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Psittacine Beak and Feather Disease Virus Nucleotide Sequence Analysis and Its Relationship to Porcine Circovirus, Plant Circoviruses, and Chicken Anaemia Virus

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Cloning and sequencing of the circular, single-stranded DNA of one isolate of psittacine beak and feather disease virus (BFDV) demonstrate a genome composed of a circular molecule of 1993 nucleotide bases. An analysis of the assembled replicative form demonstrated seven open reading frames (ORFs) (three in the virion strand and four in the complementary strand), potentially encoding seven viral proteins of >8.7 kDa. High amino acid sequence similarity was demonstrated between a potential 33.3-kDa protein product of ORF1 of BFDV and the replicase-associated protein of porcine circovirus (PCV), subterranean clover stunt virus, and faba bean necrotic yellows virus. However, significant similarity in nucleotide or amino acid sequences was not present between BFDV and chicken anaemia virus. A potential stem–loop structure similar to that found in PCV and plant circoviruses was present in the putative encapsidated strand of the BFDV genome. At the top of this structure, a nonanucleotide motif (TAGTATTAC) similar to that of PCV, plant circoviruses, and geminiviruses also was recognised. Comparison of the deduced amino acid sequences of ORF2 of BFDV and PCV demonstrated 29.1% identity, and in both viruses, ORF2 is located on the complementary strand, beginning close to or within the hairpin stem. Our findings provide further evidence of a close relationship among BFDV, PCV, and plant circoviruses but not chicken anaemia virus.

INTRODUCTION

Psittacine beak and feather disease (PBFD) is the most commonly recognised viral disease of wild and captive *Psittaciformes* in Australia (Pass and Perry, 1984), and the disease is common in captive psittacine birds in other countries. In most species, the disease is characterised by chronic progressive, symmetrical feather dystrophy and occasional beak deformity (Pass and Perry, 1984). The causative agent of the disease, PBFD virus (BFDV), is nonenveloped with isometric or spherical symmetry and is 14–16 nm in diameter with a circular single-stranded DNA (ssDNA) genome of <2 kb (Ritchie *et al.,* 1989). Three BFDV proteins with approximate molecular masses of 26.3, 23.7, and 15.9 kDa have been reported (Ritchie *et al.,* 1989).

Efforts to propagate BFDV *in vitro* have not been successful (Pass and Perry, 1985), and this has frustrated research into the molecular genetics of the virus. However, based on the size of the virion and nucleic acid characteristics, BFDV, along with chicken anaemia virus (CAV) and porcine circovirus (PCV), have been tentatively placed in the *Circoviridae* family (Lukert *et al.,* 1995; Studdert, 1993), in which plant circoviruses such as subterranean clover stunt virus (SCSV), banana bunchy top virus (BBTV), and coconut foliar decay virus (CFDV) have also been considered to be unassigned members (Lukert *et al.,* 1995). The DNA sequence of the BFDV genome has not been determined, and this information is important for defining the phylogenetic relationship among the animal and plant circoviruses. We report an analysis of the complete nucleotide (nt) sequence of the genome of one isolate of BFDV and discuss its relationship with PCV, CAV, and the plant circoviruses.

RESULTS

Cloning, sequencing, and computer analysis of PCR products

PCR primer sets A′/A′′ and B′/B′′ amplified a 368-bp segment and a 1664-bp segment, respectively, from peripheral blood mononuclear cells (PBMCs) of the PBFD-affected cockatoo but did not amplify any product from the PBMCs of a healthy cockatoo, six galahs, a chicken, and a pigeon. The nt sequence analysis of cloned segments revealed paired overlapping ends, indicating a circular molecule of 1993 nt. This sequence is depicted as a linearised molecule in Figure 1. Analysis of sequence data demonstrated seven potential open reading frames (ORFs) in the putative replicative form (Fig. 2). A sequence potentially able to form a hairpin structure was detected at nt 1976–

The nt sequence data reported here have been submitted to the GenBank nt sequence database and have been assigned accession No. AF08060.

