DISSERTATION

Phylogenetic Analysis of Borna Disease Virus Strains from Naturally Infected Animals

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Meiner geliebten Mutter

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KURZFASSUNG

Phylogenetische Untersuchungen an Borna Disease Virus-Stämmen von natürlicherweise infizierten Tieren

Schon im 19. Jahrhundert wurde das Krankheitsbild der Bornaschen Krankheit (englisch: Borna Disease; BD) ausführlich bei Pferd und Schaf beschrieben. Detaillierte elektronenmikroskopische und molekularbiologische Untersuchungen des Erregers [als „Borna Disease Virus" (BDV) bezeichnet] erfolgten allerdings erst im letzten Jahrzehnt, wobei eine Reihe von Besonderheiten festgestellt wurde. Auf grund des Replikationsmechanismus des Virus sowie virusspezifischer Charakteristika wurde BDV einer eigenen Virusfamilie (*Bornaviridae*) innerhalb der Ordnung Mononegavirales zugeordnet. Durch Sequenzanalysen ließen sich die für das Virus typischen Struktur-Proteine in sechs offenen Leserahmen (ORFs) ermitteln: ORF I (Nukleoprotein, p40), ORF II (Phosphoprotein, p24), ORF III (Matrixprotein, gp18), ORF IV (Glycoprotein, gp94) und ORF V (RNA-abhängige RNA-Polymerase, p190) und ORF VI (X-Protein, p10). Im Gegensatz zu anderen Vertretern der Ordnung Mononegavirales wie Paramyxo-, Rhabdo- und Filoviren repliziert und transkribiert BDV im Kern der infizierten Zelle und benutzt zur Herstellung seiner messenger-RNA die splicing-Mechanismen der Wirtszelle.

BDV weist ein sehr weites Wirtsspektrum auf, das neben den Hauptwirten Pferd und Schaf eine Reihe weiterer Tierarten wie z.B. Rind, Kaninchen und Hund umfaßt. Experimentell sind praktisch alle Warmblüter (von Huhn bis Primaten) infizierbar, wobei die meisten Versuche mit Ratten durchgeführt wurden. Seit etwa 20 Jahren wurde BDV auch mit einer Reihe verschiedener psychischer Krankheiten des Mensch in Verbindung gebracht.

Eine der vielen Besonderheiten der Bornaschen Krankheit ist ihr Vorkommen in bestimmten Endemiegebieten in Deutschland (Bundesländer Sachsen, Sachsen-Anhalt, Bayern, Thüringen, Baden-Württemberg), der Schweiz (Kantone Graubünden und Sankt Gallen), dem Fürstentum Liechtenstein und Österreich (Bundesland Vorarlberg), wobei eine Häufung der auftretenden Fälle im Frühling und Frühsommer beobachtet wurde. In manchen Jahren wurden deutlich mehr Krankheitsfälle beobachtet als in anderen. Die Ursachen dafür sind unbekannt. Diese Beobachtungen legen jedoch ein Virusreservoir in anderen Tieren, z.B. Nagetieren, nahe. Die Pathogenese der Bornaschen Krankheit beruht auf einer T-Zell-vermittelten immunpathologischen Reaktion. Ein hoher Prozentsatz der erkrankten Tiere stirbt.

BDV enthält RNA als Nukleinsäure. Eine Besonderheit von BDV im Vergleich zu anderen RNA-Viren ist die extrem hohe Konservierung des Genoms. Mit einer Ausnahme sind alle bisher bekannten BDV-Isolate auf Nukleinsäureebene zu 95-100% ident. Die eine Ausnahme betrifft ein in der Steiermark an BD erkranktes Pferd, bei dem das daraus isoliert Virus (mit „No/98" bezeichnet) nur etwa 85% Homologie zu allen anderen BDV-Isolaten aufweist. Diese Virusvariante ging in die Literatur als neuer BDV-Genotyp oder -Subtyp ein.

In der vorliegenden Arbeit wurden mehrere Ziele verfolgt: Es wurde erstmals eine größere Anzahl von BDV-Isolaten (33) von natürlicherweise an BD erkrankten Tieren molekularbiologisch untersucht. Die Viren wurden im Zeitraum 1985 - 1998 von Pferden, Schafen, Esel und Hirsch aus Deutschland, der Schweiz und Liechtenstein isoliert. Mittels Reverser Transkriptase-Polymerasekettenreaktion (RT-PCR) wurden bei allen Isolaten die Genregionen des gesamten p40, p10 und p24 sowie die intergenische Region zwischen p40 und p10 amplifiziert und deren Nukleinsäuresequenz bestimmt. Die ermittelte Nukleinsäuresequenz umfaßte 20,5% des gesamten Virusgenoms. Die Nukleinsäuresequenzen der verschiedenen Isolate wurden verglichen und Stammbäume wurden für die einzelnen Regionen erstellt. Mittels dieser Methode („Phylogenie / molekulare Epidemiologie") können Verwandtschaftsverhältnisse zwischen den Virusisolaten dargestellt werden.

Über 30 Jahre lang wurde in Deutschland ein abgeschwächter BDV-Lebendimpfstoff zur Immunisierung von Pferden verwendet. Dieser induzierte jedoch nur einen unzureichenden Impfschutz und stand sogar lange Zeit in Verdacht Impfdurchbrüche zu verursachen. Dieser Impfstoff („Dessau-Vakzine") wurde in die Untersuchungen einbezogen, da er in Literaturberichten als eine mögliche Ursache der Ausbreitung von BDV angesehen wurde (wobei bisher kein Beweis dafür vorlag). Ausserdem wurden in die Untersuchungen die wesentlichen weltweit für Experimente herangezogenen BDV Labor-Stämme inkludiert.

Die Sequenz-Vergleiche und phylogenetische Analysen bestätigten frühere Ergebnisse, dass BDV genetisch außergewöhnlich stabil ist und sehr konservierte Nukleinsäuresequenzen enthält. Der maximale Unterschied zwischen den verschiedenen Virusisolaten betrug nur 4%, unabhängig von der Tierart, aus der das Virus isoliert wurde und dem Jahr der Isolierung. Es konnte keine neue Virusvariante mit unterschiedlicher genetischer Komposition identifiziert werden. Auch Labor-Stämme bzw. BDV-Isolate, welche über Jahre in Zellkulturen oder in Versuchstieren passagiert wurden wiesen nur einige wenige Punktmutationen auf. Wie die Virus-Stammbäume zeigten, war nur ein Virusisolat genetisch eng mit dem Impfstamm verwandt, d.h. einerseits, dass es vermutlich tatsächlich zu einer Verbreitung des Impfvirus gekommen ist jedoch bei weitem nicht in dem Ausmaß wie befürchtet.

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I.

DISSERTATION:

Phylogenetic analysis of Borna disease virus strains from naturally infected animals

JOLANTA KOLODZIEJEK

SUMMARY

The aim of this study was to gain more detailed insights into the genetic evolution and variability of Borna Disease Virus (BDV) under field conditions. We performed phylogenetic analysis of thirty-three Borna disease (BD) field viruses from different species including horse, sheep, donkey and other animal hosts such as deer, originating from naturally infected animals in endemic areas of Germany, Switzerland and the Principality of Liechtenstein.

Additionally, since it has been suggested that German BD cases may have been associated with the BDV live-attenuated vaccine "Dessau", we included this strain in our study as well. Also, we traced the origin of seven classical and widely used BDV laboratory strains (of which nucleotide sequences were already available in the GenBank database) by genetic analysis, literature search and personal investigations. To our knowledge, this is the first complete documentation of the evolution and history of these reference strains.

Four regions of the BDV genome were analysed: the complete p40, p10 and p24 genes as well as the intergenic region between p40 and p10. Five selected phylogenetic trees are presented and discussed.

Phylogenetic analysis confirmed previous findings that BDV is genetically remarkably stable and contains highly conserved sequences. Sequence comparisons showed that BDV strains originating from the same host species are not closer related to each other than to isolates from other host species, e.g. equine field strains did not show a higher degree of homology to each other than to isolates from sheep, donkeys or other hosts.

On the other hand, sequence analysis revealed that isolates from the same geographic area (e.g. Bavaria, Germany, or Graubuenden, Switzerland) showed a high degree of identity to each other at both nucleotide and amino acid level, independent of host species and year of isolation (up to 13 years,1985-1998).

Furthermore all field strains investigated clearly segregated from the newly described and highly divergent BDV strain No/98, which originated from a non-endemic area in Austria.

Although the BDV vaccine strain has been incriminated to be possibly involved in the spread of BDV in Germany, we identified only one BDV field strain, which exhibited high sequence identity to the live-attenuated vaccine strain.

In relation to their origin and history, we were able to show that passaging of BDV laboratory strains (strain He/80, strain V and strain H1766) in cell culture or in experimental animals over years or even decades only led to few and punctual mutations in the viral genome. Interestingly, the controversially discussed isolate RW98 segregated clearly from strain He/80.

INTRODUCTION

Borna Disease (BD) was first described in the 18th century in horse in south-eastern Germany. The name Bornasche Krankheit (BD) has been derived from the city of Borna near Leipzig, Saxony, Germany, where a large number of horses died during an epidemic between 1894 and 1896. The English name Borna disease (BD) was subsequently adopted in the beginning of the 1970s.

The etiologic agent, Borna Disease Virus (BDV), exhibits several unique characteristics. Genome organization and nucleotide sequences of BDV are similar to other Mononegavirales, but due to features such as nuclear localisation of replication and transcription (53), the unusual high level of sequence conservation (20, 32) and the wide host range (probably including human beings; 7, 8, 25) BDV has been classified as the prototypic and only member of a new viral family, *Bornaviridae*, within the order Mononegavirales, related to *Paramyxo-, Rhabdo-* and *Filoviridae*. The new virus family *Bornaviridae* was officially first accepted in 1996 [International Congress of Virology, Jerusalem (18)].

One of the first detailed data on the ultrastructure and morphogenesis of BDV particles were obtained by Kohno et al. in 1999 (Fig. 1). The virus particles that were observed are about 100-130 nm in diameter, and they are covered by an envelope with approximately 7 nm-long spikes (29) (Fig. 2 B). Genome properties and organization of BDV were first determined in 1994 (10, 13). BDV RNA contains six open reading frames (ORFs), which, employing the RNA splicing machinery, encode six structural proteins (57). The nucleoprotein p40, occurring in two isoforms p38 and p40, contains a nuclear localization signal and is involved in export activity (70). The phosphoprotein p24 is localized in the nucleus of infected cells (62). Further structural proteins are the matrix protein gp18, a transcriptional activator, the surface glycoprotein gp94 which form the "spikes" and is possibly involved in viral penetration and the fusion of the viral and cellular membranes, and the viral RNA-dependent RNA polymerase p190 (9, 22, 48). The recently described sixth protein labelled X protein or p10 overlaps with p24 and mediates the associaton with the viral phospho- and nucleoproteins (7) (Fig. 2 A).

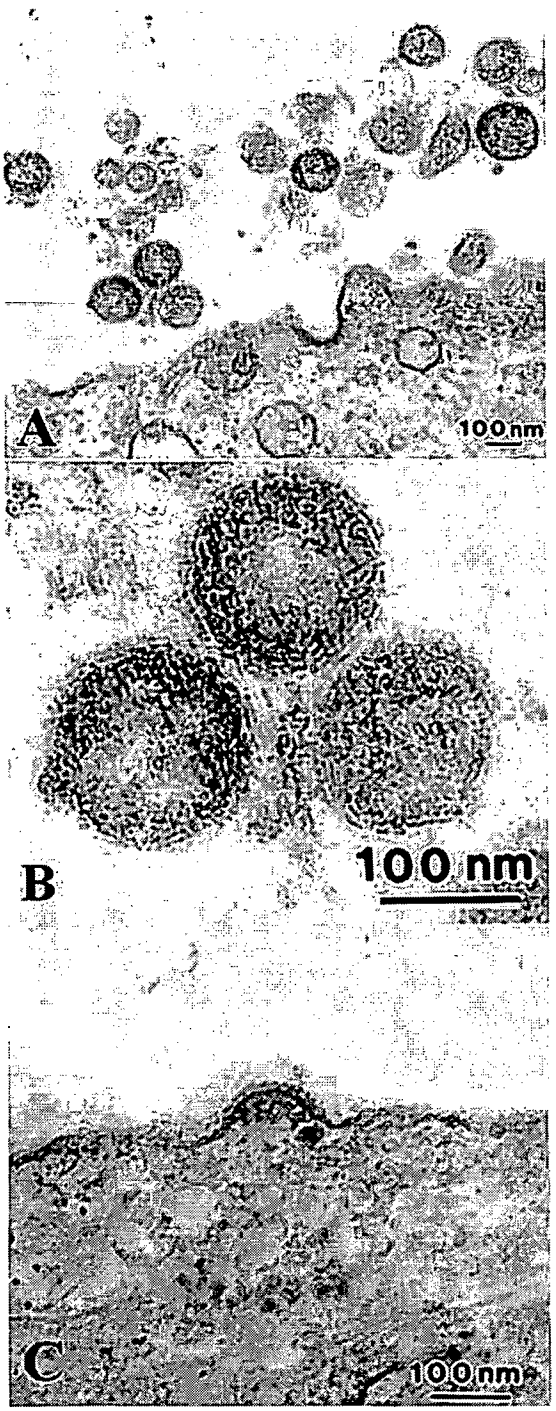

FIGURE 1. Electron micrographs showing BDV (B), its entry into the cell (A) and its release (C) (Kohno et al. Journal of Virology 1999; 73:764).

BDV enters the host cell via endocytosis. Entry of an enveloped virus into animal cells usually requires membrane-fusing activity of viral surface glycoproteins (gp 94 and gp 18). The virus-like particles reproduce by budding at the cell surface (Kohno, 16).

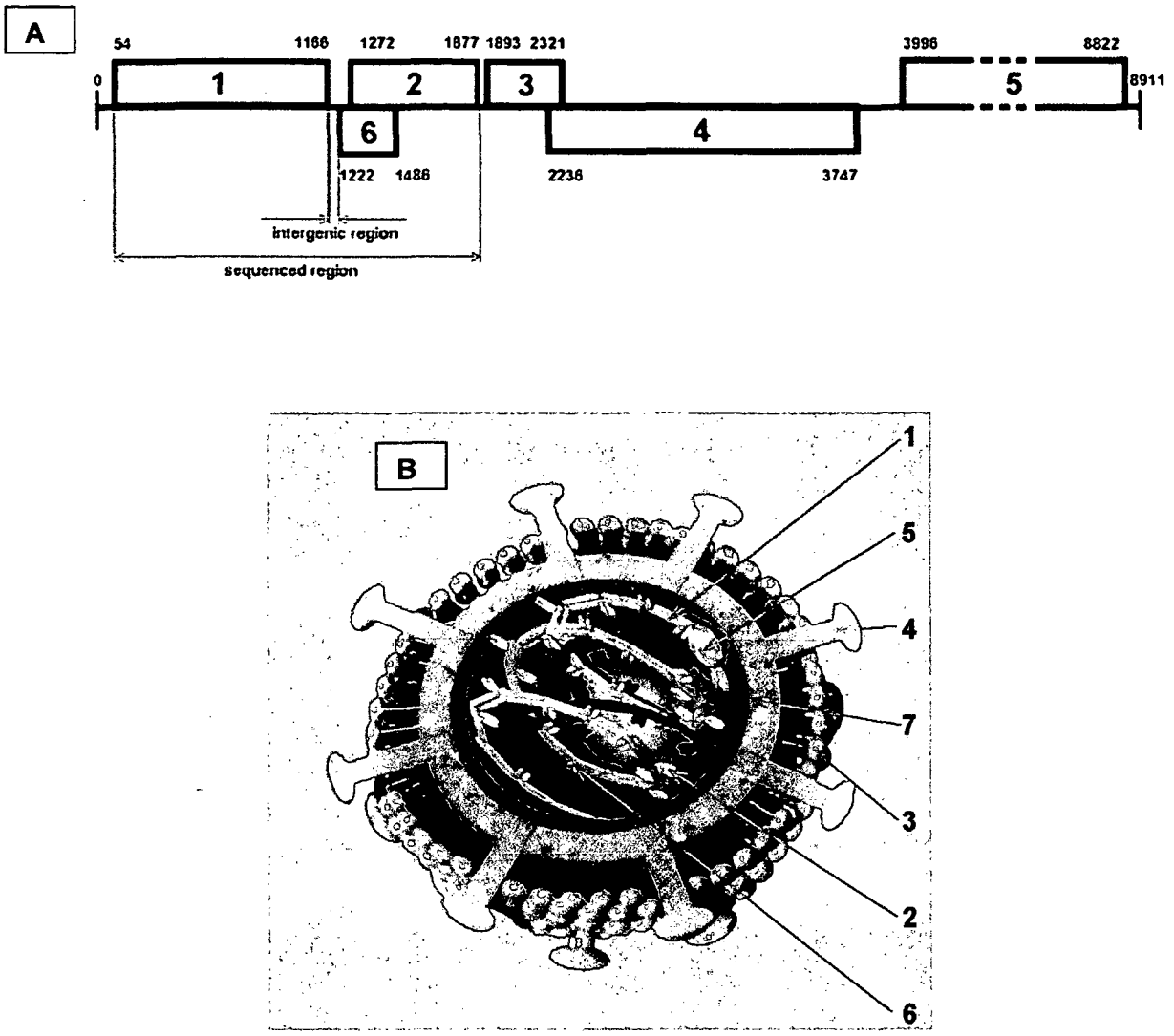

FIGURE 2. (A) Gene order of BDV. Overlapping RT-PCR products of BD viruses corresponding to nucleotide positions 54 to 1877 of reference strain V were sequenced. The resulting 1824 bp long fragments include the complete coding sequences for the viral proteins p40, p10 and p24 as well as a non-coding intergenic region. The sequenced region and the intergenic region are indicated by arrows.

(B) Computer generated image of BDV morphology (Ludwig and Bode, Intervirology 1997; 40: 188): The virus particle is about 100-130 nm in diameter and it is covered by an envelope with approximately 7 nm-long spikes.

Legend to FIG. 2A and 2B: 1, p40 (N protein, nucleoprotein, ORF I); 2, p24 (P protein, phosphoprotein, ORF II); 3, gp18 (M protein, Matrixprotein, ORF III); 4, gp84/94, p57 (G protein, Glycoprotein/Envelope, ORF IV); 5, p190 (L protein, L-Polymerase, ORF V); 6, p10 (X protein, ORF VI); 7, lipid bilayer

Although BDV infection was first described only in horses and sheep, mainly in German areas (18), it is currently known that BDV is able to infect a wide variety of animal species and it seems to be geographically more widespread than previously thought (Asia, North America). BDV endemic areas in central Europe include Germany (federal states of Bavaria, Hesse, Saxony, Saxony-Anhalt, Lower Saxony, Thuringia and Baden-Wurttemberg), the eastern part of Switzerland, mainly along the Swiss border to Austria and Liechtenstein (the kantons Graubuenden and Sankt Gallen), the Principality of Liechtenstein, and more recently the most western federal state of Austria (Vorarlberg) (11, 28, 66, 67, 68, 75, 76) (Fig. 3).

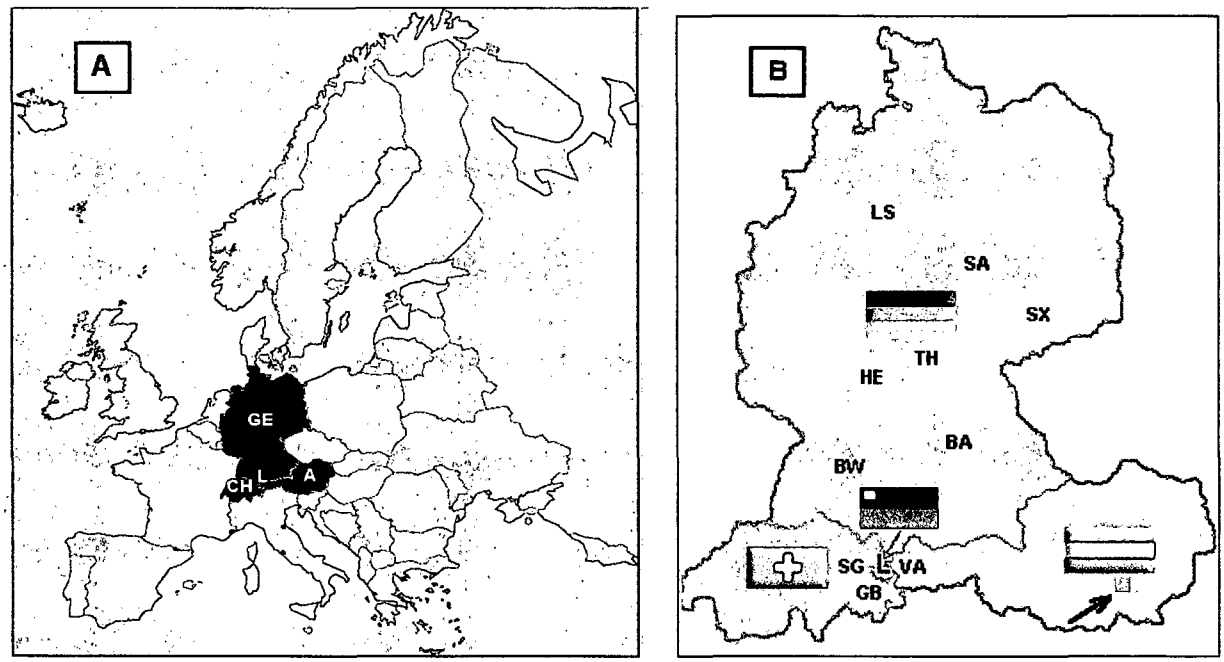

FIGURE 3. (**A**) Map of Europe. The four countries with BDV endemic areas (GE, Germany; CH, Switzerland; L, Liechtenstein, A: Austria) are highlighted. (**B**) BDV endemic areas in Germany, Switzerland, Austria and the Principality of Liechtenstein are indicated by letters: Abbreviations used: LS, Lower Saxony (Germany); SA, Saxony-Anhalt (Germany); SX, Saxony (Germany); HE, Hesse (Germany); TH, Thuringia (Germany); BW, Baden-Wurttemberg (Germany); BA, Bavaria (Germany); SG, Sankt Gallen (Switzerland); GB, Graubuenden (Switzerland); L, Principality of Liechtenstein; VA, Vorarlberg (Austria). The red square indicates the place of the pony, from which the novel BDV subtype No/98 was isolated [non-endemic area of Austria (federal state of Styria)].

