Minute Virus of Canines NP1 Protein Interacts with the Cellular Factor CPSF6 To Regulate Viral Alternative RNA Processing

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ABSTRACT The NP1 protein of minute virus of canines (MVC) governs production of the viral capsid proteins via its role in pre-mRNA processing. NP1 suppresses polyadenylation and cleavage at its internal site, termed the proximal polyadenylation (pA)p site, to allow accumulation of RNAs that extend into the capsid gene, and it enhances splicing of the upstream adjacent third intron, which is necessary to properly enter the capsid protein open reading frame. We find the (pA)p region to be complex. It contains redundant classical *cis*-acting signals necessary for the cleavage and polyadenylation reaction and splicing of the adjacent upstream third intron, as well as regions outside the classical motifs that are necessary for responding to NP1. NP1, but not processing mutants of NP1, bound to MVC RNA directly. The cellular RNA processing factor CPSF6 interacted with NP1 in transfected cells and participated with NP1 to modulate its effects. These experiments further characterize the role of NP1 in parvovirus gene expression.

IMPORTANCE The *Parvovirinae* are small nonenveloped icosahedral viruses that are important pathogens in many animal species, including humans. Unlike other parvoviruses, the bocavirus genus controls expression of its capsid proteins via alternative RNA processing, by both suppressing polyadenylation at an internal site, termed the proximal polyadenylation (pA)p site, and by facilitating splicing of an upstream adjacent intron. This regulation is mediated by a small genus-specific protein, NP1. Understanding the *cis*-acting targets of NP1, as well as the cellular factors with which it interacts, is necessary to more clearly understand this unique mode of parvovirus gene expression.

KEYWORDS RNA processing, bocavirus, parvovirus

Infection with minute virus of canines (MVC), a member of the *Bocaparvovirus* genus (1), can cause abortion and stillbirth in pregnant dogs, as well as mild gastroenteritis and respiratory disease in puppies (2–5). Recently, emerging infections of bocaviruses have been identified in dog, cat, sea lion, and nonhuman primates (2, 6–10).

MVC generates a single pre-mRNA from a promoter at the left-hand end of the genome (P6) that is processed via alternative splicing and alternative polyadenylation into multiple nonstructural- and capsid-encoding transcripts (11, 12). MVC contains two polyadenylation sites, one at the right-hand end of the genome, the distal polyadenylation [(pA)d] site, and another complex site, the proximal polyadenylation [(pA)p] site, within the capsid-coding region. As with other parvoviruses, an open reading frame (ORF) in the left half of the genome encodes nonstructural (NS) proteins, while an ORF in the right half encodes the capsid proteins VP1 and VP2 (13).

Parvoviruses use multiple mechanisms to maximize the coding potential from their compact genomes, including alternative transcription initiation, alternative splicing, alternative polyadenylation, and alternative translation initiation mechanisms (13–15).

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The bocaparvoviruses encode a small, genus-specific protein, NP1, which governs access to the viral capsid gene via its role in alternative polyadenylation and alternative splicing of the single MVC pre-mRNA (16). NP1 is required for efficient read-through of the internal polyadenylation site (pA)p and splicing of the adjacent upstream intron. Additionally, three essential nonstructural proteins are encoded by mRNAs that excise the NP1-regulated MVC intron immediately upstream of the internal polyadenylation site (pA)p, and so generation of these proteins is also regulated at the RNA processing level by NP1 (17). A number of other viral proteins, including herpesvirus simplex virus (HSV-1) ICP27 (18–20) and Kaposi sarcoma-associated herpesvirus (KSHV) ORF57 (21–24), are known to regulate similar processes.

Alternative RNA processing is mediated by coordinated interactions between polyadenylation and splicing complexes and by their interaction with defined *cis*-acting elements in pre-mRNAs (25). Pre-mRNA 3'-end processing is catalyzed by a large multienzyme complex (26). In addition to the poly(A) polymerase, the complex includes the multisubunit cleavage and polyadenylation specificity factor (CPSF) which recognizes and binds to the AAUAAA hexamer motif (26–28), the multisubunit cleavage stimulation factor (CstF), which typically recognizes and interacts with a G/U-rich downstream element (26), and the RNA cleavage factors I and II (CFIm and CFII) (26). The tetrameric CFIm complex contains two small CPSF5 subunits and two large subunits of either CPSF7 or CPSF6 (also called CFIm68). CPSF6 has recently been shown to regulate alternative polyadenylation (29–32). Additionally, CPSF6 has been shown to interact with the mRNA export receptor NXF1/TAP to promote mRNA export (33). In a presumably separate function, CPSF6 has also been shown to bind the HIV-1 capsid and has been suggested to be involved in HIV nuclear transport and targeting of HIV-1 preintegration complexes (34–39).

While NP1 plays a critical role in MVC gene expression, the *cis*-acting signals it targets and the cellular factors it interacts with to perform its functions have not yet been well defined. In the manuscript we have more closely defined the *cis*-acting sequences within (pA)p that mediate alternative RNA polyadenylation and responsiveness to NP1. In addition, we show that the cellular alternative RNA processing factor CPSF6 plays an important role in the ability of NP1 to suppress internal polyadenylation at (pA)p, in influencing viral mRNA export, and, thus, in modulating MVC gene expression.

RESULTS

Characterization of the cleavage and polyadenylation sites in (pA)p. The (pA)p region of MVC is the target of regulation by NP1. The region is complex and has not yet been well defined. Inspection of the region identified three consensus AAUAAA hexamer motifs, as well as associated U- and G/U-rich regions that could function as cleavage factor I (CFI)-binding upstream elements (USEs) and cleavage stimulatory factor (CSTF)-binding downstream elements (DSEs) (Fig. 1A). To begin to characterize this region in detail, we first performed 3' rapid amplification of cDNA ends (RACE) experiments on RNA generated following MVC infection of WRD cells to identify the specific cleavage sites used within (pA)p. Four cleavage and polyadenylation sites were identified, labeled A to D in Fig. 1A, at nucleotides (nt) 3291, 3279, 3258, and 3195, respectively (data not shown). To confirm these cleavage sites and determine the approximate frequency of their use, we performed RNase protections using three different probes (Fig. 1A). A probe spanning nucleotide (nt) 3107 to 3333 was designed to display cleavage at all the sites identified by 3' RACE. Following transfection of the wild-type (WT) MVC infectious clone into permissive 293FT cells, MVC RNA, as previously seen (40), primarily extended beyond the (pA)d site into the capsid gene [presumably to be polyadenylated to (pA)d] (Fig. 1B, lane 1). However, the RNAs that utilized the internal (pA)p site primarily used sites A and B at approximately similar levels and, to a lesser extent, sites C and D, also to approximately similar levels (Fig. 1B, lane 1). Consistent with these results, the probe spanning nt 3200 to 3333 detected cleavage sites B and C (Fig. 1B, lane 2), and the probe spanning nt 3107 to 3222

