JOURNAL OF VIROLOGY, Jan. 1996, p. 635–640 Vol. 70, No. 1
0022-538X/96/$04.00+0

Sequence Variability of Borna Disease Virus Open Reading Frame II Found in Human Peripheral Blood Mononuclear Cells

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Received 17 August 1995/Accepted 11 October 1995

A cDNA fragment of the Borna disease virus (BDV) open reading frame II (ORF-II), which encodes a 24-kDa phosphoprotein (p24 [P protein]), was amplified from total RNA of peripheral blood mononuclear cells (PBMC) from three psychiatric inpatients. The amplified cDNA fragments were cloned, sequenced, and analyzed. A total of 15 clones, 5 from each patient, were studied. Intrapatient divergencies of the BDV ORF-II nucleotide sequence were 4.2 to 7.3%, 4.8 to 7.3%, and 2.8 to 7.1% for the three patients, leading to differences of 7.7 to 14.5%, 10.3 to 17.1%, and 6.0 to 16.2%, respectively, in the deduced amino acid sequence for BDV p24. Interpatient divergencies among the 15 clones were 5.9 to 12.7% at the nucleotide level and 12.8 to 28.2% at the amino acid level. Thus, in p24, BDV in human PBMC of the patients undergoes mutation at high rates in vivo. Additionally, we found that the nucleotide sequence of the 15 human BDV ORF-II cDNA clones differed from those of the horse strains V and He/80-1 by 4.2 to 9.3%. However, comparison of the consensus amino acid sequence deduced from the 15 human clones with those of the horse strains revealed no human-specific amino acid residue, suggesting that the BDV infecting humans may be related to that infecting horses.

Borna disease virus (BDV), which is a causative or related agent of progressive encephalopathy (Borna disease) found in members of the family *Equidae* and sheep, is a not yet classified, enveloped, negative, nonsegmented, single-stranded (NNS), RNA virus (reviewed in reference 14). This virus has not been well characterized, since cell-free virus cannot be detected in infected animals, although brain homogenates are infectious. Additionally, it has been proposed that BDV ribonucleoprotein complexes may play a role in spreading the viral infection in vivo (12). By use of this infection system, it has been shown that BDV, differing from other NNS-RNA animal viruses, replicates and transcribes in the nuclei of infected cells (9, 11, 12, 15). Recent molecular cloning studies have shown that the genetic organization of BDV is similar to those of other NNS-RNA viruses (14), such as rhabdoviruses and particularly vesicular stomatitis virus (3). The BDV RNA genome contains at least five open reading frames (ORFs) (10, 13). To date, three viral proteins, i.e., 40 (p40)-, 24 (p24)-, and 18 (gp18)-kDa proteins, have been mapped to ORF-I, -II, and -III, respectively. The BDV p24 phosphoprotein (P protein) has a high Ser-Thr content with phosphorylation in the serine residues (14, 33). Similarly to the phosphoprotein transcription activator found in the infectious ribonucleoprotein complexes of NNS-RNA viruses (2, 3, 34), the N terminus of the BDV P protein contains a cluster of acidic amino acids.

Whereas Borna disease occurs naturally in horses and sheep, it can be experimentally transmitted to both avian and mammalian species (reviewed in reference 27). Animals, including rodents and nonhuman primates, give variable clinical and pathological manifestations after experimental infection with BDV (20, 21, 23, 26; for a review, see reference 27). By use of reverse transcription-PCR (RT-PCR), the nucleic acid sequence of BDV p40 can be detected in peripheral blood mononuclear cells (PBMC), bone marrow cells, and thymus stromal cells of experimentally infected rats (29, 31), suggesting that the virus is not only neurotropic but hematotropic as well. Accumulated seroepidemiologic data also suggest that BDV or a related virus may infect humans, in whom it has been associated with certain mental disorders (1, 5–7, 17, 25, 28, 35, 36). By use of nested RT-PCR and primers specific to BDV p24 to detect BDV in the PBMC of healthy horses, it has been shown that BDV infection may be more widespread than previously thought and in subclinical form (22). As many as 29.8% of the healthy horses tested carried BDV p24. Recent demonstration of the genetic footprint of BDV p24 (8, 19) and p40 (8) in the PBMC of psychiatric patients and of healthy blood donors (unpublished observation) by nested RT-PCR provides further impetus for studies to determine the origin of BDV infection in humans.