Until the early 1990s, Austria was considered as BDV free. The first Austrian BD case originated from a horse in the federal state of Vorarlberg and was diagnosed in 1993, followed by another case in 1997 (67, 76). Following further clinical BD infections in this region (three horses and one dog), it was suggested to accept Vorarlberg as new area in central Europe with endemic BD (67), extending the Swiss/Liechtenstein area. Recently, a BDV strain with a highly variant genome (differing from GenBank reference strains by more than 15%) was isolated from a pony, originating from the Austrian federal state of Styria, where no cases of BD had previously been recorded (44).

In Switzerland the disease was first described in 1976 (37). In 1978, two cases of spontaneous BD occurred in rabbits (36). Between 1990 and 1998 twenty-nine cases of BDV encephalitis were reported in *Equidae* (horses, donkeys), twenty-three in sheep, two in goats and two in cattle (11).

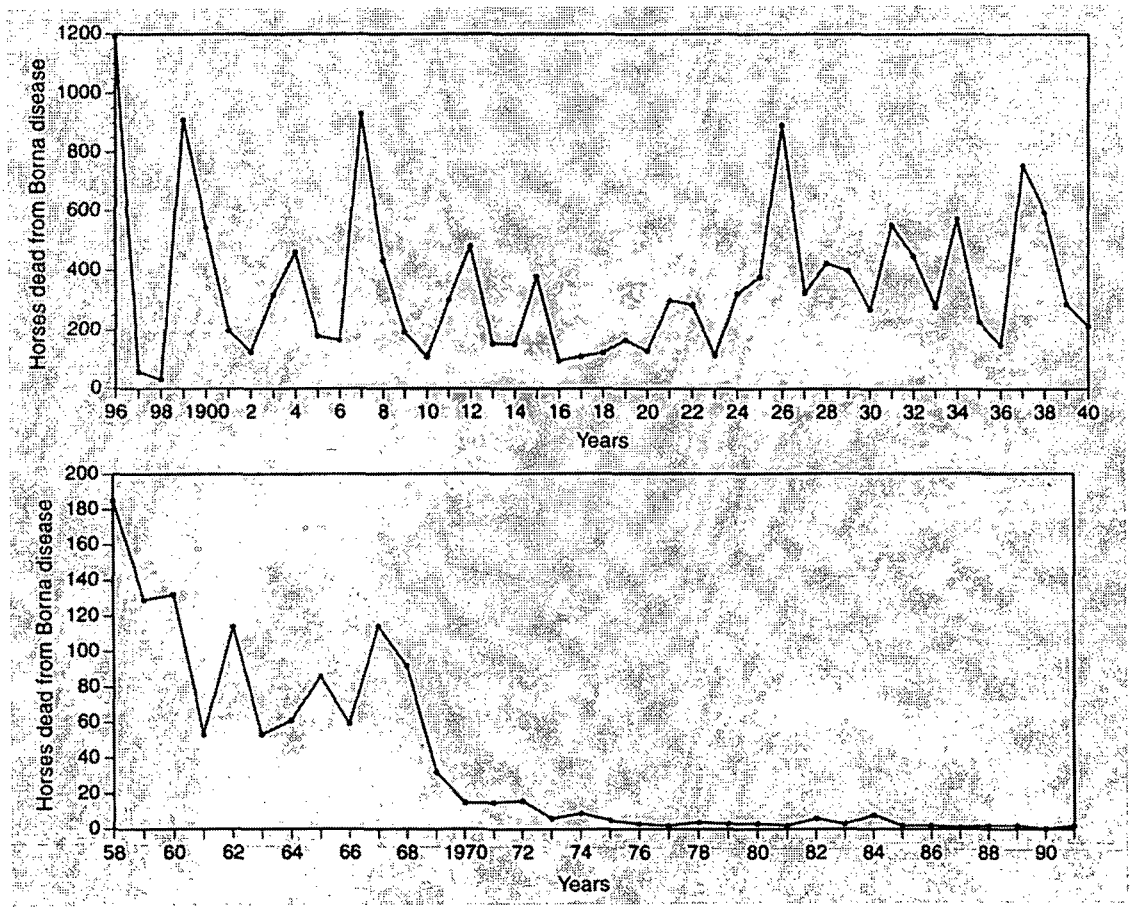

FIGURE 4. The decrease in Borna disease incidence in horses, Saxony, Germany, Dürrwald and Ludwig, 1997 (18).

In recent years, the total number of BDV cases in the endemic areas of central Europe was low (fewer than 100 cases per year) (59). BDV infections occur sporadically in non endemic regions as well. Furthermore, studies of BDV infections have shown that most cases occurred in spring and early summer (28). The seasonal and regional occurrence of BD is a further unique characteristic of this virus infection.

In the past, most of the BD cases have been reported from Germany, e.g. from 1896 to 1940 16600 horses died of BD in Germany (415/year). Until the 1960s, periodical BD outbreaks had an epidemical character but subsequently the BD incidence decreased rapidly. Whereas from 1958 to 1991 in Saxony alone, 1216 horses died of BD (18), between 1992 and 1998 only 55 BD cases were diagnosed in Germany (44 horses, 9 ponies, 2 donkeys) (23) (Fig. 4).

Clinical BD in sheep was first reported by Walter at the end of the 19th century. Since then, BDV infections in sheep were reported from Germany, Switzerland, Liechtenstein, Italy, China and Japan (73). In the central European endemic areas, BD in sheep has been diagnosed mainly with a seasonal accumulation between March and September (11; 18).

Although BD is not strictly limited to horses and sheep, the frequency at which other animals are infected or succumb to the disease appears to be very low. BDV was found in donkeys, goats, cattle and dogs (2 canine cases, one in Austria and a suspected case in Japan), in rabbits (2 cases in 1978) and in zoo animals in Erfurt (Thuringia, Germany) (4, 6, 11, 12, 17, 36, 47, 58, 69, 75, 78). With a few notable exceptions, however, including the reports of BD in an Austrian dog (75), in France in lynxes (15), in Japan in horses (24), domestic cats (39) and a dog (47), investigators reporting infection in new host species did not typically include virus isolation, experimental infection or detailed neuropathology in their studies (26). Also the question of whether BDV induces "staggering disease" in cats is not easily to be answered (33, 74). Although natural BDV infections in cats may occur occasionally, the virus is probably not the etiological agent of staggering disease (41, 45).

Since to date, the virus has not been found in reptiles, amphibias or insects. In birds, BDV has yet only been detected in ostriches (34), as reported from Israel: a neurological disease affecting a large number of ostriches is believed to have resulted from an infection with BDV or a BDV-like virus (35, 66).

First hints that BDV could also be associated with certain human diseases emerged from serological studies beginning in 1985 (55). In 1995, Bode presented the first direct evidence that BDV might infect humans using a highly sensitive reverse transcriptase-nested PCR (RT-nPCR) (8).

Nevertheless the epidemiology of human BD remains unresolved. The seroprevalences determined in various groups of psychiatric patients range from 0 to 100% and from 0 to 32% in control groups (26, 63). Similar to the conflicting serological results mentioned above, BDV-RNA and BDV-antigen analysis of peripheral blood mononuclear cells (PBMCs) and of human brain samples revealed a variation in prevalence rates of 0 to 100% and of 0 to 80%, respectively, and of 0 to 35% in control groups (26, 60). Furthermore, it has been demonstrated that human bornaviruses are often strongly related to animal BDV isolates or virus strains used for experiments in the laboratories reporting the human BDVs, thus questioning a true human origin of some isolates known to date (50, 56, 61). Notably, the BDV sequence determined from PBMCs of a chronic fatigue syndrome patient in Austria (accession no. AF094478) could not be allocated to any of the laboratory strain clusters (31, 42, 43, 50). The detection of both BDV-specific antigen and BDV-RNA in four autopsied human brains with hippocampal sclerosis and astrocytosis in the United States (de la Torre, re-investigated by Czygan; 14, 71), also sustains the theory that BDV can infect human brain tissue. Interestingly, however, the nucleotide sequences derived from these PCR amplification products showed a close relationship to the sequence of BDV strain He/80.

Although experimental infection of animals (in particular the rat, which is the most used model, but also rabbits, cattle, donkeys, dogs, macaques and birds) is successfully achieved by intracerebral, intraocular, intranasal and intramuscular application of BDV, the natural source of infectious virus has not yet been determined (66). There is no clear evidence for BDV transmission from horse to horse. One report described a possible vertical infection (24). Reports of BDV in PBMCs also indicate the possibility of hematogenous transmission (2, 26, 54).

Also the virus reservoir is yet unknown. The territorial factor, the enhanced frequency of BD and the seasonal periodicity would all be compatible with the

existence of a rodent reservoir, but since to date no evidence of BDV infection of these animals in the field has been identified (72). Ticks and wild birds may play a possible role as BDV vectors. In addition, various secretions can be potential sources of BDV infection for other animals and humans.

BDV pathogenesis is multifactorial and until to date exact biochemical and physiological mechanisms of the infection still remain unclear. Disorders of movement and behavior in adult infected rats are associated with dysfunction in dopamine (DA) circuits, as seen also in many neuropsychiatric disorders (64). BDV persistence in the central nervous system (CNS) is characterized by the infection of astrocytes (Figure. 5). Astrocytes are the most common cell type in the brain and play an essential role in the maintenance of a CNS microenvironment compatible with proper neuronal activity. Disturbances in astrocyte function and in consequence in astrocyte glutamate transporters function (neurotransmitters function) lead by complex process to neurotoxicity and cell death (3). On the other hand the pathogenesis of BD is mediated by a T-cell-dependent immune mechanism. The immune response in the acute phase of the disease is characterized by a cellular response in which $CD8^+$ T cells are responsible for the destruction of virus-infected brain cells. $CD4^+$ T cells serve as helper cells and support the production of antiviral antibodies (21, 46, 51).

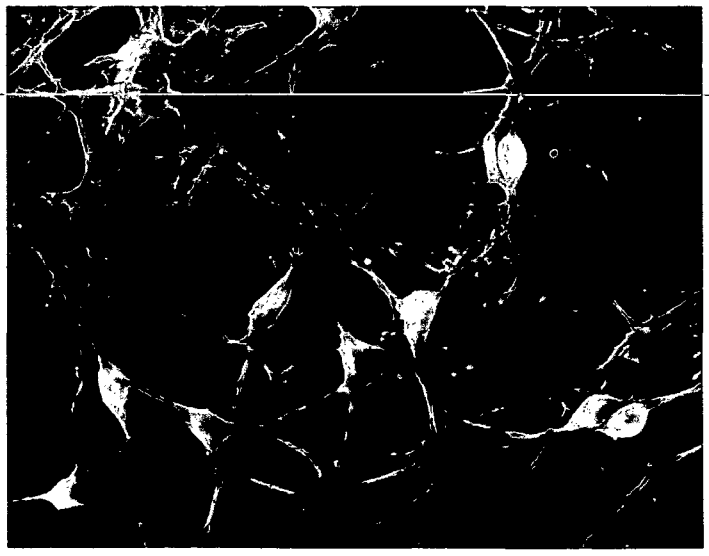

FIGURE 5. Neurones (yellow) and an astrocyte (green) are specific targets of BDV. BDV is highly neurotropic. The highest virus concentration is often found in neurons and astrocytes. Disturbances in astrocyte function lead by complex process to neurotoxity and cell death. (Picture: www.stanford.edu)

In animals, BDV is known to persist in the limbic system, a subarea of the CNS. As the limbic system affects mood, behavior and memory, BDV infection has also been associated with human psychiatric disorders, including schizophrenia, paranoid psychosis and major depression.

Clinical features of natural and experimental diseases of animals include hyperactivity, movement and posture disorders, stereotypic and abnormal social behaviours (63); the course of the disease can range from dramatic to subtle or even inapparent and depends on genetic factors, immune status and age of the host, as well as on viral factors (3).

Typical clinical symptoms result from a nonpurulent meningoencephalitis and have common features: reduced appetite, ataxia, depression, blindness, anxiety, collapsing, paresis with paralytic symptoms, circular movement and, finally, complete inability to move, loss of appetite and, in late stages, paralysis followed by death. In infected sheep, mortality is higher than 50% and in horses varies between 80 to 100% (9).

For the control of BD in horses and sheep a live-attenuated vaccine was developed in the 1960s (strain "Dessau"; 19, 38), and widely used until 1992 when investigations suggested that field cases of BD might be correlated with the live vaccine. Although some promising results to control BDV infection and disease using antiviral drugs such as ribavirin, amantadine sulfate or 1-beta-D-arabinofuranosylcytosine have since been achieved (1, 5, 16, 27), attempts to establish a new BDV vaccine continued (21, 30).

More recent research indicated that some BDV strains isolated from horses, sheep, cats and humans in Japan, Taiwan, Iran, the United Kingdom and the United States proved to be almost identical to laboratory strains derived from central European isolates. These results affected deeply the basics of hence widely accepted common knowledge about BDV. The remote possibility exists that the non-pathogenic vaccine strain "Dessau" gave rise to present-day field isolates. Alternatively, it remains possible that reports on the detection of BDV or BDV antibodies in animals and people from non-European countries represent either artefacts resulting from accidental contamination of samples with laboratory strains (66) or result of cross-reactions with non-BDV antibodies (60).

The aim of our study was to gain more detailed insights in the genetic evolution and variability of BDV by investigating BDV isolates and brain tissue samples from naturally infected animals. We performed phylogenetic analysis of thirty-three BD field viruses from different species including horse, sheep, donkey and other animal hosts such as deer, originating from naturally infected animals from Germany, Switzerland and the Principality of Liechtenstein.

Additionally, since it has been suggested that German BD cases may have been associated with the BDV live-attenuated vaccine "Dessau", we introduced this strain in our study as well.

Also, we traced the origin of seven classical and widely used BDV laboratory strains, of which nucleotide sequences have already been deposited in the GenBank database, by genetic analysis, literature search and personal investigations. We are to our knowledge the first to present a complete documentation of the evolution and history of these reference strains. In the course of our investigations we were able to obtain the original brain suspension of a German horse, an isolate which in the following had been established and widely used as laboratory strain H1766, and to compare the sequences of the original isolate and of its laboratory strain passaged for years.

Four regions of the BDV genome were analysed: the complete p40, p10 and p24 genes as well as the intergenic region between p40 and p10. Five selected phylogenetic trees are presented and discussed.

MATERIALS and METHODS

Samples. BDV infected brain tissues and BDV isolates from twenty-five horses, five sheep, two donkeys and one deer were analysed in this study. All originated from natural spontaneous cases of BD from Germany (eighteen samples), Switzerland (fifteen samples) and the Principality of Liechtenstein (two samples). They were collected by collaborating laboratories between 1985 and 1998 and sent to us for analysis. Before euthanasia or death the majority of these animals had been diagnosed as BDV infected based on disease symptoms and serological investigations (indirect immunofluorescence assay). BDV diagnosis was confirmed post mortem by histology and BDV-specific immunohistochemistry (IHC); for the German specimens, virus isolation in cell culture was performed.

One of the German BDV samples analysed, the original brain suspension of horse no. 1766 was the ancestor of strain H1766 (GenBank acc. no. AJ311523).

In addition, the vaccine strain Dessau, charge no. 198 11 90, was incorporated in the study. This live-attenuated vaccine, kindly provided by Dr. Ralf Duerrwald, Impfstoffwerk Dessau-Tornau, Rodleben, Germany, was widely used in Germany from the 1960th to 1992.

Characteristics of all BDV isolates, laboratory strains and the vaccine strain analysed in our study are listed in Table 1.

Due to the low incidence of BD, Austrian field isolates could not be included in the study. Retrospective investigations were unfortunately not possible since all attempts to extract sufficient amounts of RNA from paraffin-embedded tissues [PET(s)] were unsuccessful.

Isolation of RNA. Brain tissue samples were homogenized using sterile sand and resuspended in distilled and diethyl pyrocarbonate-treated (DEPC) water. The lyophilized vaccine was resuspended in DEPC water. All suspensions were frozen at –80°C for 30 min, thawed and centrifuged at 1,700×g for 5 min. A volume of 140 µl of each supernatant was used for RNA extraction, employing the QIAamp Viral RNA Purification Kit (QIAGEN, Valencia, USA) according to the manufacturer's instructions, except that the elution buffer AVE was equilibrated to 80°C before eluting the total RNA.

TABLE 1. Characteristics of thirty-three BDV isolates, one vaccine strain and seven known laboratory strains used in the phylogenetic study

No.	Name	GenBank access. no.	Host	Country of origin	Area of isolation	Year of isolation
1	H 446	**AY374534**	horse	**Germany**	Hesse	1992
2	H 544	**AY374535**	horse	**Germany**	Bavaria	1993
3	H 639	**AY374536**	horse	**Germany**	Lower Saxony	1993
4	H 640	**AY374537**	horse	**Germany**	Lower Saxony	1993
5	H 1499	**AY374520**	horse	**Germany**	Baden-Wurtt.	1994
6	H 1505	**AY374521**	horse	**Germany**	Bavaria	1994
7	H 1766	**AY374522**	horse	**Germany**	Lower Saxony	1994
8	H 3053	**AY374523**	horse	**Germany**	Bavaria	1996
9	H 3321	**AY374524**	horse	**Germany**	Bavaria	1997
10	H 3452	**AY374525**	horse	**Germany**	Bavaria	1997
11	H 3515	**AY374526**	horse	**Germany**	Bavaria	1997
12	H 3519	**AY374527**	horse	**Germany**	Bavaria	1997
13	H 3575	**AY374528**	horse	**Germany**	Bavaria	1997
14	H 3915	**AY374529**	horse	**Germany**	Bavaria	1998
15	H 3940	**AY374530**	horse	**Germany**	Bavaria	1998
16	H 3950	**AY374531**	horse	**Germany**	Bavaria	1998
17	H 4026	**AY374532**	horse	**Germany**	Bavaria	1998
18	H 4050	**AY374533**	horse	**Germany**	Bavaria	1998
19	E85-0795	**AY374552**	horse	**Switzerland**	Graubuenden	1985
20	S88-2297	**AY374551**	horse	**Switzerland**	Graubuenden	1988
21	S89-2224	**AY374550**	horse	**Switzerland**	Graubuenden	1989
22	S91-1307	**AY374542**	sheep	**Principality of Liechtenstein**		1991
23	S91-1350	**AY374543**	sheep	**Switzerland**	Sankt Gallen	1991
24	S91-1460	**AY374544**	horse	**Switzerland**	Graubuenden	1991
25	S91-1539	**AY374546**	sheep	**Principality of Liechtenstein**		1991
26	S91-1552	**AY374547**	horse	**Switzerland**	Graubuenden	1991
27	S93-1186	**AY374540**	sheep	**Switzerland**	Sankt Gallen	1993
28	S95-1114	**AY374539**	donkey	**Switzerland**	Graubuenden	1995
29	S95-1466	**AY374545**	donkey	**Switzerland**	Graubuenden	1995
30	S96-0868	**AY374538**	horse	**Switzerland**	Graubuenden	1996
31	S96-1202	**AY374541**	horse	**Switzerland**	Graubuenden	1996
32	S96-1924	**AY374548**	deer	**Switzerland**		1996

33	S98-2042	**AY374549**	sheep	**Switzerland**	Graubuenden	1998
34	Vaccine	**AY374519**	horse[a]	**Germany**	Saxony-Anhalt	

BDV laboratory strains included

No.	Name	GenBank access. no.	Host	Country of origin	Area of isolation	Year of isolation / sequencing
35	Strain V[10,18]	**U04608**	horse[a,b]	**Germany**	Lower Saxony	1929/1994
36	Strain V/FR[52]	**AJ311521**	horse[b]	**Germany**	Lower Saxony	1929/2001
37	He/80 [13,40]	**L27077**	horse[b]	**Germany**	Baden-Wurtt.	1980/1994
38	He/80/FR[52]	**AJ311522**	horse[b]	**Germany**	Baden-Wurtt.	1980/2001
39	RW98[d,49,50]	**AF158629-33**	horse[a,b,c]	**Germany**		1999/1999
40	Strain H1766[52]	**AJ311523**	horse[b]	**Germany**	Lower Saxony	1994/2001
41	No/98[44,52]	**AJ311524**	horse[b]	**Austria**	Styria	1998/2001

[a] passaged in rabbits; [b] cell culture adapted, [c] passaged in rat brains, [d] BDV-4p (presumably a derivate of He/80); with grey underlay: new BDV subtype, originating from a non-BD-endemic area. Baden-Wurtt., Baden-Wurttemberg

Primer design. Two different primer pairs, which proved best for the detection of classical BDV as well as for a highly variant strain, were used for screening the specimens. The primers amplifying a partial sequence of the p40 gene were previously described by Sorg and Metzler (65). Furthermore, seven primer pairs were designed in order to amplify overlapping PCR products comprising the complete N (p40), X (p10) and P (p24) protein encoding regions of the genome as well as the intergenic region between N and X, respectively. The oligonucleotides were derived from the sequence of BDV strain V (GenBank accession no. U04608) using the Primer Designer Program (Scientific & Educational Software, version 3, 0) and synthesized by Invitrogen (Life Technologies). The selected primers are shown in Table 2.

