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Sequence Conservation in Field and Experimental Isolates of Borna Disease Virus

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Coding and noncoding sequences were analyzed from field and experimental isolates of Borna disease virus. For a 24-kDa protein, maximum divergence was 1.5% at the predicted amino acid level and 3.1% at the nucleotide level. For a 40-kDa protein, maximum divergence was 1.1% at the predicted amino acid level and 4.1% at the nucleotide level. The highest variability in sequence (10%) was found in a 40-nucleotide stretch of genomic RNA between coding sequences for the 40- and 24-kDa proteins. The degree of sequence conservation in these isolates, passaged in various host species in vivo and in vitro over a period of 64 years, is unusual for negative-strand RNA viruses.

Borna disease virus (BDV) is a neurotropic, negative-strand RNA virus (3, 7, 18) that causes encephalitis in horses, donkeys, and sheep primarily in Europe (20). Strains have been adapted to cause neurologic disease in a wide range of host species including birds, rats, mice, rabbits, and primates (20). Recent studies suggest that both the natural host range and geographic distribution of BDV may be larger than previously appreciated; cats in Sweden (21) and cattle in Germany have been reported to be naturally infected with BDV (19); antibodies to BDV proteins have also been found in horses in North America (16). Whether BDV is a natural pathogen in humans remains to be determined. Several groups have reported detecting antibodies to BDV proteins in human subjects with a broad spectrum of neuropsychiatric diseases (1, 10, 28); however, infectious virus has not been isolated from human tissues or body fluids.

Because BDV grows only to low titer and is cell associated, it has been refractory to isolation and characterization. By application of subtractive molecular cloning methods with template from experimentally infected animals and cultured cells, cDNAs were identified that encoded two viral proteins, p40 (18) and p24 (18, 32). Though probes derived from these cDNAs have hybridized to RNA in naturally infected horse brain sections (11, 25), indicating some level of sequence conservation between field and experimental isolates, there has been no direct sequence analysis of BDV field isolates.

In this paper we report the molecular cloning and sequencing of BDV RNAs isolated from the brains of naturally infected horses. Sequences from these field isolates are compared with sequences obtained in this and other laboratories from experimental isolates. Our analysis reveals a high degree of conservation that is atypical for most RNA virus systems.

MATERIALS AND METHODS

Sources of viral sequence. Field isolate BDV RNA was extracted from the brains of three horses naturally infected with BDV from three different locations in Germany (WT-1, Halle B1/91; WT-2, Anje 106/91; and WT-3, S1062/92) (34) by homogenization in guanidinium isothiocyanate and centrifugation through cesium chloride (5) or acid-phenol-chloroform extraction (6). These extracts were used as template for reverse transcriptase-polymerase chain reaction (RT-PCR). Strain V BDV was originally isolated from naturally infected horse brain in 1929 (35) and was then passaged approximately 55 times in rabbits, 6 times in rats (13), and 25 times in a human oligodendrocyte cell line (Oligo/TL) prior to RNA extraction (3). Strain V sequence was obtained through the isolation of cDNA clones (D1/6-2 and 5.82) from an Oligo/TL-derived pSPORT (GIBCO-Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.) plasmid library. He/80 was originally isolated from infected horse brain in 1980 (12). It was passaged twice in rabbits, three times in rabbit fetal brain cells, and twice in Lewis rats (12, 24). He/80-1 was passaged two additional times in rats before RNA extraction (4) and cDNA cloning (23) or RT-PCR. Sequences for He/80-2 (p40 and p24) and He/80-3 (p24) were obtained from references 26 and 31, respectively. He/80-2 and He/80-3 were passaged in MDCK cells before RNA extraction and cDNA cloning. Details of passage histories for He/80-2 and He/80-3 in MDCK cells are not known (Table 1).

RT-PCR. (i) Reverse transcription. Two micrograms of total RNA in 6 μl of water was heated to 67°C for 10 min and then cooled to 4°C on ice. Four microliters of reaction mixture (50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1.5 mM each deoxynucleoside triphosphate [dNTP], 10 U of RNasin, 100 ng of primer 1 [for cloning p40 and p24 message] or primer 2 [for cloning genomic RNA between coding sequences for p40 and p24], 20 U of Moloney murine leukemia virus RT [Promega, Madison, Wis.]) was added, and the mixture was incubated at 37°C for 1.5 h. The reaction mixture was heated to 67°C for 15 min and diluted to 150 μl with water.