In this study, we used BDV p24-specific nested RT-PCR to obtain cDNA clones from the PBMC of three psychiatric patients. Sequence analyses of the cDNA clones suggested that the BDV in humans might have originated from those infecting horses.

Cloning of cDNA fragments corresponding to BDV ORF-II from the peripheral blood of psychiatric patients. Oligonucleotide primers specific to the published sequence (30) for ORF-II of He/80-1, a horse-derived BDV isolate, were used in nested RT-PCR throughout this study. Previous studies had used these BDV-specific primers successfully to detect the genetic footprint of BDV in the PBMC of horses (22) and humans (19). These specific primers were 5′-TGACCCAAC CAGTAGACCA-3′ at nucleotides (nt) 1387 to 1405 and 5′-GTCCCATTCATCCGTTGTC-3′ at nt 1865 to 1847 for RT-

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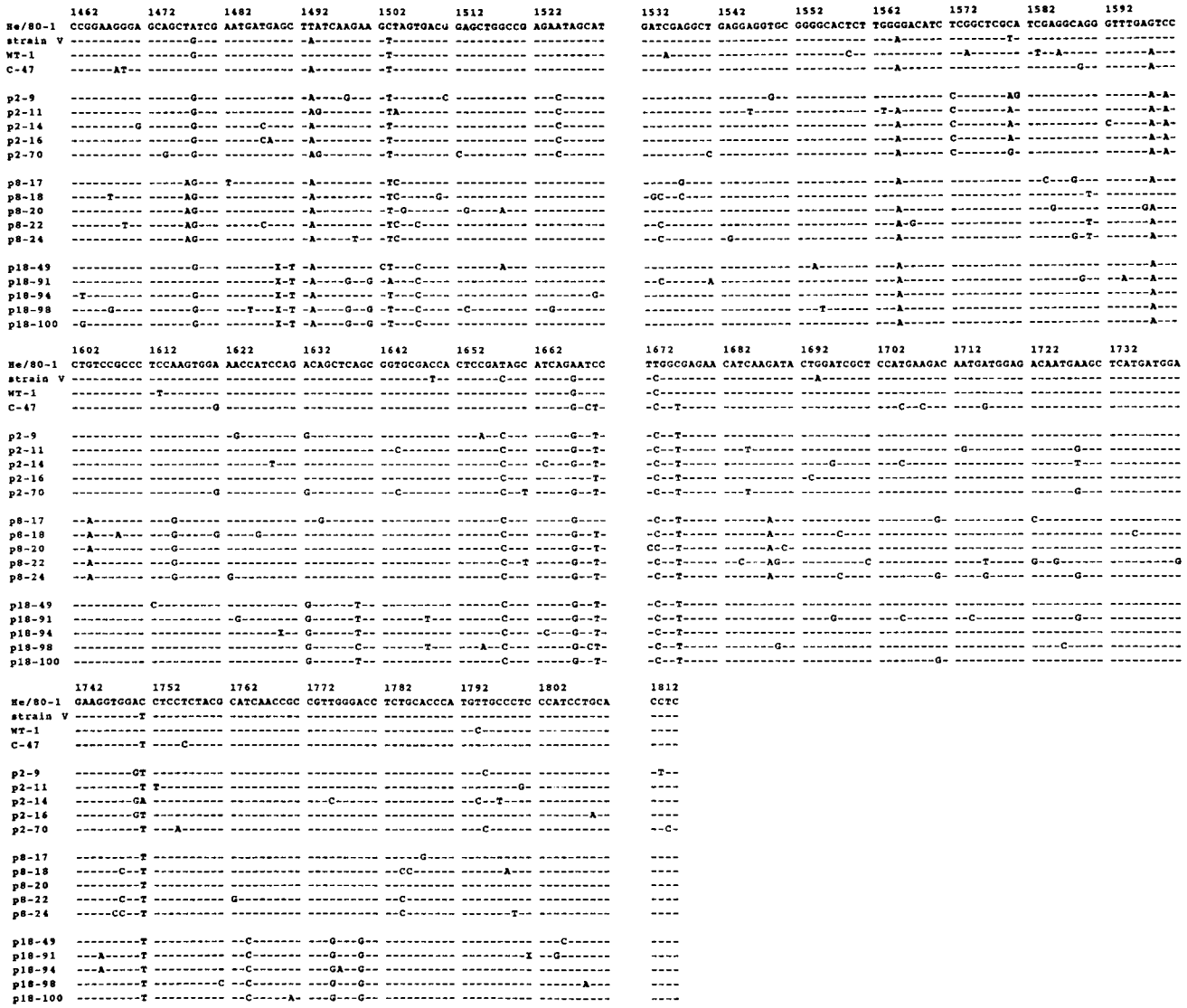

