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Nuclear Localization of the Protein from the Open Reading Frame x1 of the Borna Disease Virus Was through Interactions with the Viral Nucleoprotein

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Previous studies have predicted the presence of a small open reading frame (ORFx1) located between ORF-1 and ORF-2 of the Borna disease viral (BDV) genome. The ORFx1 is expressed as a p10 protein that is localized in the nucleus and cytoplasm of BDV-infected cells. In this study, we cloned the nucleotide sequence of ORFx1 into expression vectors and showed that it is expressed as p10. An anti-p10 serum gave nuclear and cytoplasmic staining of cells persistently infected with BDV. Immunoprecipitation of p10 from BDV-infected cells coprecipitated the p40 nucleoprotein N and the 24-kDa viral phosphoprotein P. Transient transfection of noninfected cells showed that p10 and p40 can be coprecipitated and revealed that p10 localized in the cytoplasm was imported into the nucleus in the presence of the BDV p40 N. *In vitro* protein–protein interaction studies on solid phase showed the direct interaction of the p10 with the BDV N protein. The subcellular distribution of p10 and its interaction with p40 suggest that this protein may play a role in the nuclear replication and/or transcription of BDV.

INTRODUCTION

Borna disease (BD) is a transmissible progressive polioencephalomyelitis caused by an enveloped, negative, nonsegmented, single-stranded (NNS) RNA virus (BDV) (Gosztonyi and Ludwig, 1984, 1995; de la Torre *et al.,* 1990; Ludwig *et al.,* 1993; Briese *et al.,* 1994; Cubitt *et al.,* 1996). This virus naturally infects a wide range of hosts, including horses, sheep, ostrich, to give diseases of the central nervous system. BDV has recently been isolated from humans by cocultivation of a human oligodendroglia cell line with the peripheral blood mononuclear cells (PBMCs) from three hospitalized psychiatric patients but not from any of the healthy control subjects (Bode *et al.,* 1996). Partial nucleic acid sequence of the BDV isolates from humans also suggested a high degree of sequence conservation (0.07–0.83% divergences) with respect to horse BDV (de la Torre *et al.,* 1996). It has been proposed that BDV infection in humans may be associated with certain psychiatric disorders, including affective disorders (Amsterdam *et al.,* 1985; Rott *et al.,* 1985, 1991; Bode *et al.,* 1993, 1994, 1995; Fu *et al.,* 1993; Kishi *et al.,* 1996). BDV antigens and RNAs have been detected in PBMCs of neuropsychiatric patients (Bode *et al.,* 1994, 1995; Kishi *et al.,* 1996). The prevalence of plasma antibodies to BDV also is higher in these patients than in the general populations studied (Amsterdam *et al.,* 1985; Bode *et al.,* 1993; Fu *et al.,* 1993).

The genome of BDV is approximately 8.9 kb in length with complementary 5′ and 3′ termini (Briese *et al.,* 1994; Cubitt *et al.,* 1994). It has at least five major open reading frames (ORFs) (Briese *et al.,* 1994; Cubitt *et al.,* 1994). ORF-1 encodes a 38/40-kDa protein (p40), and ORF-2 encodes for a phosphoprotein, p24 (Briese *et al.,* 1994; Cubitt *et al.,* 1994). ORF-3 encodes a glycoprotein, gp18, which has an unusual N-linked glycosylation pattern (Kliche *et al.,* 1994), and ORF-4 encodes the heavily N-linked glycosylated gp84, which has been suggested to be important for virus entry into susceptible cells. The protein encoded by ORF-5 has not been isolated, but this most 5′ ORF, which encompasses more than half the genome, has the capacity to encode for a protein of 170/180 kDa and is likely the RNA-dependent RNA polymerase (i.e., the L protein) of BDV (Briese *et al.,* 1994; Cubitt *et al.,* 1994). The subgenomic RNAs of BDV have been mapped to the viral genome and show overlaps of transcription units, transcription signals, a read-through of transcription termination signals, and RNA splicing (Cubitt *et al.,* 1994a; de la Torre, 1994; Schneider *et al.,* 1995; Schneemann *et al.,* 1995). These features suggest that BDV is the prototype of a new family of animal viruses in the order of *Mononegalovirales* (de la Torre, 1994; Schneider *et al.,* 1995). Also, unique among known animal NNS RNA viruses, BDV replicates and transcribes in the nucleus (Briese *et al.,* 1992; Cubitt and de la Torre, 1994). Thus viral proteins found in the nucleus of BDV-

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infected cells are likely to play a central role in the nuclear replication of the virus.