(ii) Amplification. One microliter of the reverse transcription reaction mixture was amplified with gene-specific primers in a 100-μl PCR mixture containing 1× Stoffel buffer, 3.5 mM $MgCl_2$, 0.2 mM each dNTP, 500 ng of each primer, and 5 U of Stoffel fragment *Taq* polymerase (Perkin-Elmer Cetus, Irvine, Calif.). The mixture was overlaid with mineral oil (Sigma

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TABLE 1. Origin of Borna disease virus isolates

Virus	Yr of original isolation	Region of origin in Germany	Experimental hosts[a]	Source of RNA[b]
He/80-1[c]	1980	Hessen	Rabbit, RFG,[d] rat	Rat
He/80-2[c]	1980	Hessen	Rabbit, RFG, rat, MDCK[e]	MDCK
He/80-3[c]	1980	Hessen	Rabbit, RFG, rat, MDCK	MDCK
Strain V	1929	Niedersachsen	Rabbit, rat, Oligo/TL[f]	Oligo/TL
WT-1	1991	Sachsen-Anhalt		Horse
WT-2	1991	Sachsen-Anhalt		Horse
WT-3	1992	Thüringen		Horse

[a] Sequence of BDV passages in experimental hosts. See Materials and Methods for details on numbers of passages.
[b] Last host from which RNA was extracted.
[c] He/80-1, -2, and -3 represent different passage levels of the original He/80 strain.
[d] RFG, rabbit fetal glial cells.
[e] MDCK, Madin-Darby canine kidney cell line.
[f] Oligo/TL, human oligodendrocyte cell line.

Chemicals, St. Louis, Mo.) and cycled in a DNA thermal cycler for 30 cycles of amplification (94°C, 1.5 min; 55°C, 30 s; 60°C, 50 s; 72°C, 3 min).

Oligonucleotide primers used in this study were (1) 5′-GAATTCAGGATCCGCGGCCGCTTTTTTTTTTTTTTT-3′, (2) 5′-GTCACGGCGCGATATGTTC-3′, (3) 5′-ACACGCAATGCCACCCAAGA-3′, (4) 5′-GATCCTATCACAACCCCA-3′, (5) 5′-GGGAACAGACTGTCGTTAAG-3′, (6) 5′-CTTCTTACTCCAGTAAAACGC-3′, (7) 5′-CAGATGACTACGTACACTAC-3′, (8) 5′-TTGAATTAGTCAGGAGGCTCAATGG-3′, and (9) 5′-CTGAGATCATGGAGGGGTTC-3′.

Because of the length of p40 mRNA, it was amplified with three sets of primer pairs (3 and 4, 5 and 6, and 7 and 1) to produce overlapping fragments spanning the entire message. p24 mRNA was amplified with primers 8 and 1. Genomic RNA between coding sequences for p40 and p24 was amplified with primers 7 and 9 (Fig. 1). These primers were selected on the basis of the observation that coding sequence for p40 is 3′ to p24 on the viral genome (4). As positive and negative controls for these experiments, RT-PCR was performed with RNA from BDV-infected and uninfected rat brain, respectively.

Cloning and sequencing of PCR products. PCR products were size fractionated by electrophoresis in 1% agarose–40 mM Tris-acetate–1 mM EDTA gels and recovered by using a USBioclean purification kit (U.S. Biochemical, Cleveland, Ohio) according to the manufacturer's protocols. The purified PCR products were cloned into Bluescript SKII+ (Stratagene, La Jolla, Calif.) prepared with 3′ T-overhangs (22), and plasmids containing inserts were selected. Nucleotide sequence was determined for both strands by the dideoxy chain termination method (29) with T7 polymerase (Sequenase version 2.0; U.S. Biochemical). Sequence analysis was carried out by using the GCG software package (Genetics Computer Group Inc., Madison, Wis.) available through the University of California, Irvine, Office of Academic Computing.