FIG. 1. Nucleotide sequences of BDV ORF-II cDNA clones isolated from the PBMC of three psychiatric patients. cDNA fragments (354 bases long) of the BDV p24 region, amplified from total RNA extracted from the PBMC of three psychiatric patients by nested RT-PCR, were cloned and sequenced. The sequences presented are in antigenomic polarity (positive sense). Clones p2-9, -11, -14, -16, -17; p8-17, -18, -20, -22, -24; and p18-49, -91, -94, -98, -100 were derived from patients 2, 8, and 18, respectively. Nucleotide numbering is according to that previously reported for a horse-derived BDV infecting the C6BV cell line (13). Sequences for the horse-derived He/80-1, WT-1, and V strains had previously been reported (30). C-47 was a BDV ORF-II cDNA clone derived from MDCK/BDV cells (18). X, deletion; bars, nucleotides identical to those of He/80-1.

PCR and 5′-TCAGACCCAGACCAGCGAA-3′ at nt 1443 to 1461 and 5′-AGCTGGGGATAAATGCGCG-3′ at nt 1834 to 1816 for nested-primer PCR. For nested RT-PCR, 1 μg of total RNA was reverse transcribed and amplified by two-step PCR in a Thermal cycler as previously described (19). A 354-bp cDNA fragment (nt 1462 to 1815), corresponding to a region in ORF-II (nt 1272 to 1977) which codes for p24 of BDV, was amplified from total RNA of PBMC from three institutionalized male patients diagnosed to have schizophrenia. Patients 2 (61 years old), 8 (63 years old), and 18 (41 years old) had been hospitalized for 21, 23, and 22 years, respectively. As previously reported (19), patients 8 and 18 were seropositive for antibodies to BDV p24 as detected by Western blotting (immunoblotting), whereas patient 2 was seronegative. Total RNA from Madin-Darby canine kidney (MDCK) cells uninfected and persistently infected with BDV (MDCK/BDV) (18) were used as negative and positive controls, respectively. An additional negative control consisted of total RNA from the PBMC of a healthy individual seronegative for BDV. In keeping with a previous report (19), total RNA from uninfected MDCK cells did not give any BDV-specific product in nested RT-PCR. Likewise, no BDV-specific product was amplified from total RNA of PBMC from the seronegative healthy control. Each of the cDNA fragments amplified from the patients was cloned into the pCR™II plasmid vector (Invitrogen Corporation, San Diego, Calif.). Five cDNA clones were derived from each patient, i.e., p2-9, -11, -14, -16, and -70 from patient 2; p8-17, -18, -20, -22, and -24 from patient 8; and p18-49, -91, -94, -98, and -100 from patient 18. Clone C-47 was obtained from the control MDCK/BDV cells. Each clone was sequenced by use of the AmpliTaq Cycle sequencing kit (The Perkin-Elmer Corporation, Foster City, Calif.) in a 373A DNA