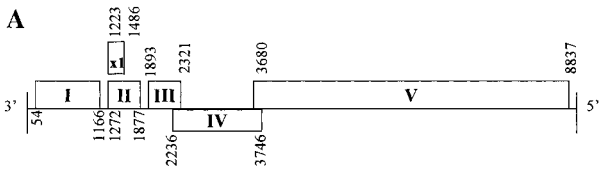
The p40 nucleoprotein N encoded by ORF-1 carries a nuclear localization signal (NLS). It is found in the nucleus of BDV-infected cells and of cells transfected with an expression vector carrying the ORF-1 nucleotide sequence (Pyper and Gartner 1997; Kobayashi *et al.,* 1998). Coprecipitation experiments have shown that the p24 phosphoprotein P encoded by ORF-2 interacts with the p40 N (Hsu *et al.,* 1994). Hence, p24 P also may play a role in the nuclear replication and/or transcription of the virus. Recently, cDNA expression and nucleotide analyses of the BDV genome suggest the presence of a small ORFx1 overlapping with ORF-2 (Vande Woude *et al.,* 1990; Pyper *et al.,* 1993; Cubitt *et al.,* 1994). This ORFx1 has 87 codons and is expressed as a 10-kDa protein (Wehner *et al.,* 1997). Immunofluorescence studies of BDV-infected cells have shown that this novel p10 is detected in the nucleus and the cytoplasm of BDV-infected cells (Wehner *et al.,* 1997). Likely, p10 also may play a role in the replication and/or transcription of BDV in the nucleus. Computer analyses of the p10 codon sequence have failed to reveal the presence of an amino acid motif previously identified as an NLS to known nuclear proteins. p10 has been reported to interact with p24 (Schwemmle *et al.,* 1998), which putatively possesses an NLS (Thierer *et al.,* 1992). However, Wehner *et al.* (1997) reported the colocalization of p10 and p40 in BDV-infected cells. Thus the direct interaction of p10 with p40 N cannot be ruled out (Schwemmle *et al.,* 1998). In this study, we cloned the nucleotide sequence of ORFx1 into expression vectors. We also prepared specific serum to p10. Immunoprecipitation of cell lysate from BDV-infected cells with our anti-p10 antibody gave p10 + N + P. Immunoprecipitation of cell lysate from transiently transfected cells with the anti-p10 antibody recovered the p40 N and p10. Immunofluorescence experiments revealed that p10 was expressed in the cytoplasm of the transfected cells, but becomes localized in the nucleus in the presence of p40 N. Protein–protein interaction studies *in vitro* confirmed the direct interaction of p10 with p40 N; thus direct interaction with the p40 N will mediate the nuclear localization of p10.

RESULTS AND DISCUSSION

The expression of BDV ORFx1 as p10

The genomic organization of BDV is given in Fig. 1. To express ORFx1, its cDNA was amplified from total RNA of the BDV persistently infected Madin-Darby canine kidney (MDCK/BV) cells by reverse transcription-polymerase chain reaction (RT-PCR) and cloned in-frame to the nucleotide sequence of glutathione-*S*-transferase (GST) in the pGEX 4T-3 vector. The GST-fusion protein with an apparent molecular mass of 39 kDa was purified from the bacterial lysate by use of a glutathione affinity column. Western blot analyses revealed that this purified 39-kDa protein reacted with the anti-GST antibody and with the BDV-infected rabbit serum (Fig. 2A). The anti-GST antibody detected two protein bands: the 39-kDa GST-fusion protein and a 29-kDa GST protein, suggesting that ORFx1 encodes for a protein with an apparent molecular mass of approximately 10 kDa (p10). The BDV-infected rabbit serum detected only the 39-kDa GST-p10 fusion protein band and not the 29-kDa GST protein band. Noninfected rabbit serum was not reactive (Fig. 2A). The fusion protein also reacted with the BDV-infected rat serum but not with the noninfected rat serum (data not shown). The rat sera did not react to GST alone. The presence of naturally occurring antibodies to p10 provided evidence for *in vivo* expression of this viral gene.

To study the expression of ORFx1 in BDV-infected cells, the GST-p10 fusion protein purified by glutathione affinity chromatography was used to immunize rabbits to raise specific antibodies to p10. The rabbit anti-p10 serum was reactive to GST and the GST-p10 fusion protein (data not shown). This anti-p10 rabbit serum was then used to examine the BDV-infected rat glial tumor cells (C6BV) and the noninfected C6 cells by Western blotting. A protein band with an apparent molecular mass of 10 kDa (Fig. 2B) was detected in the cell lysate of the C6BV cells stained with anti-p10 serum but not in the cell lysate of C6. The preimmune serum from the rabbit did not detect the 10-kDa protein band in the cell lysate of the C6BV cells. The preimmune serum also did not react



B

MGSDLRLTLLELVRRLNGNATIESGRLPGGRRRSPDTTTGTIGVTKTTEDPKECIDP
TSRPAPEGPQEEPLHDLRPRPANRKGAAIEDYKDDDDK

FIG. 1. BDV ORFx1 and its codon sequence. (A) The 3′ terminal of ORFx1 overlaps with the 5′ end of ORF-2 in the BDV genome (adapted from Cubitt *et al.,* 1994). The pcORFx1-FLAG plasmid was constructed by use of RT-PCR with primer 1 (5′-CGGGAATTCACCATGGGTTCCGACCTCCGG-3′) containing an *Eco*RI site (underlined) and primer 2 (5′-TGCCTCGAGTCA**CTTGTCATCGTCGTCCTT**GTAGTATTCGATAGCTGCTCCC-3′) containing an *Xho*I site (underlined) and the sequence (bold) encoding the FLAG epitope in-frame to the 3′ end of ORFx1. Total RNA from MDCK/BV cells was used as template. The amplified cDNA fragment was digested with *Eco*RI and *Xho*I and cloned into pcDNA3. Sequencing to determine that there was no PCR and insertion error was performed by the method of Sanger *et al.* (1977). Computer analysis of the nucleotides to give the codon sequence of the ORFx1-FLAG (B) was performed by use of the PCgene software (Intellegenetic Suite, CA). There are four serine phosphorylation sites and one threonine phosphorylation site. The FLAG codons are underlined. The nucleotide sequence of pcORFx1-FLAG has been submitted to the GenBank (accession number 030353).