RESULTS

Coding sequences for two BDV proteins, p40 and p24, and the genomic sequence between them, were amplified from naturally infected horse brain RNA and compared with sequence from virus propagated in experimental systems.

p40 cDNA was amplified from horse brain RNA (WT-1), by using three sets of primer pairs, to produce overlapping fragments spanning the entire message. A total of five clones were sequenced: two clones for nucleotides 1 through 450, two clones for nucleotides 350 through 925, and one clone for nucleotides 700 through 1150. WT-1 p40 sequence was compared with those of BDV strain V, He/80-1, and He/80-2 (Fig. 2A). Divergence of WT-1 p40 at the nucleotide level was 3.9% for strain V and 4.1% for both He/80-1 and He/80-2. Ninety percent of the substitutions were purine transitions. Divergence of WT-1 p40 at the deduced amino acid level was 0.8% for strain V and He/80-2 and 1.1% for He/80-1 (Fig. 3A; Table 2).

p24 cDNA was amplified from WT-1 horse RNA; two clones were analyzed and found to have identical sequences. WT-1 p24 was compared with those of BDV strain V, He/80-1, He/80-2, and He/80-3 (Fig. 2B). Though sequences for He/80-2 and He/80-3 were reported from different laboratories, the isolates have the same origin and are likely to have similar passage histories (Table 1). Divergence of WT-1 p24 at the

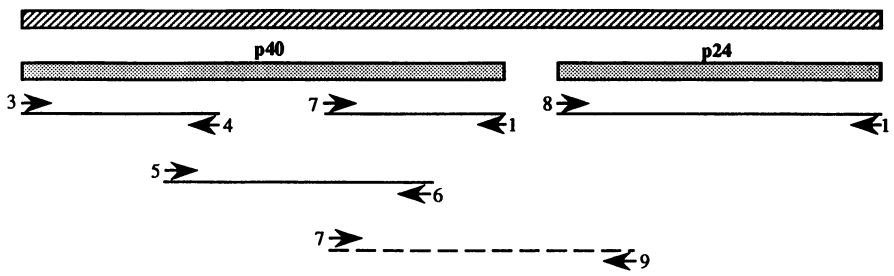

FIG. 1. Positions of cloned BDV fragments. The hatched box indicates genomic RNA, and the grey boxes indicate ORFs encoding p40 and p24. Solid lines indicate positions of cloned fragments amplified from mRNA template, and the dashed line indicates the position of the cloned fragment amplified from genomic RNA template. Numbers identify primers used for amplification. Primer 2, not shown, was used for the reverse transcription of genomic RNA. (See Materials and Methods.)