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   1  ACCCGCCGCC TGGGGCACCG GGGCACCGCA GCTATTGGCT GCTCTGCCGA
  51  GGTGCCCGCC CCTAGGGAGG AGTAAATGGC GGCGTTATAC GCCGCCGTAA
 101  TCTCCGGAGG ATCACACCCG CCCGGGAACC ATGCCGTCCA AGGAGGGCTC
 151  CGGCTGTCGC CGTTGGTGTT TCACCCTTAA CAACCCTACA GACGGCGAGA
 201  TCGAATTCGT CCGTTCTCTG GGGCCTGACG AATTCTACTA TGCCATCGTT
 251  GGACGCGAAA AGGGTGAGCA AGGCACCCCC CATTTGCAAG GCTACTTTCA
 301  TTTTAAAAAT AAGAAGCGAC TGAGCGCGCT TAAGAAACTG CTGCCGCGAG
 351  CCCATTTTGA GCGCGCTAAA GGTAGCGATG CTGATAATGA GAAGTATTGC
 401  AGTAAAGAGG GGGACGTTAT ACTCACCCTG GGCATTGTGG CGAGAGACGG
 451  TCACCGCGCG TTCGACGGAG CTGTTGCTGC CGTGATGTCC GGACGCAAAA
 501  TGAAGGAAGT CGCGCGAGAG TTCCCAGAAG TCTACGTAAG GCATGGGCGG
 551  GGCTTACATA ACCTCTCGCT ATTGGTTGGT TCCAGCCCAC GTGACTTCAA
 601  GACTGAGGTT GACGTCATZT ACGGGCCACC GGGGTGTGGC AAGAGTAGAT
 651  GGGCCAATGA GCAGCCTGGG ACTAAATATT ATAAAATGCG CGGTGAATGG
 701  TGGGATGGAT ATGACGGGGA AGATGTCGTC GTCTTGGACG ATTTTTATGG
 751  GTGGCTACCT TATTGCGAGA TGCTCCGCCT CTGCGACCGT TATCCACATA
 801  AAGTGCCAGT CAAGGGCGCT TTTGTGGAGT TTACCAGCAA GAGGATAATT
 851  ATCACGAGCA ATAAGCCCCC CGAGACCTGG TACAAGGAGG ACTGTGACCC
 901  TAAGCCACTG TTCCGGAGGT TCACACGTGT TTGGTGGTAC AACGTGGACA
 951  AGTTGGAACA AGTCCGGCCT GACTTCCTCG CCCACCCCAT CAATTACTGA
1001  TAGTCTCGGT GATGTTTTCA TAAAGCTAGG CGTCGGGCCG AAGGCCCGAT
1051  GCCGCAGGGG GGGACCCCCT GCCGGAGGGC TCGCAGGGCC GTCAGGCCCG
1101  AYAGACCGAC CAGCCCGGAG GGCGTARTCT GTGTCGGGGG GGGGGCCCCG
1151  GGGGGTCCCC CCGACACGAC GAACACGGTC AGCGCCGAAG GCGCCAATAA
1201  ACACTCGAAA AGGTATTTGC TGTCTGAGTC TTTATTAAGT GCTGGGATTG
1251  TTAGGGGCAA ACTGACGGAA TTGAACATAC AAAGTGAGCT TGGTCACATA
1301  AGTGATCGTT TGTTCTGGCT GGGGGAAGCT GAATGCAATG CCGTAGTGCC
1351  GGACTTTCGT TCCCGCTGAG TTGGGTCCTC CTTGTAGTGG GATCCAGCCG
1401  GTTCTGGCGC TGTTTAGCCA CAATGCCGCA GACTGGTTCG CTGTGRTCAG
1451  GTCCTCTATC GTTATTTGTG GTTTGGGTCT GAGGAGGCGT TTGAATCCCC
1501  TGCTAACAAA CCATTTCTTG GCACCGTCGA AAGGTGCCAG AGGGTCTTGT
1551  GTTTGGTCTG CAGTAGTTTT AAATTTAGTT ATTCTGGAGT CTTGGATTAC
1601  GGCCGTGTGG CCGAATCCGT TTGATTGTAC GGTGTAATGT CCCCCTGTGG
1651  GCCTCATTTC CATTTTAGCT AACTTAATAC GGTAGTCTTC GAAATTCAGT
1701  GCGTGTGGGT TTGGGACGGC TTGTAGGAAG TCGTCCAACG CAAATGTTAT
1751  GTAGTCAGCA TTAAAAATTA GGTTGCCGAC ACTGGTGGTT TGTTTTTGAA
1801  TTTTGAATTG GAATTGGCGT GTGAGTCGGA GAGTGTAGAC CCTATTGGTT
1851  GTGAAACGGC GCCTGCGGAA GTGCCTACGT CGCCTGCGGT ATCGCCTGAT
1901  GTGACGTCTG CGGTATGGGC GGGCATATCT TCGTCTAATC TGAAATTTAG
1951  CGCATGCGCA GTTAGAGGTG CCCCACAGGC GGCGGTTAGT ATT
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FIG. 1 The nt sequence of the encapsidated BFDV genome containing a potential stem–loop sequence (underlined) encompassing a nonanucleotide sequence (bold). The largest ORF (ORF1) is boxed and potentially encodes a replicase-associated (Rep) protein (see Fig. 3) similar to the Rep proteins of other circoviruses and geminiviruses. Two potential TATA boxes were detected; the first (TATA) at nt 86–89, and the second (TATAAAA) at nt 680–686. Two potential polyadenylation signals at nt 1019–1024 (CATAAA) and 1196–1201 (AATAAA) are also shown (bold). A third polyadenylation signal (AATAAA) was present in the complementary strand at nt 1236–1231 (not shown). Y represents A or G; R represents G or T; and Z represents C or T, respectively.