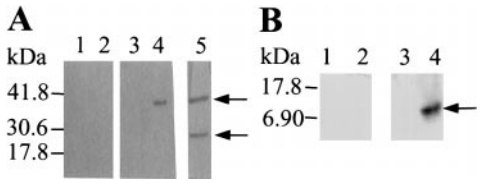


FIG. 2. Expression of ORFx1 as protein. Total RNA (10 μg) from MDCK/BV cells was used for RT-PCR (primer 1: 5′-TGTGAATTCAATGAGTTCCGACCTCCGG-3′; primer 2: 5′-TGCCTCGAGTCATTCGATAGCTGCTCCC-3′; *Eco*RI and *Xho*I enzyme sites are underlined) to isolate the ORFx1 DNA fragment. The amplified product was digested with *Eco*RI and *Xho*I and cloned in-frame to, and immediately downstream of, the GST sequence in the pGEX 4T-3 vector to give the new construct pGEX-ORFx1. The pGEX-ORFx1 and pGEX 4T-3 plasmids were used to prepare purified GST-p10 and GST proteins, respectively. The proteins were purified from the soluble fractions of each bacterial lysate by affinity chromatography with a glutathione Sepharose 4B column. (A) The purified proteins (100 ng) were analyzed by Western blotting with noninfected and BDV-infected rabbit sera. Anti-GST antibodies were used as controls. Serum from the infected rabbit (1:200 dilution) reacted with the GST-p10 fusion protein as indicated by the top arrow (lane 4) but not with the GST protein (lane 3). Serum from the noninfected rabbit (1/200 dilution) did not react to both proteins (lanes 1 and 2). In contrast, the positive control, anti-GST antibodies (1:10 dilution), reacted with both the GST (indicated by the bottom arrow) and GST-p10 (indicated by the top arrow) proteins (lane 5). The purified GST-p10 fusion protein was used to immunize a rabbit. (B) The p10-specific serum was used in Western blotting to analyze cell lysate from C6 (lane 3) and C6BV cells (lane 4). The arrow indicates the p10 protein detected by the p10-specific serum in lane 4. C6 and C6BV cell lysate did not react to a prebleed serum from the same rabbit before injection with the purified GST-p10 protein (lanes 1 and 2). Protein A cross-linked to alkaline phosphatase reactive to BCIP/NBT was used to detect the antibody–antigen interactions.

with the cell lysate of C6 cells. This finding that BDV ORFx1 is expressed as p10 in BDV-infected cells is consistent with that previously reported by Wehner *et al.* (1997). Our results also showed that our rabbit anti-p10 serum detected only p10 in C6BV cells.

Nuclear localization of p10 in BDV-infected cells

To study the subcellular localization of p10, the anti-p10 rabbit serum was then used to examine the BDV-infected C6BV cells and the noninfected C6 cells by indirect immunofluorescence (IFA). Figure 3A shows that the anti-p10 staining was localized in the cytoplasm and the nucleus of the C6BV cells. The C6 cells were not stained by IFA (Fig. 3B). Both C6BV and the C6 cells were not stained by the preimmune sera (Figs. 3C and 3D).

In vivo interaction of p10–p40

In a previous study, Schwemmle *et al.,* 1998 used a two-hybrid system to show interaction between p10 and BDV p24. They also showed by cotransfection experiments that p10 is localized in the nucleus in the presence of BDV p24 P. However, Wehner *et al.* (1997) reported the colocalization of p40 N and p10 in BDV-infected cells, and Schwemmle *et al.* (1998) reported that p10, p40 N, and p24 P could be coprecipitated from the BDV-infected cells. Although the interaction of p24 with p40 N may explain the coprecipitation of the three proteins, the possibility that p10 and p40 can interact has not formally been excluded (Schwemmle *et al.,* 1998). To confirm BDV