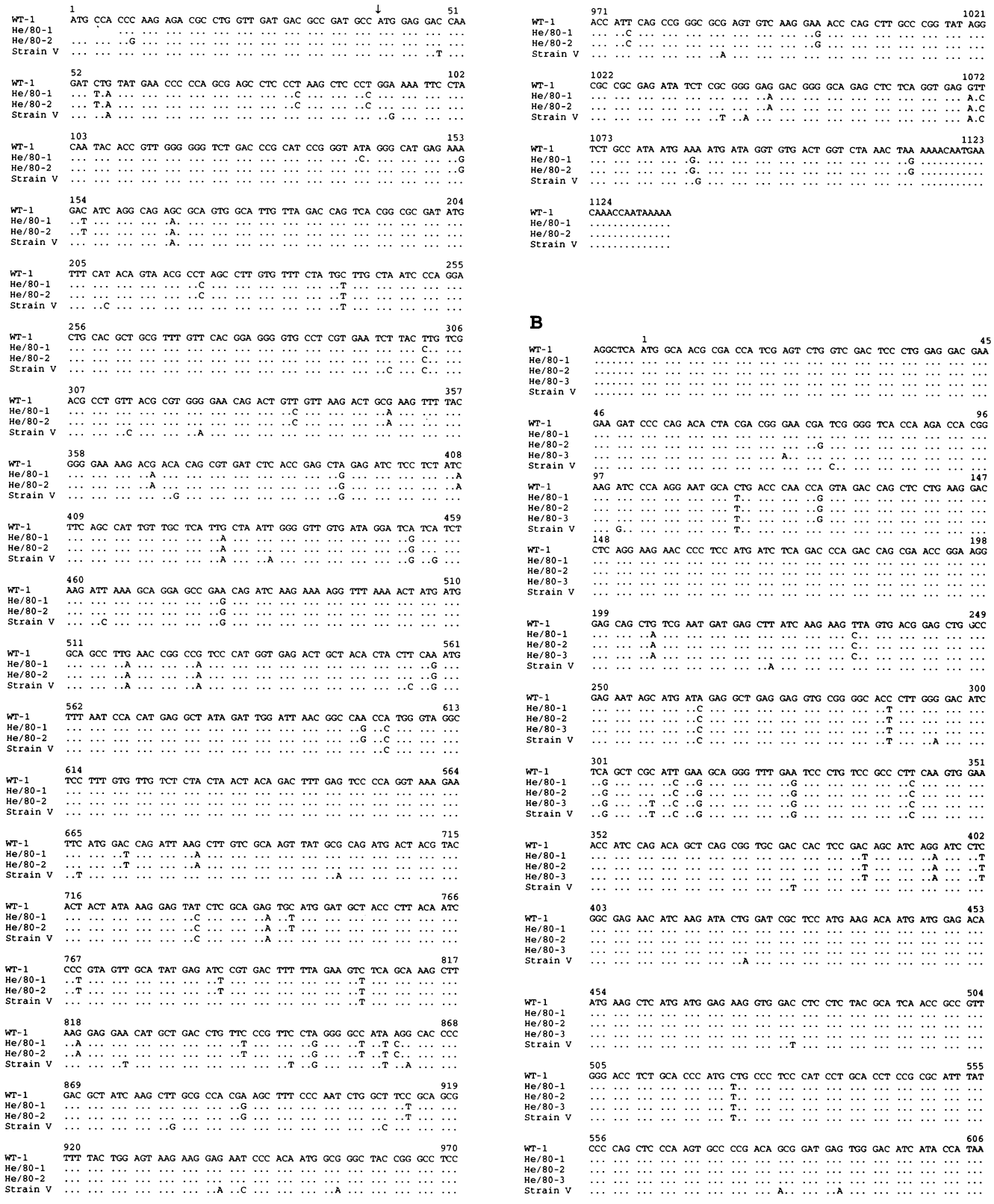

FIG. 2. Nucleotide sequences for p40 (A) and p24 (B) in BDV field (WT-1), He/80 (23, 26, 31), and strain V isolates. Numbers above the sequences indicate the nucleotide residue positions from the first potential translational start site. The arrow indicates a second potential translational start site for p40. Dots indicate nucleotides unchanged with respect to the field isolate.