FIG 1 Characterization of the cleavage and polyadenylation sites in (pA)p. (A) At top is a partial map of MVC transcripts showing the proximal polyadenylation sites [(pA)p sites], the upstream adjacent third intron, the second intron, the location of the three AAUAAA hexamers (marked 1 to 3), the initiating AUG of VP1, and the stop codon TAA of NP1. The positions of the RNase protection probes for (pA)p (nt 3107 to 3333, 3107 to 3222, and 3200 to 3333) and probe 2A/3D (nt 2344 to 2550) are indicated. The alignments of the nucleotide sequence around the (pA)p region in wild-type MVC or, as indicated, in the (pA)p mutants show the four cleavage sites (marked A, B, C, and D, and located at nt 3291, 3279, 3258, and 3195 of the MVC genome, respectively), three AAUAAA hexamers (in red and underlined) or their mutations (in italics and uppercase), and their corresponding downstream elements (DSEs) (dotted underline and uppercase). (B). RPAs of total RNA extracted 48 h following transfection of 293FT cells with pMVC using the three (pA)p probes as indicated (nt 3107 to 3333, 3200 to 3333, and 3107 to 3222). The sizes of the probes

(Continued on next page)

detected primarily RNAs cleaved at site D (Fig. 1B, lane 3). 3' RACE assays of RNAs generated from an MVC Rep-Cap construct (MVCRC) (containing only the "flip" right-hand end configuration) identified two primary cleavage sites at the (pA)d signal, at nt 5305 and 5279 (data not shown). These sites lie within the region of the hairpin that switches configuration; the "flop" orientation contains an AAUAAA hexamer approximately 120 nt downstream of that in the flip orientation and so would generate an RNA with an extended 3' end.

To determine which AAUAAA motif(s) programed which cleavage and polyadenylation events for (pA)p, we made mutations of these motifs either individually or in combination (Fig. 1C and D). When AAUAAA hexamer 1 (nt 3170 to 3175) was mutated [mutant 1M(pA)p] as shown in Fig. 1A, the relative amount of MVC RNA polyadenylated at (pA)p was diminished; however, the relative levels at which the individual sites were used was not significantly altered (Fig. 1C, compare lanes 1 and 3). When NP1 was mutated in this background (mutant 5X, which contained an inactivating insertion of 5 proline residues into NP1 at nt 2780, 81 amino acids downstream of its initiating codon, as previously characterized [40] and as described also in Materials and Methods), read-through of (pA)p was still abolished as expected (Fig. 1C, compare lanes 3 and 4). When AAUAAA hexamer 2 (nt 3228 to 3233) was mutated [mutant 2M(pA)p], the internal polyadenylation was again significantly diminished, and cleavage was much more heterogenous, with a particular reduction in cleavage at site D upstream (Fig. 1C, compare lane 5 to lane 1). Again, additional mutation of NP1 led to a dramatic reduction in RNAs extending past (pA)p, primarily using (pA)p cleavage site A (Fig. 1C, compare lanes 5 and 6). When the AAUAAA hexamer 3 (nt 3258 to 3263) was mutated [mutant 3M(pA)p], internal polyadenylation was essentially abolished (Fig. 1C, compares lanes 1 and 7). However, in this case in the absence of functional NP1, accumulation of read-through transcripts was lost, and internal cleavage was regained at (pA)p sites B, C, and D (Fig. 1C, compare lanes 7 and 8). These results suggested a complex, and likely partially redundant, relationship for cleavage and polyadenylation within (pA)p. AAUAAA hexamer 1 likely played a minor role in (pA)p cleavage and polyadenylation, while AAUAAA hexamer 2 seemed to act primarily on upstream cleavage site D. AAUAAA hexamer 3 was likely the primary determinant for (pA)p recognition and polyadenylation and was an essential element for cleavage at site A. It is interesting that the profile of cleavage at (pA)p in the presence and absence of NP1 was substantively similar for wild-type (pA)p and single mutations at the first and second sites. Mutant 3M(pA)p, however, prevented cleavage at (pA)p in the presence of wild-type NP1 yet allowed cleavage at sites B, C, and D in the absence of functional NP1. This suggested that cleavage site A was likely the primary target of NP1 suppression of (pA)p. A mutant in which all three AAUAAA hexamers were mutated (mutant 123M) generated no products cleaved specifically at (pA)p, either in the presence or absence of functional NP1 (Fig. 1C, lanes 9 and 10).

Splicing of the MVC third intron, which lies immediately upstream of (pA)p, has previously been shown to be dependent upon MVC NP1 (16) yet functional when (pA)p was replaced with a heterologous polyadenylation sequence (16). Consistent with these results, mutations of the individual AAUAAA motifs showed no significant effect on splicing of the upstream third intron when assayed using a probe across the second intron acceptor and third intron donor (Fig. 1D, compare lanes 3, 5, and 7 to lane 1). In

FIG 1 Legend (Continued)

and various protected bands are shown on the left. The protected bands representing RNA species extending through (pA)p to (pA)d or cleaved at the various cleavage sites as described are indicated to the right. P, probe alone. (C) RPAs of total RNA extracted 48 h following transfection of 293FT cells with wild-type pIMVC and AAUAAA hexamer mutants as indicated using the wild-type or AAUAAA mutant (pA)p probes diagrammed in panel A. The size of the probes and various protected bands are shown on the left. The protected bands representing RNA species extending through (pA)p to (pA)d or cleaved at the various cleavage sites as described are indicated to the right. (D) RPAs of total RNA extracted 48 h following transfection of 293FT cells with wild-type pIMVC and AAUAAA hexamer mutants as indicated using the 2D/3A probe as indicated and as described in Materials and Methods. The sizes of the probe and various protected bands are shown on the left. Bands representing individual RNA species (unspliced, read-through of the second intron acceptor [2A] and third intron donor [3D]; 2Aspl/3Dun, spliced second intron acceptor and unspliced third intron; 2Aspl/3Dspl, spliced second intron acceptor and spliced third intron) are indicated to the right.

these cases, splicing remained dependent on NP1 (Fig. 1D, lanes 4, 6, and 8). However, a mutation of all three hexamer motifs exhibited a significant reduction in upstream intron splicing (Fig. 1D, compare lanes 1 and 9) that remained dependent on NP1 (Fig. 1D, lane 10). These results suggested that, while not dependent on (pA)p specifically, upstream splicing required some functional downstream polyadenylation signal. This is addressed further below.

