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Molecular Basis for the Differential Subcellular Localization of the 38- and 39-Kilodalton Structural Proteins of Borna Disease Virus

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Borna disease virus (BDV) is a nonsegmented negative-strand (NNS) RNA virus that is unusual because it replicates in the nucleus. The most abundant viral protein in infected cells is a 38/39-kDa doublet that is presumed to represent the nucleocapsid. Infectious particles also contain high levels of this protein, accounting for at least 50% of the viral proteins. The two forms of the protein differ by an additional 13 amino acids that are present at the amino terminus of the 39-kDa form and missing from the 38-kDa form. To examine whether this difference in amino acid content affects the localization of this protein in cells, the 39- and 38-kDa proteins were expressed in transfected cells. The 39-kDa form was concentrated in the nucleus, whereas the 38-kDa form was found in both the nucleus and cytoplasm. Inspection of the extra 13 amino acids present in the 39-kDa form revealed a sequence (Pro-Lys-Arg-Arg) that is very similar to the nuclear localization signals (in both sequence homology and amino-terminal location) of the VP1 proteins of simian virus 40 and polyomavirus. Primer extension analysis of total RNA from infected cells suggests that there are two mRNA species encoding the two forms of the nucleocapsid protein. In infected cells, the 39-kDa form is expressed at about twofold-higher levels than the 38-kDa form at both the RNA and protein levels. The novel nuclear localization of the 39-kDa nucleocapsid-like protein suggests that this form of the protein is targeted to the nucleus, the site for viral RNA replication, and that it may associate with genomic RNA.

Borna disease virus (BDV) causes severe neurological disease in a wide variety of species (13, 32). BD was initially described in sheep and horses in Central Europe more than a century ago. More recently, natural infections have also been documented in cats and cattle in Europe. Many species can be infected experimentally, although symptoms vary across species. In horses, the disease is severe, characterized clinically by incoordination leading to paralysis and death. In contrast, experimentally infected primates (*Tupaia glis*) show only a subtle modification of social interactions (35). The disease has been best characterized in an experimental rat system (32, 36). Adult immunocompetent rats develop a severe immunopathological disease that leads to destruction of much of the neuropil (24). However, neonatal and immunosuppressed adult rats develop a persistent tolerant infection with minimal neuronal damage that is localized mostly to the hippocampus (23). These animals exhibit some neurological deficits such as impaired maze-learning ability (12) and behavioral abnormalities (1). These results suggest that although the viral infection can cause alterations in behavior, the host immune response plays a major role in determining the severity of the disease.

BDV is a nonsegmented negative-strand (NNS) RNA virus (4, 26, 28). It is unique among animal NNS viruses in that replication occurs in the nucleus (4, 8) and that some of the viral mRNAs are spliced (9, 34). The genomic organization, as predicted by nucleotide sequence analysis (5, 9), is reminiscent of that of other NNS viruses. The structural proteins are encoded in the 3′ portion of the genomic RNA, with the putative nucleocapsid protein encoded at the 3′ end. Two forms of this protein are expressed at high levels in infected cells: a 38-kDa protein and a 39-kDa protein. These proteins also represent at least 50% of the viral proteins present in infectious particles (26). The relationship between these two forms of the protein has not been clarified since both are recognized by the same monoclonal antibodies. Immunofluorescence assays (IFAs) of infected cells, using monoclonal antibodies specific for the 38/39-kDa protein (Bo18) (14), revealed a characteristic staining pattern: punctate nuclear staining (that does not colocalize with nucleoli) and more diffuse staining in the rest of the nucleus and sometimes in the cytoplasm as well. A similar staining pattern is seen in IFAs in which polyclonal sera from infected animals are used, suggesting that the 38/39-kDa protein is the major viral protein expressed in infected cells. The high levels of this protein in the nuclei of infected cells led us to investigate whether it could be responsible for targeting the viral nucleocapsid to the nucleus for replication.

Viruses that replicate in the nucleus must specifically transport the replication apparatus (the genome and any required proteins) to the nucleus. This has been well documented for simian virus 40 (SV40) and polyomavirus, two DNA viruses that replicate in the nucleus. Most negative-strand RNA viruses replicate in the cytoplasm, since no host cell replicative enzymes are required. Exceptions are the orthomyxoviruses, which are negative-strand viruses that replicate in the nucleus. In this case, the viral polymerase "snatches" caps from cellular mRNAs to synthesize viral mRNAs (3, 6). There is no evidence that BDV uses a cap-snatching mechanism. However, some BDV mRNAs are spliced (10, 34), and this may explain the need for nuclear localization.