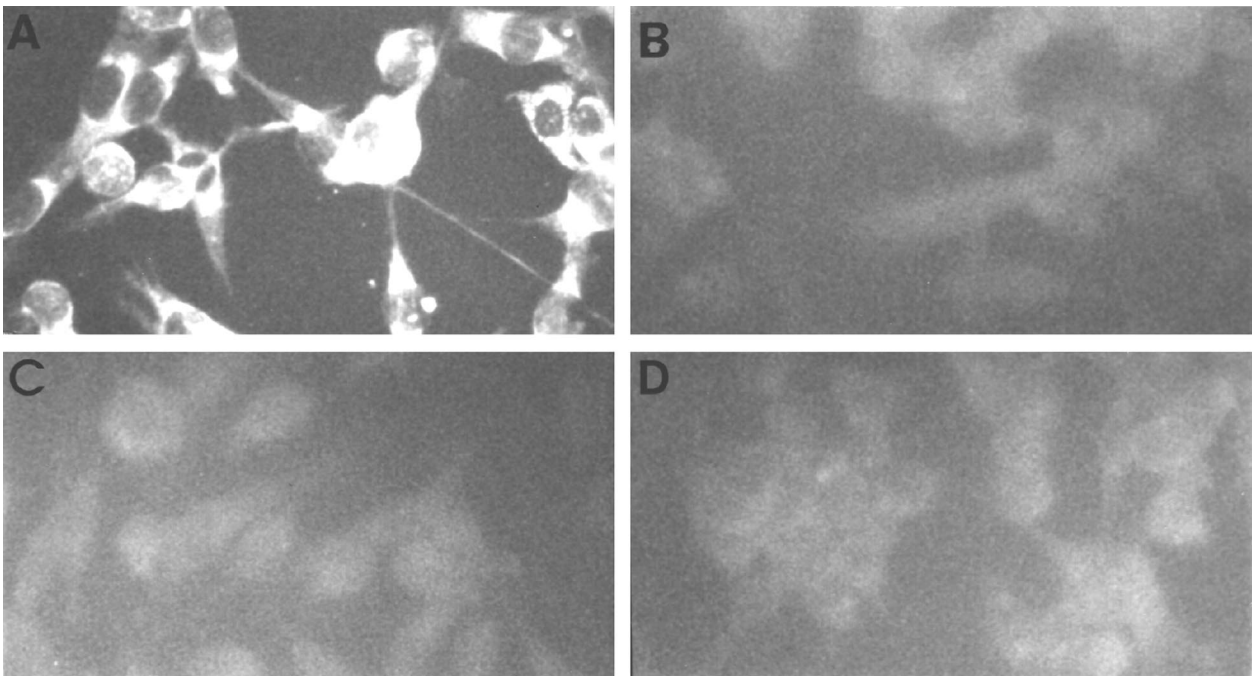


FIG. 3. Subcellular localization of p10 in BDV-infected cells. The p10-specific antiserum was used to stain C6BV (A) and C6 (B) cells by IFA. C6BV (C) and C6 (D) cells were also stained with prebleed serum as control. The FITC-conjugated protein A was used as second layer antibody. The stained cells were examined using an epifluorescence microscope (magnification, 260×).

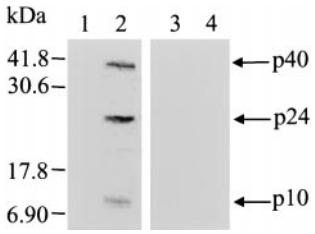


FIG. 4. Immunoprecipitation of BDV proteins from BDV-infected cells. C6 and C6BV cells (2.5×10^5 cells/35-mm-diameter dish) were maintained overnight at 37°C in an atmosphere of 5% CO_2 as described in Materials and Methods. The cells were harvested by multiple freeze–thaw in lysis buffer. The soluble cell lysate from each was collected after centrifugation. Cell-free lysate from C6 (lane 1) and C6BV (lane 2) cells was immunoprecipitated with the anti-p10 rabbit serum (1:20 final dilution) and analyzed by Western blotting with a serum from an infected rabbit with antibodies to p40/38, p24, and p10. As control, cell-free lysate from C6 (lane 3) and C6BV (lane 4) cells was also immunoprecipitated with a prebleed serum from the same rabbit before injection with the purified GST-p10 protein (1:20 final dilution) and analyzed by Western blotting with a serum from an infected rabbit with antibodies to p40/38, p24, and p10. Protein A cross-linked to alkaline phosphatase reactive to BCIP/NBT was used to detect the antibody–antigen interactions.

protein interaction with p10 in the BDV-infected cells, soluble cell lysate from C6BV cells was immunoprecipitated with the anti-p10 serum. The precipitate was recovered by protein G beads. Analyses of the immune precipitate with a BDV-infected rabbit serum showed that three proteins, p10, p40, and p24, were coprecipitated by the anti-p10 serum (Fig. 4, lane 2). Preimmune serum did not immunoprecipitate proteins from the soluble cell lysate of C6BV cells (Fig. 4, lane 4).

To determine whether p10 interacts directly with p40, the eukaryotic expression vectors pcORFx1-FLAG and pDL-N.WILD were cotransfected into Cos-7 cells. The Cos-7 cells were used because they allow plasmid replication of the pcORFx1-FLAG, which contains the SV40 origin of replication, and thereby increased expression of p10. Soluble cell lysate from the cotransfected Cos-7 cells was immunoprecipitated with the anti-p10 serum. The precipitate was recovered by protein G beads. Analyses of the immune precipitate with a BDV-infected rabbit serum showed that p10 and p40 were coprecipitated by the anti-p10 serum (Fig. 5, lane 2). Preimmune serum did not immunoprecipitate p10 and p40 from the soluble cell lysate of cotransfected Cos-7 cells (Fig. 5, lane 4). The coprecipitation of p40 with p10 by the anti-p10 serum is highly suggestive that p10 may interact with p40.