TABLE 2. Oligonucleotide primers used for amplification and sequencing of the p40, p10 and p24 genes as well as of the intergenic region between p40 and p10 of the BDV strains analysed in this study. Each primer name contains its position and orientation on the genome. The nucleotides are numbered according to the corresponding position on BDV strain "V" [GenBank accession no. U04608 (10)].

[a] f, forward; r, reverse. IGR, intergenic region

Primer name / position / orientation[a]	**Primer sequence (5′ to 3′)**	**Length of amplified product (bps)**	**Amplified region**
BDV 1 f	GTTGCGTTAACAACMAACCA		
BDV 652 r	TGGCCGTTAATCCAATCTAT	652	p40
BDV 218 f	GAACGCAGTGGCATTGTTAG		
BDV 796 r	CAYTCTGCGAGGTACTCCTT	579	p40
BDV 587 f	TGGTGAGACTGCTACACTAC		
BDV 1161 r	TTAGACCAGTCACACCTATC	575	p40
BDV 738 f	GTCGCAAGTTATGCRCAGATG		p40, IGR,
BDV 1274 r	CATTGAGCCTCCTGACTARTT	537	p10, p24
BDV 778 f	AGGAGTACCTCGCAGAATG		p40, IGR,
BDV 1518 r	CCAGCTCCGTCACTARCTT	741	p10, p24
BDV 1327 f	AGACACTACGACGGGAACGA		
BDV 1837 r	TGGGAGCTGGGGATAAATGC	511	p10, p24
BDV 1695 f	GATCGCTCCATGAAGACAAT		
BDV 2138 r	GAAGTCGTCAATCTGGAAGT	444	p24

Detection of BDV RNA by RT-PCR. Copy DNA (cDNA) synthesis and PCR were carried out in a single step using a commercially available Kit (OneStep RT-PCR Kit, QIAGEN, Valencia, USA) according to the manufacturer's recommendations. The annealing temperature of 60°C was employed for 45 PCR

cycles. Each reaction contained 0, 8 µM (final concentration) of each of the primers and 2.5 µl volume of RNA extract (corresponding to 10% of the total reaction volume). Additionally 4 units Rnasin ribonuclease inhibitor (Promega) were used. All amplifications were performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer) or in a Mastercycler gradient (Eppendorf).

Following gel electrophoresis and ethidium bromide staining (Fig. 1 and 2), two different methods were employed for the purification of the amplification products: when after gel electrophoresis one specific band of the expected size was visible, DNA was extracted directly from the PCR product using PCR Kleen Spin Columns (BIO-RAD) according to the manufacturer's instructions. If necessary, bands visualised under UV-light were excised from the gel and DNA was extracted from the gel slices using the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocol.

Sequencing and sequence analysis. The ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, United Kingdom) diluted in an ABI PRISM® BigDye™ 5× Sequencing Buffer (Applied Biosystems, United Kingdom) in the ratio 2 : 3 was employed for sequencing PCR. The primer concentration used for this reaction was 4 pmol/20 µl. For removal of unincorporated dye terminators directly from sequencing reactions the spin columns of the DyeEX™ 2.0 Spin Kit (QIAGEN) were used. Clean reaction products purified by one of these methods were sequenced in both directions employing the automatic sequencing system ABI Prism 310 genetic analyzer (Perkin Elmer).

The obtained BDV sequences were aligned using the Align Plus program (Scientific & Educational Software, version 3.0, serial no. 43071), and their genetic identity was compared with the sequences of seven published BDV laboratory strains (Table 2).

Phylogenetic studies. Two phylogenetic trees were constructed of each protein (p40, p10 and p24) as well as of the intergenic region, with and without the variant strain No/98 (acc. no. AJ311524), respectively. As the sequence of the intergenic region of strain RW98 was not available in the GenBank database and therefore could not be included in the phylogenetic analyses, the tree of this region is not shown.

Phylogenetic studies were performed using the Phylogeny Inference Program package, PHYLIP (Felsenstein, J. 1993. PHYLIP, version 3,57c. Distributed by the author. University of Washington, Seattle).

The stability of the trees was tested by bootstrap resampling analysis of 100 replicates computed with the SEQBOOT program. Genetic distances between each pair of sequences were calculated using the DNADIST (for nucleotide sequences) and PROTDIST (for amino acid sequences) programs based on the Kimura two-parameter model with a transition/ transversion ratio of 2. From these distance matrices the phylogenetic trees were generated by the neighbour-joining method of the NEIGHBOR program and the best tree was displayed by the program DRAWGRAM. An isolate from Switzerland named S 96 1202 (GenBank accession no. AY374541) was used as outgroup.

RESULTS

Amplification results

The two primer pairs employed for BDV screening were able to detect classical BDV strains as well as the highly variable strain No/98 (Fig. 6).

The seven primer pairs designed for this study and amplifying the complete p40, p10 and p24 genes of each BDV sample yielded DNA amplicons of the expected sizes (Fig. 7). In total, a 1824 bp long fragment corresponding to 20,5% of the whole BDV genome was analysed.

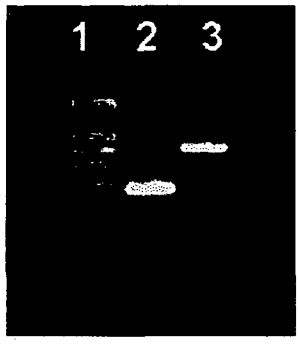

FIGURE 6. Agarose gel electrophoresis of RT-PCR products obtained with two primer pairs, which proved best for the detection of classical BDV and a highly variable strain, and which were used for screening PCR.

Lane 1, DNA Size Standard (100 bp, BioRad); lane 2, PCR product obtained with primers published by Sorg and Metzler (65) amplifying a 270 bp fragment of the viral p 40 protein gene; lane 3, PCR product of 511 bp obtained with primers amplifying a segment of the p24 protein gene (1327f/1837r).

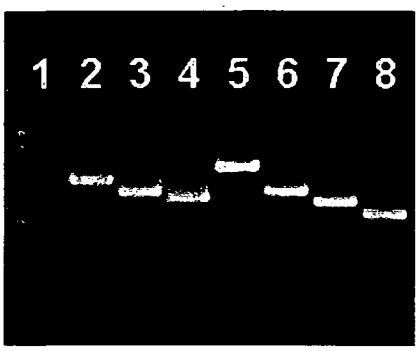

FIGURE 7. Electrophoretic profiles of DNA amplicons obtained by RT-PCR. The seven primer pairs used to amplify the complete p40, p10 and p24 genes of each positive BDV sample yielded DNA amplicons of the expected sizes.

Lane 1, DNA Size Standard (100 bp, BioRad); lane 2, primers 1f/652r (652 bp); lane 3, primers 218f/796r (579 bp); lane 4, primers 738f/1274r (537 bp); lane 5, primers 778f/1518r (741 bp); lane 6, 587f/1161r (575 bp); lane 7, primers 1327f/1837r (511 bp); lane 8, primers 1695f/2138r (444 bp).

Results of sequence analysis

Multi-way alignments of the forty BD field viruses (without strain No/98) and laboratory strains analysed showed overall nucleotide sequence identities of 96 - 99% in the p40, of 98 - 100% in the p10 and of 97 - 99% in the p24 regions. These data correspond to amino acid identities of 98 - 100% in the p40, of 96 - 100% in the p10, and of 98 - 100% in the p24 region. Strain No/98 occupies always a unique position (Fig. 8-10) and shows identities at the nucleotide level of 84 - 85%, 88 - 90% and 85 - 88% in the p40, p10 and p24 genes, respectively, corresponding to amino acid identities of 97 - 98%, 80 - 82% and 96 - 97% in the p40, p10 and p24 proteins, respectively, compared to other BDV strains.

Results of phylogenetic analysis

The samples investigated clearly segregated in two major clusters: German BD field viruses and laboratory strains on the one hand and Swiss/Liechtenstein BD isolates on the other (Fig. 8-12).

FIGURE 8. Phylogenetic tree of the complete p40 gene nucleic acid sequences of various BD viruses (thirty-three BDV samples from naturally infected animals, one vaccine strain and seven BDV laboratory strains, including the novel BDV subtype No/98). Analyses were performed using the PHYLIP phylogeny program, version 3,57c package (SEQBOOT, DNADIST, NEIGHBOR and CONSENSE). The tree was outgrouped to the swiss BDV sequence designated S 96 1202 (GenBank accession no. AY374541).

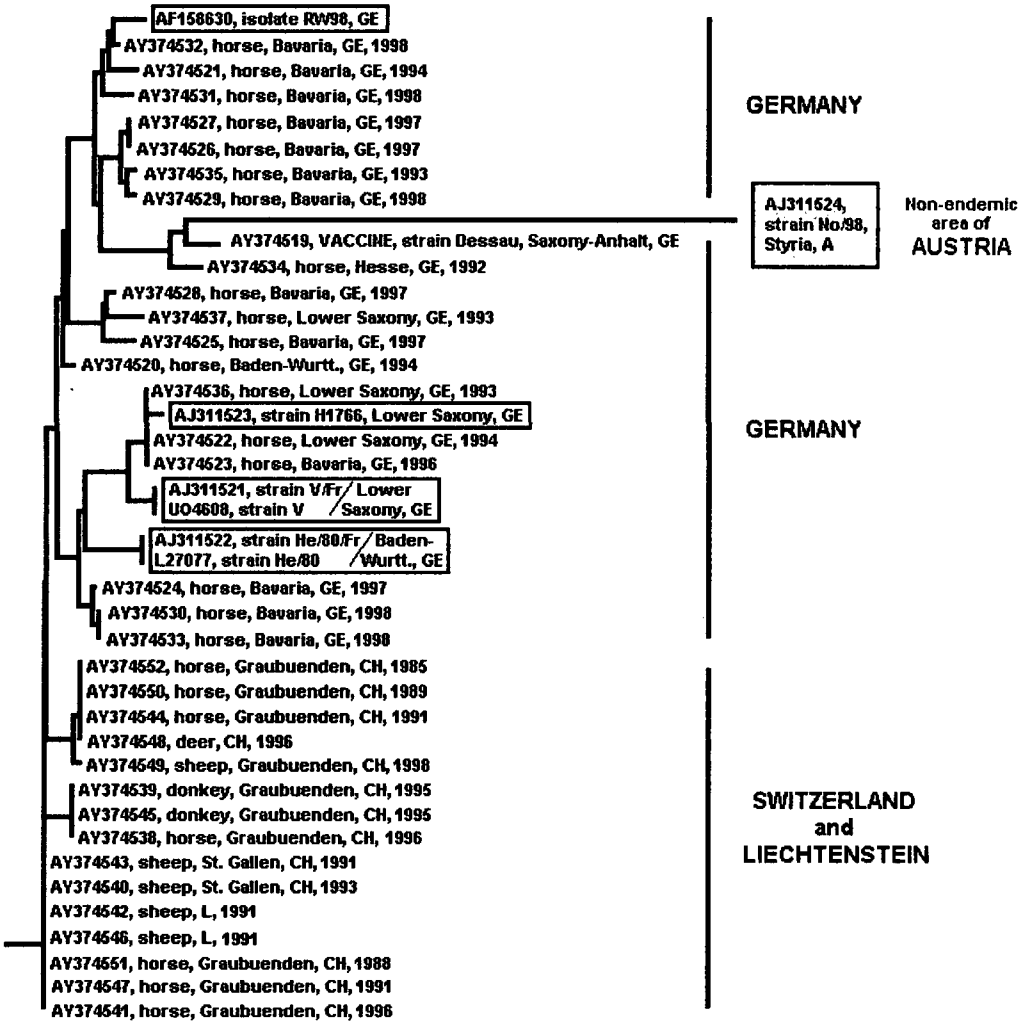

FIGURE 9. Phylogenetic tree of the complete p24 gene nucleic acid sequences of various BD viruses (thirty-three BDV samples from naturally infected animals, one vaccine strain and seven known BDV strains, including the variant strain No/98). The tree was constructed as described in FIGURE 8.

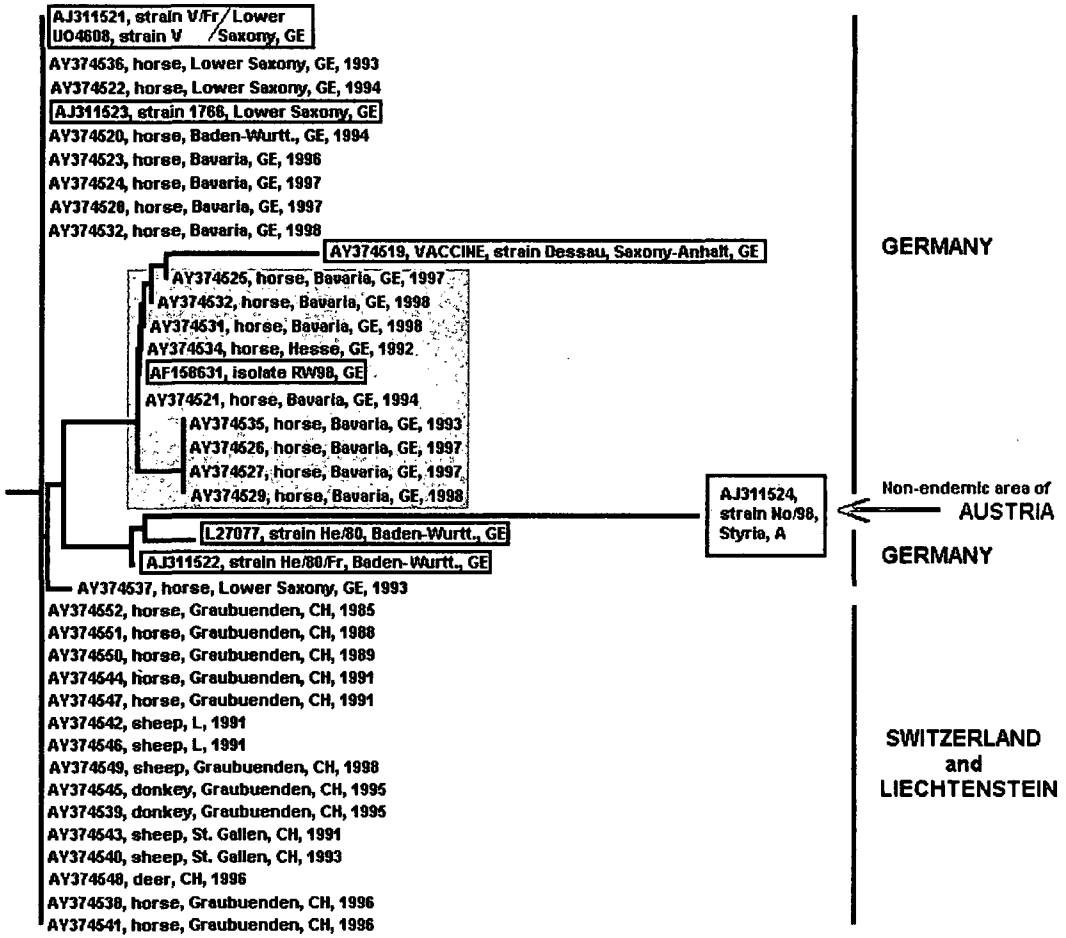

FIGURE 10. Phylogenetic tree of the complete p10 gene amino acid sequences of various BD viruses (thirty-three BDV samples from naturally infected animals, one vaccine strain and and seven BDV laboratory strains, including the novel BDV subtype No/98). The tree was constructed as described in FIGURE 8, except that the PROTDIST program was used (instead of DNADIST).

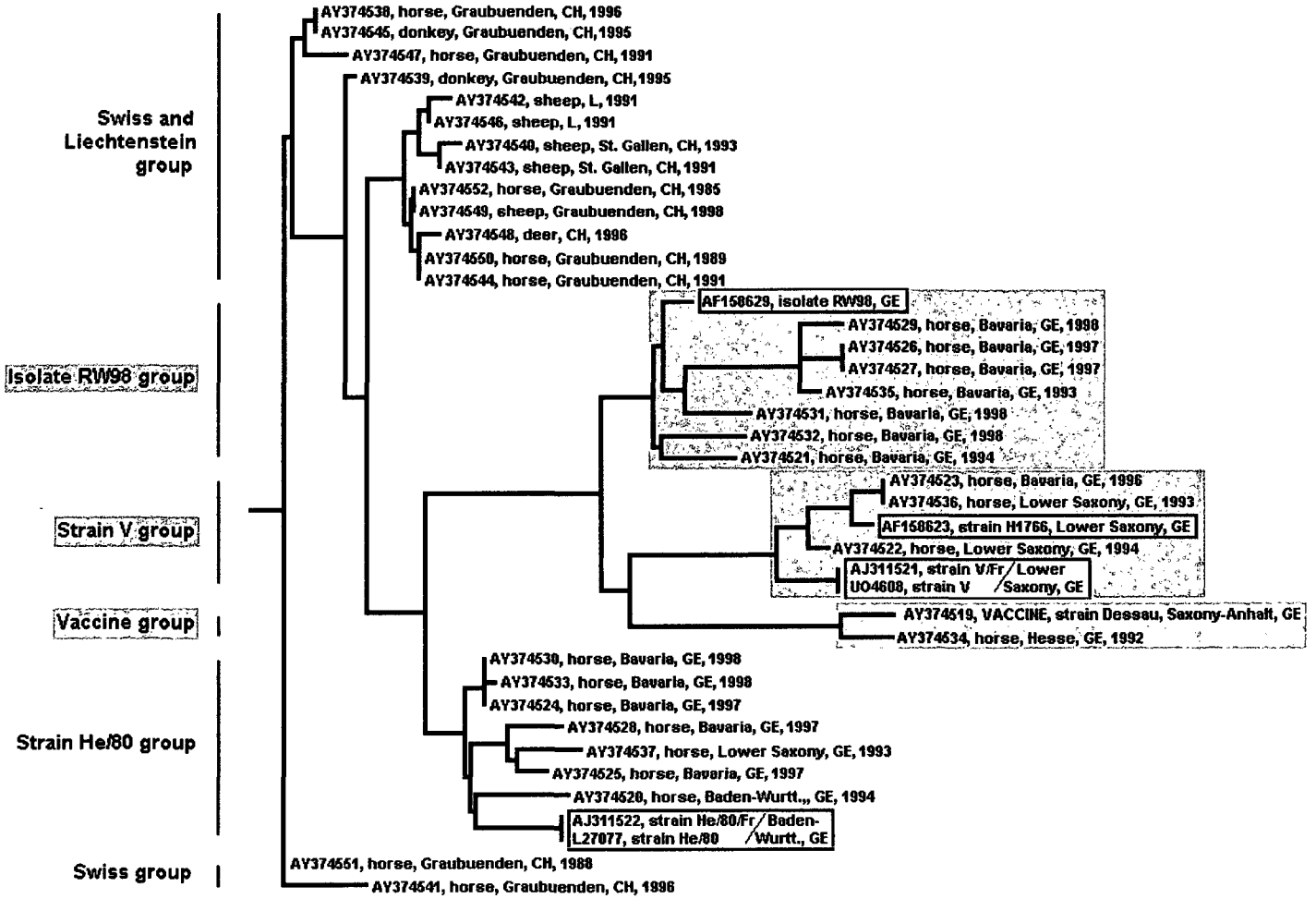

FIGURE 11. Phylogenetic tree of the complete p40 gene nucleic acid sequences of various BD viruses [thirty-three BDV specimens from naturally infected animals, one vaccine strain and six BDV laboratory strains, without the variant strain No/98 (in order to enhance the resolution of the phylogenetic analysis)]. The tree was constructed as described in FIGURE 8.

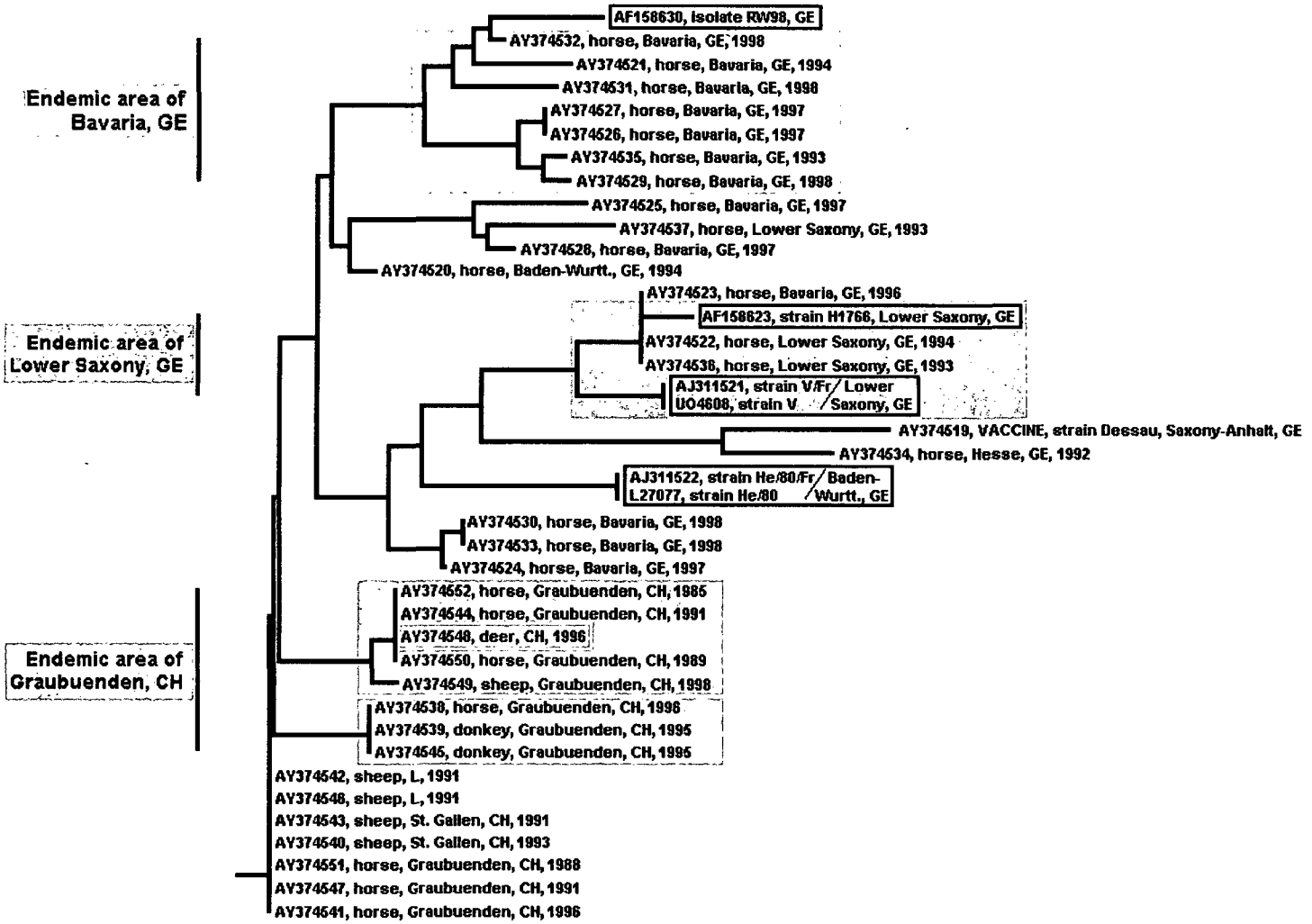

FIGURE 12. Phylogenetic tree of the complete p24 gene nucleic acid sequences of various BD viruses (thirty-three BDV specimens from naturally infected animals, one vaccine strain and six known BDV strains, without strain No/98). The tree was constructed as described in FIGURE 8.