Among the NNS viruses, the ribonucleoprotein core (RNP) consists of the polymerase proteins P and L and the genomic RNA, which is tightly encased by the nucleocapsid protein N. The major protein in this complex is the nucleocapsid protein; targeting this protein to the nucleus could provide a mechanism to transport the complex to the nucleus. Using cells trans-

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fected with expression vectors, we show that the 39-kDa protein is targeted to the nucleus while the 38-kDa protein is found in both the nucleus and the cytoplasm. Translational initiation of the 38-kDa form of the protein occurs at a second in-frame ATG, and this protein is predicted to be missing 13 amino acids that are present in the 39-kDa protein. This region contains a sequence that is very similar to the nuclear localization signals (NLS) of the major capsid proteins of polyomavirus and SV40. In infected cells and in partially purified virions (26), the 39-kDa protein is expressed at approximately twofold-higher levels than the 38-kDa form; this difference in expression reflects differences in the levels of mRNAs for the two proteins. The differences in cellular localization and levels of expression suggest that the two forms of the nucleocapsid have different viral functions.

MATERIALS AND METHODS

Cell culture and metabolic labeling. Fetal rabbit brain cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (15). They were infected with brain homogenate from acutely infected rats. One week after infection, the cells were fixed and BDV antigens were detected by IFAs, as described previously (26).

SKNSH cells (human neuroblastoma cells) (2) were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Persistently infected SKNSH cells were maintained in the same medium (26).

For metabolic labeling, the cells were depleted of methionine by a 1-h incubation in RPMI 1640 that lacked methionine (Gibco) followed by a 6-h incubation in the same medium supplemented with 0.25 mCi of [^{35}S]methionine and [^{35}S]cysteine (TransLabel; ICN) per ml. The cells were washed in phosphate-buffered saline before extracts were prepared. For whole-cell extracts, the cells were lysed in 1× IP lysis buffer (1% Zwittergent, 0.5% sodium deoxycholate, 0.5 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 25 mM Tris-HCl [pH 7.6]) and sonicated three times, and the insoluble material was removed by centrifugation. Nuclear and cytoplasmic fractions were prepared by a Nonidet P-40 (NP-40) lysis procedure. The cell pellet was resuspended in 0.4 ml of NP-40 lysis buffer (0.65% NP-40, 0.15 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.8]) and incubated on ice for 2 min. Lysis of the cells was confirmed by microscopic examination. The nuclei were pelleted at 4°C by centrifugation for 5 min at 2,200 rpm in an SH-MT rotor (Sorvall). The supernatant (cytoplasm) was transferred to a fresh tube and clarified by centrifugation at 10,000 rpm in the SH-MT rotor. The clarified supernatant was transferred to a fresh tube, and an equal volume of 2× IP lysis buffer was added. The nuclear pellet was gently washed in NP-40 lysis buffer and pelleted at 2,200 rpm in the SH-MT rotor. The washed nuclei were resuspended in 0.4 ml of NP-40 lysis buffer, and an equal volume of 2× IP lysis buffer was added. The nuclear and cytoplasmic lysates were sonicated and clarified as described above.

Immunoprecipitations. BDV-specific proteins were immunoprecipitated with pooled anti-BDV rat sera as described previously (27) and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunofluorescence and immunocytochemistry assays. The cells were fixed for 20 min at room temperature in 3.7% formaldehyde in phosphate-buffered saline and permeabilized for 30 min in 1% Triton X-100. For immunofluorescence, BDV-specific proteins were detected as described previously (26), with pooled anti-BDV rat sera and fluorescein isothiocyanate-conjugated anti-rat antibodies (Dako) or Bo18 monoclonal antibody (14) and fluorescein isothiocyanate-conjugated anti-mouse antibodies (Dako). For immunocytochemistry, pooled anti-BDV rat sera and horseradish peroxidase-conjugated anti-rat antibodies (Dako) were used followed by diaminobenzidine detection.

Construction of expression vectors. PCR was used to generate the BDV inserts for the expression vectors. The 5′ and 3′ ends have *Not*I sites for insertion into a cytomegalovirus (CMV) promoter-driven expression vector in which BDV sequences replace a β-galactosidase sequence in the original vector (CMV–β-gal; Clontech). The oligonucleotides used for the constructs were as follows: for CMV-p39, 1122 (5′ TATAGCGGCCGCGGAACACACGCAATGCCACC 3′) and 1123 (5′ TATAGCGGCCGCTTACTAGTTTAGACCAGTCACACCTAT 3′); and for CMV-p38, 1124 (5′ TATAGCGGCCGCGGAACACACGCAATG GAGGACCAAGATTTATATGAA 3′) and 1123. Reactions (30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) were performed in reaction mixtures containing both *Taq* (Perkin-Elmer) and *Pfu* (Stratagene) polymerases to minimize the introduction of mutations. The mutations in CMV-p39(mut) were generated by using overlapping PCRs. Oligonucleotides 1122 and 1126 (5′ TAG TTTTAAACCTTTGCTGGATCTGCTCGGCTCC 3′) were used to synthesize the 5′ half, and oligonucleotides 1123 and 1127 (5′ GGAGCCGAGCAGATCC AGCAAAGGTTTAAAACTA 3′) were used to synthesize the 3′ half. The reaction products were gel purified with low-melting-point agarose, and an aliquot from each reaction product was used with oligonucleotides 1122 and 1123 in a 20-cycle reaction to generate the full-length insert. Sequences were verified for each construct.