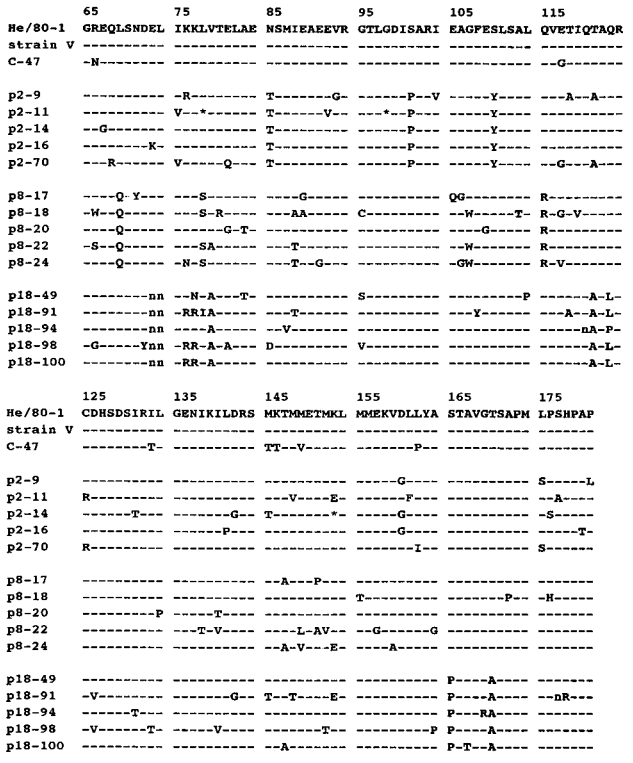

FIG. 2. Deduced amino acid sequences of the isolated BDV p24 cDNA clones. By using the Geneworks 2.2 (Intelligenetics Inc.) computer program, the amino acid residues were deduced from the nucleotide sequences of the 15 BDV ORF-II cDNA clones shown in Fig. 1. Residue numbering is according to that previously reported for a horse-derived BDV infecting the C6BV cell line (13). The amino acid sequences for the horse-derived He/80-1, WT-1, and V strains had previously been reported (30). C-47 was a BDV ORF-II cDNA clone derived from MDCK/BDV cells (18). Bars, residues identical to those of He/80-1; n, amino acid residue which is not determined because of the presence of nucleotide deletion within a corresponding codon; asterisk, a stop codon caused by nucleotide substitution. Amino acid sequence following a stop codon or frameshift is supplied as if this change did not occur to illustrate sequence alignment.

Sequencer. The nucleotide numbering used in this report follows that previously reported for a horse-derived BDV infecting the C6BV cell line (13).

Composition of the BDV ORF-II cDNA clones isolated from the PBMC of psychiatric patients. Nucleotide sequences for each of the 15 ORF-II cDNA clones from the patients are given in Fig. 1. There was no significant homology with other mammalian cDNA sequences in the nucleic acid (GenBank) data bank. In contrast, nucleotide sequences of the ORF-II cDNA clones from patients 2, 8, and 18 varied on average by 7.0% (range, 5.4 to 7.9%), 7.7% (range, 6.2 to 9.3%), and 7.9% (range, 6.8 to 9.3%), respectively, compared with that previously reported for the horse-derived He/80-1 (30) and by 5.4% (range, 4.2 to 6.2%), 6.4% (range, 4.8 to 7.9%), and 6.6% (range, 5.4 to 8.8%), respectively, compared with that of strain V (30), which is another BDV strain that is derived from horses. Figure 1 shows that some of the nucleotide mutations found in the ORF-II cDNA clones were specific to the patients. For example, a C at nt 1525 and 1572 and an A at nt 1600 were found in all clones derived from patient 2, but not in any of the clones derived from other patients or in horse-derived BDV. Likewise, an A at nt 1477, 1604, and 1688 and a G at nt 1615 were specific to patient 8, whereas a single base deletion at nt 1489, a G at nt 1775 and 1779 and a C at nt 1764 were specific to patient 18.

