears to influence mainly a posttranslational step(s) of the JSRV replication cycle. JSE-SP could facilitate assembly per se or Gag trafficking to a cellular compartment where viral particles are assembled or released more efficiently. In a similar manner, the HIV Rev protein has been found to facilitate, beside RNA export, other posttranscriptional events in the viral life cycle, such as protein expression (5, 17) and encapsidation (8, 27, 28).

JSE-SP function depends on functional Crm1 and B23 pathways similarly to the HIV Rev and other Rev-like proteins (1, 15, 22, 38, 65). The possible association between JSE-SP and B23 suggests that B23 may be involved directly in JSE-SP shuttling. JSE-SP function depends, as mentioned above, on a *cis*-acting region (the SPRE) present at the 3' end of the viral genome. However, our studies have also shown that the 5' portion of the *env* region may contain other *cis*-acting regions important for RNA stability, as identified in other retroviruses (73, 75), or affect the JSE-SP-SPRE interaction. Indeed, both the exogenous JSRV Δ BS deletion mutant (Fig. 3B, lane 4) and a similar construct derived from enJS-2 (data not shown), despite retaining the SPRE, expressed very little or no Gag even in the presence of JSE-SP-HA. Results obtained with the HIV Gag-Pol vectors (Fig. 4) would tend to rule out the possibility that the 5' region in *env* functions as a CTE-like element, although more studies will be needed to address this specific point. It is also possible that disruption of the *env* mRNA splice acceptor could alter JSE-SP function.

In conclusion, our studies have shown yet another mechanism that retroviruses have evolved to control posttranscriptional viral gene expression.

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