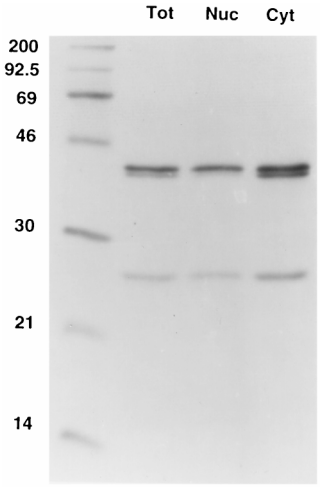

FIG. 1. Immunoprecipitations of BDV proteins from nuclear and cytoplasmic fractions of persistently infected cells. Metabolically labeled proteins were immunoprecipitated from total-cell lysates or from lysates prepared from nuclear and cytoplasmic fractions and separated on an SDS–15% polyacrylamide gel. Size markers are shown on the left. Lanes: Tot, total-cell lysate; Nuc, lysate prepared from the nuclear fraction; Cyt, lysate prepared from the cytoplasmic fraction.

Electroporation of cells. Subconfluent SKNSH cells were trypsinized and resuspended at 0.2×10^7 to 1×10^7 cells/ml in medium lacking antibiotics. The Bio-Rad gene pulser was used to electroporate plasmid DNA into the cells. Three hundredths of a milliliter of cells and 20 μg [CMV-p39 and CMV-p39(mut)] or 30 μg (CMV-p38) of plasmid DNA were incubated in 0.4-cm cuvettes at room temperature for 10 min. The cells were electroporated at room temperature (250 V and 250 μF), placed on ice for 10 min, and then plated in complete medium with antibiotics. The medium was changed on the following day, and the cells were used for experiments 72 h postelectroporation. Cells in chamber slides were fixed for IFAs, and cells in six-well dishes were metabolically labeled.

Primer extension analysis of RNA. Oligonucleotide 498 (5′ TATAAGCTTC CAACGGTGTATTGTAGGAAT 3′) was end labeled with [γ-^{32}P]ATP and used in a primer extension reaction with 10 μg of total RNA from persistently infected SKNSH cells as previously described (27). In parallel, oligonucleotide 498 was used to prime a sequencing reaction of cloned BDV DNA (RT-PCR 5′/3′, obtained from Juan Carlos de la Torre [9]) with a Sequenase kit (United States Biochemical).

RESULTS

Cell fractionation of persistently infected SKNSH cells. To determine whether the 38- and 39-kDa proteins are differentially localized in infected cells, SKNSH cells persistently infected with BDV were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Lysates from nuclear and cytoplasmic fractions were immunoprecipitated with pooled sera from infected rats, and BDV proteins were resolved by SDS-PAGE (Fig. 1). Both nuclear and cytoplasmic fractions contained the 39-kDa form. However, the nuclear fraction contained greatly reduced levels of the 38-kDa protein relative to the 39- and 24-kDa proteins. In the total-cell lysates from infected cells, there is approximately twice as much 39-kDa protein as 38-kDa protein. Analysis of additional autoradiograms of immunoprecipitated BDV proteins from infected cells further supports this observation. Autoradiograms from seven gels were scanned and quantitated with ImageQuant (Molecular Dynamics). The ratio of the 39-kDa protein to the 38-kDa protein ranged from 1.64 to 2.09 (mean, 1.83 ± 0.17).

Since there was a difference in the localization patterns of the two proteins, the effectiveness of the fractionation protocol