Sequences in (pA)p outside the classical polyadenylation motifs are important for NP1 regulation. To investigate which *cis*-acting signals within (pA)p are responsive to NP1-mediated suppression of internal polyadenylation, we performed the following experiments. We first replaced the complete MVC (pA)p region with a heterologous sequence from the capsid gene of minute virus of mice (MVM), not known to contain any regulatory sequences (Fig. 2A, MVC/MVM₁). As expected, no internal polyadenylation was seen (Fig. 2B, lanes 3 and 4). Splicing of the upstream third intron was also reduced (Fig. 2C, lanes 3 and 4) although the levels of NP1 generated were similar to the wild-type level (Fig. 2D, lanes 3 and 4). When the three AAUAAA hexamers plus associated DSEs were added back into the heterologous MVM background in such a way that they were located at approximately same positions they occupy in MVC (pA)p (Fig. 2A, MVC/MVM₁ins), we found a surprising increase in internally polyadenylated RNA in the presence (as well as the absence) of NP1 (Fig. 2B, compare lane 5 to lane 1 and to Fig. 1B, lane 1, and Fig. 1C, lane 1). These results suggested that sequences within the wild-type (pA)p region outside the specific classical elements played an important role in NP1 regulation of the region but not directly for NP1-independent polyadenylation and cleavage. These results were accompanied by both an expected increase in NP1 (Fig. 2D, lane 5), which is translated primarily from internally polyadenylated RNAs (16) and by a relative increase in splicing of the NP1-dependent upstream third intron (Fig. 2C, lane 5). Adding back all MVC sequences which lie upstream of the third hexamer to MVC/MVM₁ins returned both polyadenylation and splicing to wild-type profiles (MVC/MVM3') (Fig. 2B and C, compare lanes 7 and 8 to lanes 1 and 2).

NP1 specifically binds to MVC RNA. Considering that *cis*-acting sequences within the (pA)p region modulate NP1 suppression of internal polyadenylation, it seemed possible that NP1 interacted directly with MVC RNA. As can be seen, following cross-linking either with formaldehyde (Fig. 3B) or directly with UV irradiation (Fig. 3C), anti-hemagglutinin (HA) antibody but not anti-FLAG antibody specifically immunoprecipitated MVC RNA that was generated following transfection of an MVC Rep-Cap (MVCRC) clone in which wild-type NP1 was tagged with HA (Fig. 3B and C, MVCHANP1). RNA was detected by PCR using the probes as indicated and diagrammed in Fig. 3A. Immunoprecipitation (IP) of functionally inactive (5X) NP1 tagged with HA did not pellet MVC RNA, and anti-HA antibody did not immunoprecipitate MVC RNA under either cross-linking protocol when NP1 was left untagged (MVCRC) (Fig. 3B and C). Comparative protein levels are shown in Fig. 3D. Following UV cross-linking, FLAG-tagged NP1, but not a previously characterized mutant of the NP1 arginine-serine (SR) motifs deficient for splicing the upstream third intron (16), bound specifically to MVC RNA following their transient transfection (Fig. 3E). Protein levels are shown in Fig. 3F. Whether these latter results implicate NP1 interaction with MVC RNA specifically for its splicing enhancement is under investigation.

Many nucleic acid binding proteins can form multimers and are often found as oligomers within cells. This is the case for NP1. As can be seen in Fig. 3G, Western analysis following gentle extraction indicated that NP1 can form oligomers of at least tetramer size (Fig. 3G, lane 2). Isolation following boiling in SDS to diminish protein-protein interactions collapses the oligomers to unit-length size (Fig. 3G, compare lanes 2 and 4). The N terminus of NP1 is intrinsically disordered (16), a feature that favors oligomerization and interactions common in many RNA and DNA binding proteins that modulate RNA processing and viral genome replication (41–44).

The cleavage and polyadenylation specificity factor CPSF6 interacts with NP1 and affects MVC alternative RNA processing and export. Pre-mRNA 3'-end process-

FIG 2 Sequences in (pA)p outside the classical polyadenylation motifs are important for NP1 regulation. (A) Partial map of MVC transcripts showing a diagram of various MVC (pA)p mutation constructs (MVC/MVM_j, MVC/MVM_jins, and MVC/MVM3'), as described in Materials and Methods, or wild-type (pA)p and the upstream third intron. Positions of the RNase protection probes (pA)p (nt 3107 to 3333) and 3A (nt 2910 to 3110) are indicated. The MVM_j fragment (MVM nt 3502 to 3744) inserted in MVC (between MVC nt 3168 and 3333) is shown in blue. Three MVC AAUAAA core elements are shown in red vertical rectangles as described in the legend of Fig. 1A. (B) RPAs of total RNA extracted 48 h following transfection of 293FT cells with wild-type pMVC or the AAUAAA hexamer mutants (MVC/MVM_j, MVC/MVM_jins, and MVC/MVM3') as indicated, using the wild-type or MVMp heterologous sequence (pA)p probes as described in Materials and Methods. The sizes of the probe and various protected bands are shown on the left. The protected bands representing RNA species extending through (pA)p to (pA)d or cleaved at the various cleavage sites as described are indicated to the right. (C) RPAs of total RNA extracted 48 h following transfection of 293FT cells with wild-type pMVC and AAUAAA hexamer mutants (MVC/MVM_j, MVC/MVM_jins, and MVC/MVM3') as indicated, using the 3A probes as indicated and as described in Materials and Methods. The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species (3Aunspliced, unspliced at the third intron acceptor [3A]; 3Aspl, third intron acceptor spliced) are indicated to the right. (D) 293FT cells transfected with constructs described for panels B and C were harvested and analyzed by immunoblotting using antibodies directed against MVC NS proteins and NP1 (the epitopes were described in Materials and Methods). Immunoblotting for tubulin (Tub) was used for a loading control.

ing is catalyzed by a multienzyme complex, including the multisubunit cleavage and polyadenylation specificity factor (CPSF) which binds to the AAUAAA hexamer motif (26–28), the multisubunit cleavage stimulation factor (CstF) (26), which typically recognizes a G/U-rich downstream element, and the cleavage factors I and II (CFIm and