The coprecipitation of the NLS-carrying p40 N with p10 suggested that this protein might play a role in the nuclear localization of p10. To study this aspect, Cos-7 cells were cotransfected with the pcORFx1-FLAG and the pDL-N.WILD plasmids, and cells singularly transfected or not transfected served as controls. The pcORFx1-FLAG and pDL-N.WILD plasmids both contain an SV40 origin of replication, and thus their expression is amplified in the SV40 T-antigen expressing Cos-7 cells. Figure 6A shows cytoplasmic staining of Cos-7 cells transfected with the pcORFx1-FLAG plasmid alone and examined with the anti-p10 serum. Cos-7 cells cotransfected with the pcORFx1-FLAG and the pDL-N.WILD plasmid and stained by IFA with a rabbit anti-p10 (Fig. 6D) or a mouse anti-p40 antiserum (Fig. 6C) gave nuclear staining, suggesting that both p10 and p40 were present in the nucleus. Two-tone IFA by use of the p10- and p40-specific sera revealed the two viral proteins colocalized in the nucleus of the cotransfected cells (Fig. 6E). As controls, Cos-7 cells transfected with the p40-expressing pDL-N.WILD plasmid alone gave nuclear staining (Fig. 6B). No staining was observed with the nontransfected Cos-7 cells (Figs. 6F and 6G). Although p10 is a small protein, we (Fig. 6A) and others (Schwemmle *et al.,* 1998) have not observed p10 to simply diffuse into the nucleus of singularly transfected cells, and hence our results are highly suggestive that p40 N plays a role in importing p10 into the cell nucleus.

In vitro confirmation of p10–p40 interaction

To confirm that p10 can directly interact with p40, we performed *in vitro* protein–protein interaction studies with the use of recombinant p10 and p40. The pcORFx1-FLAG vector was *in vitro* transcribed by the T7 polymerase and translated to give the ^{35}S-labeled p10-FLAG, which could be immunoprecipitated with the anti-FLAG monoclonal antibody (Fig. 7). The GST-p40 fusion protein from pGEX-N.WILD-transformed bacteria was cross-linked to glutathione 4B beads. The labeled p10-FLAG protein was then allowed to interact with the GST-p40

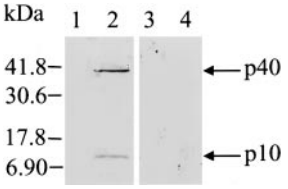


FIG. 5. Coprecipitation of p10 and p40 from cell lysate of cells cotransfected with BDV p10 and p40 N. The Cos-7 cells were transfected with the pcORFx1-FLAG and the pDL-N.WILD vectors as described in Materials and Methods. Mock-transfected Cos-7 cells were used as controls. The cells were harvested by multiple freeze–thaw in lysis buffer. The soluble cell lysate from each was collected after centrifugation. Cell-free lysate from the Cos-7 (lane 1) and the Cos-7 cells transfected with the pcORFx1-FLAG and the pDL-N.WILD vectors (lane 2) was immunoprecipitated with the anti-p10 rabbit serum (1:20 final dilution) and analyzed by Western blotting with serum from a BDV-infected rabbit. As control, cell-free lysate from Cos-7 (lane 3) and the Cos-7 cells transfected with the pcORFx1-FLAG and the pDL-N. WILD vectors (lane 4) was also immunoprecipitated with a prebleed serum from the same rabbit before injection with the purified GST-p10 protein (1:20 final dilution) and analyzed by Western blotting with a serum from a BDV-infected rabbit. Protein A cross-linked to alkaline phosphatase reactive to BCIP/NBT was used to detect the antibody–antigen interactions.