Endemic areas and genetic clusters of German BD field viruses

German BD viruses and laboratory strains can be divided in the endemic areas of Bavaria, Lower Saxony and a third mixed area including parts of Bavaria, Baden-Wurttemberg, Lower Saxony and Hesse (Fig. 12).

Furthermore, phylogenetic analysis of the p40 gene indicated that German BDV isolates may to some extent be grouped around the three laboratory strains: V, He/80 and RW/98 (Fig. 8 and Fig. 11).

Strain V group. The majority of samples in this cluster originated from the endemic area of Lower Saxony independently of the year of isolation (1993 - 1996). The homologies of the field isolates in this cluster to strain V amounted to 98%, 98 - 100% and 98% for the nucleotide sequences of the p40, p10 and p24 regions, respectively, and to 100%, 97% and 99% for the corresponding amino acid sequences (Fig. 8 and Fig. 11).

He/80 group. In the genetic cluster of strain He/80, which itself originates from Baden-Wurttemberg, equine isolates from different German endemic areas are localised: Bavaria (five specimens), Lower Saxony (one specimen) and Baden-Wurttemberg (one specimen), isolated between 1993 and 1998 (Fig. 8 and Fig. 11). The identities of this cluster to strain He/80 amount to 98 - 100%, 97 - 99% and 97 - 98% for nucleotide sequences in the p40, p10 and p24 regions, respectively, and to 99 - 100%, 95 - 98% and 97 - 98% for the corresponding amino acid sequences.

RW98 group. RW98 is localised in a subgroup of samples collected without any exception in the endemic area of Bavaria between 1993 and 1998 (Fig. 8 - 12). Nucleotide identity rates in this group were 98 - 99% for p40, 99 - 100% for p10 and 97 - 99% for p24 region; amino acid identity rates were 99 - 100%, 98 - 100%, and 99%, respectively.

Endemic areas and genetic clusters of Swiss and Liechtenstein BDV field viruses

Two subgroups of Swiss and Liechtenstein BD field viruses were observed (Fig. 8-12). The first subgroup with two small clades incorporates 8 BD viruses originating from the Swiss endemic area of Graubuenden only, isolated between 1989 and 1998 from four horses and two donkeys such as from one sheep and one deer. The homologies within this clade are 98 - 100%, 100%, and 98 - 99% for the

nucleotide sequences of the p40, p10 and p24 regions, respectively, and 100% for all 3 corresponding amino acid sequences.

The second subgroup contains BD viruses of different host species (horse, sheep), year of isolation (1988-1996), but also of different geographic regions (Graubuenden, St. Gallen, Liechtenstein). Nucleotide identity rates in this group were 98 - 99% for p40, 99 - 100% for p10, and 99 - 100% for the p24 region; the amino acid sequences exhibited 99%, 100%, and 100% identity rates, respectively.

Phylogenetic analysis of BDV laboratory strains

Strain He/80/Fr is identical in the p40 gene with its ancestor **He/80** and differs in only 1 nucleotide in the p10 and p24 gene regions, leading to an amino acid sequence homology of 99%.

Strain V and V/Fr are 100 % identical in the p40, p10 and p24 genes, respectively.

Strain H1766. Comparative sequence analyses revealed only few differences between the original BD brain suspension and its corresponding laboratory strain H1766. In the p40 and p24 genes differences of 9 and 3 nucleotides, respectively (99% identity), did not lead to changes at the amino acid level (100% identity), the p10 genes were identical on both nucleotide and amino acid levels.

RW 98. Sequence alignments of RW98 and its believed ancestor He/80 revealed in the p40 gene 40, in the p10 gene 6 and in the p24 gene 20 nucleotide changes reflecting a homology of only 96%, 97% and 96%, and amino acid homologies of 98%, 95% and 96%, respectively.

Vaccine strain "Dessau" and natural cases of BD infection

Comparative sequence analysis of the live-attenuated vaccine strain „Dessau" (acc. nr. AY374519) with all BDV isolates originating from spontaneous natural cases of BD revealed one BDV isolate, which proved to be genetically closely related to the vaccine strain.

The intergenic region

The sequence of the intergenic region was not available for strain RW/98. Among the BD viruses sequenced in this region including strain strain No/98 nucleotide sequence identities varied between 96 and 100%.

DISCUSSION

Geographical regions and endemic areas of BDV field isolates.

The pairwise genetic distances calculated for all aligned sequences clearly show two major clusters of country-specific sequence diversity: German BD field viruses and laboratory strains on the one hand and Swiss/Liechtenstein BD viruses on the other hand (Fig. 8-12).

These two major geographical regions can be further divided into endemic areas as follows: German BD horse isolates and laboratory strains clearly segregate into the endemic areas of Bavaria, Lower Saxony, and a third mixed area incorporating parts of Bavaria, Baden-Wurttemberg, Lower Saxony and Hessen (Fig. 12). Swiss and Liechtenstein BD viruses can be separated into two regional subgroups: the endemic area of Graubuenden (Fig. 12) and a second area including parts of Graubuenden, St. Gallen and Liechtenstein. It is noteworthy that genetic clustering proved to be independent of the animal species (horse, sheep, donkey, deer), from which the isolate was derived, and the year, in which the virus was isolated (1985 – 1998).

Strain No/98, so far one single exception in the family *Bornaviridae*. Strain No/98 occupies always a unique position in all phylogenetic trees (Fig. 8-10).

The BDV strain No/98 was isolated in 19**98** from a pony stallion originating from the Austrian federal state of Styria, where previously no cases of BD had been recorded. This area is several hundred kilometers away from the Austrian BD endemic area in Vorarlberg. This strain was virologically characterized by **No**wotny and Kolodziejek: Using frozen brain material of the pony and applying an array of primer pairs, overlapping PCR products were generated and directly sequenced; by doing so, a large fragment of the No/98 genome, spanning almost half of the entire BDV genome, has been sequenced by the Vienna BDV research group (acc. no. AF136236) (44). It turned out that this strain exhibited only approx. 85% nucleotide identity to the other known BDV strains, thus representing a novel BDV genotype. The virus was isolated in primary young rabbit brain cells, and co-cultivated with Vero cells; these investigations have been carried out jointly in Freiburg and in Vienna. Subsequently, the entire No/98 genome was sequenced by the Vienna BDV group and confirmed by the Freiburg group (acc. no AJ311524) (52). While sequence

homologies among common laboratory strains amount to more than 95%, the overall sequence identities between No/98 and other BDV reference strains are only about 85%. Surprisingly the nucleotide exchanges have little effect on most of the viral protein structures with the exception of the X protein (p10), which is only 81% identical to its counterpart in reference strains. Although the No/98 sequence is strictly colinear with that of other reference strains it contains a unique three-nucleotide deletion in the N gene and a single nucleotide deletion as well as a three-nucleotide insertion located in the first intergenic region between the N and X genes. Therefore, the genome of strain No/98 consists of 8911 nt, whereas the genome of other laboratory strains contains 8912 nt. Also, the L protein of No/98 differs at 7% of its amino acid positions from the polymerase in the other BDV strains (44, 52). Because of these unique characteristics it was suggested to classify the BDV strain No/98 as a novel BDV subtype.

Genetic clustering of German BDV field and laboratory strains as well as of vaccine strain Dessau

Phylogenetic analysis of the p40 gene indicated that German BDV isolates may to some extent be grouped around the three laboratory strains: V, He/80 and RW/98 (Fig. 8 and Fig. 11).

Strain V group. The oldest BDV strain V (accession no. UO4608), which is still used today, was derived at the end of the 1920s from a diseased horse after several passages in rabbits by Zwick and his co-workers in Giessen. The isolate was used for several experiments to induce the disease in rabbits and rats. It served as vaccine for active immunization of horses and sheep in Germany for half a century (17, 18, 19, 38).

In Berlin, strain V served as reference strain in the laboratory of Ludwig at the Free University Berlin. RNA from this material allowed establishing the first sequence of Borna disease virus in the laboratory of Lipkin in the United States in 1994 (acc. no. UO4608 (10, 18). Seven years later Schwemmle and colleagues from the Freiburg BDV research group sequenced the whole genome of strain V again and designated this new sequence as strain V/Fr (acc. no. AJ311521) (52).

The origin of the oldest BDV strain V could be confirmed, the majority of isolates in this cluster could be allocated to the endemic area of Lower Saxony independently of the year of isolation (1993 - 1996) (Fig. 8 and Fig. 11).

He/80 group. The abbreviation He/80 stands for strain **Her**zog/80. The virus was isolated in 19**80** from a horse in Baden-Wurttemberg, Germany (horse 7) and identified by cell culture isolation and by histological and immunohistological examination of the brain. The isolate was first mentioned in a study by Narayan at al. as He/80 (40 and personal communication Sibylle Herzog). The virus was grown in Madin Darby Canine Kidney cells (MDCK) by Herzog and Rott and then continuously used by researchers in Giessen and in Narayan`s and Cabone`s laboratories in Baltimore, USA, from there going to La Jolla, USA (18). The complete nucleotide sequence and genome organization of this BDV strain had been determined by de la Torre´s research group in 1994 (acc. no. L27077) (13) and the sequencing was repeated by Schwemmle in 2001. This new He/80 BDV sequence was named He/80/Fr (acc. no. AJ311522) (52).

In the genetic cluster of strain He/80, which itself originates from Baden-Wurttemberg, equine isolates from different German endemic areas are localised: Bavaria (five specimens), Lower Saxony (one specimen) and Baden-Wurttemberg (one specimen), isolated between 1993 and 1998 (Fig. 8 and Fig. 11).

RW98 group. The controversially discussed strain RW98 (acc. no. AF158629-158631), previously described as a possible new BDV strain isolated from the blood of a psychiatric patient (49) has in the following been interpreted as the fourth rat passage of Giessen strain He/80 and designated as BDV-4p, a derivate of He/80 (50). It seems that this strain was originally isolated from a horse, and propagated in rabbits, different cell lines, and passaged several times in newborn and in adult rat brains (also named rat-BDV, accession no. AJ250177-8). This strain was used worldwide in many laboratories, also in the Tuebingen laboratory (49, 50). However, phylogenetic analysis of several human and animal BDV sequences including strains RW98, He/80 and V performed by the Tuebingen group showed, that BDV-4p (RW98) did not cluster in the He/80 group (50). Sequencing data also revealed that BDV-4p was identical to RW98, but both viruses were different from He/80. Due to the long passage history of He/80, it was almost impossible to determine the reasons that led to the described sequence differences (50). The third passage of the Giessen strain He/80 in Lewis rats by intracerebral inoculation (named BDVRp3) was also used for investigations in Cubitt´s and de la Torre´s laboratories in 1994 but they did not sequence this strain (13).

The RW98 subgroup represents especially interesting aspects. RW98 is localised in a subgroup of samples collected without any exception in the endemic area of Bavaria between 1993 and 1998 (Fig. 8 - 12). This picture became even clearer when analysing p10 amino acid sequences (Fig. 10).
RW98 has been designated as forth passage of strain He/80 in rat brain cells (BDV4p), but our results suggest that RW98, which has been shown not to be of human origin, is much closer related to Bavarian field isolates than to He/80.

Evolutionary tracing and sequence variability of BDV laboratory strains

Strain He/80/Fr sequenced in 2001 is identical in the p40 gene with its ancestor He/80 (sequenced in 1994) and differs in only 1 nucleotide, which resulted in an amino acid change in the p10 and p24 gene regions (99% identity).
Strain V and V/Fr, as well sequenced in 1994 and 2001, respectively, are 100 % identical in all 3 genomic regions analysed.

To investigate the high stability of the BDV genome even after years and decades of passaging, we compared nucleotide and amino acid sequences of strain H1766 sequenced by Schwemmle in 2001 (acc. no. AJ311523) and of the original brain suspension of the horse from which the strain was isolated (originated from Lower Saxony, 1994; acc. no. AY374536).
Strain H1766 was originally isolated in 1994 from brain material of a horse from Lower Saxony, Germany. One aliquot of this original brain suspension was stored and made available for this study (acc. no. AY374536). By co-cultivation of this isolate in MDCK for years a new BDV laboratory strain, strain H1766, also known as BDV-MDCK, had emerged, which is up to date frequently used for experiments in Japanese laboratories (29, 52 and personal communication S. Herzog). The complete genome sequence of this BDV isolate was determined by Schwemmle and colleagues in 2001 (acc. no. AJ311523) (52).

Comparative sequence analyses revealed only few differences between the original BD sample and its corresponding laboratory strain H1766. In all three genomic regions analysed, the two strains were identical on the amino acid level and to 99 – 100% in their nucleotide sequence (12 nucleotide changes). In the phylogenetic trees, both BD viruses are located close to each other (Fig. 8-12).

On the other hand, when comparing sequences of RW98 and its initially suggested ancestor He/80 we find that 66 (!) nucleotide changes reflect an unusually

low homology of only 96 - 97% and amino acid homologies of 95 - 98%, respectively. Sequence analysis of the other laboratory strains (as mentioned above) do point towards the fact that such frequent nucleotide changes are more than unusual for BDV strains, even after several years of in vitro or in vivo passaging. We therefore suggest as more probable explanation that RW98 has not been derived from He/80 but from a Bavarian field isolate, which by accident has been established to a laboratory strain.

Possible involvement of Vaccine strain "Dessau" in German BD cases?

Strain V, also named rabbit-adapted vaccine strain (18), was used as vaccine for active immunization of horses and sheep in Germany for half a century (17, 18, 19, 38). Since inactivated brain suspensions of experimentally infected animals first used in challenge experiments showed no protective effects, two live BDV vaccines were employed, the socalled "Zwick-vaccine" and the "Dessau-vaccine" (28).The Dessau-vaccine (charge no. 198 10 90) was included in this study. It had originally been derived from an equine isolate (Saxony-Anhalt, 1960) (personal communication R. Dürrwald), was subsequently adapted to rabbits and had been in use as an attenuated live vaccine until 1992 in some parts of Germany. Because its efficacy was questionable, the use of this vaccine was discontinued around 1980 in West Germany and a few years later in East Germany (66). Since it had been suggested, that these BDV live-virus vaccines may have considerable effects on the prevalence of BDV infection in natural animal hosts, the vaccination was consequentially stopped by the end of June 1992.

Comparative sequence analysis of the live-attenuated vaccine strain „Dessau" (acc. nr. AY374519) with all BDV isolates originating from spontaneous natural cases of BD revealed only one BDV isolate, which proved to be genetically closely related to the vaccine strain. All other field isolates were rather unrelated to the vaccine strain (Fig. 8-12). The closely related field isolate with the number H446 originated from a BD-endemic area in Germany, Hesse; the horse, harbouring this virus, was euthanized in July 1992, shortly after vaccinations had been stopped.

These data sustain the possibility, that the vaccine strain had been involved in certain natural cases of BD in Germany, however, not at all at such a high incidence, as suggested by other investigators.

The intergenic region

In respect to a unique insertion as well as deletion in the intergenic region between the p40 and p10 genes of strain No/98, we included this part of the genome in our analysis (data not shown). Unfortunately, the sequence of this intergenic region was not available for strain RW/98. Among the BD viruses sequenced in this region including strain strain No/98 nucleotide sequence identities varied between 96 and 100%.

Further work should focus on the detection of BD in non-endemic areas because such BD viruses may have a variant genome, as we could demonstrate for the isolate No/98. Also, we intend to determine the complete nucleotide sequence of the vaccine strain "Dessau", which may help us to get a better understanding of the complex relationship between the vaccine and naturally occurring BD viruses. Another future goal would be to investigate why BDV has - although being an RNA virus - such an incredible conserved genome. And finally, extensive and detailed studies should be carried out to identify the natural animal host(s) for BD. Of course, the question of human BDV infection, its possible link to psychiatric disorders, and the way human beings may be infected (is BDV a zoonotic agent?) has to be investigated thoroughly, too.

Nucleotide and protein sequence accession numbers. The nucleotide and protein sequences described in this paper were submitted to the GenBank database under accession numbers AY374519-AY374552 (listed in Table 2).

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II.

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	12. / 13. 11. 2000	Teilnahme am Kongress „PCR-Methoden und Anwendungen" an der Justus-Liebig-Universität in Gießen / Deutschland
	08. 10. 2001	Teilnahme am PCR-Seminar von Applied Biosystems
	WS 2000	Inskription des Doktoratsstudiums Chemie an der Technischen Universität Wien
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Wissenschaftliche Publikationen, Vorträge und Posterpräsentationen

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III.

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1. Weissenböck, H., Nowotny, N., Caplazi, P., Kolodziejek, J., Ehrensperger, F. (1998): Borna disease in a dog with lethal meningoencephalitis. **J. Clin. Microbiol. 36**, 2127-2130.

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ERLÄUTERUNG DES ARBEITSANTEILS IN DEN EINZELNEN PUBLIKATIONEN

Publ. Nr. 1:

1. Isolierung von RNA aus 5 formalin-fixierten, in Paraffin eingebetteten Geweben (PET)
2. Durchführung der RT-PCR
3. Sequenzierung der PCR-Produkte
4. Vergleichs-Analysen der erhaltenen Nukleinsäuresequenzen miteinander und mit den entsprechenden schon in der GenBank existierenden BDV-Sequenzen mit Hilfe des Align-Programmes
5. Feststellung und Diskussion des Identitätsgrades
6. Beschreibung der Methodik

Publ. Nr. 2:

1. Durchführung des gesamten praktischen Teiles der Studie (phylogenetische Analyse der Nukleinsäure-Sequenzen aus der Publ. Nr. 3 unter Inkludierung von in der GenBank vorhandenen BDV-Sequenzen mittels des PHYLIP-Phylogenie-Programms)
2. Beitrag zur Diskussion

Publ. Nr. 3:

1. Durchführung des gesamten praktischen Teiles der Studie wie Isolierung der viralen RNA aus der menschlichen Blutprobe unter S3 Sicherheits-Bedingungen, Durchführung der RT-PCR und Sequenzierung der PCR-Produkte
2. Einfügung der erhaltenen Nukleinsäure-Sequenzen in die GenBank
3. Beschreibung der Methodik

Publ. Nr. 4:

1. Aufbereitung der Probe (No/98)
2. Isolierung der RNA
3. Design (mit Hilfe des Primer-design-Programms) und Austestung einer großen Anzahl von Primerpaaren
4. Sequenzierung des 4210 bp langen BDV Genom-Stückes (47,2% des gesamten Genoms)
5. Durchführung der vergleichenden Sensitivitätsuntersuchungen (unter Verwendung konventioneller und modifizierter RT- und nested PCR-Primer)
6. Beschreibung der Methodik
7. Einreichung der erhaltenen Sequenz in die GenBank

Publ. Nr. 5:

1. Design (mittels des Primer-design-Programms) und Austestung einer großen Anzahl von Oligonukleotiden zur Amplifizierung der zweiten Hälfte des Genoms des BDV-Stammes No/98
2. Sequenzierung der zweiten Hälfte des No/98-Genoms
3. Einfügung der erhaltenen Sequenz in die GenBank
4. Beitrag für die Diskussion

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Borna Disease in a Dog with Lethal Meningoencephalitis

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A dog was euthanatized because of progressive neurological signs. Histologically, a nonsuppurative meningoencephalitis was found. By immunohistochemistry, in situ hybridization, and nested PCR procedures, Borna disease virus (BDV) antigen and BDV-specific RNA were demonstrated in brain tissues of the dog. The nucleotide sequence of the PCR product showed 94 to 98% homology to published BDV sequences. This is the first description of Borna disease in a dog.

Borna disease (BD) is a fatal disease of the central nervous system that was originally recognized in horses and sheep in certain areas of Germany (6). Subsequently, naturally occurring BD has also been diagnosed in other animal species, such as rabbits, cattle, and certain zoo animals (2, 4, 11, 21). Cases of spontaneous BD have been found only in Germany (6), Switzerland (12), Liechtenstein (unpublished data), and, recently, Austria (24). A condition discovered in cats of Sweden and Austria, known as feline nonsuppurative meningoencephalitis or staggering disease, and a paretic syndrome in ostriches of Israel have also been linked to an infection with Borna disease virus (BDV) (9, 10, 15). Furthermore, BDV antibodies and BDV nucleic acid sequences have been detected in specimens from clinically healthy horses (7, 13) and cats (14) in the United States and Japan, where classical BD has never been seen. BD has become of increasing interest to human medicine since BDV-specific antibodies, antigen, and RNA were found in humans with certain mental disorders (3, 5, 16, 20) but also in individuals without neurological disease (1). The relevance of these findings is controversially discussed (17), and formal proof of the causative role of BDV in psychiatric diseases is still lacking. Another important but still unsolved question is whether BDV can be transmitted from animals to humans; this question would be of particular interest if BDV infections were to be demonstrated in pet animal species. In addition, BDV infection achieved significance in biomedical research by serving as a model for immune-mediated disease of the central nervous system (23).