1993 and 1–12 (Fig. 2B). A nonanucleotide motif (TAGTATTAC) with a sequence similar to that of PCV, plant circoviruses, and geminiviruses was demonstrated at the top of this structure (Fig. 2C). Accordingly, the convention of numbering the nt was adopted from that used for geminiviruses (Tan *et al.,* 1995) and PCV (Meehan *et al.,* 1997) in which the "A" residue immediately downstream of the putative nick site in the nonanucleotide motif was designated nt position 1 (Tan *et al.,* 1995). There was a repeated octanucleotide motif (GGGCACCG) immediately downstream of the hairpin structure (Fig. 2B).

The seven ORFs demonstrated in the replicative form potentially encode seven proteins of >8.7 kDa. The respective nt length and size of predicted proteins of these ORFs were 867 nt and 33.3 kDa for ORF1, 741 nt and 28.9 kDa for ORF2, 480 nt and 17.7 kDa for ORF3, 318 nt and 11.2 kDa for ORF4, 303 nt and 10.7 kDa for ORF5, 264 nt and 9.7 kDa for ORF6, and 258 nt and 8.7 kDa for ORF7. Except for ORF2, which had a start codon of CTG, the start codon for all other ORFs was AUG. The position and orientation of each potential ORF are shown in Figure 2A. In the virion strand, two potential TATA boxed were detected (Fig. 1). The first (TATA) was present at nt 86–89, 45 bp upstream of the start codon of ORF1. The second (TATAAAA) was present at nt 680–686. Two potential polyadenylation signals (Fig. 1) were present at nt 1019–1024 (CATAAA) and 1196–1201(AATAAA) of the virion strand, respectively, ~22 and ~198 nt positions downstream of the stop codon for ORF1. A polyadenylation signal was also present in the complementary strand at nt 758–763 (AATAAA), 1 nt downstream of the stop codon for ORF2.