Computer analyses revealed that the nucleotide mutations would have led to patient-specific changes in the amino acid residues of the ORF-II encoded BDV p24 detected in the patients (Fig. 2). For example, the nucleotide substitution at nt 1525 and 1572 found in all of the ORF-II cDNA clones derived from patient 2 (Fig. 1) would have replaced an N with a T at amino acid residue 85 and an S with a P at residue 101 of BDV p24, respectively (Fig. 2). Likewise, nucleotide substitution at nt 1477 and 1615 common to the p24 cDNA clones from patient 8 would have replaced an L with a Q at residue 69 and a Q with an R at residue 115, respectively. The nucleotide substitution at nt 1764 and 1779 in all clones from patient 18 would have replaced an S with a P at residue 165 and a T with an A at residue 170. In addition, the common deletion at nt 1489 caused a frameshift, converting an E to a G at residue 73, a stop codon at residue 74, and truncation of the BDV p24 in the PBMC of patient 18. Hence, all ORF-II cDNA clones from patient 18 were defective for p24.

Apart from these patient-specific mutations, 1- or 2-base substitutions and 1-base deletion (represented by X in Fig. 1) were found, resulting in codon substitution and changes in the amino acid residues at the protein level. For example, a 1-base substitution at nt 1534 (T to C) in clones p8-22 and p8-24 (Fig. 1) changed residue 88 of p24 in these clones from an I to a T (Fig. 2), whereas the 2-base substitution in clone p8-18 (AT to GC in Fig. 1) changed residue 88 to an A instead (Fig. 2). In clone p2-11, the substitution of CT by TA at nt 1504 and 1505 (Fig. 1) gave a stop codon (TAA) and consequent truncation of p24 at residue 78. Likewise, the A-to-G substitution at nt 1728 in clone p2-14 gave the TAG stop codon (Fig. 1) and premature termination of p24 at residue 153. Hence, two of the five ORF-II cDNA clones from patient 2 were defective in BDV p24.

Intrapatient and interpatient variabilities of BDV ORF-II. The nucleotide and deduced amino acid sequences of ORF-II cDNA clones isolated from the PBMC of each of the patients were compared. For this comparison, all amino acid residues in each clone were deduced (Fig. 2) without consideration of premature termination of translation created by nucleotide substitutions or deletions. Figure 3 shows that the pairwise nucleotide distances of the p24-coding ORF-II cDNA clones found in patient 2 ranged from 4.2 to 7.3% (mean, 5.5%). This gave a 7.7- to -14.5% (mean, 11.0%) divergence at the amino acid level. Comparable sequence divergence was observed among the ORF-II cDNA clones from patient 8 and from patient 18. The pairwise nucleotide distances were 6.4% (range, 4.8 to 7.3%) in clones from patient 8 and 4.9% (range, 2.8 to 7.1%) in clones from patient 18, which corresponded to 13.9% (range, 10.3 to 17.1%) and 11.2% (range, 6.0 to 16.2%) divergence at the deduced amino acid level, respectively.

Among the patients, pairwise nucleotide distances of the ORF-II cDNA clones ranged from 5.9 to 12.7%, with an average of 9.0%, leading to 12.8 to 28.2% (mean, 19.6%) differences at the amino acid level. The highest divergence was found between clone p8-18 from patient 8 and clone p18-91 from patient 18. The distance found was 12.7% at the nucleotide level and 28.2% at the amino acid level.

Consensus amino acid sequence of the p24-coding ORF-II cDNA clones found among the three patients. The consensus amino acid sequence of BDV p24 encoded by the 15 ORF-II cDNA clones isolated from the three patients was analyzed by use of the GeneWorks 2.2 (Intelligenetics, Inc., Mountain View, Calif.) computer program. An amino acid residue found to be conserved in more than 8 of the 15 clones is shown as a consensus amino acid residue in Fig. 4. This figure also shows that the region between amino acid residues 73 and 79 appeared to be highly divergent, with an average index of 4.7