FIG 3 NP1 specifically binds to MVC RNA. (A) Diagram of the MVC second intron, third intron, and (pA)p region showing the locations of the specific primers used for first-strand synthesis (3337-R) and the primers across the third intron for qRT-PCR analysis (F-2386 and R-3100R). (B and C) qRT-PCR analysis of the RNA-IP experiment showing the percent MVC RNA immunoprecipitated from 293FT cells transfected with the constructs as indicated, followed by cross-linking with 0.5% formaldehyde (B) or cross-linked with UV irradiation (C). Anti-Flag antibody was used as an irrelevant antibody control. Random primers were used for synthesis of the first-strand cDNA, and forward primer F-2386 and reverse primer R-3100 were used for quantitative PCR. (D) Western blot of protein fractions from the experiments from panel B and C are shown. Rabbit anti-HA antibody was used to detect HA-tagged MVC NP1. (E) RT-PCR analysis of the product of the RNA-IP experiment showing the MVC RNA immunoprecipitated from 293FT cells cotransfected with MVC Rep-Cap constructs and the indicated constructs, following cross-linking by UV-irradiation. Specific primer R-3337 was used for synthesis of the first-strand cDNA, and forward primer F-2386 and reverse primer R-3337 were used for quantitative PCR. (F) A Western blot of protein fractions from the experiments from panel E is shown. Rabbit anti-Flag antibodies were used to detect Flag-tagged MVC NP1. The asterisk on the right indicates the light chain of mouse IgG. (G) MVC NP1 protein oligomer analysis of 293FT cells transfected with pMVC (lane 2) or nontransfected 293FT cells (lane 1). The Western blot shows the monomer, dimer, and tetramer oligomers of the MVC NP1 protein. The protein markers are shown on the left.

CFIm) (26). The tetrameric CFIm complex contains two small CPSF5 subunits and two large subunits of either CPSF7 (CFIm59) or CPSF6 (also called CFIm68). CPSF6 has recently been shown to be influential in regulating alternative polyadenylation (29, 30) and promoting mRNA export (33), and, thus, we examined its role in MVC RNA processing.

293T cells that lack CPSF6 were generated and showed an altered polyadenylation profile of cellular mRNAs (38). Following transfection of either MVC Rep-Cap or the infectious clone pIMVC (IMVC-WT) into these cells [CPSF6(-)293T], the ratio of internal polyadenylation of MVC RNA at (pA)p to distal polyadenylation at (pA)d was significantly reduced (Fig. 4B, compare lanes 3 and 4 and lanes 1 and 2). Interestingly, in the absence of functional NP1, we found little difference in alternative polyadenylation levels of MVC RNAs in the two cell types: RNAs that extended beyond (pA)p and were polyadenylated at (pA)d did not substantially accumulate in either cell type (Fig. 4B, compare lanes 5 and 6). These results suggested that CPSF6 likely decreased putative inhibition of (pA)p by NP1, reducing read-through transcripts rather than directly enhancing polyadenylation at (pA)p.

The increase in third intron splicing associated with NP1 (Fig. 4D and E, compare lanes 5 to lanes 1 and 3) was also further enhanced in cells lacking CPSF6 (Fig. 4D and E, compare lanes 2 and 4 to lanes 1 and 3). Again, however, in the absence of functional NP1, the presence or absence of CPSF6 made little difference: the lack of NP1 resulted in little splicing in either cell type (Fig. 4D and E, compare lanes 5 and 6). This suggested that CPSF6 lessened NP1's ability to enhance third intron splicing, rather than directly inhibiting its splicing. Taken together, these results suggested that CPSF6 tempered the two main effects of NP1 on MVC RNA processing, namely, inhibition of the internal polyadenylation site (pA)p and enhancement of splicing of the upstream adjacent third intron. These effects would result in reducing access to the capsid gene ORF and might be necessary to maximize overall infection efficiency.

These results also implied that that CPSF6 may have interacted with NP1 to affect alternative polyadenylation and not the polyadenylation machinery directly. In support of this possibility, we found that HA-tagged NP1, expressed from a Rep-Cap derivative of the MVC infectious clone, interacted specifically with endogenous CPSF6, as detected by immunoblotting, under low-salt but not high-salt extraction followed by subsequent immunoprecipitation with anti-HA antibody (Fig. 4C, lane 3).

Surprisingly, in cells lacking CPSF6, we observed an increase in levels of NP1, both in the presence (Fig. 4F, compare lanes 2 and 4 to lanes 1 and 3) and absence (Fig. 4F, compare lanes 5 and 6) of a functional NP1. NP1 is encoded from an RNA that spans the third intronic region, and, thus, splicing of the third intron would be predicted to reduce the level of NP1 encoding RNA (diagrammed in Fig. 4A). An increase in NP1 protein levels has also previously been observed in the characterization of the 5-proline insertion mutation of NP1 (16, 40) and attributed to a decrease in splicing of the third intron (16). However, in CPSF6-deficient cells, NP1-dependent splicing of the third intron was surprisingly increased, which would be expected to reduce NP1-encoding RNA and, thus, would be incompatible with an increase in NP1 levels.

To investigate this seeming inconsistency further, we analyzed the export of various MVC RNAs in the presence and absence of CPSF6. There is a close connection between RNA processing in the nucleus and the export of mRNAs, and, as mentioned, CPSF6 has been shown to interact with the mRNA export receptor NXF1/TAP to promote mRNA export (33). A difference in the abundances of cytoplasmic MVC mRNAs could contribute to differences in MVC protein abundances. Interestingly, we found that in the absence of CPSF6, both internally polyadenylated RNAs and RNAs that extended through (pA)p were exported to greater levels than exported in 293FT cells (Fig. 5A, compare lanes 2 and 6 to lanes 1 and 5; note quantification below). In addition, in the absence of CPSF6, RNAs unspliced at the third intron were preferentially exported to the cytoplasm compared to export of those spliced at the third intron (Fig. 5B, compare the ratio of lane 2 to 6 to that of lane 5 to 1; note quantification below). Such differences would provide increased availability in the cytoplasm of NP1-encoding RNAs. In addition, an examination of the temporal accumulation of NP1 indicated that it accumulated faster at early times posttransfection in CPSF6(-)293T cells than in 293FT cells. Although the transfection efficiency in CPSF6(-)293T cells was significantly less than that in 293FT cells, NP1 accumulated faster at earlier times posttransfection than a cotransfected enhanced green fluorescent protein (EGFP) control reporter. Such

FIG 4 CPSF6 affects MVC alternative RNA processing. (A) Transcription profile of MVC showing the P6 promoter, transcription starting site (TSS), splice donors (D) and acceptors (A), and proximal [(pA)p], and distal [(pA)d] polyadenylation sites. The annotated nucleotides delineate the boundaries of the transcription landmarks indicated within the MVC genome. The position of the RNase protection probes, 2A/3D (nt 2344 to 2550), 3A (nt 2910 to 3110), and (pA)p (nt 3107 to 3333), are indicated. NP1 and NS-66 mRNA species polyadenylated at (pA)p or (pA)d are shown. (B) RPAs of total RNA extracted 48 h following transfection of 293FT or CPSF6(-)293T cells with MVC Rep-Cap (lanes 1 and 2), IMVC-WT (lanes 3 and 4), and IMVC-5X (lanes 5 and 6) using the (pA)p probe. The sizes of the probe and various protected bands are shown on the left. The