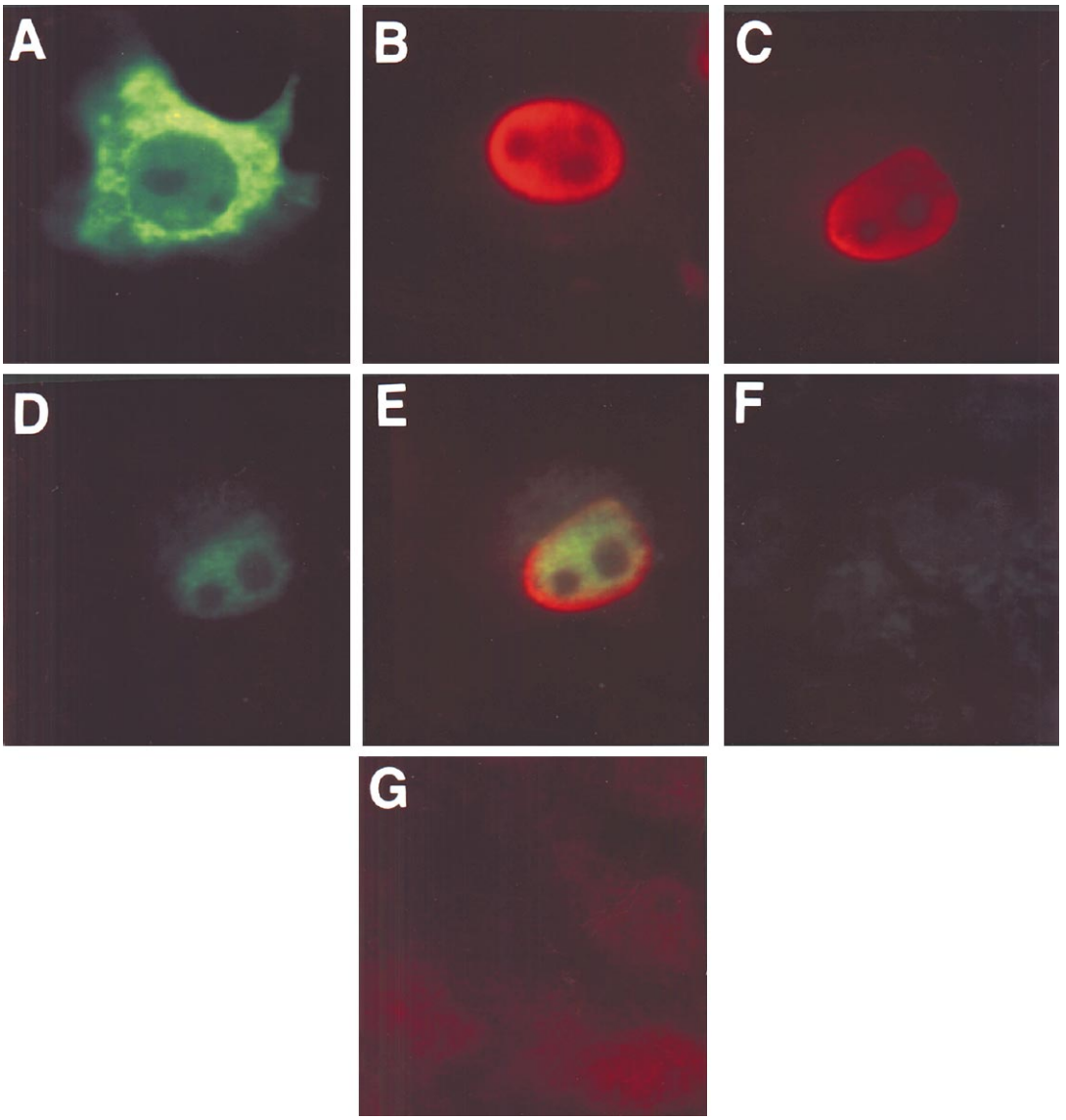


FIG. 6. Subcellular localization of p10 in cells cotransfected with BDV p40 N. The Cos-7 cells were transfected with the pcORFx1-FLAG vector alone (A), the pDL-N.WILD vector alone (B), or with both constructs (C–E), as described in Materials and Methods. Mock-transfected Cos-7 cells (F and G) were used as controls. Test and controls were stained with the rabbit anti-p10 serum (A, D, and F), the mouse anti-p40 serum (B, C, and G), or both antisera (E). An FITC-conjugated donkey anti-rabbit serum was used to detected staining by the anti-p10 antibodies, and a Cy3-conjugated donkey anti-mouse serum was used to detect the anti-p40 staining by confocal microscopy by use of argon illumination (A, D, and F), krypton illumination (B, C, and G), or both (E). Magnification of A–G is 100×. Cotransfected cells not examined with the anti-p10 and anti-p40 sera, but stained with the conjugated donkey antisera, did not give any staining (data not shown).

bound to solid phase. Analyses of the bound proteins showed that the labeled p10-FLAG protein had bound to the GST-p40 protein (Figs. 8A and 8B). Thus the *in vitro* studies showed that p10 could directly interact with BDV p40 N.

Recent studies (Wehner *et al.,* 1997, Schwemmle *et al.,* 1998) have shown that BDV ORFx1 is expressed as p10 in BDV-infected cells. Here, we show that in the sera of BDV-infected animals, naturally occurring antibodies to p10 encoded by the BDV ORFx1 are present. This further supports the hypothesis that ORFx1 is indeed expressed *in vivo*. Consistent with earlier reports (Wehner *et al.,* 1997, Schwemmle *et al.,* 1998), we also demonstrated that the ORFx1 is expressed as p10 in BDV-infected cells and can be found in the nucleus. The p10 could be coprecipitated with p40 N and p24 P from the soluble lysate of the BDV-infected cells. Schwemmle *et al.* (1998) reported the interaction of p10 with p24, which carries a putative NLS, and suggested that such an interaction may be sufficient to import p10 into the nucleus. The p40 N also has an NLS (Pyper and Gartner, 1997; Kobayashi *et al.,* 1998). Previously, Wehner *et al.* (1997) showed that p10 is colocalized with p40 in the nucleus of BDV-infected cells. Hence p40 N also may play a role in the

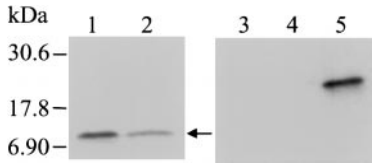


FIG. 7. Fluorograph of *in vitro* transcribed and translated p10-FLAG. The pcORFx1-FLAG was *in vitro* transcribed and translated by use of the TNT-coupled rabbit reticulocyte lysate system and analyzed by SDS–PAGE. Arrow denotes the ^{35}S-labeled p10-FLAG (lane 1), which could be immunoprecipitated by the anti-FLAG monoclonal antibody (1μg) (lane 2). *In vitro* transcribed and translated pcDNA3 lysate either by itself or immunoprecipitated with anti-FLAG monoclonal antibody served as negative control (lanes 3 and 4). The *in vitro* transcribed and translated CAT gene from pc-CAT served as positive control (lane 5).