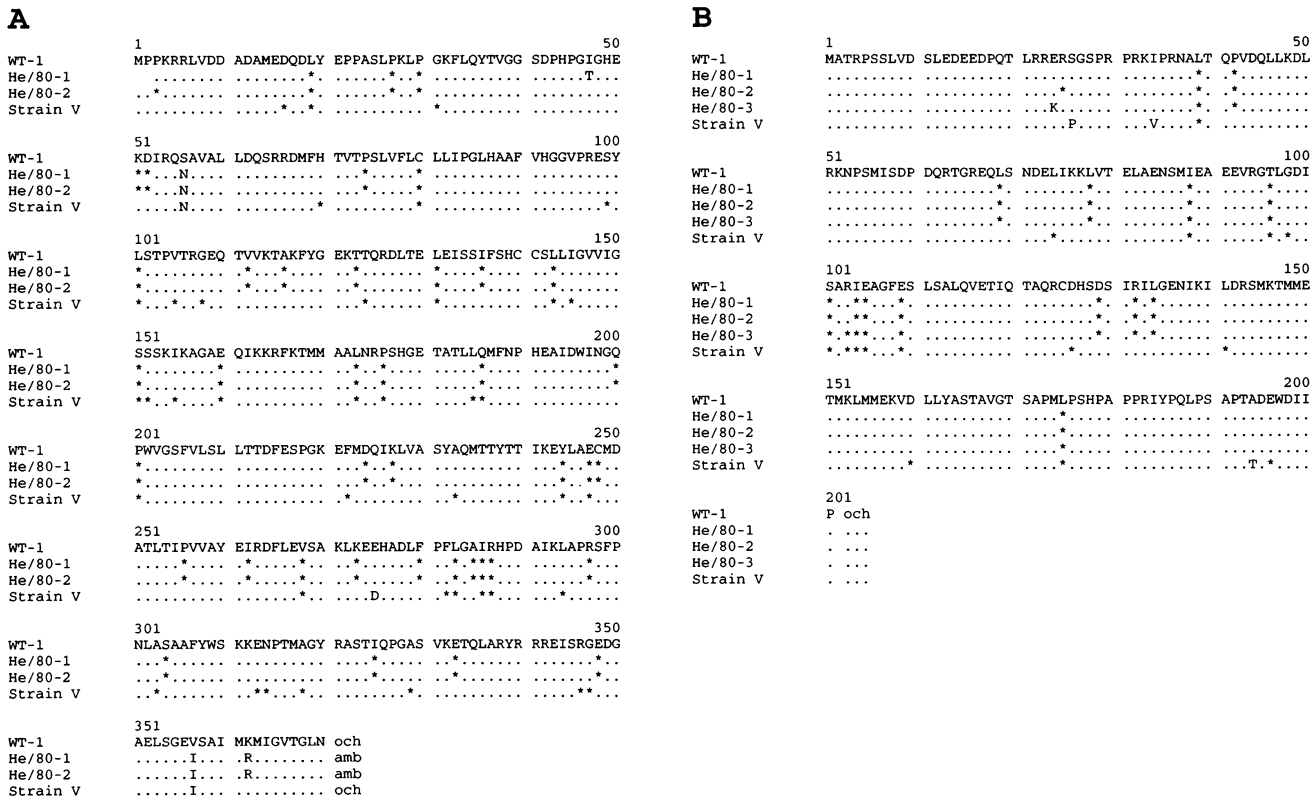

FIG. 3. Deduced amino acid sequences for p40 (A) and p24 (B) in BDV field, He/80 (23, 26, 31), and strain V isolates. Numbers above the amino acid sequences indicate the positions from the first methionine. Asterisks indicate positions of nucleotide substitutions that do not alter the amino acid sequence (silent mutations).

nucleotide level was 3.1% for strain V, 2.5% for He/80-1, 2.6% for He/80-2, and 2.8% for He/80-3. Ninety-eight percent of the substitutions were purine transitions. Divergence of WT-1 p24 at the deduced amino acid level was 1.5% for strain V, 0% for He/80-1 and He/80-2, and 0.5% for He/80-3 (Fig. 3B; Table 2).

Genomic RNA was used as a template for the amplification of the region between the p40 and p24 open reading frames (ORFs). A total of nine independent clones were isolated and sequenced. Two identical clones were isolated from both WT-1 and WT-2. Two different sequences were amplified from WT-3; two clones contained genome A, and three clones contained genome B. Divergence was as high as 10% between the stop codon of p40 and the putative translational start of p24. The majority of the divergence was clustered between nucleotides 43 and 84. Seventy-four percent of the substitutions were purine transitions (Fig. 4).

TABLE 2. Comparative sequence analysis of He/80 and strain V with reference to the WT-1 field isolate[a]

Virus	p40		p24	
	No. of nucleotide substitutions[b]	No. of amino acid substitutions[b]	No. of nucleotide substitutions	No. of amino acid substitutions
He/80-1	47 (4.1)	4 (1.1)	15 (2.5)	0
He/80-2	47 (4.1)	3 (0.8)	16 (2.6)	0
He/80-3	NA[c]	NA[c]	17 (2.8)	1 (0.5)
Strain V	44 (3.9)	3 (0.8)	19 (3.1)	3 (1.5)

[a] Nucleotide and deduced amino acid sequences were aligned by the BESTFIT program (Genetics Computer Group Inc.).

[b] Numbers in parentheses indicate divergence as a percentage of total sequence for either p40 or p24.

[c] NA, not available.