	p2-					p8-					p18-				
	9	11	14	16	70	17	18	20	22	24	49	91	94	98	100
	amino acid divergency														
p2-9		17 14.5	13 11.1	10 8.5	12 10.3	20 17.1	26 22.2	19 16.2	25 21.4	23 19.7	20 17.1	25 21.4	20 17.1	25 21.4	18 15.4
p2-11	21 5.9		15 12.8	13 11.1	13 11.1	20 17.1	26 22.2	19 16.2	25 21.4	22 18.8	23 19.7	27 23.1	23 19.7	30 25.6	23 19.7
p2-14	22 6.2	22 6.2		9 7.7	15 12.8	19 16.2	24 20.5	17 14.5	24 20.5	22 18.8	21 17.9	25 21.4	19 16.2	28 23.9	21 17.9
p2-16	15 4.2	16 4.5	17 4.8		12 10.3	16 13.7	22 18.8	15 12.8	21 17.9	20 17.1	27 23.1	26 22.2	17 14.5	24 20.5	17 14.5
p2-70	21 5.9	16 4.5	26 7.3	20 5.6		20 17.1	24 20.5	18 15.4	25 21.4	23 19.7	20 17.1	29 24.8	20 17.1	26 22.2	20 17.1
p8-17	29 8.2	27 7.6	31 8.8	24 6.8	31 8.8		17 14.5	12 10.3	16 13.7	12 10.3	20 17.1	28 23.9	20 17.1	28 23.9	18 15.4
p8-18	35 9.9	34 9.6	41 11.6	32 9.0	37 10.5	25 7.1		18 15.4	20 17.1	19 16.2	25 21.4	33 28.2	26 22.2	32 27.4	26 22.2
p8-20	26 7.3	25 7.1	28 7.9	21 5.9	27 7.6	17 4.8	25 7.1		16 13.7	16 13.7	17 14.5	27 23.1	19 24.8	25 21.4	19 16.2
p8-22	37 10.5	35 9.9	37 10.5	30 8.5	37 10.5	25 7.1	26 7.3	25 7.1		16 13.7	23 19.7	29 24.8	23 19.7	26 22.2	23 19.7
p8-24	34 9.6	30 8.5	35 9.9	29 8.2	34 9.6	18 5.1	21 5.9	22 6.2	22 6.2		24 20.5	27 23.1	24 20.5	21 17.9	21 17.9
p18-49	26 7.3	27 7.6	29 8.2	23 6.5	28 7.9	27 7.6	35 9.9	22 6.2	33 9.3	32 9.0		16 13.7	9 7.7	14 12.0	7 6.0
p18-91	34 9.6	37 10.5	36 10.2	34 9.6	34 9.6	39 11.0	45 12.7	36 10.2	43 12.1	40 11.3	22 6.2		18 15.4	19 16.2	13 11.1
p18-94	27 7.6	28 7.9	28 7.9	24 6.8	29 8.2	28 7.9	36 10.2	25 7.1	34 9.6	33 9.3	11 3.1	21 5.9		17 14.5	9 7.7
p18-98	31 9.0	36 10.2	38 10.7	32 9.0	37 10.5	36 10.2	43 12.1	32 9.0	40 11.3	41 11.6	20 5.6	25 7.1	21 5.9		9 7.7
p18-100	24 6.8	27 7.6	29 8.2	23 6.5	28 7.9	24 6.8	35 9.9	24 6.8	33 9.3	30 8.5	10 2.8	18 5.1	10 2.8	16 4.5	
	nucleotide divergency														

FIG. 3. Intrapatient and interpatient variabilities of BDV p24. The nucleotide and deduced amino acid sequences of the 15 BDV ORF-II cDNA clones from the PBMC of three psychiatric patients were compared. For this comparison, all amino acid residues of each clone were deduced (shown in Fig. 2) without consideration of premature termination of translation created by nucleotide substitutions or deletions. The numbers above the diagonal line indicate divergent amino acid residues. The numbers below the diagonal line indicate divergent nucleotides. The numbers in the upper lines are the numbers of divergent amino acid residues from a total of 117 or the number of divergent nucleotides from a total of 354. The numbers in the lower lines are percent divergence values.

residues. However, comparison of the consensus sequence with the amino acid sequences of p24 of horse-derived He/80-1 (30), strain V (30), and WT-1 (30) BDV revealed no human-specific amino acid residue.