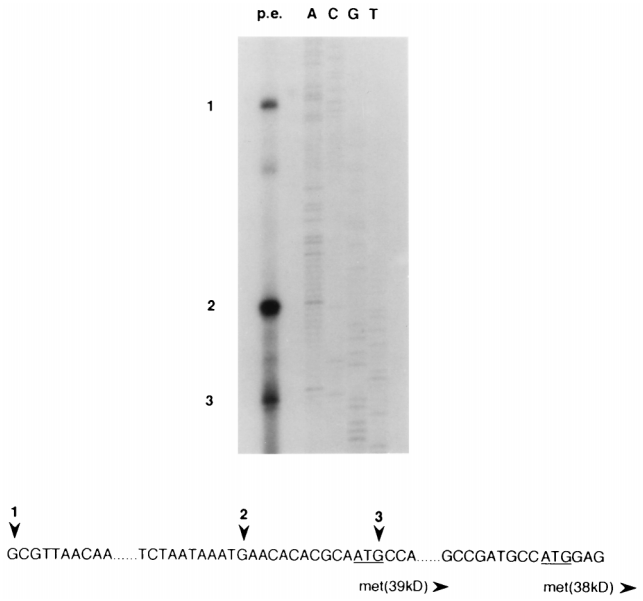

FIG. 2. Primer extension analysis of RNA. Oligonucleotide 498 was used for both primer extension analysis of BDV RNA from infected cells and a sequencing reaction with cloned BDV cDNA as template. The primer extension reaction (p.e.) is shown on the left, with the major products indicated (bands 1, 2, and 3). The sequencing reaction is shown on the right (ACGT). The bottom schematic shows the RNA endpoints mapped by primer extension analysis and their relation to the initiating methionine codons.

was examined. The localization of two cellular nuclear proteins was examined after fractionation by the NP-40 procedure. Lamin, a nuclear protein that forms a network beneath the nuclear envelope, was found to be strictly associated with the nuclear fraction (data not shown). However, the 36-kDa TATA binding protein was found mostly in the cytoplasmic fraction (data not shown). Because small (<60-kDa) proteins without NLS equilibrate between the nuclear and cytoplasmic compartments (25), it is possible that the 38-kDa protein was lost from nuclei during isolation or that it is a nonnuclear protein. The differential patterns of expression suggest that the 39- and 38-kDa proteins may perform different functions during viral replication. The observation that the 39-kDa protein is expressed at a twofold-higher level than the 38-kDa form led to an investigation of the mRNA(s) encoding them.

Primer extension analysis of RNA encoding the 38/39-kDa proteins. The basis for the size difference of the two forms of the nucleocapsid protein has not been identified. However, in vitro translation of a plasmid containing the 39-kDa coding region results in the production of both the 39- and 38-kDa forms of the protein, as well as additional smaller proteins resulting from initiation of translation at internal ATGs (27). Primer extension analysis was used to identify the 5′ end of viral mRNAs responsible for the production of the 38/39-kDa protein in infected cells (see Materials and Methods). The primer extension products from RNA isolated from infected SKNSH cells were electrophoresed adjacent to products from a sequencing reaction in which the same primer was used (Fig. 2). Three major primer extension products can be identified (bands 1, 2, and 3). Product 1 corresponds to the full-length antigenome, and product 2 corresponds to the first transcriptional start site previously identified by Schneeman et al. (33). Product 3 identifies the 5′ end of an RNA species that initiates with the G residue in the first ATG; such a species would be unable to encode the 39-kDa protein but could direct synthesis of the 38-kDa protein. Quantitative analysis showed that the intensity of band 2 was approximately twice that of band 3 (NIH ImageQuant analysis of a scanned autoradiogram). These results suggest that infected cells contain separate mRNAs directing the synthesis of the 39- and 38-kDa proteins and that there is a twofold-higher level of the mRNA encoding the 39-kDa protein than of that encoding the 38-kDa protein.

Subcellular localization of the 38/39-kDa proteins. To investigate the targeting of the 38/39-kDa protein in cells at an early time after infection, fetal rabbit brain cells were infected with BDV-infected rat brain homogenate. At 1 week after infection, the cells were fixed for immunofluorescence detection of BDV proteins with Bo18 (14), a monoclonal antibody that detects the 38/39-kDa protein (Fig. 3a), and pooled polyclonal sera from infected rats (Fig. 3b). In both cases, some cells had only punctate nuclear staining, some had intense staining throughout the nuclei, and some were stained in both the nucleus and cytoplasm. These results suggest that early in infection, the 38/39-kDa protein is localized in the nucleus, whereas later in infection, the antigen is expressed more diffusely.

To clarify the targeting of the 38/39-kDa proteins, we generated a panel of expression vectors to identify their location in the absence of other viral components. Examination of the predicted amino acid sequence for the 38/39-kDa protein revealed two regions with clusters of basic residues similar to known NLS. The first region is at the amino terminus of the protein. At the corresponding 5′ end of the open reading frame encoding the 38/39-kDa proteins, there are two in-frame ATGs 13 codons apart which could account for the approximately 1-kDa size difference detected by SDS-PAGE. Within this region is a sequence (Pro-Lys-Arg-Arg) that is very similar to sequences present in the nuclear targeting domains of polyomavirus VP1 (Pro-Lys-Arg-Lys) (7) and SV40 VP1 (Pro-Thr-Lys-Arg-Lys) (18, 38). The expression vector CMV-p39 initiates translation from the first ATG. The expression plasmid CMV-p38 initiates translation from the second ATG, eliminating expression of this putative NLS. The second possible NLS (codons 167 to 171, Lys-Lys-Arg-Phe-Lys) is in the middle of the protein and has previously been predicted to be a nuclear targeting motif (22). To test the nuclear targeting activity of this region, the first two Lys residues (amino acids 167 and 168) in the second potential NLS were mutated to glutamine in the expression vector CMV-p39(mut).