DISCUSSION

This study was initiated to compare BDV sequences from field and experimental isolates with the goal of identifying conserved motifs. For this purpose, we analyzed coding sequences for two proteins, p40 and p24, and genomic sequence between them. Both p40 and p24 are expressed at high levels in infected cells in vitro (20, 26) and in vivo (20) and are the predominant proteins detected in viral particles (17, 26). p40 has regions of sequence similarity to L genes of members of the families *Paramyxoviridae* and *Rhabdoviridae* (23), indicating likely interaction with RNA. Though the roles of p40 and p24 in the virus life cycle are not known, our analysis suggests stringent structural and functional constraints: the majority of the nucleotide substitutions in coding sequences of both p40 and p24 were in the third position of the codon; thus, most nucleotide substitutions did not affect predicted amino acid sequence.

Three regions were identified that may be important to regulation of gene expression or interaction with regulatory elements. (i) Comparison of p40 and p24 revealed a 21-nucleotide region of 81% identity. Sequence similarity was not found at the protein level. The region of identity had a similar

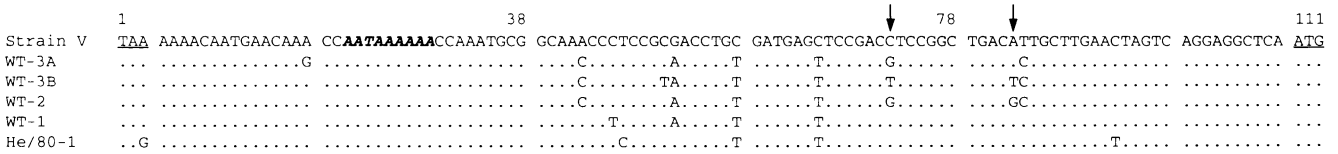

FIG. 4. Genomic sequence between ORFs encoding p40 and p24 of BDV field (WT-1, WT-2, WT-3A, and WT-3B), He/80-1, and strain V isolates. Numbers above the sequence indicate the nucleotide residue position counted from the stop codon of p40 to the first potential start codon of p24 (stop and start are underlined). Arrows indicate hypervariable residues, and boldface italics indicate a motif consistent with a transcription termination signal.

location in both ORFs (33 nucleotides downstream from the first AUG in p40 and 27 nucleotides downstream from the first AUG in p24). (ii) An 18-nucleotide palindromic sequence was found within a conserved region in p40 beginning at nucleotide 1024; whether this represents a *cis*-acting regulatory element (8, 9) is unknown. (iii) Genomic RNA between coding sequences for p40 and p24 contained a stretch of 40 nucleotides (a 40-nucleotide variable region [40VR]) with as many as eight substitutions flanked by conserved regions.

Within 40VR is the start of a potential ORF for a 9-kDa protein. It has been suggested (26, 27, 32) that this potential ORF corresponds to the 14.5-kDa protein described by Schädler et al. (30). The amino acid sequence predicted by this potential ORF showed less conservation than p40 or p24. Though divergence in the 9-kDa potential ORF between isolates was similar to that for p40 and p24 at the nucleotide level (2.6 to 4.6%), the divergence at the predicted amino acid level was significantly higher (3.4 to 6.9%). Further, variability of 40VR was observed within a single host. By using WT-3 RNA as template, two species of clones representing this region that contained three nucleotide substitutions (7.5% divergence) were obtained (Fig. 4; WT-3A and WT-3B).

Mutation rates at a given position in single-stranded RNA genomes have been estimated to be 10^{-3} to 10^{-4} per round of replication (14). In members of the families *Paramyxoviridae* and *Rhabdoviridae*, sequence conservation in the face of this high mutation rate is due to environmental pressure(s) that selects emergence of viral variants (15). Though the number of rounds of replication separating the BDV isolates cannot be determined, passage in various host species, in vivo and in vitro, over a period of 64 years has resulted in extraordinary conservation of genomic sequence. Analysis of coding sequence for maximum divergence at the nucleotide level showed values of 3.1% for p24 and 4.1% for p40 (1.5 and 1.1%, respectively, at the amino acid level); 40VR showed a maximum of 10% divergence (eight nucleotide substitutions). Whether conservation of noncoding sequence is due to structural constraints (2) or other factors, like enhanced polymerase fidelity (33), remains to be determined.

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