Conclusions. Recently, nested RT-PCR has been used to detect BDV RNA in the PBMC of experimentally infected rats (31), in the brains of horses with Borna disease (38), in the PBMC of healthy horses (22), and in the PBMC of humans

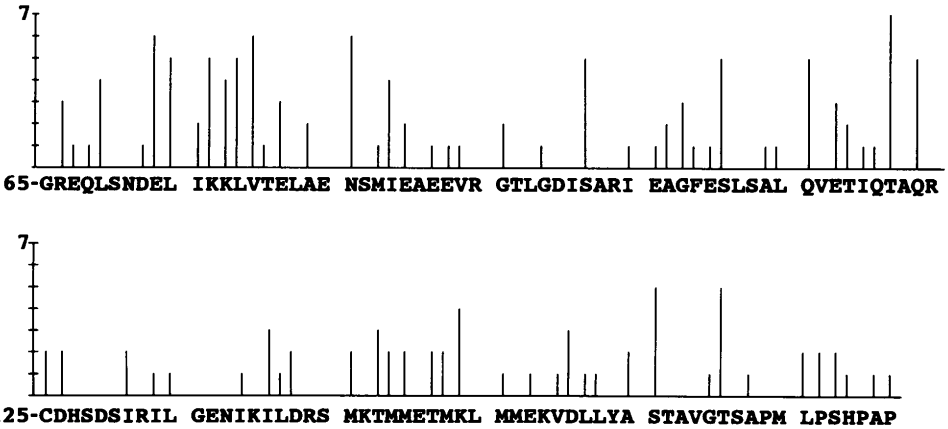

FIG. 4. Consensus amino acid sequence and sequence divergency among the 15 BDV p24 clones. The amino acid sequences of the 15 BDV p24 clones were analyzed by the Geneworks 2.2 (Intelligenetics Inc.) computer program. An amino acid residue found to be conserved in more than 8 of the 15 clones is shown as a consensus amino acid residue. Vertical columns indicate the numbers of divergent amino acid residues. The numbers at the left are amino acid positions previously reported for a horse-derived BDV infecting the C6BV cell line (13).

with mental disorders (8, 19). Here, we coupled nested RT-PCR with cDNA cloning and sequencing to study the quasispecies of BDV p24 in three mental patients. We also calculated the sequence variability of BDV p24 among patients and between humans and horses. Patient-specific sequence variations were detected among the 15 p24 cDNA clones isolated from the PBMC of the three patients, giving rise to patient-specific amino acid residues in BDV p24. Stop codons were detected in two of five clones from patient 2 but in all clones from patient 18. These truncations would have rendered the encoded p24 defective, and the BDV ribonucleoproteins containing these p24 would be nonreplicative and noninfectious. One of the two clones from patient 2 and two of the five clones from patient 18 had two stop codons each. How a replication-defective BDV acquired the second stop codon in ORF-II is unclear. It is unlikely that both stop codons were acquired in a single round of division, because as high as 3 (20%) of 15 clones had two stop codons. For the same reason, it is also unlikely that both stop codons were introduced during reverse transcription of the RNA in the creation of the corresponding cDNA. One way in which a p24-defective BDV might have acquired the second stop codon is via continued replication due to complementation in *trans* by functional p24 produced by a coinfecting BDV carrying a functional ORF-II. *trans* complementation is a mechanism commonly used by RNA viruses to acquire genetic heterogeneity and requires the coinfection of the same cell by two genetically distinct viruses. Whether *trans* complementation had occurred to give a second stop codon in ORF-II in this study remains a conjecture, especially with patient 18, whose ORF-II clones were all defective in p24.