importation of p10 into the nucleus. In this study, we further extended the observations of Wehner *et al.* (1997) by showing p10 and p40 N colocalization in the nucleus after cotransfection of Cos-7 cells with the p10 and p40 expression constructs. The p10 was observed in the cytoplasm of cells transfected with the p10-expressing plasmid alone, but became imported to the nucleus on cotransfection with p40 N. Previous studies by use of IFA have shown that the nuclear staining patterns of BDV p40 N (Wehner *et al.,* 1997; Kobayashi *et al.,* 1998) and p10 (Wehner *et al.,* 1997, Schwemmle *et al.,* 1998) in BDV-infected cells are spot-like with focal appearance. In contrast, in cells transfected with N alone, the nuclear staining of p40 appeared as an intensive smear (Kobayashi *et al.,* 1998). In this study, the nuclear staining of p10 in BDV-infected cells and in p10 + p40 cotransfected cells closely resembled the nuclear staining patterns previously reported for p40 (Kobayashi *et al.,* 1998) (i.e., focal spotty staining in BDV-infected cells and diffuse smear staining in the cotransfected cells). Thus the colocalization of p10 and p40 is not limited to BDV-infected cells but also includes p10 + p40 cotransfected cells. The reason for the focal nuclear staining in infected cells in contrast to the smear staining in the transfected cells is not clear. However, the focal staining in the infected cells might represent staining of the BDV protein in the ribonucleoprotein complex as previously proposed (Kobayashi *et al.,* 1998).

The subcellular colocalization of BDV p10 and p40 in the BDV-infected cells and in the cotransfected cells is highly suggestive that these two viral proteins may interact. This was confirmed by the coprecipitation of the p10 and p40 proteins in the cotransfected cells by use of the anti-p10 serum. This coprecipitation of the two transfected proteins, however, does not rule out the possibility that p10 may interact with a yet-to-be-determined cellular protein, which in turn interacts with p40. The *in vitro* protein–protein binding studies by use of affinity solid phase left no doubt that p10 and p40 can directly interact. In an earlier study by use of a two-hybrid system and an *in vitro* protein binding assay, Schwemmle *et al.* (1998) reported the interaction of p10 with p24 but failed to observe p10–p40 interaction. The reason why they did not detect p10–p40 interaction as seen in this study is unclear. The differences in the experimental approaches and protocols between the two studies may have contributed to the experimental outcome.

Studies on the replication of NNS RNA viruses have shown that the minimal replicative and infectious unit is the ribonucleoprotein containing the genomic RNA tightly associated with the N, P, and L proteins (Schnell *et al.,* 1994; Lawson *et al.,* 1995; Radecke *et al.,* 1995). Although BDV may be a member of a new family of mammalian *Mononegalovirales* (Cubitt *et al.,* 1994a; de la Torre, 1994; Schneider *et al.,* 1995; Schneemann *et al.,* 1995) and replicates in the nucleus (Briese *et al.,* 1992; Cubitt and de la Torre, 1994), being an NNS RNA virus, its N, P, and L proteins associated with the RNA genome may still represent its minimal replicative and infectious unit. Our observation that p10 is found in the nucleus and is closely associated with p40 N suggests that it also may play an important role in the replication and/or transcription of BDV and deserves additional study.

MATERIALS AND METHODS

Cell lines

MDCK/BV were kindly provided by Dr. R. Rott (Justus-Leibig-Universitat Giessen, Germany). Rat glial tumor cells not infected (C6) and persistently infected with BDV (C6BV) were kindly provided by Dr. J. C. de la Torre (The Scripps Research Institute, La Jolla, CA). Cos-7 cells were purchased from the American Type Culture Collec-

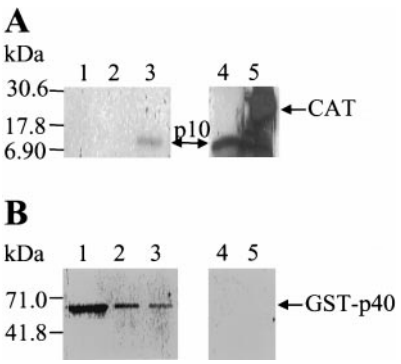


FIG. 8. Protein–protein interaction of p10 with p40 *in vitro*. Interaction of the p10-FLAG protein with the GST-p40 fusion protein bound to the solid phase was performed as described in Materials and Methods. The bound protein was resolved by SDS–PAGE, electrotransferred onto nitrocellulose membrane, Western blotted (B) with p40-specific antisera to detect the p40 protein, and fluorographed (A) to detect the ^{35}S-labeled p10-FLAG protein. Lane 1 is solid phase-bound GST-p40 alone. GST-p40 reaction with *in vitro* transcribed and translated pc-CAT lysate served as negative control (lane 2). The GST-p40 protein interacting with the p10-FLAG protein is given in lane 3. Lanes 4 and 5 are the *in vitro* transcribed and translated lysates from pcORFx1-FLAG and pc-CAT, respectively.

tion (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD).