Dogs have never been suspected as being possible host species of BDV infection, and neither natural nor experimental BD has ever been described in the dog to date (19). Also, there is no report of serological surveys of BDV infection in canines. This communication reports the first known case of naturally occurring BD in a dog. During a retrospective study of etiologically unclear cases of nonsuppurative encephalitis in dogs, we came upon a case from September 1994. A 2-year-old female husky from Vorarlberg, the most western federal state of Austria, was presented to a veterinarian with a clinical history of anorexia and lethargy. After 2 days of unsuccessful treatment of symptoms with electrolytes, glucose, vitamins, amino acids, a nonsteroidal antiphlogistic agent, and antibiotics, the dog developed severe central nervous signs and was euthanatized 1 day later because of clinical signs suspicious of rabies or canine distemper. A neuropathological examination revealed a severe nonsuppurative meningoencephalitis that was characterized by the following: lymphocytic meningitis (Fig. 1A); perivascular lymphomonocytic cuffs (Fig. 1B); neuronal necrosis in the neocortex, allocortex, and hippocampus; subpial endothelial cell swelling (Fig. 1A); and focal gliosis. The lesions were most severe in the piriform lobe, rostral neocortex, periventricular gray matter, hippocampus, and mesencephalon, while minor changes were found in the occipital neocortex, medulla oblongata, and cerebellum. Some neurons contained single or several eosinophilic intranuclear Joest-Degen inclusion bodies (Fig. 1B, inset), which are considered characteristic of BD. In Vorarlberg, BD had already been diagnosed in horses, and this federal state is in close proximity to the area in eastern Switzerland in which BD is endemic. However, the histological lesions of the dog, in particular the widespread neuronal necroses and the swelling of endothelial cells, do not fully correspond with the changes associated with BD seen in horses; in the latter, large perivascular cuffs with infiltration of the inflammatory cells into the adjacent neuroparenchyma, without significant neuronal changes, are conspicuous. It may be assumed that the changes seen in the dog were due to ischemia of the cerebral cortex, perhaps as a result of seizures, rather than to primary viral effects.

The histological diagnosis was confirmed by immunohistochemistry (IHC), in situ hybridization (ISH), and nested PCR procedures and by sequencing of the PCR product. Serum or cerebrospinal fluid (CSF) was not available for this retrospectively diagnosed case; therefore, it was not possible to determine BDV antibodies.

For IHC, we used an avidin-biotin complex technique and monoclonal antibody BO 18, directed against the 38/40-kDa protein (p38/40) of BDV, as the primary antibody (18). Brain tissue samples from two different horses with BD were taken as positive controls, while brain tissue samples from a normal dog and from various dogs with different nonsuppurative encephalitides (i.e., rabies, canine distemper, tick-borne encephalitis, and etiologically unresolved cases) served as negative controls. Another negative control was carried out by replacing the first antibody with a monoclonal antibody directed against bovine viral diarrhea virus. In brain tissue of the reported dog, strong immunoreactivity was confined to areas with severe encephalitis: the piriform lobe, the medio- and laterobasal areas of the neocortex, the basal ganglia, the hippocampus, the basal area of the thalamus, the hypothalamus, and the mesencephalon. In

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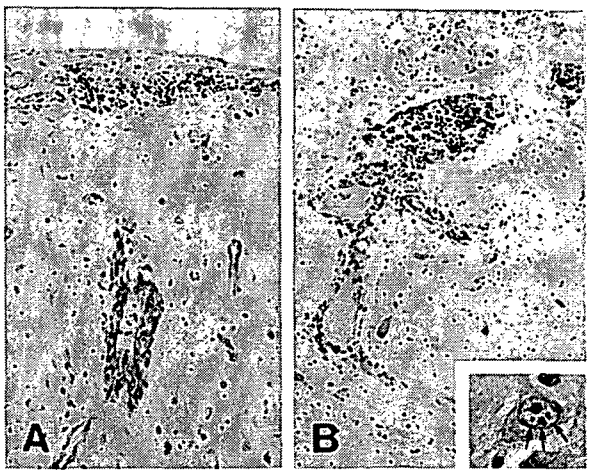

FIG. 1. (A) Cresyl echt violet staining of neocortex tissue, showing lymphocytic meningitis and endothelial cell swelling of a subpial blood vessel. Magnification, ×110. (B) Cresyl echt violet staining of thalamus tissue, showing lymphomonocytic perivascular cuffing. Magnification, ×110. The inset shows hematoxylin- and eosin-stained thalamus tissue, with the arrows indicating intranuclear Joest-Degen inclusion bodies in a neuron (magnification, ×510).

these regions, there was diffuse immunostaining of the neuropil and white matter but also of the nuclei, perikarya, and neurites of many neurons, astrocytes, and oligodendrocytes (Fig. 2A to D). The immunoreactivity in the remaining brain regions (the upper portions of the neocortex and thalamus, as well as pons, medulla oblongata, and cerebellum), which also displayed minor inflammatory changes, was less diffuse and restricted to groups of neurons and glial cells. Diffuse labelling of the neuropil was rarely found, and it was only focally present in these areas. This pattern of immunostaining was largely consistent with that of tissues from horses with BD examined with the same antibody (24).

ISH was carried out on a paraffin-embedded sample of mesencephalon, using a digoxigenin-labelled RNA probe specific for open reading frame (ORF) I of BDV. The probe was obtained by runoff synthesis after subcloning of the BDV ORF I cDNA into a transcription vector (pGEM 3z; Promega, Wallisellen, Switzerland) containing the viral RNA polymerase promoters T7 and Sp6. Briefly, the conditions for ISH were as follows: pretreatment with 0.1 N HCl for 10 min at room temperature, digestion with 0.1% (wt/vol) pepsin in 0.1% HCl at 37°C for 20 min, hybridization with partially hydrolyzed probe at 50°C overnight, and washing with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) at room temperature (twice, for 15 min each time) and with 0.1× SSC at 50°C (twice, for 20 min each time). Specific hybrids were detected by standard IHC using alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer, Rotkreuz, Switzerland) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate as the chromogen.

ISH revealed intense dark-blue cytoplasmic staining of numerous neurons (Fig. 2E) and astrocytes. Such a reaction was absent from the same tissue sample when it was hybridized with an irrelevant probe (Fig. 2F), and it was also not evident in tissue samples from uninfected animals when the specific probe was used.

To detect BDV-specific RNA, we applied a nested PCR assay to formalin-fixed, paraffin-embedded brain tissue specimens from the dog, since there was no frozen material available. RNA extraction was performed according to the method described by Sorg and Metzler (22); in order to extract as much nucleic acid as possible, proteinase K digestion was extended to 5 days at 37°C. For cDNA synthesis and the first PCR, the Titan One Tube reverse transcription-PCR system (Boehringer, Mannheim, Germany) was used. The primers for PCR and nested PCR as well as the conditions for the nested PCR were the same as those described by Sorg and Metzler (22), yielding a 212-bp PCR product within the ORF encoding p38/40. Formalin-fixed, paraffin-embedded brain tissue specimens from a horse from Vorarlberg and a horse from Bavaria, both with confirmed cases of BD, served as positive controls, and formalin-fixed, paraffin-embedded brain tissue specimens from dogs and horses without neurological diseases served as negative controls. Subsequently, the PCR products were sequenced in both directions by using an ABI Prism 310 genetic analyzer (Perkin-Elmer, Norwalk, Conn.), and the sequences were compared with already published sequences. For the suspected canine case, as well as the horses with BD from Vorarlberg and Bavaria, nested PCR resulted in PCR products of the expected size of 212 bp. In contrast, PCR of the brain tissue specimens from the dogs and horses which died due to other etiologies proved to be negative. Automated DNA sequencing of the amplicons showed a high degree of homology between dog and horse sequences from the same area (only two single-base changes; 99% homology at the nucleotide level and 100% identity at the amino acid level), although the canine case was from 1994 and the equine case was from 1997; the sequence of the Bavarian horse showed 97% nucleotide homology and 100% amino acid identity to the BDV sequence from the canine host. Comparison with sequences deposited in the GenBank database revealed nucleotide homologies of between 94 and 98%; the corresponding amino acid identities were between 97 and 100%. Although we have sequenced only a small fragment, it seems likely that the same virus strain is responsible for the BD cases in the dog and in horses in this area.

The reports of a tentative BDV infection in another carnivore—staggering disease in cats—differ significantly from the observations in this canine case. The cats did not originate from areas in which BD is endemic, and did not show the typical signs of classic BD, and only single positive cells were detected by IHC, which is not consistent with the huge amount of antigen found in horses and the present canine case (8). Taken together, the results of neuropathology, IHC, ISH, PCR, and sequencing studies provide evidence that the reported case is the first confirmed case of BD in a dog, caused by a BDV strain that is endemic to western Austria.

It is presently unknown whether this was just an exceptional single case or whether BD is more widespread in dogs. Paraffin blocks of specimens from 14 other dogs with nonsuppurative encephalitis of unresolved etiology were also examined immunohistologically for BDV antigen, and all proved to be negative. No serum or CSF was available for any of these animals. To gain additional insight into the prevalence of BDV infection in dogs, an epidemiological survey is being carried out in the federal state of Vorarlberg. Also, as a consequence of this case, BD has to be taken into account as a potential differential diagnosis in dogs with symptoms of neurological disease, and dogs with nonsuppurative encephalitis of unknown etiology should be checked for the presence of BDV antigen or nucleic acid, at least in endemic areas.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this article have been deposited in the GenBank database under accession no. AF054275 (BDV, dog, Vorarlberg), AF054276 (BDV, horse, Vorarlberg), and AF054277 (BDV, horse, Bavaria).

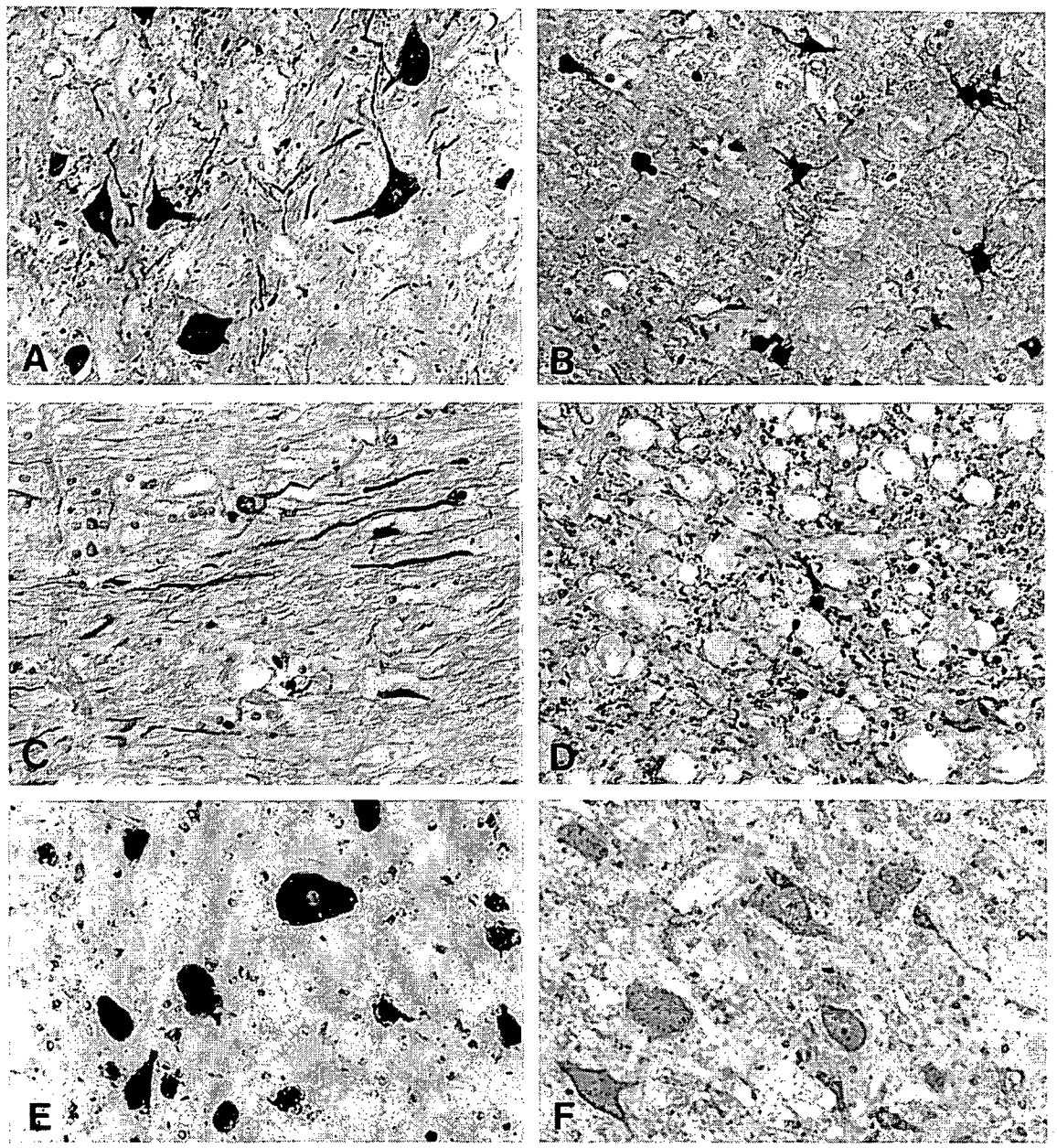

FIG. 2. Use of the avidin-biotin complex technique to show specific labelling of neurons (A), astrocytes (B), neurites (C), and white matter (D) with monoclonal antibody BO 18, which specifically recognizes BDV p38/40. Magnification, ×350. (E) In situ hybridization with an RNA probe specific for BDV ORF I reveals that positive signals are predominantly in the cytoplasm of neurons. Magnification, ×570. (F) In situ hybridization with an RNA probe specific for bovine herpesvirus 1 shows the absence of specific signal. Magnification, ×570.

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Human bornaviruses and laboratory strains

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Human bornaviruses and laboratory strains

Sir—Schwemmle and colleagues[1] give convincing evidence that nearly all published human borna-disease-virus (BDV) isolates and PCR amplification products seem to be contaminations of laboratory strains. We agree that the isolation of BDV from human blood or evidence that BDV nucleic acid has been found in human blood is highly controversial.

BDV is a neurotropic virus, and it is not clear as yet, even in BDV-infected animals, whether there is a period of viraemia during the course of infection, during which it would be possible to detect BDV or BDV nucleic acid in blood. There is the possibility that following nasal uptake of BDV, the virus does not only infect nerve cells, and proceeds via the olfactory bulb to the brain, but also gets into a few immune cells in the nasal mucosa. Thus, during the early stage of infection, and maybe during further acute periods, BDV may be present in blood.

There is clear serological evidence for the existence of BDV (or a closely related virus) in human beings. Nearly all laboratories investigating BDV worldwide have found antibodies to BDV or BDV proteins in psychiatric patients and also—at a lower prevalence—in healthy individuals.

There is a fundamental difference between BDV infection in naturally infected animals and human beings. BDV infection in animals leads to a severe and often lethal meningo-encephalomyelitis, but such inflammation has never been seen in human beings. In human beings only subtle changes are suspected and these may lead to psychiatric disorders. Also, in animals that succumb to BDV infection, BDV, BDV antigen, and nucleic acid can easily be detected using standard techniques including non-nested PCR. In human blood, BDV nucleic acid is only detectable with nested PCR, which has a high chance of becoming contaminated.

We have detected BDV nucleic acid sequences, amplified from peripheral blood mononuclear cells, of a patient with chronic fatigue syndrome (CFS).[2] The nucleotide sequences from this patient are unique, showing several nucleotide changes that have not been seen in any other BDV. Also, the sequences found are not related to the sequences of any laboratory strain or BDV isolate handled in our laboratory. We constructed a phylogenetic tree (see http://www.thelancet.com) of the nucleotide sequences of the N and P proteins of BDV and included all the sequences that Schwemmle and colleagues used and added the sequences of the patients with CFS (GenBank accession numbers AF094477 [N protein] and AFO94478 [P protein], respectively). The sequences do not group together with any laboratory strain in the N protein region, and show a significant distance from strain He/80 in the P protein gene region. Schwemmle and colleagues have not included our sequences in their analyses, although the sequences were already available in the GenBank database at the time when they prepared their manuscript.

BDV has been seen as genetically invariable and to form a single type. Consequently, all assays for the diagnosis of BDV are based on this single type. However, as we have shown,[3] other subtypes may exist and most of the standard reverse transcriptase-PCR protocols used to date for the diagnosis of human and animal BDV would fail to detect such a novel strain.

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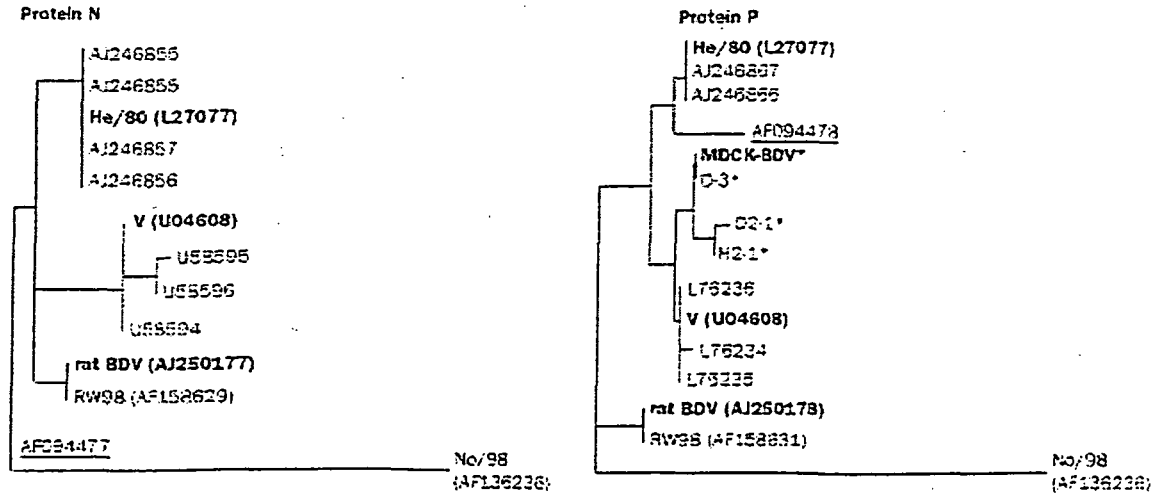

Phylogenetic tree of the nucleotide sequences of the N and P proteins of BDV

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Demonstration of Borna Disease Virus Nucleic Acid in a Patient with Chronic Fatigue Syndrome

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CORRESPONDENCE

Demonstration of Borna Disease Virus Nucleic Acid in a Patient with Chronic Fatigue Syndrome

To the Editor—Czygan et al. [1] reported the detection of Borna disease virus (BDV) nucleic acid in 3 cases of a rare form of hippocampal degeneration, whereas the brains of patients with other neuropsychiatric disorders tested negative for BDV. Chronic fatigue syndrome (CFS) is another, more frequently diagnosed neuropsychiatric disease that is associated with BDV infection. However, the published findings are highly controversial. Nakaya et al. [2, 3] and Kitani et al. [4] showed both BDV-specific antibodies and RNA in a high percentage of Japanese patients with CFS. Bode et al. [5] isolated BDV from peripheral blood mononuclear cells (PBMC) of an American patient with CFS; however, in an earlier publication, Bode et al. [6], as well as Evengård et al. [7] and Yamaguchi et al. [8] in recent publications, did not find serologic evidence for BDV in patients with CFS. A possible explanation for the controversial results is that the term "chronic fatigue syndrome" probably includes several similar clinical conditions that may have different etiologies. In the study by Czygan et al. [1], brain tissue samples from patients who had CFS were not included. Unfortunately, none of the BDV sequences of the CFS cases mentioned above are available in the GenBank database.

We report a 30-year-old man who was diagnosed with CFS. After a sudden onset 5 years ago of a disease with influenza-like symptoms, mild fever, myalgia, and muscle weakness, he had debilitating fatigue, impaired memory and concentration, and recurrent headache and was unable to work efficiently. Phases of temporary recovery alternated with relapses. During a more acute course of the disease, he saw a psychiatrist and had an EDTA blood draw. PBMC were separated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), aliquoted, and stored frozen.