The three constructs CMV-p39, CMV-p38, and CMV-p39(mut) were electroporated into SKNSH cells. Three days after electroporation, these cells were stained by immunocytochemistry to detect expression of the 38/39-kDa proteins (Fig. 4). Cells electroporated with CMV-p39 or CMV-p39(mut) expressed high levels of antigen that was concentrated in the nucleus. In contrast, cells electroporated with the CMV-p38 vector showed lower levels of diffusely distributed antigen in both the nucleus and cytoplasm. These results indicate that the amino terminus of the 39-kDa protein is responsible for targeting to the nucleus.

Immunoprecipitation of proteins expressed in transfected cells. To confirm that proteins of the expected sizes were expressed, cells electroporated with each construct were metabolically labeled and the proteins were immunoprecipitated from total-cell lysates with pooled BDV rat sera (Fig. 5). Proteins expressed from CMV-p39 and CMV-p39(mut) showed similar mobility to the 39-kDa protein from infected cells, whereas protein expressed from the CMV-p38 construct comigrated with the 38-kDa protein from infected cells. It should be noted that expression from either CMV-p39 or CMV-p39(mut) resulted in the production of only the 39-kDa form of the protein. As is generally true for other eukaryotic mRNAs, apparently the

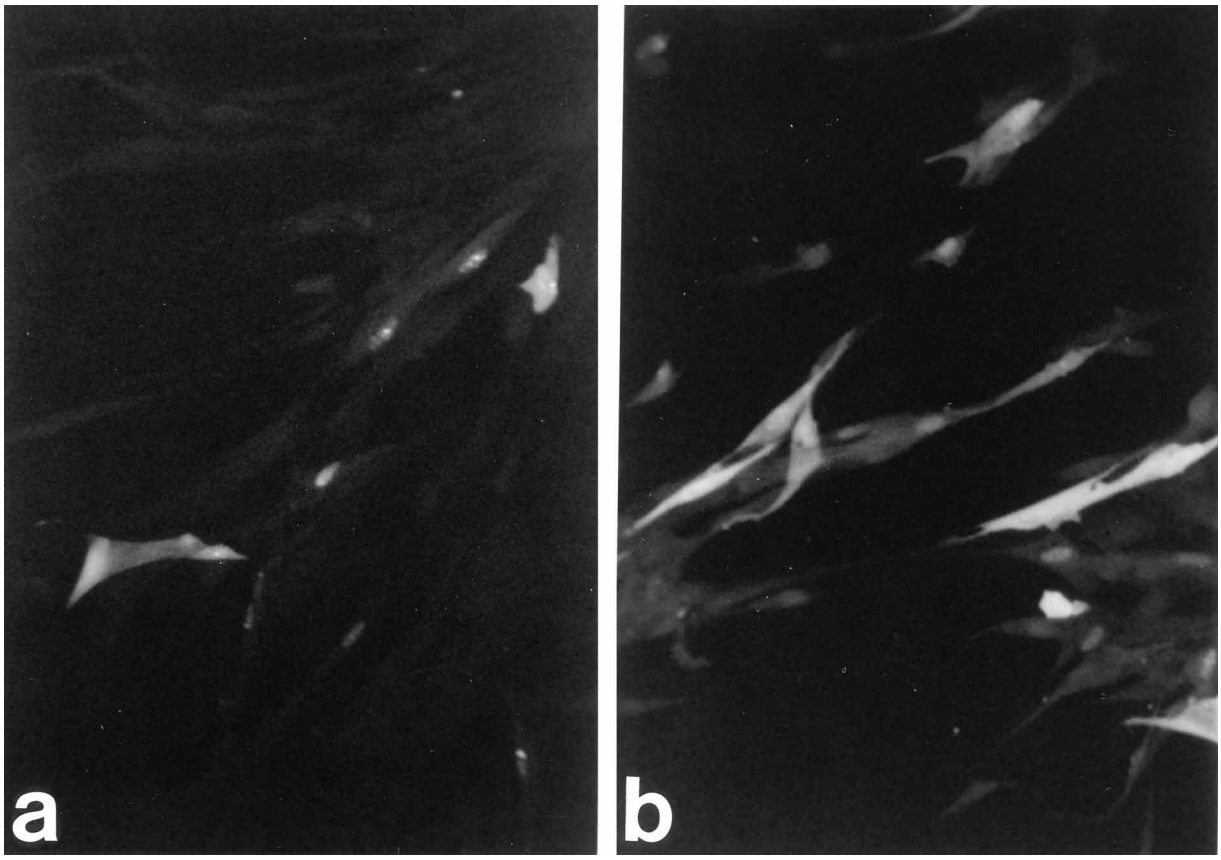

FIG. 3. Immunofluorescence detection of BDV antigen in infected fetal rabbit brain cells. One week after infection, the cells were fixed for IFA. (a) Cells were stained with Bo18 monoclonal antiserum. (b) Cells were stained with pooled sera from infected rats.

second ATG is not used if the first is present (19). This supports the results from the primer extension experiment indicating that there was a unique mRNA species encoding the 38-kDa protein.

DISCUSSION

In this study, we have shown that the two forms of the BDV nucleocapsid protein differ in their inherent intracellular distribution and that they are translated from different mRNAs. We also showed that at both the RNA and protein levels, there is approximately twofold-greater expression of the 39-kDa protein than of the 38-kDa protein. We confirmed this relationship by comparing ratios of the 38- and 39-kDa proteins in infected cells with the ratios in proteins translated in vitro from RNA isolated from infected cells (data not shown). In all cases, the 39-kDa protein was expressed at an approximately twofold-higher level than was the 38-kDa protein. This strongly suggests that expression is controlled at the level of transcription and not translation.