(Continued on next page)

kinetics could also allow for its accumulation before additional enhancement of NP1-dependent splicing and concomitant reduction of NP1-encoding RNA (Fig. 5C, compare lanes 2, 4, and 6 to lanes 1, 3, and 5; note the quantification at right). RNAs encoding the NS 66-kDa (NS-66) protein are also reduced when the third intron is spliced, and there was a similar increase in the levels of the NS 66-kDa protein in CPSF6(-)293T cells at 48 h posttransfection when NP1 was nonfunctional and splicing was diminished (Fig. 4F, compare lanes 5 and 6). This was consistent with a role in increased export of unspliced RNA. However, in contrast to NP1 levels, a decrease in the expression of the 66-kDa NS protein at this time point was observed in these cells in the presence of a functional NP1, similar to levels of expression from the wild-type clone (Fig. 4F, compare lanes 3 and 4). How a functional NP1 contributed to differential expression of the 66-kDa-encoding RNA versus the NP1-encoding RNA at this time point in the CPSF6(-)293T cells is not yet clear; however, it should be noted that the processing history of these two RNAs is different. Consistent with this observation, the rapid early accumulation seen for NP1 was not seen for the NS 66-kDa protein (Fig. 5C, right panel). Taking these observations together, CPSF6, in addition to providing a tempering function for NP1, influenced MVC gene expression by modulating the export of MVC RNA.

To investigate further how CPSF6 might influence the function of NP1 at (pA)p in MVC RNA processing, we replaced (pA)p with the strong polyadenylation signal from the bovine growth hormone gene (bGH) (diagrammed in Fig. 6A). In the presence of either the wild-type or nonfunctional NP1, all MVC RNAs were polyadenylated internally at the bGH site in 293FT cells (Fig. 6B, lanes 1 and 3; also as previously reported [16]), as well as in CPSF6(-)293T cells (Fig. 6B, lanes 2 and 4). Splicing of the third intron was enhanced in the bGH replacement in the infectious clone containing a wild-type NP1 when transfected into CPSF6(-)293T cells compared to the level in 293FT cells (Fig. 6C and D, compare lanes 1 and 2). This was similar to results in the presence of (pA)p and supported a role for CPSF6 in tempering NP1's enhancement of splicing. However, in contrast to the situation in which internal polyadenylation was programmed by (pA)p (Fig. 4D and E, compare lanes 5 and 6), in the presence of nonfunctional NP1, bGH substitution supported a significant increase in splicing in CPSF6(-)293T cells compared to that in 293FT cells (Fig. 6C and D, compare lanes 3 and 4). This suggested that in the absence of NP1, when bGH was resident rather than (pA)p, either CPSF6 could itself inhibit splicing of the MVC third intron, or, alternatively, CPSF6 could functionally inhibit upstream splicing in conjunction with factors associated with the strong bGH polyadenylation signal adjacent downstream. How the different internal polyadenylation signals affect CPSF6's role in splicing of the adjacent upstream intron in the absence of NP1 is currently being investigated. As seen for the results with the authentic (pA)p in place (Fig. 4D and E), expression of constructs with the bGH polyadenylation signal in place of (pA)p showed a greater level of NP1 expression in the

FIG 4 Legend (Continued)

protected bands representing RNA species extending through (pA)p to (pA)d or cleaved at the various cleavage sites as described for (pA)d and (pA)p are indicated to the right. Quantifications below show the ratio of (pA)p/(pA)d RNAs from transfected CPSF6 knockout cells compared to the level in 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (C) Immunoprecipitation analysis reveals the association of CPSF6 and MVC NP1 in 293FT cells. Equal amounts of cell lysates were immunoprecipitated (IP) using protein-G magnetic beads (lane 2) or anti-HA magnetic beads (lane 3), as described in Materials and Methods, followed by immunoblotting with antibodies against CPSF6 and HA-tagged NP1. (D) RPAs of total RNA as described for panel B using the 3A probe. The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species (3Aunsp, unspliced at the third intron acceptor [3A]; 3Aspl, third intron acceptor spliced) are indicated to the right. Quantifications below show the ratio of spliced/unspliced RNAs from transfected CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (E) RPAs of total RNA as described for panels B and D using the 2A/3D probe (nt 2910 to 3100). The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species [(pA)d, read-through of the second intron acceptor (2A) and third intron donor (3D); 2Aspl/3Dun, the second intron acceptor spliced but third intron unspliced; 2Aspl/3Dspl, both second intron acceptor and third intron spliced] are indicated to the right. Quantifications below show the ratio of 2Aspl/3Dspl to read-through RNAs from transfected CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (F) 293FT or CPSF6(-)293T cells transfected with constructs described for panels B, D, and E were harvested and analyzed by immunoblotting using antibodies directed against CPSF6, NS, and NP1 (the individual epitopes are described in Materials and Methods). Immunoblotting for β -actin was used as loading control.

FIG 5 CPSF6 affect MVC viral RNA export. (A) At top are shown results of RPAs of cytoplasmic (Cyto) and nuclear (Nuc) RNA extracted 48 h posttransfection of 293FT and CPSF6(-)293T cells with pIMVC (IMVC-WT) using the (pA)p probe. The sizes of the probe and various protected bands are shown on the left. The protected bands representing RNA species extending through (pA)p to (pA)d or cleaved at the various cleavage sites as described for (pA)d and (pA)p are indicated to the right. Below, the quantification shows the ratio of cytoplasmic/nuclear RNA which was polyadenylated at (pA)d or (pA)p in CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (B) RPAs of cytoplasmic and nuclear RNA as described for panel A using the 3A probe. The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species (3Aunspl, unspliced at the third intron acceptor [3A]; 3Aspl, third intron acceptor spliced) are indicated to the right. The quantification shows the ratio of cytoplasmic/nuclear RNA which was spliced or unspliced in CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (C) 293FT or CPSF6(-)293T cells transfected with pIMVC (IMVC-WT) were harvested at 6, 18, and 36 h and analyzed by immunoblotting using antibodies directed against NS and NP1 (the epitopes are described in Materials and Methods) (left). Immunoblotting for tubulin was used as a loading control, and transfection with an enhanced green fluorescent protein (EGFP)-expressing construct was used to monitor transfection efficiency. Quantification of the Western blot showing the relative NP1 and NS-66 levels at indicated times posttransfection is also shown (right).