To test for the presence of BDV nucleic acid, RNA was extracted by use of the QIAamp Viral RNA kit (Qiagen, Chatsworth, CA), and a 1-tube reverse-transcriptase (RT) polymerase chain reaction (PCR) system (Titan; Boehringer Mannheim/Roche Diagnostics, Mannheim, Germany) was used for reverse transcription and PCR. Subsequently, nested and seminested PCR assays resulted in 3 PCR products of 212, 691, and 724 bp in length (figure 1). To minimize possible PCR contamination, RNA extraction was done in a biosafety level 3 (P3) laboratory on a different floor, and RT-PCR and seminested and nested PCR were done in laboratories that had not previously handled BDV. Finally, the whole procedure was repeated with another aliquot of PBMC that had been stored frozen, with the same results. As a negative control, PBMC from a healthy person who was serologically and virologically negative for BDV was tested in parallel (figure 1). The PCR products were sequenced in both directions by direct automated sequencing. The sequences represent part of the p38/40 gene,

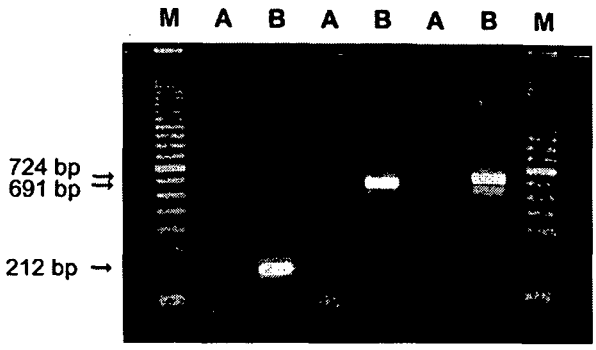

Figure 1. Detection of Borna disease virus (BDV)–specific nucleic acid in peripheral blood mononuclear cells (PBMC) of a man with chronic fatigue syndrome (CFS), by nested and seminested polymerase chain reaction assays. Lanes: A, BDV-negative PBMC; B, BDV-specific amplicates from PBMC of patient with CFS; M, size marker (100-bp DNA ladder).

the complete p23/24 gene, the complete gp18 gene, and part of the p57 gene of BDV for a total of 1398 bp of sequence information. When compared with BDV sequences in the GenBank database, the sequences showed an overall nucleotide identity rate of 96.2%–97.7%. In contrast to the sequences amplified from 3 brains with hippocampal degeneration, which proved to be virtually identical to the sequence of a BDV laboratory strain [1], there was no identity with any human, animal, or laboratory BDV strain in the sequence of the CFS case that we describe.

The BDV sequence of this case exhibits several point mutations, 2 of which lead to amino acid changes, that have not been seen in any other BDV case. Also, 3 different primer pairs were used independently, amplifying different regions of the BDV genome, and all resulted in specific amplifications by PCR.

The patient was retested 4 months later, during a chronic stage of the disease. At this point, no BDV nucleic acid was detected in his PBMC. This corresponds with the finding of Bode and Ludwig [9], who also detected BDV during acute disease only. With regard to the discussion of whether BDV infection should be considered a zoonosis, we note that the patient we describe had no close contact with animals and does not live in an area in which BDV is endemic in animals.

The sequences described in this paper have been reported to the GenBank database (accession numbers AF094477 and AF094478) and are available to the scientific community.

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Isolation and Characterization of a New Subtype of Borna Disease Virus

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Borna disease virus (BDV), the causative agent of severe meningoencephalitis in a wide variety of animal species, has been considered to be genetically invariable and to form a single type within the genus *Bornavirus* of the family *Bornaviridae*. BDV infections are of particular interest, because for the first time a virus infection appears to be linked to human psychiatric disorders. We now describe a new subtype of BDV isolated from a horse which was euthanatized due to severe, incurable neurological disease. The nucleotide sequence of this new strain, named No/98, differs from the reference strains by more than 15%, and the subtype is difficult to detect by standard reverse transcriptase PCR protocols. The nucleotide exchanges of the novel BDV isolate have surprisingly little effect on the primary structures of most viral proteins, with the notable exception of the X protein (p10), which is only 81% identical to its counterpart in reference strains. Our data indicate that the genome of BDV is far more variable than previously assumed and that naturally occurring subtypes may escape detection by currently used diagnostic assays.

Borna disease virus (BDV) is the causative agent of severe meningoencephalitis in horses, sheep, and other animal species in central Europe (7, 13, 21, 22), and it is suspected to contribute to human psychiatric disorders worldwide (1, 2, 6, 8–12, 14, 15, 20). The pathogenesis of Borna disease is mediated by a T-cell-dependent immune mechanism. All natural isolates of BDV that have been available to date, independent of species (humans, horses, sheep, cats, dogs, etc.), area (Europe, the United States, Japan), and year of isolation (1929 to 1998), show a remarkably high sequence conservation of the 8.9-kb RNA genome (5, 11, 16, 17). Human BDV isolates are 95 to 100% identical to animal-derived BDV at the nucleotide level and are 97 to 100% identical at the amino acid level (5, 11, 16). BDV is therefore considered a potential zoonotic agent. Because serum antibody titers are frequently low in naturally infected individuals and BDV serology has several other limitations, reverse transcriptase PCR (RT-PCR) technology using primers that match sequences of the viral N or P genes (1, 2, 5, 6, 8, 9, 12, 15, 16, 17, 19) is now widely used for the diagnosis of BDV infection. In this report, we describe a novel subtype of BDV which escapes detection by currently used diagnostic RT-PCR protocols.

MATERIALS AND METHODS

Immunohistochemistry. Paraffin-embedded brain sections were stained with the monoclonal antibody BO18, directed against the N protein, and a polyclonal mouse antiserum, directed against the X protein of BDV (3), as described previously (21, 22). Staining of cultured cells grown on glass coverslips was carried out according to standard procedures, by using polyclonal antisera raised against purified recombinant X and P proteins of BDV He/80.

Sample preparation and RNA extraction. Samples from hippocampus and rhinencephalon were homogenized by using liquid nitrogen and were resuspended in diethyl pyrocarbonate-treated water. Following low-speed centrifugation, 140 μl of supernatant was used for RNA extraction, employing the QIAamp viral RNA kit (QIAGEN).

PCR assays. In order to amplify overlapping genome fragments of the causative BDV strain, named No/98, RT-PCR was performed with a large number of different oligonucleotide primer pairs. cDNA synthesis and PCR were carried out in a single step by using the Titan One Tube RT-PCR Kit (Boehringer Mannheim/Roche). A 5-μl volume of extract, containing 50 pmol of RNA (approximately 0.2 μg of RNA), was used in each RT-PCR. For the experiments shown below (see Fig. 4A and B), the primer pairs described by Sorg and Metzler (19) were employed, because PCR assays using these oligonucleotide primers proved best for the detection of classical BDV in a recent multicenter study. Nested PCR (see Fig. 4B) was carried out by using 3 μl of the RT-PCR product, *Taq* polymerase from Promega, and a $MgCl_2$ concentration of 1.5 mM. For the RT-PCR experiment shown below (see Fig. 4C), the forward primer was 5′-CC TGGCATCCTGTGACTATT-3′, and the reverse primer was 5′-ATCTGCTCT TGGCTGTGTCT-3′ (nucleotide positions 3863 to 3882 and 4254 to 4235 of strain V, respectively).

Sequence analysis. PCR products were usually sequenced directly (in both directions) without subcloning into plasmid vectors, by using an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Perkin-Elmer); only a 0.65-kb fragment of the BDV isolate No/98 was cloned into pCR2.1 by using the TA-ligation procedure as described by the manufacturer (Invitrogen). The software package DNAStar 3 was used for sequence alignment and for construction of the phylogenetic trees.

Isolation of BDV No/98 from frozen brain material. Specimens from hippocampus and rhinencephalon were homogenized and added to early-passage cultures of young rabbit brain cells. After foci of infected cells were visualized by indirect immunofluorescence analysis, uninfected monkey Vero cells were added. After six cell passages, most rabbit cells were lost and the virus isolate had infected a high percentage of the Vero cells.

Nucleotide sequence accession number. The sequence of the genome fragment of the new BDV strain described here was submitted to GenBank under accession no. AF136236.

RESULTS AND DISCUSSION

The diseased animal was a 7-year-old pony stallion, originating from the Austrian federal state of Styria, where no cases of Borna disease had been previously recorded. Also, the animal had never been in regions in which BDV is endemic. The animal's clinical and histopathological picture matched that of classical Borna disease; this preliminary diagnosis was confirmed by immunohistochemical detection of BDV antigen in paraffin-embedded brain sections (Fig. 1). Unexpectedly, however, BDV RNA was not detected in frozen brain material by

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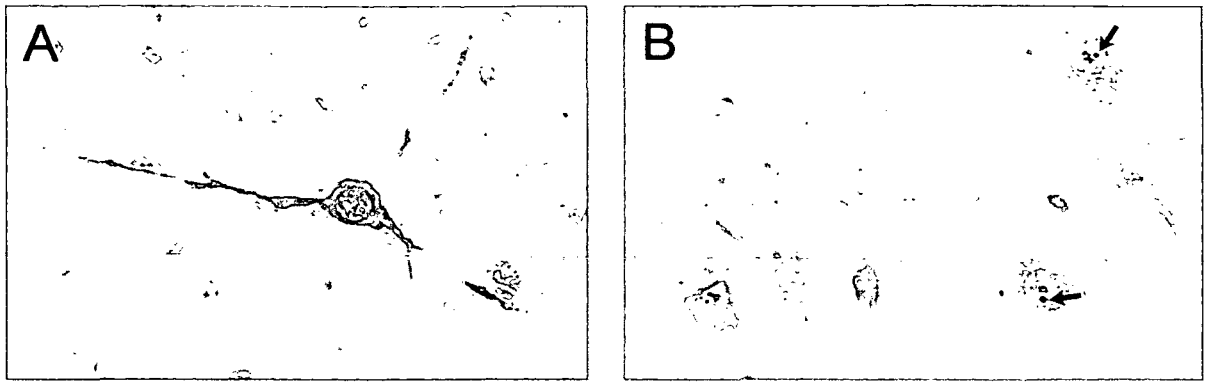
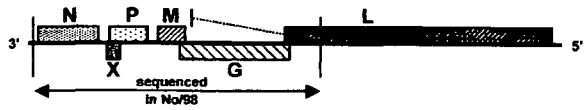


FIG. 1. Immunohistochemical staining of paraffin-embedded brain tissue by using the avidin-biotin complex technique and the monoclonal antibody BO18. (A) Specifically labeled neuron within the hippocampus region (magnification, ×335). (B) Positive immunostaining of hippocampal neurons. Note the numerous intranuclear Joest-Degen inclusion bodies (arrows), which are considered to be characteristic of BDV infection (magnification, ×335).

RT-PCR with several primer pairs that routinely give reliable results, indicating major sequence differences from previously isolated BDV strains. To verify this, we performed PCR on cDNA samples from the brain of this horse using a large array of primer pairs, including standard primers for classical BDV as well as primers specifically designed for this novel BDV strain. Using this PCR strategy, we generated overlapping fragments of the viral genome that were sequenced directly. The compiled sequence data yielded information on a large fragment of the genome of the new BDV strain, designated No/98, that corresponds to nucleotide positions 25 to 4234 of reference strain V; this fragment represents almost half of the entire BDV genome, including the complete open reading frames (ORFs) of the viral N, X, P, M and G genes and a small part of the L gene (Fig. 2A). The No/98 sequence was strictly colinear with that of reference strain V, except for a three-nucleotide deletion at positions 90 to 92 that deletes alanine 13 of the N protein, a single nucleotide deletion at position 1170, and a three-nucleotide (GCA) insertion after nucleotide 1204, both located in the first intergenic region between the N and X ORFs. The overall sequence identities between No/98 and the two reference strains V and He/80 were 84.2 and 84.0%, respectively (Fig. 2B). The nucleotide exchanges were distributed fairly evenly over the entire region that we have sequenced. Within the coding sequences of the N, P, M, G, and L genes, most nucleotide exchanges were silent, because they frequently affected the third positions of the codons. Consequently, the amino acid sequences were more than 93% identical to their counterparts in strains V and He/80. When conservative amino acid substitutions were taken into account, similarities were more than 96% (Fig. 2B). A different situation emerged for the X protein that is encoded by an ORF which overlaps the P ORF. Here, most nucleotide exchanges were not silent, and the X protein of No/98 exhibited only about 81% identity to its counterparts in strains V and He/80 (Fig. 2B). More careful inspection (Fig. 2C) demonstrated a highly biased distribution of nucleotide exchanges which strongly favored conservation of the P ORF over the X ORF, indicating that the latter protein can tolerate more variation. It is of interest to note, however, that the N-terminal region of the X protein, which harbors a nuclear export sequence (M. Salvatore, R. E. O'Neill, M. Schwemmle, P. Palese, and W. I. Lipkin, Abstr. Bornavir. Meet. 1998, abstr. V3, 1998) and a domain that mediates interactions with the P protein (Salvatore et al., Abstr. Bornavir. Meet. 1998), was completely invariant. A more variable region within the second transcrip-

A



B

		V vs He/80		V vs No/98		He/80 vs No/98	
all	nt	95.5		84.2		84.0	
N	nt	95.4		85.0		85.0	
	aa	99.5	(100.0)	97.8	(99.2)	97.8	(99.2)
X	nt	97.3		88.6		89.8	
	aa	94.3	(95.4)	80.6	(83.9)	81.6	(85.1)
P	nt	97.2		86.5		87.0	
	aa	98.0	(98.5)	96.0	(98.5)	97.0	(99.0)
M	nt	96.3		88.6		88.8	
	aa	99.3	(100.0)	97.9	(100.0)	98.6	(100.0)
G	nt	95.8		82.9		82.3	
	aa	97.8	(98.8)	93.6	(96.2)	93.4	(96.2)
L	nt	95.8		83.0		81.7	
	aa	98.4	(99.5)	96.2	(98.4)	94.5	(97.8)

C

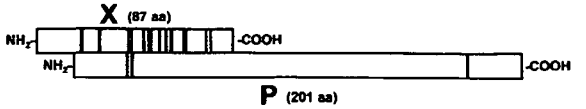


FIG. 2. Comparison of nucleotide and amino acid sequences of BDV No/98 and common laboratory strains. (A) Gene order and coding strategy of BDV. Overlapping RT-PCR products of No/98 corresponding to nucleotide positions 25 to 4234 of reference strain V (3) were sequenced. This fragment includes the complete coding sequences for the viral proteins N, X, P, M, and G and part of the L gene, as indicated. (B) Comparison of nucleotide (nt) and amino acid (aa) sequences of No/98 and laboratory strains V (3) and He/80 (4). Percentage of overall sequence identity (all) or sequence identities of the particular gene products at nucleotide and amino acid levels are indicated. Numbers in parentheses show percentage of sequence similarity when conserved amino acid exchanges are taken into account. Amino acid exchanges were rated conservative when the affected residues had similar biochemical properties. Any exchanges within the following groups of amino acids were considered conservative (single letter code): AILVM, STC, FY, NQ, WF, DE, and KR. Numbers boxed with gray highlight the striking dissimilarity of the X gene product. (C) Strongly biased conservation of the P protein sequence in the X-P gene overlap region. Positions of nonconservative amino acid exchanges in the X (n = 14) and P (n = 3) proteins are indicated by vertical bars. The comparison of strains V and No/98 is shown.

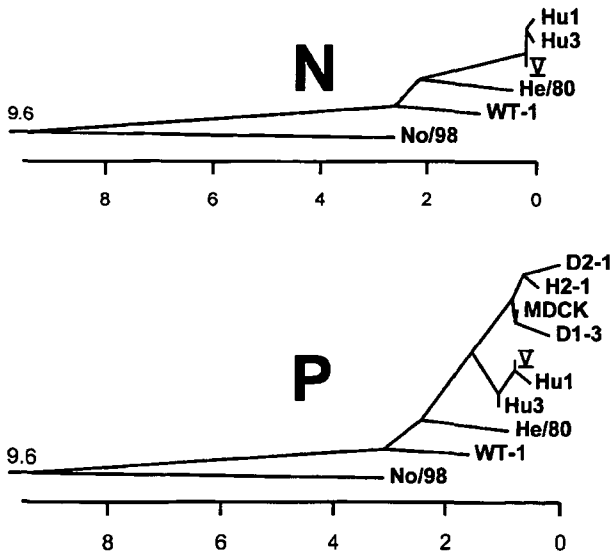


FIG. 3. Phylogenetic trees of BDV strains. Nucleotide sequence comparisons of fragments from the N gene coding region (corresponding to nucleotides 262 to 829 of reference strain V) and from the P gene coding region (corresponding to nucleotides 1482 to 1814 of reference strain V) were performed. Distances between strains indicate percentage of sequence divergence. Sequence information used to construct these trees was taken from the following sources: No/98, this report and GenBank no. AF136236; V, reference 3 and GenBank no. U04608; He/80, reference 4 and GenBank no. L27077; WT-1 N gene, reference 17 and GenBank no. S67502; WT-1 P gene, reference 17 and GenBank no. S67507; Hu1 N gene, reference 1 and GenBank no. U58594; Hu1 P gene, reference 5 and GenBank no. L76234; Hu3 N gene, reference 1 and GenBank no. U58596; Hu3 P gene, reference 5 and GenBank no. L76236. Sequences designated MDCK, D1-3, D2-1, and H2-1 were taken from the work of Iwata et al. (9).

tion unit was also described in highly conserved BDV isolates (11, 17).

To elucidate the relationship between No/98 and previously known BDV strains, we constructed phylogenetic trees based on published nucleotide sequences of N and P gene fragments (Fig. 3). This comparison included the prototype strains V (3), He/80 (4), WT-1 (17), and MDCK (9) and various BDV-derived nucleotide sequences found in blood samples of humans from Germany (1, 5) and Japan (9). As previously noted by others (5, 11, 16, 17), all BDV strains that had been previously recognized had shown highly similar nucleotide sequences. In sharp contrast, No/98 occupies a unique position in both phylogenetic trees, indicating that it may represent the first member of a novel BDV subtype.

To study the biological properties of No/98, we isolated this virus from frozen brain material by using cultures of primary young rabbit brain cells. Viral replication was monitored by indirect immunofluorescence analysis. The newly isolated virus could be transmitted to cultures of Vero monkey cells, in which it spread quickly without inducing a cytopathic effect. Vero cells infected with No/98 showed strong nuclear staining with monospecific antiserum raised against either X or P of strain He/80. To verify that the Vero cells were indeed infected with No/98, we performed RT-PCR on RNA from the persistently infected Vero cell culture. The amplified 0.65-kb fragment (corresponding to nucleotide positions 1202 to 1856) included the complete X ORF and part of the intergenic region between the N and X genes. Its sequence precisely matched the No/98 sequence previously established by direct analysis of horse brain material. All nucleotide exchanges and the three-base insertion after position 1204 were also present in the virus

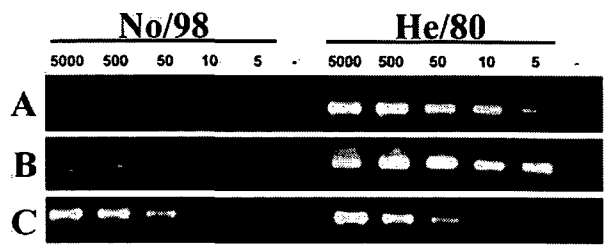


FIG. 4. No/98 is poorly recognized by conventional diagnostic RT-PCR assays. To examine at which sensitivity levels a published RT-PCR assay is able to detect the novel BDV subtype compared to the classical subtype, we prepared cell mixtures, each containing 5×10^5 uninfected Vero cells and the indicated numbers of Vero cells infected with either BDV No/98 or He/80 and subjected them to PCR analysis. (A) Conventional RT-PCR (19); (B) conventional nested PCR (19); (C) modified RT-PCR using primer pairs (described above) that amplify both No/98 and He/80 genomes.

isolate, indicating that the genome of No/98 did not rapidly acquire major alterations during replication in Vero cells.

Sequence comparisons revealed that currently used diagnostic RT-PCR assays employ primer pairs that only poorly match the No/98 sequence. To test if they would still detect this virus subtype, we prepared mixtures of 5×10^5 uninfected Vero cells and various numbers of cells infected with either BDV strain He/80 or No/98, extracted RNA from these cell mixtures, and used samples for reverse transcription. Standard (nonnested) PCR for detection of transcripts from the BDV N gene yielded the expected amplification products with all cell mixtures containing between 5,000 and 5 He/80-infected cells (Fig. 4A). By contrast, no amplification products were observed with cell mixtures containing high or low numbers of No/98-infected cells (Fig. 4A). Only when nested PCR was performed (Fig. 4B) did the samples containing 5,000 and 500 No/98-infected cells become weakly positive. When other primer pairs that better match both viral genomes were used, No/98- and He/80-infected cells were detected at similar sensitivities (Fig. 4C). The above experiment was repeated with other published RT-PCR protocols and showed results very similar to those presented in Fig. 4A and B; they either failed completely to amplify BDV No/98 sequences or exhibited significantly lower sensitivities in detecting this novel BDV subtype, even when altering the PCR conditions.

The identification of a new BDV field isolate that escapes detection by currently used diagnostic assays has far-reaching implications for proper diagnosis of BDV in, for example, human neuropsychiatric disorders. The new findings imply that previous studies, which relied on RT-PCR technology, might have underestimated the true prevalence rates of human BDV infections and might also have missed etiological correlations between BDV infection and certain neuropsychiatric disorders. The isolation of a BDV with a highly variant genome disproves the general opinion of high sequence conservation of all BDV genomes; the possibility that further, yet unidentified, BDV subtypes do exist should be considered seriously.

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Conservation of coding potential and terminal sequences in four different isolates of Borna disease virus

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We determined the complete nucleotide sequences of two poorly characterized strains of Borna disease virus (BDV) and compared them to reference strains V and He/80. Strain H1766 was almost 98% and 95% identical to strains V and He/80, respectively, whereas strain No/98 was only about 81% identical to both reference strains. In contrast to earlier reports, we found an additional A residue at the extreme 3′-end of the single-stranded RNA genome in all four BDV strains. The exact numbers of nucleotides in the four BDV genomes could not be determined due to a micro-heterogeneity at the 5′-end. If our longest sequence is a correct copy of the viral RNA, the two ends of the BDV genome would show almost perfect complementarity. All three transcription start sites, all four termination sites, both splice donor sites and both major splice acceptor sites are highly conserved, whereas a minor alternative splice acceptor site is not. The L protein of No/98 differs at 7% of its amino acid positions from the polymerase in the other strains, with most differences mapping to the C-terminal moiety of the molecule. Re-evaluation of L protein sequences of strains V and He/80 revealed differences at several positions compared to published information, indicating that variant forms of the viral polymerase have previously been characterized. These results are important because correct structures of genome ends and of the polymerase gene are the most critical parameters for the future development of techniques that will permit the genetic manipulation of BDV.