Construction of expression vectors

Total RNA was isolated from MDCK/BV cells. By use of specific primers and RT-PCR, the ORFx1 cDNA was generated. The cDNA was digested with *Eco*RI and *Xho*I and cloned in-frame to the GST sequence, in the pGEX 4T-3 vector (Pharmacia, Piscataway, NJ), to give the pGEX-ORFx1 prokaryotic expression vector. Likewise, the cDNA of p40 N was generated by RT-PCR with specific primer pairs. Cloning of the amplified p40 cDNA fragment into the *Bam*HI site of the pGEX-5X-3 vector (Pharmacia) gave the pGEX-N.WILD construct. To produce the eukaryotic expression vector, pcORFx1-FLAG (Fig. 1), the ORFx1 cDNA was generated by use of RT-PCR with ORFx1-specific primers. One of the primers contained a sequence encoding the FLAG epitope tag. The ORFx1-FLAG fragment (GenBank accession number 030353) was cloned into the *Eco*RI–*Xho*I sites of the pcDNA3 vector (InVitrogen, San Diego, CA). The construction of the eukaryotic expression vector pDL-N.Wild for p40 N has been described previously (Kobayashi *et al.,*1998).

Antibodies

To prepare antiserum to the protein (p10) encoded by ORFx1, soluble lysate was prepared from isopropyl-1-thio-β-D-galactopyranoside-induced, pGEX-ORFx1-transformed JM109 bacteria. The GST-p10 fusion protein was purified from the soluble lysate by use of a glutathione 4B column (Pharmacia). The purified GST-p10 protein was used to immunize New Zealand White rabbits (Coast Scientific) to prepare the p10-specific antiserum. Likewise, an anti-p40 serum was prepared in mice by immunization with the GST-p40 protein purified by use of a glutathione 4B column. Sera from noninfected and BDV-infected rabbit and rat were kindly provided by Dr. K. M. Carbone (Division of Viral Products, FDA, Bethesda, MD). The anti-FLAG monoclonal antibody was from Eastman Kodak (Rochester, NY). The anti-GST antibody was from Pharmacia. The fluorescein isothiocyanate (FITC)-conjugated protein G was from Sigma Chemical (St. Louis, MO); alkali phosphatase-conjugated protein A/G were from Pierce (Rockford, IL), and the dichlorotriazinyl amino fluorescein-conjugated goat anti-rabbit IgG antibody was from Immunotech. The 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) reactive to the alkaline phosphatase-conjugated protein-A/G was purchased from Sigma. The FITC-conjugated donkey anti-rabbit IgG serum and the Cy3-conjugated donkey anti-mouse IgG serum were from Jackson ImmunoResearch Lab (West Grove, PA).

Eukaryotic expression

Cells were seeded at a concentration of 2.5×10^5 cells/35-mm-diameter tissue culture dish or 5×10^4 cells/microscope glass slide. After overnight culture at 37°C, the cells were transfected with 2 μg of plasmid DNA by use of Lipofectamine (Life Technologies). Cells were fixed 24–48 h post-transfection with 4% paraformaldehyde and stained by IFA with the appropriate antibodies. Cells not transfected or mock-transfected but treated the same way were used as controls. Immunofluorescence was detected by use of an epifluorescence microscope (Nikon) or a confocal laser scanning microscope (Meridian Instrument Inc.) by use of a krypton and/or an argon lamp.

Immunoprecipitation

Transfected or persistently infected cells were lysed by freeze–thaw in a buffer containing 10 mM Tris (pH 8.0), 2 mM EDTA, 0.5% Nonidet P-40, and 1 mM PMSF. After centrifugation, the soluble fraction was reacted with anti-p10 serum for 2 h at 4°C, and the precipitate was then recovered by incubation with protein G beads for 24 h at 4°C. After thorough washing, proteins bound to the beads were resolved by SDS–PAGE (15%) and analyzed by Western blotting with the appropriate antibodies.

Protein–protein interaction

In vitro transcription/translation of pcORFx1-FLAG was performed by use of the TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI). The *in vitro* products were resolved by denaturing 15% SDS–PAGE and analyzed after fluorography, or immunoprecipitated by use of the anti-FLAG monoclonal antibody before SDS–PAGE and fluorography. The GST-p40 fusion protein was purified by glutathione column chromatography of lysate from the pGEX- N.WILD-transformed bacteria. The purified protein was cross-linked to glutathione 4B beads (Pharmacia) to provide a solid phase. For protein–protein interaction, 1–2 μg of GST-p40 was then mixed with the ^{35}S-labeled *in vitro* transcribed/translated p10-FLAG protein. The mixture was incubated for 24 h at 4°C. Incubation of 1–2 μg of GST with the ^{35}S-labeled *in vitro* transcribed/translated p10-FLAG protein served as control. After thorough washing, proteins bound to the beads were resolved by SDS–PAGE (15%) and analyzed by Western blotting with the appropriate antibodies. In addition, the blot was subjected to autoradiography to detect any bound ^{35}S-labeled proteins.

Data processing

Images of gels and autoradiograms were scanned into an IBM-compatible PC and digitally generated for publication by use of Adobe Photoshop 4.0 and Microsoft Power-point 97.

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