The third primer extension product (Fig. 3) identified a transcription initiation site that bears only limited similarity to the previously identified initiation sites (33). All four transcription initiation sites have the sequence UUAC, where transcription initiates from the C residue. However, the other three sites have six or seven Us (of nine nucleotides) before the C and three Us (of four residues) after the C; the newly identified site has only four Us in nine residues before the C and one U in five residues after the C. There are only 12 nucleotides between the two initiation sites. The U-rich region of the first site may serve to engage the polymerase without necessarily initiating transcription, with either the first or second UUAC signaling the site for initiation. The first site is likely to be used preferentially since it conforms more precisely to the consensus sequence and is more proximal to the U-rich region. This preference then leads to the production of twofold-higher levels of the 39-kDa protein.

The CMV-p38 construct was not expressed as efficiently as the other constructs. Even when more DNA was transfected into the cells, the level of protein detected was lower than for the CMV-p39 and CMV-p39(mut) constructs. In Fig. 4, the p38 panel shows a cluster of cells expressing high levels of protein; such intensely staining clusters were rare in cells transfected with CMV-p38. Most cells transfected with CMV-p38 expressed much lower levels of the 38-kDa protein. In contrast, a large proportion of cells expressed high levels of protein from the CMV-p39 and CMV-p39(mut) vectors. Two possibilities for the low level of 38-kDa expression are that (i) transcription and translation are not equivalent, and (ii) the 38-kDa protein is less stable and is degraded more rapidly in the cell. We have not directly addressed the first possibility, but indirect evidence suggests that transcriptional efficiency is not solely responsible. Several constructs with different upstream sequences have been made for the expression of the 38-kDa protein (two in the context of the second ATG and one in the context of the ATG that efficiently initiates translation of the 39-kDa protein). None of these constructs produced high levels of protein expression in transfected cells.

However, some preliminary experiments suggest that the additional amino acid sequence of the 39-kDa form may confer