CPSF6(-)293T cells (Fig. 6E, compare lanes 1 and 2 and lanes 3 and 4). Levels of the NS 66-kDa protein remained relatively constant.

DISCUSSION

Unlike other parvoviruses, the bocaviruses control access to their capsid ORFs via alternative splicing and alternative polyadenylation (16, 45, 46). MVC and human bocavirus (HBoV) alternative pre-RNA processing is governed by a small nonstructural protein, NP1, that is unique to this genus, via interaction with the viral internal polyadenylation site (pA)p (45, 46). Here, we further characterized the *cis*-acting signals within (pA)p and further examined the function of NP1 in conjunction with the cellular factor CPSF6.

It is clear that the (pA)p region itself is complex. While there seemed to be some redundancy in the AAUAAA hexamers that govern the various cleavages within this region, hexamer 3 (nt 3258 to 3263) played the predominant role. It also was the controlling element for cleavage at site A. Interestingly, while mutation of AAUAAA hexamer 3 dramatically reduced internal polyadenylation overall, when NP1 was additionally mutated in this background, cleavage at sites B, C, and D occurred. This suggested that cleavage site A was likely the main target of NP1's suppression of polyadenylation at (pA)p.

We have previously shown that when the MVC (pA)p region was replaced with the strong polyadenylation signal from the bGH gene, splicing of the small intron was still supported and enhanced in the presence of NP1 (16). Experiments here show that mutation of the (pA)p AAUAAA hexamer elements individually had little effect; however, consistent with previous results, mutation of all three combined dramatically reduced upstream splicing in the presence of NP1. Interestingly, the reduced splicing in these constructs was still NP1 dependent, suggesting that NP1 may have had direct effects on the splicing machinery.

Sequences other than the classical polyadenylation elements were necessary to mediate NP1 regulation of the region. When the MVC (pA)p AAUAAA hexamers and associated DSEs were added back to a heterologous substitution that itself did not support polyadenylation, efficient polyadenylation was regained; however, it was no longer suppressed by NP1. These results indicated that while the classical motifs retained all function necessary to program the basic polyadenylation and cleavage reaction, additional elements within the region were necessary for NP1 suppression of these events, allowing the accumulation of read-through products.

NP1 was found to form a complex with MVC RNA, as detected following either formaldehyde or UV cross-linking. The proline insertion mutation of NP1, which inactivated NP1 for its roles in both suppressing internal polyadenylation and enhancing splicing of the upstream third intron (16), as well as the SR mutations of NP1 which allowed (pA)p suppression but prevented third intron splicing (16), did not bind MVC to detectable levels. UV cross-linking suggested that NP1 could bind directly to MVC RNA; however, it should be noted that even though the primers used for PCR detection lie within the (pA)p region, these results did not necessarily localize binding to this

FIG 6 Legend (Continued)

CPSF6(-)293T cells with IMVC/bGH (lanes 1 and 2) and 5X/bGH (lanes 3 and 4) using the 3A probe. The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species 3Aunspliced [unspliced at the third intron acceptor (3A)] or 3Aspl (third intron acceptor spliced) are indicated to the right. Quantifications below show the ratio of spliced/unspliced RNAs from transfected CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (D) RPAs of total RNA as described for panel B using the 2A/3D probe. The sizes of the probe and various protected bands are shown on the left. Bands representing RNA species (RT, read-through of the second intron acceptor [2A] and third intron donor [3D]; 2Aspl/3Dun, second intron acceptor spliced but third intron unspliced; 2Aspl/3Dspl, both second intron acceptor and third intron spliced) are indicated to the right. Quantifications below show the ratio of 2Aspl/3Dspl to read-through RNAs from transfected CPSF6(-)293T cells compared to the value for 293FT cells, which was set to 1. Standard errors were derived from at least three independent experiments. (E) 293FT or CPSF6(-)293T cells transfected with constructs described for panels B, C, and D were harvested and analyzed by immunoblotting using antibodies directed against CPSF6, NS, and NP1 (the epitopes are described in Materials and Methods). Immunoblotting for β -actin was used as a loading control. The asterisk on the right indicates the MVC NS-50 protein.

region. Since MVC transcripts were unlikely to be fragmented in this assay, the primers would detect MVC RNA immunoprecipitated with NP1 bound anywhere on these molecules. Identification of the specific region of MVC bound by NP1 is currently in progress. It is not yet clear whether NP1 binds to, and perhaps affects the processing of, cellular RNAs. Additionally, as with other nucleic acid binding proteins, we found that NP1 forms oligomers, up to at least tetramer levels, in cells transfected with the infectious clone of MVC. This oligomerization could be detected only when cells were extracted under mild conditions (described in Materials and Methods) and found to collapse into a single band upon boiling.

The cellular tetrameric RNA cleavage factor I (CFIm) complex contains two small CPSF5 subunits and two large subunits of either CPSF7 or CPSF6 (also called CFIm68). CPSF6 has recently been shown to be important in regulating alternative polyadenylation via interaction with polyadenylation factors (32, 47, 48), components of the U2 snRNP (49), SR proteins (SRSF3 and SRSF7), and the nuclear methylation reader YTHDC1 (48, 50). Additionally, CPSF6 has been shown to interact with the mRNA export receptor NXF1/TAP to promote mRNA export (33). For these reasons we examined the potential role for CPSF6 in MVC alternative RNA processing, using a cell line in which the CPSF6 gene had been knocked out using CRISPR/Cas9 (38). These experiments led to the observation that CPSF6 played a role in MVC alternative polyadenylation. Importantly, our results indicated that CPSF6 tempered the ability of NP1 to perform both of its defined functions, namely inhibition of internal polyadenylation at (pA)p and enhancement of upstream third intron splicing. In the absence of CPSF6, both of these functions were enhanced. Additionally, CPSF6 was found to suppress the export of unspliced MVC RNAs. Consistent with these observations, we have found that NP1 directly interacted with CPSF6. A more precise mapping of this interaction is under way.

It is not fully clear why CPSF6 functions to temper NP1's suppression of internal polyadenylation and enhancement of third intron splicing. One possibility is that at early times, CPSF6 function results in promoting expression of the MVC NS genes. Then, as more NP1 is generated over time, perhaps it either overcomes or eludes CPSF6 inhibition to enhance (pA)p read-through and upstream third intron splicing in order to enhance capsid gene expression. Since in the presence of (pA)p NP1 function is affected by CPSF6 and since the role of CPSF6 is abrogated in the absence of functional NP1, it is likely that CPSF6 modifies the effects of NP1 on MVC RNA processing.