Introduction

Borna disease virus (BDV) is a non-segmented negative-strand RNA virus that persistently infects the central nervous system of a broad variety of animals and, possibly, humans (Staeheli *et al.*, 2000). Natural and experimental BDV infections can result in severe immune-mediated neurological disease (Richt & Rott, 2001; Rott & Becht, 1995; Stitz *et al.*, 1993). Unlike the related animal rhabdo- and paramyxoviruses, BDV transcribes and replicates its approximately 8900 nt long genome in the nucleus of infected cells and uses the splicing machinery to regulate the expression of viral proteins (Briese *et al.*, 1992; Cubitt *et al.*, 1994*b*; Jehle *et al.*, 2000; Schneider *et al.*, 1994*b*). Mainly because of these features, BDV has recently been classified as the prototype of a new virus family, *Bornaviridae*, within the order *Mononegavirales*. At least six viral proteins are expressed in BDV-infected cells, namely nucleoprotein (N), X-protein (X or p10), phosphoprotein (P), putative matrix protein (M), glycoprotein (G) and polymerase (L). Synthesis of a shorter form of P, designated P′, is initiated at the second in-frame AUG of the P ORF (Kobayashi *et al.*, 2000). Splicing of two introns regulates the balanced expression of M, G and L (Cubitt *et al.*, 1994*b*; Jehle *et al.*, 2000; Schneider *et al.*, 1994*b*, 1997). Whereas intron 1 is located within the M ORF, intron 2 is found in the ORFs of G

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and L (Cubitt *et al.*, 1994*b*; Schneider *et al.*, 1994*b*). Within the coding region of the L protein, the presence of an alternative intron 2 splice acceptor site was recently described (Cubitt *et al.*, 2001; Tomonaga *et al.*, 2000). Alternative splicing of intron 2 gives rise to two new ORFs with coding capacities for 8·9 kDa and 165 kDa proteins, respectively. Whether these two proteins are indeed produced in BDV-infected cells and whether they serve vital functions remain to be determined. To date, it is not known whether the genomes of all BDV strains have the capacity to encode these additional proteins.

Little is known about the conservation of genome size, coding potential and terminal sequences in different isolates of BDV. Until now, the complete genetic information of only two closely related BDV strains, named He/80 and V, has been available (Briese *et al.*, 1994; Cubitt *et al.*, 1994*a*). The two genomes show 95% sequence identity, and they have identical organization of transcription units and ORFs. However, the L protein of He/80 was reported to lack 24 amino acids at the C terminus, and the last 3 nt at the 5′-ends differ completely. Since genome ends are of critical importance for efficient replication and transcription of non-segmented negative-strand RNA viruses (Conzelmann, 1998), it remained possible that the unique end sequences may point to strain-specific differences in the 5′-promoter region.

To better differentiate between conserved and strain-specific features, we determined the complete genome sequences of two additional BDV strains. No/98 originates from a horse that acquired Borna disease outside the well-known endemic region of BDV in central Europe (Nowotny *et al.*, 2000). Partial nucleotide sequence analysis indicated that No/98 differs from reference strains He/80 and V by about 15%. Strain H1766 (also known as MDCK-BDV) is frequently used for experiments in Japanese laboratories (Nakamura *et al.*, 1999; Shoya *et al.*, 1997). We show here that the two reference strains and the newly characterized strains harbour a 3′-terminal A residue and most likely a 5′-terminal G residue. We further show that the alternative intron 2 splice acceptor site is not conserved in strain No/98. Re-analysis of the L ORF revealed that the polymerase of strain He/80 has the same length as its counterparts in strains H1766, V and No/98.

Methods

■ **BDV strains and cells.** Vero and OL cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (FCS). OL cells were kindly provided by Georg Pauli (Robert Koch Institute, Berlin, Germany). Persistent BDV infections were established by infecting 10^5 cells with 10^4 focus-forming units of appropriate virus stock followed by continued passage for at least 5 weeks. Complete infection of the cultures was confirmed by indirect immunofluorescence as described (Formella *et al.*, 2000).

■ **Virus stock preparation and titration.** Virus stocks were prepared from OL cells persistently infected with either BDV strain He/80 (Cubitt *et al.*, 1994*a*), strain H1766 (Shoya *et al.*, 1997) or strain V (Briese *et al.*, 1994), and from Vero cells persistently infected with strain No/98 (Nowotny *et al.*, 2000) essentially as described (Briese *et al.*, 1992). Briefly, 25 confluent 90 mm dishes were washed with 20 mM HEPES (pH 7·4) and incubated with 10 ml of 20 mM HEPES (pH 7·4) containing 250 mM $MgCl_2$ and 1% FCS for 1·5 h at 37 °C. Subsequently, supernatants were harvested and centrifuged twice at 2500 *g* for 5 min to remove cell debris. Virus particles were concentrated by ultra-centrifugation for 1 h at 20 °C at 80000 *g* onto a 20% sucrose cushion containing 20 mM HEPES (pH 7·4) and 1% FCS. Virus-containing pellets were either re-suspended in PBS to approximately 10^7 focus-forming units/ml or were directly used to prepare RNA.

■ **Preparation of viral RNA.** Viral RNA was isolated with TRIzol (Gibco/BRL) according to the manufacturer's instructions. Briefly, virus particles in 1 ml of a concentrated BDV stock (about 10^7 focus-forming units) were precipitated for 30 min at 4 °C in a TL120 centrifuge (Beckman) at 70000 r.p.m. The virus pellet was lysed by dissolving in 1 ml of TRIzol solution and incubating for 5 min at room temperature. The RNA was then extracted with chloroform, and precipitated from the aqueous phase with isopropanol and glycogen (Boehringer Mannheim/Roche). Finally, the RNA was washed with ethanol, dried and re-suspended in water.

■ **Determination of 3′- and 5′-terminal sequences of BDV genomic RNA by RNA ligation.** RNA recovered from partially purified virus particles (1 µg) was self-ligated in a volume of 20 µl of ligation buffer (75 mM Tris–HCl, pH 7·5; 0·1 mM ATP; 10 mM $MgCl_2$; 5 mM dithiothreitol; 10%, v/v, dimethyl sulfoxide; 50 U RNasin) with 50 U of T4 RNA ligase (NEB). After incubation at 25 °C for 90 min, the volume was adjusted to 200 µl with TE buffer (10 mM Tris–HCl, pH 8·0 and 1 mM EDTA). The RNA was then extracted once with phenol and chloroform, precipitated with isopropanol/glycogen and centrifuged for 15 min at 4 °C. Finally, the RNA was washed with ethanol, dried and re-suspended in 13 µl of water. The material was subjected to reverse transcription using Superscript II (GIBCO/BRL) as recommended by the manufacturer using 2·5 pmol of primer GSP1 (5′ ATTATAGTTTTG-TCATGGACCTC 3′). Primer GSP1/No98 (5′ ATGGCTTCTTGATG-GACTTGGTC 3′) rather than GSP1 was used for RNA from BDV strain No/98. The reaction was stopped by incubation at 70 °C for 10 min. Then RNase H (GIBCO/BRL) was added and the sample was incubated at 37 °C for another 60 min. Samples (5 µl) were amplified using Taq polymerase (Boehringer Mannheim/Roche) and 0·4 pmol of primer GSP1 (for H1766, V and He/80) or GSP1/No98 (for No/98) and primer GSP3 (5′ CGTGACTGGTCTAACAATGC 3′; for H1766, V, No/98 and He/80). Nested PCR was performed with 2 µl samples of PCR products using primer GSP2 (5′ GCTTGTGGTAGGACAGCACATC 3′; for H1766, V and He/80) or GSP2/No98 (5′ GTTGGTGGTAG-GGCAGTACATC 3′; for No/98) and primer GSP4 (5′ GAGCTTA-GGGAGGCTCGCTG 3′; for H1766, V and He/80) or GSP4/No98 (5′ GAAAGCTTGGGAAGGCTTGCTG 3′; for No/98). The final PCR products were cloned into vector pCR4 TOPO (Invitrogen) and sequenced using a MegaBASE 1000 sequencer (Amersham Pharmacia Biotech) and the PHRED base-calling algorithm (STATEN Software package). Sequences were analysed using the DNASTAR (Lasergene) software package.

■ **3′-RACE analysis.** To determine the 3′-end of the BDV genome, 1 µg of RNA from partially purified BDV particles was tailed with A or C residues using poly(A) polymerase (Pharmacia) as recommended by the manufacturer. The A-tailing reaction was stopped after 15 min, whereas the C-tailing reaction was stopped after 1 h. RNA was purified by phenol and chloroform extraction, precipitated with isopropanol/glycogen, washed, dried and re-suspended in 11 µl of water. A-tailed RNA was reverse-transcribed with Superscript II (GIBCO/BRL) and oligo(dT)

adapter primer from the 3′-RACE kit (GIBCO/BRL). C-tailed RNA was reverse-transcribed using the inosine/guanosine adapter primer provided with the 5′-RACE kit (GIBCO/BRL). PCR was performed with 5 µl of cDNA samples, adapter primer [oligo(dT) adapter primer from the 3′-RACE system for A-tailed RNA and inosine/guanosine adapter primer from the 5′-RACE system for C-tailed RNA] and primer GSP3 (for strain H1766, No/98, V and He/80). For semi-nested PCR, 2 µl samples of the first PCR were amplified a second time with the respective adapter primers described above and primer GSP4 (for strain H1766, V and He/80) or GSP4/No98 (for No/98). PCR products were cloned into vector pCR4 TOPO before sequencing.

■ **Determination of the 5′-terminal sequences of BDV genomic RNA by 5′-RACE.** A standard 5′-RACE system (GIBCO/BRL) was used according to the manufacturer's instructions. Briefly, reverse transcription was done with primer GSP1 (for strain H1766, V and He/80) or GSP1/No98 (for No/98) and Superscript II. RNA was then digested with RNase H, and the cDNA was purified with a Glass MAX kit (GIBCO/BRL). C-tails were added to the 3′-terminal end of the cDNAs with terminal deoxynucleotidyl transferase. The tailed cDNA was amplified by PCR using appropriate adapter primers and GSP2 (for H1766, V and He/80) or GSP2/No98 (for No/98). PCR products were cloned into vector pCR4 TOPO before sequencing. Determination of the 5′-terminal sequences of BDV genomic RNA of He/80 by ligation of RNA oligos and subsequent RT–PCR was carried out with GeneRacer (Invitrogen) and primer GSP2 according to the manufacturer's instructions. Tobacco acid pyrophosphatase (TAP) treatment was carried out as recommended by the manufacturer (Invitrogen). Amplification products were either cloned into vector pCR4 TOPO before sequencing or sequenced directly.

■ **Cloning and sequencing of near-full-length cDNA of strain H1766.** PCR-amplified, near-full-length cDNA derived from BDV strain H1766 that had been grown in persistently infected MDCK cells (Shoya *et al.*, 1997) was kindly provided by K. Tomonaga. The material was cloned into vector pCR-XL-TOPO (Invitrogen) according to the manufacturer's instructions. Three independent plasmids were sequenced which (according to restriction analysis) seemed to contain the complete viral genome. Sequencing primers were designed from published BDV sequences (GenBank accession nos U04608 and L27077). Regions were chosen that showed no or only little variation. In order to identify and eliminate PCR-generated mutations, overlapping sequences of each plasmid were assembled, resulting in three complete contiguous sequences which were then used to generate a single consensus sequence. Sequences at the extreme ends of the viral RNA were determined by RNA ligation, 3′-RACE and 5′-RACE using RNA isolated from partially purified particles.

■ **Sequencing of strain No/98.** The complete cDNA sequence of No/98 was determined by direct sequencing of overlapping RT–PCR amplification products using a large number of different oligonucleotides as described previously (Nowotny *et al.*, 2000). Briefly, RT–PCR reactions were carried out in a single-step reaction using the Titan One Tube RT–PCR kit, the Expand Reverse Transcriptase kit and the Expand Long Template PCR system (all Boehringer Mannheim/Roche). As template we used RNA from hippocampus and rhinencephalon of the diseased horse from which No/98 was originally isolated. PCR products were sequenced in both directions without subcloning using an automated sequencer (ABI Prisma 310 Genetic Analyser, Perkin Elmer). Sequences near the 3′- and 5′-ends were determined as described above using RNA from virus particles released from an infected Vero cell culture.

■ **Cloning of a cDNA containing the L ORF of strain He/80$_{FR}$.** Fragments of the L ORF were generated by RT–PCR using RNA from C6 cells persistently infected with the Freiburg variant of strain He/80 (He/80$_{FR}$). Reverse transcription was carried out with Superscript II (GIBCO/BRL) and strain-specific primers were derived from the published He/80 sequence (GenBank accession no. L27077). PCR was performed with Taq polymerase (Boehringer Mannheim/Roche) as described by the manufacturer. An RT–PCR fragment corresponding to nt 2393–4229 of He/80$_{FR}$ (GenBank accession no. AJ311522) was cloned into the *Eco*RI and *Pst*I sites of vector pBlue (Stratagene) followed by insertion of a second RT–PCR fragment (nt 4229–4869) into the unique *Pst*I and *Bam*HI sites. Subsequently, a third RT–PCR fragment corresponding to nt 4869–7870 was inserted between the *Bam*HI and the *Xho*I sites. In a final step, a fourth RT–PCR fragment containing nt 7870–8823 was cloned into the unique *Xba*I site, resulting in plasmid pBlue-L encoding the complete L ORF of He/80.

■ **Sequencing of the L ORF of strain V$_{FR}$.** The L ORF of the Freiburg variant of strain V (V$_{FR}$) was determined by sequencing overlapping RT–PCR fragments generated from RNA of strain V-infected Vero cells. Briefly, first-strand synthesis was carried out with Superscript II (GIBCO/BRL) as recommended by the supplier using different oligonucleotides derived from the published BDV strain V sequence (GenBank accession no. U04608). PCR was performed with Taq polymerase (Boehringer Mannheim/Roche) using a panel of suitable primers. Amplification products were gel-purified and sequenced without subcloning.

Results and Discussion

Conserved coding strategy in four independent BDV isolates

We sequenced the complete genomes of BDV isolates H1766 and No/98. Strain H1766 originates from a diseased horse in Germany. It is frequently used for experiments by Japanese laboratories. Strain No/98 represents the most distant BDV subtype known to date (Nowotny *et al.*, 2000). When compared to published sequences of standard strains V and He/80 as well as to the Freiburg variants of these strains, designated V$_{FR}$ and He/80$_{FR}$ (which differ from the parental strains at several nucleotide positions), it became clear that all four BDV strains have identical genome lengths, except for strain No/98, which lacks a single residue in the U-rich region (nt 1168–1171) that follows the N gene. Due to a micro-heterogeneity of the 5′-end (see below), the exact number of nucleotides in the BDV genome was difficult to determine. If our longest sequence represents a correct copy of the viral RNA, the genomes of BDV strains V, H1766 and He/80 would consist of 8912 nt and that of strain No/98 of 8911 nt. Since neither of these numbers corresponds to genome lengths that are a multiple of six, the 'rule of six' (Calain & Roux, 1993) does not seem to apply to members of the family *Bornaviridae*. Nevertheless, it is interesting to note that a 3 nt deletion in the N gene of strain No/98 is compensated for by a 3 nt insertion in the first intergenic region (Nowotny *et al.*, 2000), suggesting that unknown mechanisms are operating that maintain a constant number of nucleotides in the BDV genome.

An overall comparison of the four BDV genomes allowed us to establish the exact phylogenetic relationship of these

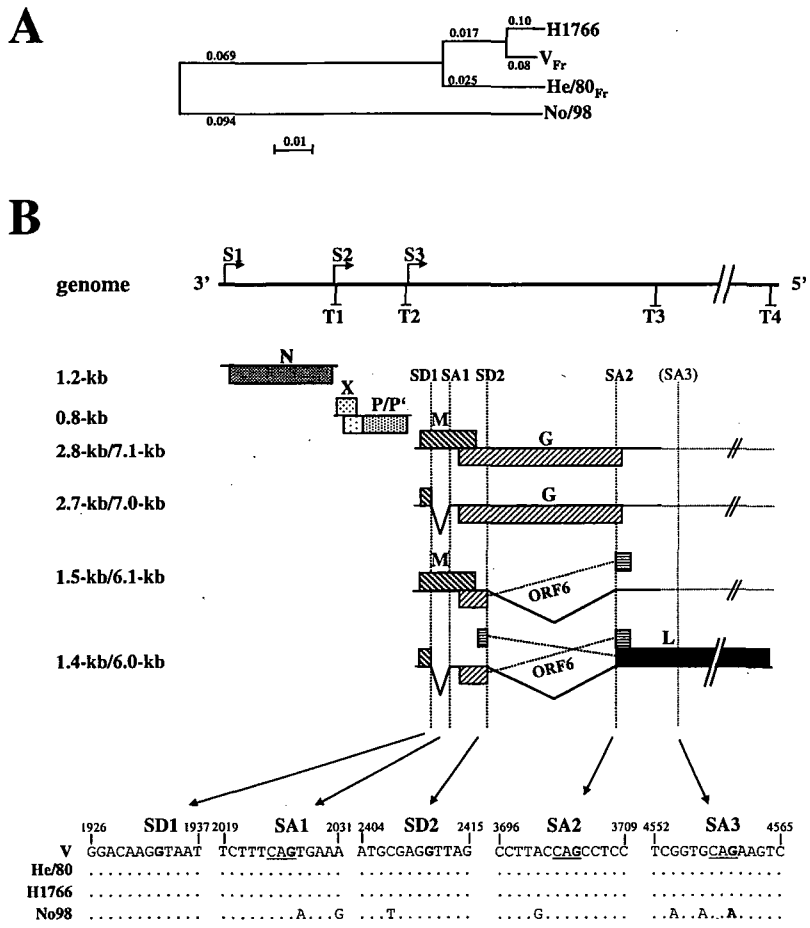

Fig. 1. Conserved coding strategy of four different BDV isolates. (A) The phylogenetic tree represents a rooted consensus tree generated by the neighbour-joining method on the genome sequences of strain No/98 (GenBank accession no. AJ311524), strain H1766 (GenBank accession no. AJ311523), strain V_{FR} (GenBank accession no. AJ311521) and strain $He/80_{FR}$ (GenBank accession no. AJ311522). Bootstrap analysis was applied using 100 values. (B) Consensus transcription map. Positions of transcription start (S1–S3) and stop (T1–T4) sites are indicated. Locations and nucleotide sequences flanking splice donor (SD1, SD2) and acceptor sites (SA1–SA3) are given. ORFs are individually marked on the various viral transcripts. Letters N, X, P, P', M, G and L indicate translation products that have been identified in infected cells by immunohistochemical methods. The putative product of ORF6 remains to be visualized. Due to the absence of a functional SA3 site in strain No/98, our consensus transcription map does not include SD2–SA3 spliced viral mRNAs and their predicted translation products.

viruses. As shown in Fig. 1(A), strain H1766 is closely related but distinct from reference strain V. These two strains differ by only 1·8% (155 nt). Interestingly, strain V was isolated from a diseased horse in southern Germany more than 70 years ago (Schneider *et al.*, 1994*a*), whereas strain H1766 was isolated in 1994 (S. Herzog, personal communication). Our results thus confirm earlier observations that the genomes of European BDV field isolates show remarkably few changes over long periods of time. As expected from earlier comparisons of incomplete viral genomes (Nowotny *et al.*, 2000), strain No/98 occupies a unique position in the phylogenetic tree (Fig. 1A). This may reflect the fact that it was isolated from a diseased horse that lived outside the classical endemic region in Europe (Nowotny *et al.*, 2000).

The coding strategy of BDV strains No/98 and H1766 is virtually identical to that of the two reference strains (Fig. 1B). All sequences previously found to define the transcription start sites S1, S2 and S3 (Schneemann *et al.*, 1994) are completely conserved. Similarly, the four transcription stop sites (Briese *et al.*, 1994; Cubitt & de la Torre, 1994; Schneemann *et al.*, 1994) are also present at corresponding positions in the genomes of strains No/98 and H1766. Similarly, the sequences flanking intron 1 and intron 2 are highly conserved (Cubitt *et al.*, 1994*b*; Schneider *et al.*, 1994*b*). The only notable difference between

the various strains maps to a site, designated SA3, that was recently reported to define an alternative intron 2 splice acceptor site (Cubitt *et al.*, 2001; Tomonaga *et al.*, 2000). The consensus motif CAG, which is present in strains V, He/80 and H1766, is not present in strain No/98, indicating that the latter virus cannot generate the corresponding alternatively spliced mRNA. This suggests that the predicted products of this new mRNA may not serve essential functions in the BDV replication cycle. However, we cannot exclude the remote possibility that atypical splice donor sites are used.

A consensus transcription map of the BDV genome (Fig. 1B) shows that all four viruses are able to direct the synthesis of proteins designated N, X, P, P′, M, G and L (Kobayashi *et al.*, 2000; Schwemmle *et al.*, 1999; Walker *et al.*, 2000; Wehner *et al.*, 1997). Interestingly, the coding regions for N and P in strain H1766 are completely identical to those of virus isolate BDVHuP2br, believed to have originated from a Japanese psychiatric patient (Nakamura *et al.*, 2000). This unusual congruence and the fact that strain H1766 (also named MDCK-BDV) is being used by the Japanese laboratory for experiments (Nakamura *et al.*, 1999; Shoya *et al.*, 1997) support our previous notion that BDVHuP2br might represent a laboratory contamination (Staeheli *et al.*, 2000). Intriguingly, all four viruses have one additional ORF generated by splicing of intron 2 that we designate ORF6. The putative product of ORF6 consists of the N-terminal 58 amino acids of viral protein G fused to a unique 21 residue polypeptide encoded by sequences immediately downstream of splice acceptor site 2. Since this putative protein carries the G-derived signal peptide but lacks a transmembrane anchor, it is expected to be secreted by BDV-infected cells. It thus seems to resemble sGP of Ebola virus (Sanchez *et al.*, 1996), which was suggested to influence the host defence (Yang *et al.*, 1998). It remains to be investigated whether the ORF6 product is indeed synthesized during BDV infection and whether it can modulate the antiviral response.