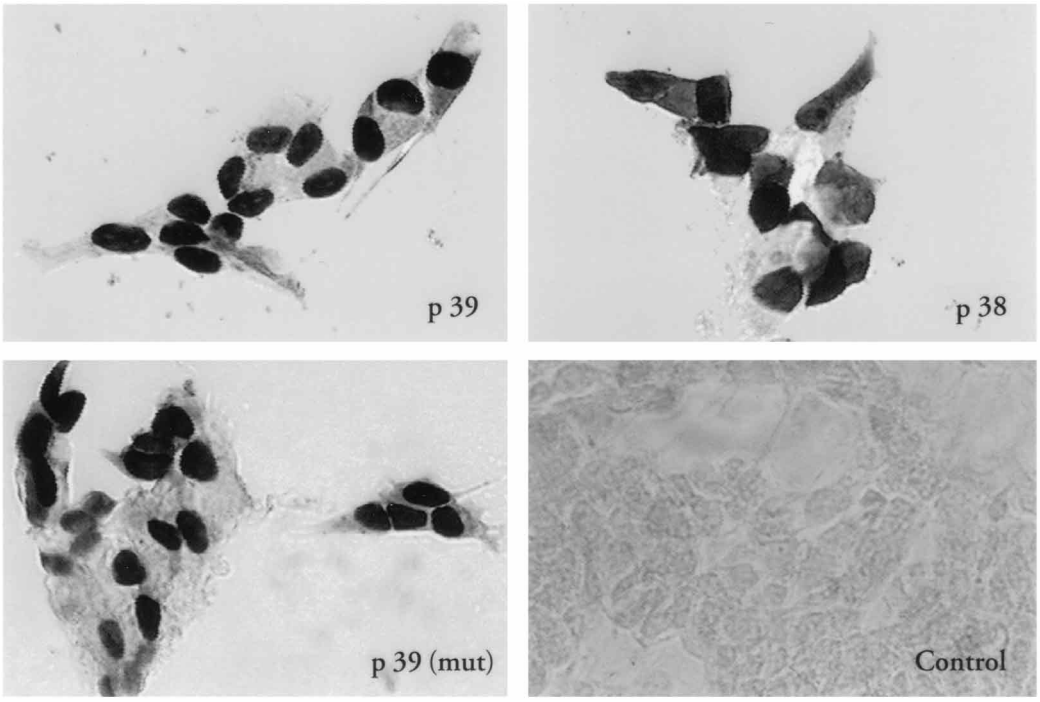

FIG. 4. Immunocytochemical detection of proteins expressed in cells transfected with the p39, p38, and p39-mut plasmids.

stability to the protein. In experiments designed to define the specific amino acids involved in nuclear targeting of the 39-kDa protein, mutations have been made in the single Lys and two Arg residues in the unique region of the 39-kDa form. Seven mutant constructs were made: three with the individual residues mutated, three with the different possible double mutations, and one with all three residues mutated. Immunocytochemical analysis of transfected cells suggested that the double and triple mutations decreased the level of protein that could be detected relative to that for either the single-site mutations or the wild type. Interestingly, none of the single mutants expressed proteins that localized specifically to the nucleus, although the levels of expression were high (data not shown). The double mutants were expressed at a significantly lower level, and the triple mutant was expressed very poorly, with antigen being detectable in only a few cells (data not shown). Since these constructs differed only in coding for the Lys and Arg residues, it is unlikely that differences in transcriptional or translational efficiency account for the decreased levels of detected protein. A more likely possibility is that the amino-terminal region is important for conferring stability to the nucleocapsid protein. However, in the context of the infected cell, interactions with other viral components may confer stability to the 38-kDa protein.

The functions of the differentially localized proteins remain unclear. The EMBL Predict Protein paradigm (29–31) did not predict any significant differences in secondary structure between the two proteins. Both proteins are predicted to have large helical regions, as might be expected for nucleocapsid proteins that are involved in structural assembly. However, this model, which is based on linear analysis of the proteins, could fail to predict long-range effects on secondary structure.

The nucleocapsid protein is likely to have several functions which have not yet been characterized fully for BDV. It is expected to bind to the viral RNA, and it is expected to interact with other viral proteins. It has been shown previously that the 38/39- and 24-kDa proteins form heterodimers (17, 37). Hsu et al. (17) showed that only the 39-kDa form dimerized with the 24-kDa protein, but the interacting domains have not been identified for either protein. Potential interactions between the 38- and 39-kDa proteins have not been examined, but now that the structural basis for the difference in size has been determined, it should be possible to investigate such interactions. Finally, in the case of viruses that replicate in the nucleus, the nucleocapsid protein is likely to be responsible for targeting the RNP core (containing the genome and virion enzymes required for replication) to the nucleus. Interestingly, polyomavirus and SV40, two DNA viruses that replicate in the nucleus, have major capsid proteins (VP1) with NLS that are very similar to the NLS of the BDV 39-kDa protein.

NLS have been defined for many nuclear proteins (11). Two basic types of NLS have been reported: the SV40 large T-type

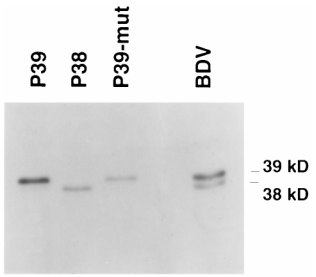

FIG. 5. Immunoprecipitation of proteins expressed from transfected cells. SKNSH cells were electroporated with the indicated plasmids and cultured for 2 days before being subjected to metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine. Proteins were immunoprecipitated and fractionated on an SDS–10% polyacrylamide gel. Lanes indicate the constructs electroporated into the cells. BDV, immunoprecipitation of lysate from metabolically labeled persistently infected SKNSH cells. Locations of the 39- and 38-kDa bands are indicated.