Interestingly, when the bGH polyadenylation signal was substituted for (pA)p, we found a role for CPSF6 in directly inhibiting third intron splicing in the absence of a functional NP1. This further highlights the complexity of the interaction between polyadenylation at (pA)p, NP1, and splicing of the adjacent upstream intron, which merits continued attention. The results we observe for regulation of MVC (pA)p appear to be quite different from the regulation of AAV5 internal polyadenylation, which is governed by U1 snRNP which is stabilized by the 5' CAP-binding protein in a distance-dependent manner (50).

Alternative polyadenylation and alternative splicing are critical RNA processing mechanisms used by parvoviruses (16, 45, 46, 51, 52) and other DNA viruses (21, 53, 54) to coordinate the temporal expression of viral proteins necessary for successful infection. A number of viral RNA binding proteins in addition to NP1, including herpesvirus simplex virus (HSV-1) ICP27 (18–20) and Kaposi sarcoma-associated herpesvirus (KSHV) ORF57 (21–23), regulate alternative polyadenylation and alternative splicing. It will be important to characterize the similarities and differences among these proteins in the future.

MATERIALS AND METHODS

Cells and transfections. All experiments were carried out in either human 293FT cells or a CPSF6 knockout 293T cell line [CPSF6(-)293T], obtained from Gregory Sowd and Alan Engelman of the Harvard Medical School (38), which were propagated as previously described (55) in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum. Transfections were performed using LipoD293 transfection reagent (SignaGen Laboratories, MD) and maintained at 37°C in 5% CO₂ for 36 to 48 h.

RNase protection assays. Total RNA was extracted using TRIzol reagent (Invitrogen), and RNase protection assays (RPAs) using the probes indicated in the figures were performed on 20 μ g of total RNA as previously described (56). RPA signals were analyzed with the Typhoon FLA9000 and quantified with Multi Gauge software (GE). Relative molar ratios of individual RNA species were calculated after adjustment for the number of 32 P-labeled uridines in each protected fragment, as previously described (56). Probes utilized in this study are as follows. The MVC 2A/3D probe, spanning MVC nucleotides (nt) 2344 to 2550 across the second acceptor (2A) at nt 2386 and the third intron donor at nt 2490, was used to analyze splicing across the MVC second and third introns as previously described (16). Appropriate homologous probes were used when the MVC proximal polyadenylation region was replaced by MVmp heterologous sequences as shown in Fig. 2A or when the AAUAAA hexamers were individually or jointly mutated as diagramed in Fig. 1A. The wild-type 3A probe (nt 2910 to 3110) was used to analyze splicing across the MVC third intron. The MVC(pA)p probe which spanned MVC nt 3107 to 3333 was generated as previously described (16). The bGH probe (bovine growth hormone gene polyadenylation sequence, nt 1021 to 1263) was amplified from pcDNA3.1(+) (Life Technologies, CA). All the probe fragments were cloned into pGEM-3Z (Promega, WI) using restriction enzymes EcoRI and XbaI as described previously (16).

Antibodies and immunoblot analysis. Transfections with the constructs indicated in the text and figure legends were performed as previously described (16). Western immunoblotting analysis was performed as previously described (17). Briefly, 293FT or CPSF6(-)293T cells were harvested and lysed at 48 h posttransfection with WL lysis buffer (50 mM Tris [pH 8.0], 400 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, and protease inhibitors) which contained protease and phosphatase inhibitors. For MVC NP1 protein oligomer analysis, 293FT cells were transfected with pMVC and subsequently harvested and lysed in nondenaturing radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris [pH 8.0], 2 mM EDTA). Samples were either boiled or not after the addition of nondenaturing loading buffer, as indicated in the figure. Polyclonal antibodies directed against the MVC NS epitope (NS ORF amino acids 687 to 700, PKKQRKTEHKVLID) and the MVC NP1 epitope (NP1 amino acids 1 to 13, MSTRHMSKRSKAR), were utilized for MVC NS protein or NP1 protein immunoblotting, as previously described (17). Rabbit anti-HA and anti-Flag antibodies were used to precipitate and subsequently detect HA-tagged NP1 and Flag-tagged NP1 proteins. Mouse anti-tubulin, anti- β -actin and rabbit anti-CPSF6 antibodies (Abcam) were utilized for tubulin, β -actin, and CPSF6 protein immunoblotting, respectively. Anti-tubulin, anti- β -actin, anti-HA, and anti-Flag antibodies were purchased from Sigma-Aldrich.

Immunoprecipitation assays. Immunoprecipitation assays were performed as previously described (57). Briefly, following transfection with HA-tagged MVC construct (MVC-NPHA), 293FT or CPSF6(-)293T cells were harvested with mild lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP-40, 0.25% sodium deoxycholate, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], protease inhibitors) or WL lysis buffer (50 mM Tris [pH 8.0], 400 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors), as noted and lysed and sheared using a syringe. After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was divided into two samples of equal volumes, treated with RNase, and immunoprecipitated with either protein G magnetic beads (Pierce/ThermoFisher) or anti-HA magnetic beads (Pierce/ThermoFisher) overnight at 4°C. The beads were then washed five times in WL washing buffer (10 mM Tris [pH 7.4], 250 mM NaCl, 0.5% NP-40, 0.25% sodium deoxycholate, 0.5 mM EDTA, 1 mM DTT, protease inhibitors). SDS-PAGE and immunoblot analysis were carried out using antibodies as indicated in the text.

3' RACE assays. Rapid amplification of cDNA ends (RACE) was conducted using a 3' RACE system (3'-Full Race Core set; TaKaRa) according to the manufacturer's protocol. The two forward primer sets, MVC (pA)p primer-F (nt 2537 to 2569, 5'-ATGTCTACGAGACATATGAGCAAGAGATCAAAA-3'; melting temperature [T_m], 58.9°C) and MVC (pA)d primer-F (nt 4801 to 4824, 5'-CATGGGATTGACGCCCATCA GCA-3'; T_m , 63.5°C) were used for the PCR amplification reaction, as described in the text.