Primary structure of the BDV polymerase

The most striking difference between the published ORFs of the L proteins of He/80 and V is a sequence heterogeneity at the 3′-ends that would seem to result in an L protein of He/80 that lacks 24 amino acids at the C terminus (Fig. 2). Our analysis showed that the L protein of strain H1766 consists of 1711 amino acids as does the L protein of strain V (Fig. 2). Furthermore, we found that the L gene of strain No/98 encodes a protein of exactly the same length (Fig. 2), suggesting that the truncated version of L in He/80 does not reflect a true strain difference but rather errors in previous cDNA cloning and sequencing experiments. To investigate this point in more detail, we re-cloned and sequenced the complete L ORF of the Freiburg variant of strain He/80 ($He/80_{FR}$) by RT–PCR as described in Methods. As shown in Fig. 2, the predicted L ORF of $He/80_{FR}$ encodes a protein of 1711 residues, like all other BDV strains. Original He/80 differs from the $He/80_{FR}$ sequence by a deletion of 2 nt (positions 8717–8718), which results in a premature stop codon. Insertion of the two missing nucleotides into the published sequence of He/80 restores the L ORF. This new C terminus of He/80 L perfectly matches the C terminus of the $He/80_{FR}$ L protein, indicating that the deletion in the published L ORF of He/80 most likely represents a cloning or sequencing artefact. In fact, these errors in the He/80 sequence have recently been corrected (March 2001, accession no. L27077). Thus, the various strains of BDV appear to have L proteins of identical lengths.

A more complete comparison of the published L protein sequence of strain He/80 with sequence information from variant strain $He/80_{FR}$ revealed nine additional differences in the amino acid sequence (Fig. 2). In all of these cases, one of the two strains possesses a residue that matches the consensus sequence of all four viruses, while the other strain has a unique residue at the corresponding position. The most simple explanation for these findings is that cDNA cloning or sequencing errors occurred. It remains possible, however, that at least some of these amino acid exchanges reflect true differences between viruses with different passage history. We further observed seven amino acid differences in the L protein of strain V and the Freiburg variant (strain V_{FR}) of this virus. As discussed above for strain He/80, these differences could represent cloning or sequencing errors or else might indicate true differences between viruses with different passage history.

Most differences between the L protein of strain No/98 and the reference strains map to the last third of the protein (Fig. 2). Within this region, three blocks of amino acids of No/98 (G_{1449}LHRRA$_{1454}$, L_{1643}IQE$_{1646}$ and G_{1656}RGPVVS-RSSRWVG$_{1664}$) are particularly poorly conserved. Whether these amino acid differences have an impact on the polymerase activity remains to be shown.

The 5′- and 3′-termini of the BDV genome

To determine the 5′-end of the viral RNA, 5′-RACE experiments were performed with RNA extracted from partially purified virus particles obtained from persistently infected cells. In this procedure, viral cDNA is synthesized and 3′-ends are elongated artificially with C residues which later serve as annealing sites for oligo(dG/dI) primers that are used to amplify the adjacent region of the viral genome by PCR. The sequence of the viral RNA at the extreme 5′ terminus is then deduced by combining the sequence information from the various cloned PCR products. Irrespective of strain origin (He/80, V, H1766 and No/98) all cDNA clones analysed were virtually identical except that they differed significantly at the fusion site that links the virus sequence to the C tail (Fig. 3 A, left panel), indicating that the viral RNA used for reverse transcription was heterogeneous at the 5′-end. A consensus derived from the majority of PCR fragments suggested that 5′ GCGCUA ... 3′ is the most likely sequence at the extreme 5′-

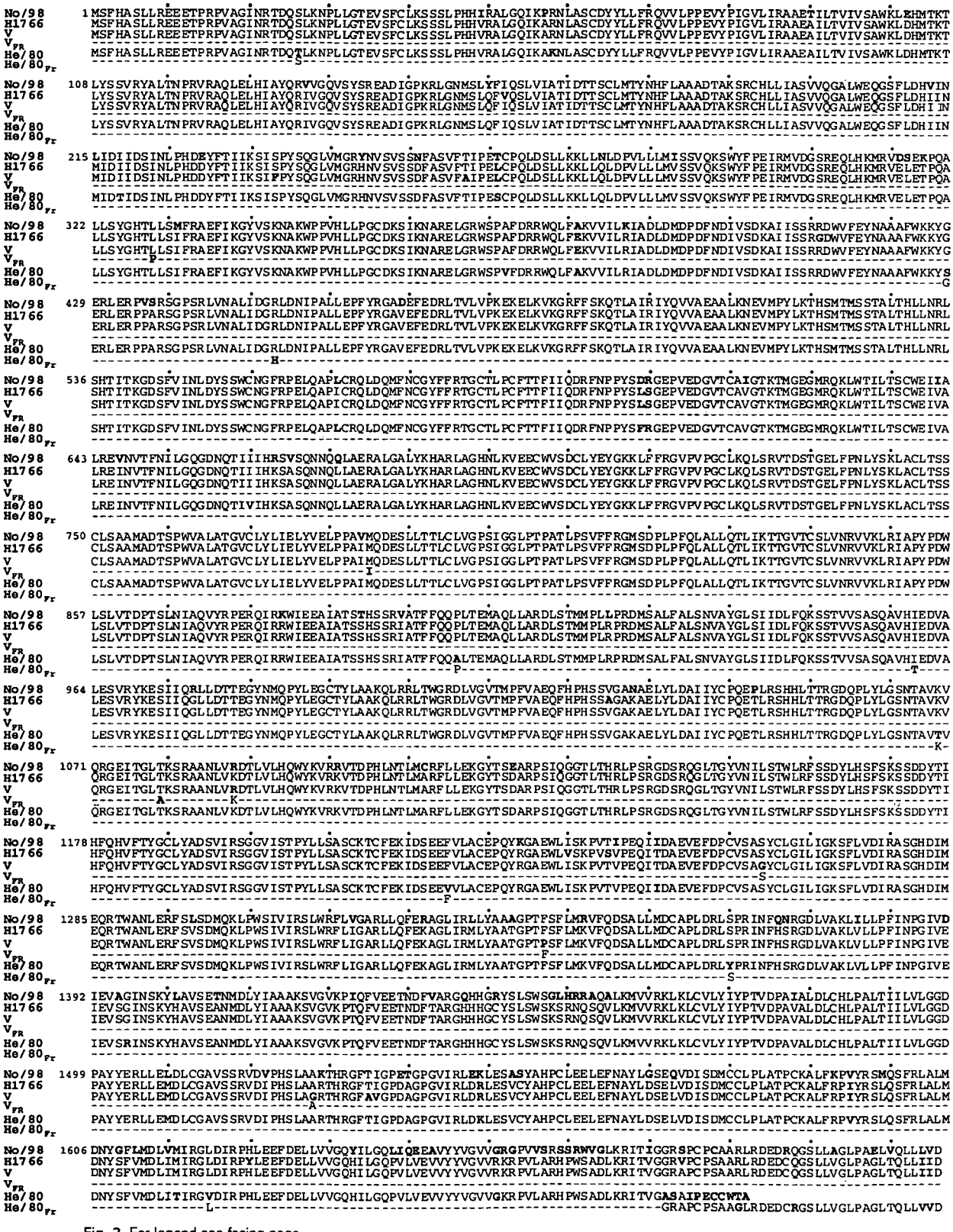

Fig. 2. For legend see facing page.

end of the viral genome, although it remains possible that additional nucleotides are present.

3′-RACE experiments were performed to define the sequence at the other end of the viral genome (Fig. 3A, right panel). Here, either A- or C-tails were added to the 3′-ends of viral RNA molecules before they were subjected to reverse transcription using oligo(dT) or oligo(dG/dI) primers. Products were amplified by PCR and cloned into a plasmid vector. Most of the resulting clones contained viral cDNA fragments that suggested that the most likely 3′-end of the viral genome might be 5′ ... CGCAACA 3′.

To confirm these data by an independent approach, we analysed a series of cDNA clones generated from viral RNA ends that were artificially fused by RNA ligase before the product was used for cDNA synthesis and PCR amplification. In this RNA-ligation/RT–PCR procedure, primers for reverse transcription were designed to drive cDNA synthesis across the junction site. Analysis of resulting cDNA clones from strains V, He/80 and H1766 revealed a rather non-uniform picture in that most of the clones appeared to carry deletions of variable lengths that seemed to map to either side of the fusion site (Fig. 3B). To generate a consensus sequence, we decided to ignore all hypothetical deletions in order to deduce the putative sequence of the longest possible RNA template. The 3′-terminal sequence of this hypothetical molecule is identical to that determined by 3′-RACE. Its 5′-terminal sequence is identical to that determined by 5′-RACE except for an extension of 4 nt. Assuming that this hypothetical molecule indeed reflects a copy of the full-length viral RNA, the proper ends of the BDV genome would be 5′ UGUUGCGCUAC-AACAAA ... and ... UUUGUUGUUAACGCAACA 3′. These two sequences show a high degree of complementarity, indicating that the BDV genome can potentially form a panhandle (Fig. 4A), as is the case for other members of the order *Mononegavirales*. However, the 5′-RACE data (Fig. 3A) do not fit this model well.

Since we cannot rule out the possibility that intrinsic problems associated with the 5′-RACE procedure were responsible for the failure to unambiguously identify the 5′-end sequence of the viral genome, we used an alternative technique for analysis that involves ligation of a defined RNA oligonucleotide to the 5′-terminus of the genomic RNA prior to performing RT–PCR. To ensure that the genome ends were mono-phosphorylated, we treated the viral RNA with TAP before ligation of the RNA oligonucleotide. Analysis of the resulting cDNA clones revealed that 5′ GCGCUA ... 3′ was the predominant sequence at the extreme 5′-end of the viral genome of He/80 (Fig. 3C, left panel). Identical results were obtained with viral RNA that was not treated with TAP (Fig. 3C, right panel), suggesting that the 5′ end of the viral genome is not tri-phosphorylated but rather mono-phosphorylated. Direct sequencing of the amplification product revealed a single prominent sequence (Fig. 3D), indicating that the vast majority of viral RNAs carry no additional nucleotides at the 5′-end. It remains to be determined whether viral genomic RNAs with these non-complementary ends are replication-competent. It is possible that the 5′-end sequences determined here reflect terminally cleaved viral RNAs. This view is compatible with the observation that the viral RNAs seemed to carry a monophosphate at the 5′-end. If correct, this situation would be similar to that observed with Seoul virus (a hantavirus; Meyer & Schmaljohn, 2000), where terminally deleted genome ends were found to be abundantly present in persistently infected cells.

The end sequences of the BDV genome that we deduced from our experiments are in conflict with data from previous reports (Briese *et al.*, 1994; Cubitt *et al.*, 1994*a*) in which the genome ends of strains V and He/80 were determined (Fig. 4B). The most striking difference is that we find an A residue at the 3′ terminus of the viral RNA, whereas the others did not (Fig. 4B). It is not entirely clear why our analysis yielded a different result. One reason might be that previous RNA-ligation/RT–PCR studies were performed in combination with 3′-RACE of A-tailed RNA, but not with C-tailed viral RNA. Such restricted analysis can, by definition, not identify any terminal A residues at the 3′-end of the viral genome.

Assuming that the 3′-end of the BDV genome is indeed occupied by an A residue, the BDV polymerase has to initiate transcription with UTP. If true, this constellation would represent a unique case among non-segmented, negative-strand RNA viruses. The polymerases of viruses from the order *Mononegavirales* all seem to initiate transcription by employing either ATP or GTP. Among other negative-strand RNA viruses, Hantaan virus (Garcin *et al.*, 1995) is known to carry an A residue at the 3′-end of the genome. However, transcription of the Hantaan virus genome is thought not to be initiated at the terminal A residue but rather at the C residue at position 3. After synthesis of a short nucleotide primer, a sophisticated 'prime-and-realign' mechanism ensures that the polymerase will synthesize a complete copy of the viral genome (Garcin *et al.*, 1995). It remains to be determined whether similar mechanisms are at work in BDV.

Closer inspection of sequences near the 3′- and 5′-ends of the genomes of various BDV strains revealed both

Fig. 2. Comparison of L polymerase sequences from various BDV strains. Sequence information was from the following sources: strain No/98 (GenBank accession no. AJ311524), strain H1766 (GenBank accession no. AJ311523), strain V_{FR} (GenBank accession no. AJ311521), strain $He/80_{FR}$ (GenBank accession no. AJ311522), strain V (GenBank accession no. U04608) and He/80 (GenBank accession no. L27077). Note that the He/80 reference sequence depicted in the figure has recently been corrected (March 2001). The new sequence features a full-length L ORF of identical length to the Freiburg subline of this virus. Variant amino acids are indicated in bold letters. Dashed lines indicate amino acids identical to the sequence above.

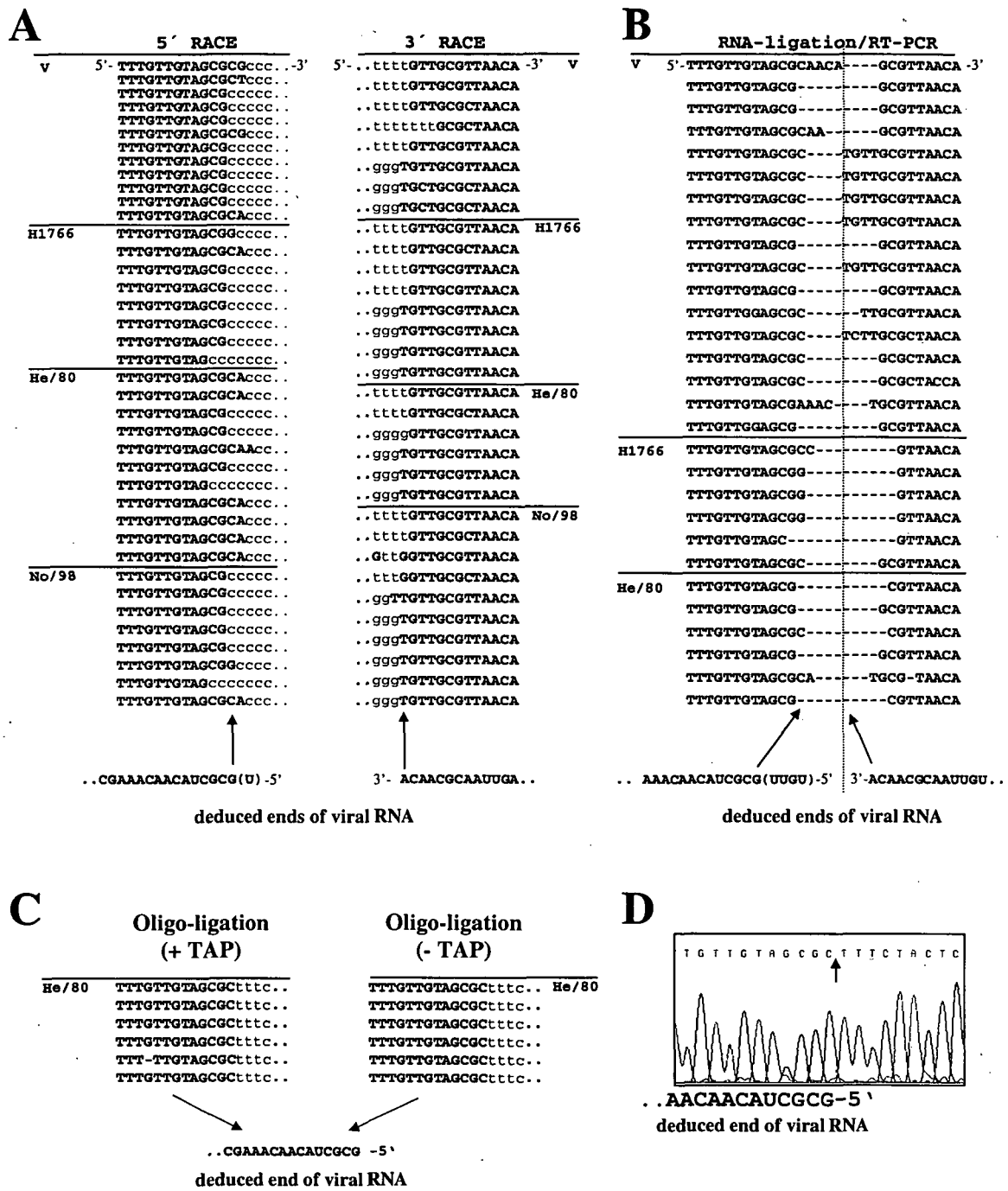

Fig. 3. Determination of the 5′- and 3′-termini of the BDV genome. (A) Terminal virus sequences deduced from the results of 5′- and 3′-RACE experiments. Lower-case letters mark terminal nucleotides that were introduced artificially during the RACE procedure. Strain origins of viral RNA used for the RACE experiments are indicated. Each lane shows the sequence (in antigenome orientation) of one individual RACE cDNA clone. Deduced ends of the viral genome are shown at the bottom. (B) Terminal virus sequences deduced from results of RNA-ligation/RT–PCR experiments. Each lane shows the sequence (in antigenome orientation) of one individual cDNA clone. Deduced ends of the viral genome are shown at the bottom. (C) Terminal virus sequences deduced from 5′-RACE experiments employing ligation of an RNA oligonucleotide to the 5′-end of the viral genome (Oligo-ligation). Prior to ligation of the oligonucleotide, the viral RNA was either treated (+TAP) or not

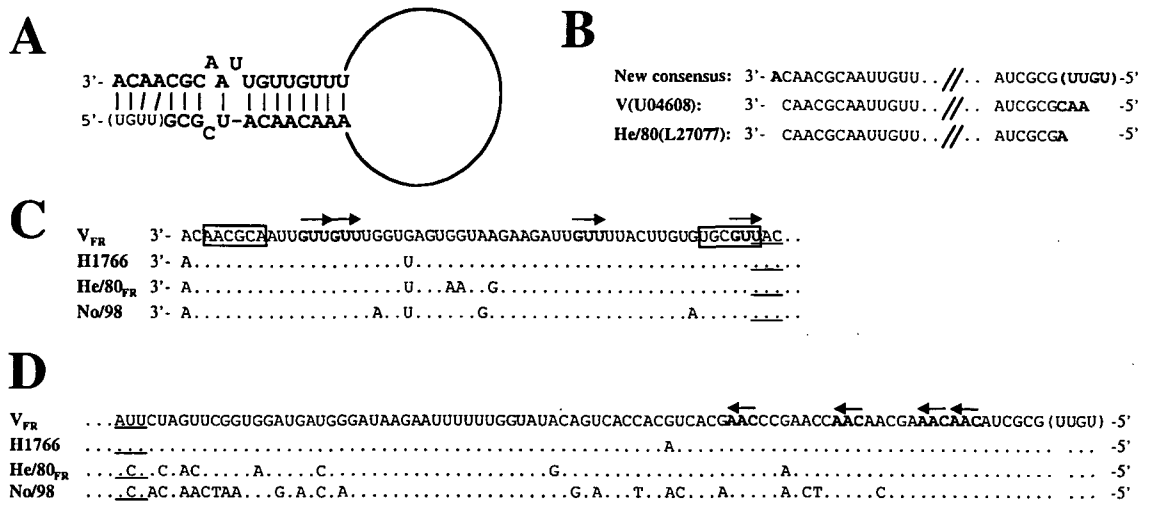

Fig. 4. Comparison of 5'- and 3'-termini of the BDV genome. (A) Potential secondary structure of paired 3'- and 5'-genome ends. Our experiments did not unambiguously prove the presence of nucleotides shown in brackets. (B) Comparison of our new consensus sequences with published genome end sequences of strain V and He/80. Variability between BDV strains in the non-coding regions near the 3' (C) and 5' end (D) of the viral genome. Arrows mark repetitive motifs that are found in complementary orientation near the 3' and 5' ends of the genome. Two inverted repeat motifs near the 5' end of the viral genome that have the potential to form a stem–loop are boxed. Nucleotides complementary to the initiation codon of N (C) or the stop codon of L (D) are underlined.

non-conserved and highly conserved regions. Multiple copies of a highly conserved 5′ CAA 3′ motif are present near the 5′-end of the viral genome and are matched by an identical number of 5′ UUG 3′ motifs near the 3′ end (Fig. 4C, D). These repeats could potentially help to stabilize an extended panhandle structure of the BDV genome. Furthermore, a well-conserved inverted repeat consisting of the elements 3′ AACGCA 5′ and 3′ UGCGUU 5′ is present near the 3′-end of the viral genome (Fig. 4C). These elements could easily fold into stem–loop structures and thus influence the architecture of the genome ends. It is not known at present which functions these various motifs might have.

Conclusions and implications

Nucleotides located near the termini of the viral genome were previously found to play a critical role in establishing a technique that permits the genetic manipulation of negative-strand RNA viruses (Conzelmann, 1998). Of similar importance are cDNA clones used to reconstitute the functional viral polymerase complex (Conzelmann, 1998). Our new sequence information on the hypothetical structure of both ends of the BDV genome might explain why various artificial 'mini-replicons' that were based on published sequence information did not replicate to detectable levels in previous experiments. Functional tests with native and reconstituted viral polymerase complexes will be necessary to determine whether artificial BDV RNA molecules with the new structural features are replication-competent.

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treated (–TAP) with tobacco acid pyrophosphatase. Lower-case letters mark terminal nucleotides that were introduced artificially during the RACE procedure. The deduced end of the viral genome is shown at the bottom. (D) Terminal virus sequences deduced by direct sequencing of the 5′-RACE amplification product obtained after ligating the RNA oligonucleotide to the viral genome. The arrow indicates the junction between virus-specific and oligo-derived sequences. The deduced end of the viral genome is shown at the bottom.

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