Schneider and colleagues (30) compared the sequences of ORF-II among WT-1, strain V, and He/80-1, -2, and -3 BDV from horses. They found a 3.1% variation at the nucleotide level and a 1.5% difference at the amino acid level. Binz and coworkers (4) also found that BDV ORF-II in tissues from four infected horses had a maximum divergence of 3.3% at the nucleotide level and 1.5% at the amino acid level. Likewise, Bode et al. (8) determined the interpatient variability of BDV p40 at the nucleic and amino acid levels as 3.1% in two clones obtained from two psychiatric patients. In the present study, a higher nucleotide divergency (5.1%) was found between ORF-II of He/80-1 (30) and C-47, an ORF-II cDNA clone that we obtained from BDV adapted to MDCK cells. The intrapatient sequence variabilities of BDV ORF-II were 2.8 to 7.3% at the nucleotide level and 6.0 to 17.1% at the deduced amino acid level. The interpatient sequence variabilities were 5.9 to 12.7% at the nucleotide level and 12.8 to 28.2% at the amino acid level. The reason for the higher variabilities detected in this study is unknown but could have resulted from (i) the higher number of clones analyzed in this study, i.e., 15 versus 3 to 6, (ii) infidelity of the reverse transcriptases used in the different studies to prepare the DNA, and (iii) in the case of the differences in p40 and p24, the two regions of BDV having possibly different mutabilities.

Previous studies with vesicular stomatitis virus, another NNS-RNA virus, at highly conserved sites have shown that nucleotide substitution frequency averaged from 10^{-4} to 4×10^{-4} substitutions per base incorporated at a single site (32). Although the frequency of BDV polymerase errors is unknown, it would likely be as high as those of rhabdoviruses. In this case, every member of a cloned BDV population would differ from each other at a number of nucleotide positions in the 8.9-kb genome. The appearance of patient-specific sequence in such variable RNA virus genomes could have only resulted from strong biological selection, which might or might not be immunologic in nature (16). A previous study reported BDV variants which were tissue specific (4). The nucleotide sequence of BDV ORF-II detected in the cerebrum and kidneys of an infected horse showed 10 mutations, whereas that obtained from the parotid gland had 20 mutations (3.3%) compared with the ORF-II of tissue culture-adapted BDV (4). No deletion or truncation of BDV ORF-II was found. In the present study, as many as 33 mutations (9.3%), including deletions and truncations, were found in BDV ORF-II from human PBMC compared with the nucleotide sequences of tissue culture-adapted BDV from horses. However, intrapatient and interpatient variations were as high as 26 (7.3%) and 45 (12.7%) mutations, respectively. These differences may reflect that BDV in PBMC is subjected to higher selective pressures in vivo, especially those that are immunologic in nature. Generation of antigenic variants via a high mutation rate may constitute a means for the virus to escape surveillance. It will be interesting to study whether BDV ORF-II in human PBMC is more divergent than that in human tissues.

Recently, we have demonstrated the presence of BDV RNA in the PBMC obtained from 29.8% of a population of healthy horses in Japan (22), suggesting that BDV infection may be more widespread than previously thought. In an epidemiologic study, we have also found that the seroprevalence of BDV among healthy blood donors from areas with neighboring horse farms was markedly higher than that from the city (unpublished observation). It has been proposed that BDV may be horizontally transmitted from animals, such as from ostriches, to humans (37). In the present study, we found that the nucleotide sequence of the 15 human BDV ORF-IIcDNA clones differed from those of the horse strains V (30) and He/80-1 (30) by 4.2 to 9.3%. Comparison of the consensus sequence derived from the deduced amino acid residues common to 8 or more of the 15 human BDV cDNA clones with those of the horse strains also revealed no human-specific amino acid residue. A study of human BDV p40 also found strong sequence homology to animal reference strains (8). In sum, the observed data suggest that BDV in humans may be related to those infecting horses; however, formal proof awaits the isolation of human BDV, so that the total viral genomes can be compared.

We thank Rudolf Rott (Justus-Liebig-Universität Giessen, Giessen, Germany) for providing MDCK/BDV cells and Yoko Ueno for excellent technical assistance.

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