NLS that consists of a short contiguous stretch of mostly basic amino acids, and the bipartite nuclear targeting motif that consists of 2 basic amino acids followed by a spacer region of any 10 amino acids and a basic cluster in which 3 of 5 amino acids must be basic (11). Few NLS that are at the amino terminus of proteins have been identified. The fiber protein of adenovirus serotype 2 has an NLS (KRAR) at amino acid residues 2 to 5, but it does not have a proline residue (16). The N-terminal NLS of the VP1 proteins of polyomavirus (7) and SV40 (18, 38) have a proline at the second position in the protein and basic residues in the third, fourth, and fifth positions (polyomavirus VP1) or in the fourth, fifth, and sixth positions (SV40 VP1). In both cases, the first two basic residues are Lys-Arg. In polyomavirus VP1, this lysine residue is critical for nuclear localization (7). The amino terminus of the BDV 39-kDa protein is very similar to VP1 of these papovaviruses. We do not know if the initiating methionine in BDV is retained in the mature protein; neither SV40 nor polyomavirus VP1 retain the initiating methionine. If this methionine is cleaved, the BDV 39-kDa protein will also have a proline in the second position followed by Lys-Arg-Arg.

The fact that there is differential subcellular localization suggests there are important functional differences between the 38- and 39-kDa proteins. Cellular fractionation of infected cells (Fig. 2) showed that the 39-kDa protein was both nuclear and cytoplasmic whereas the 38-kDa protein was largely excluded from the nucleus. However, the immunocytochemical detection of the proteins in transfected cells (Fig. 3) showed that the 39-kDa protein was localized to the nucleus while the 38-kDa form was found in both the nuclear and cytoplasmic compartments. Control experiments involving detection of lamin (a nuclear protein in a large complex) and TATA binding protein (a small nuclear protein) suggested that the NP-40 fractionation protocol allowed small proteins to leak from the nucleus but that proteins in large complexes remained associated with the nuclei. Under the same conditions for fractionation of transfected cells, both the 39- and 38-kDa proteins were found mostly in the cytoplasmic fraction (data not shown). This shows that both proteins can diffuse from the nucleus during the fractionation procedure. Since a large proportion of the 39-kDa protein remained in the nuclear fraction isolated from infected cells, it is likely that it is present in a complex which is too large to diffuse from the nucleus. This form of the nucleocapsid protein is likely to bind the viral RNA as it is synthesized. The 39-kDa protein found in the cytoplasmic fraction may represent uncomplexed protein that diffused from the nucleus during fractionation or nucleocapsid structures which have been exported from the nucleus. The role of the 38-kDa protein is less clear, although the similar 39/38-kDa ratios seen in both infected cells and partially purified virions (26) suggest that the 38-kDa protein plays an important structural role.

For any virus that replicates in the nucleus, there are important transport issues: the RNP core of the infecting virus must be delivered to the nucleus for replication, but newly synthesized RNPs must be exported from the nucleus. Signals are required to direct transport in the appropriate direction. Martin and Helenius (20, 21) have documented this for influenza virus and have shown that the viral matrix protein (M1) promotes export of the viral RNPs from the nucleus. M1 dissociates from the viral RNP in the acidic environment of the endosome. The free RNPs are then transported to the nucleus, where they are replicated and transcribed. RNPs that remain associated with M1 are unable to translocate to the nucleus. Ye et al. (39) have identified the NLS for the M1 protein. Interestingly, this domain is coincident with a previously defined RNA binding domain. This suggests that the NLS is masked when complexed with the viral RNP. Newly synthesized RNPs associate with M1 in the nucleus and are transported to the cytoplasm. It is worth noting that a large fraction of M1, possibly in a different conformation, remains in the cytoplasm of infected cells. After the RNP-M1 complex leaves the nucleus, additional cytoplasmic M1 binds to the complex. Perhaps the cytoplasmic form of M1 is conformationally different from the nuclear form and binds differently to the RNP.

BDV expresses two forms of nucleocapsid protein, which may be important in intracellular transport of RNPs. The 39-kDa nucleocapsid protein is efficiently targeted to the nucleus, where it presumably binds the virion RNA as it is synthesized. This nuclear complex may then bind the 38-kDa protein, which can diffuse into the nucleus but has no specific NLS. One could postulate that the 38-kDa protein binds to the 39-kDa protein–RNA complex in a way that masks the NLS and leads to a conformational change that promotes export from the nucleus and prevents reimport into the nucleus. IFA detection of antigen early after infection (Fig. 3) shows cells expressing different levels of protein. Antigen is detected only in the nucleus of cells expressing low levels of protein. In cells expressing higher levels, antigen is found in both nuclear and cytoplasmic locations. This suggests initial targeting to the nucleus; at later times, antigen is exported from the nucleus. Future experiments will examine the roles of the 39- and 38-kDa proteins in intracellular targeting of the RNPs.

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