RNA immunoprecipitation following *in vivo* UV or formaldehyde cross-linking. 293FT cells were grown in a 10-cm dish and transfected with the indicated MVC constructs described in the text and figure legends. UV cross-linking and RNA immunoprecipitation (RNA-IP) experiments were performed as previously described (58). Briefly, for *in vivo* UV cross-linking and RNA-IP experiments, at 36 to 48 h posttransfection with HA-tagged MVC Rep-Cap constructs or mutants, cells were exposed to UV irradiation (150 mJ/cm²) and lysed with SDS lysis buffer (0.5% SDS, 50 mM Tris [pH 6.8], 1 mM EDTA, 1 mM DTT, 4 mM vanadyl-ribonucleoside complex [VRC; NEB], 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mg/ml tRNA). After cells were harvested and washed with cold phosphate-buffered saline (PBS), the samples were heated to 65°C for 5 min, chilled on ice for 2 to 3 min, and passed through a QIAshredder column (Qiagen). The extract was centrifuged at 16,000 $\times$ g at 4°C for 30 min, and the supernatant was combined with the preincubated antibody-bound magnetic beads and rotated at 4°C for 2 h. Samples were washed with cold RIPA buffer and treated with proteinase K for 1.5 h at 37°C, and samples were extracted with phenol-chloroform followed by sodium acetate precipitation. Specifically, precipitated RNA was analyzed by quantitative reverse transcription-PCR (qRT-PCR).

RNA immunoprecipitation following formaldehyde cross-linking experiments was performed as described previously (58). Briefly, at 36 to 48 h posttransfection, formaldehyde was added to cells at 0.5% for 10 min at room temperature. Glycine (2 M; pH 7.0) was added to a final concentration of ~0.2 M for 5 min to quench the reaction, and cells were harvested and lysed in RIPA-plus buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris [pH 8.0], 2 mM EDTA, 1 mM DTT, 4 mM VRC [NEB], 1 mM PMSF, 0.5 mg/ml tRNA). After a brief centrifugation, the supernatant was combined with the preincubated antibody-bound magnetic beads and rotated at 4°C for 2 h. Following washing with RIPA-U-plus buffer (RIPA buffer containing 1 M urea), high-salt buffer (1 M NaCl, 10 mM Tris [pH 7.5], 0.5%

Triton X-100), low-salt buffer (150 mM NaCl, 10 mM Tris [pH 7.5], 0.5% Triton X-100), and RIPA buffer, cross-links were reversed by incubation at 70°C for 45 min. Samples were then treated with proteinase K for 1.5 h at 37°C, and RNA was extracted as described above. Specifically, precipitated RNA was analyzed by qRT-PCR.

RT-PCR and real-time qRT-PCR. Real-time qRT-PCR was performed in 96-well plates with iTaq Universal SYBR Green Supermix (Bio-Rad). RNA extraction was carried out with TRIzol (Invitrogen), followed by Turbo DNase treatment (Invitrogen). Synthesis of first-strand cDNA was produced with ImProm-II reverse transcriptase (Promega) according to the manufacturer's protocol with random primer or the specific viral genomic primers (nt 3315 to 3337-R, 5'-TGGCTAGGCAAGTCTGCATTTAG-3'). To quantify the RNA immunoprecipitation levels, cDNA was amplified using primer sets (nt 2386 to 2407-F, 5'-GACTCTCCAGAAACACCAACG-3', $T_m = 55.9^\circ\text{C}$; nt 3078 to 3100-R, 5'-TTTCTATTCGGAGGAGCCATCTA-3', $T_m = 55^\circ\text{C}$.) across the third intron of MVC pre-mRNA as described in the figure legends. qRT-PCR data are presented as mean values for the percentages of the input signal for at least three independent experiments (with standard errors of the means) as previously described (8).

Cytoplasmic and nuclear RNA isolation. RNA extraction from cytoplasmic and nuclear fractions was performed as previously described (59). In brief, 293FT cells were transfected with pIMVC as described above and in the figure legends. At 48 h posttransfection, cells were harvested and divided into two equal packaged cell volumes (PCV). For total RNA extraction, the cell pellets were lysed in TRIzol (Invitrogen). For cytoplasmic and nuclear RNA isolation, the cell pellet was lysed in RLN buffer (50 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1.5 mM MgCl_2 , 0.5% NP-40 substitute, 1,000 U/ml RNase inhibitor [Roche], 1 mM DTT), and the debris and nuclei were pelleted by centrifugation at $300 \times g$ for 5 min at 4°C . The supernatant cytoplasmic fraction was collected, and the cell nuclear pellet was washed in PBS and pelleted again. Cytoplasmic and nuclear RNA was separately isolated by TRIzol according to the manufacturer's instructions. Total RNA and cytoplasmic and nuclear RNA were dissolved in the same volume of diethyl pyrocarbonate (DEPC)-treated water, and the same volume of RNA was used for RNase protection assays.

Plasmid constructs. The generation of the multiple plasmid constructs used in this study was as follows. The wild-type infectious clone pIMVC (IMVC-WT) and its mutant pIMVC NP1 5X (IMVC-5X) were generated as previously described (16).

pIMVC-Ig has a heterologous sequence from MVMP (the Nae I fragment from nt 2286 to 3285; GenBank accession no. [NC_001510](#)) cloned into nt 3151 of pIMVC.

The plasmids pIMVC 1M, 2M, 3M, and 123M (pA)p (WT, 5X) have the three AAUAAA core elements within MVC (pA)p (nt 3168 to 3333) disrupted by GCA substitutions, generated in wild-type and NP1 5XPro mutant construct pIMVC SK (WT, 5X), as described previously (16).

pIMVC MVC/MVM₁ (WT, 5X), has the MVMP heterologous sequence (nt 3502 to 3744; GenBank accession number [NC_001510](#)) amplified from the MVMP infectious clone and cloned into pIMVC SK (WT, 5X), as described previously (16).

pIMVC MVC/MVM₁ins (WT, 5X), has the MVMP heterologous sequence (nt 3502 to 3744), in addition to inserts of three AAUAAA core elements and corresponding DSEs, cloned into infectious clone pIMVC SK (WT, 5X), as described previously (16).

pIMVC MVC/MVM3' (pA)p (WT, 5X) was created in the pIMVC MVC/MVM₁ins (WT, 5X) background and includes a chimeric sequence composed of upstream MVC (pA)p sequence from construct MVC/MVM3' (nt 3111 to 3320), joined to the remaining MVMP 33-nt sequence, as indicated.

MVCHANP and 5XHA were created in the wild-type pIMVC-Ig or NP1 5X mutant backgrounds by insertion of an HA epitope upstream of the NP1 termination codon.

MVC Rep-Cap (MVCRC), pIMVC bGH (IMVC/bGH), and pIMVC bGH 5X (5X/bGH) were generated as previously described (16).

To generate 3×-Flag-NP1-WT (NP1 carrying three copies of the Flag tag [Flag-NP1]), the cDNA encoding wild-type NP1 was cloned into the cytomegalovirus (CMV) 3×-Flag 7.1 expression vector (Sigma). The 3×-Flag-NP1-SRm (Flag-SRm) construct includes the DNA sequence encoding the NP1 SR mutant amplified from construct pIMVC NP1SRm1-5, as described previously (16).

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