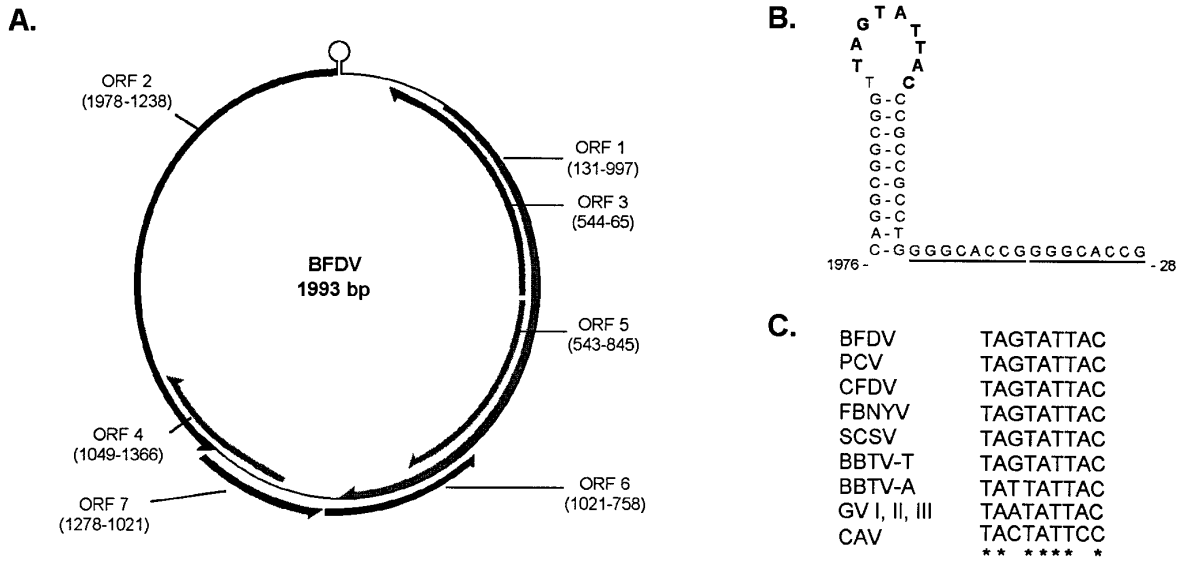

FIG. 2. (A) Schematic diagram of the putative replicative form of the BFDV genomel Seven ORFs with their nt positions are shown. The largest ORF (ORF1) potentially encodes a replicase-associated (Rep) protein (see Fig. 3) similar to the Rep proteins of other circoviruses and geminiviruses. (B) A stem–loop structure at nt 1987–11 with a nonanucleotide (TAGTATTAC) motif (bold) at the top and a repeated octanucleotide motif (underlined) immediately downstream of the hairpin structure. (C) The alignment of the nonanucleotide motifs of BFDV (accession No. AF08060, PCV (accession No. U49186) (Meehan *et al.,* 1997), CFDV (accession No. M29963) (Rohde *et al.,* 1995), FBNYV (accession No. Y11405) (Katul *et al.,* 1997), SCSV (accession No. U16731) (Boevink *et al.,* 1995), BBTV-Taiwanese isolate (BBTV-T; accession No. L32166) (Wu *et al.,* 1994), bean yellow dwarf virus (BFDV; accession No. Y11023), beet curly top virus (BFDV; accession No. U56975), bean golden mosaic virus (BGMV; accession No. M88686) [representatives of geminivirus subgroups (GV) I, II, and III (accession No. D10068)], and CAV (accession No. D10068). These nonanucleotides have been recognised as essential *cis*-acting elements in the replication of some of these viruses. ∗, An identical nt within the motifs.

Computer analysis of the amino acid sequence predicted the putative product of ORF1 (Fig. 3) would have a size of 33.3 kDa. There was marked similarity in the amino acid sequence of the putative product of the ORF1 of BFDV with the amino acid sequence of the Rep proteins of PCV and the plant circoviruses BBTV, CFDV, SCSV, and FBNYV (Table 1). There also was 29.1% identity demonstrated (Fig. 4) between the amino acid sequence of the putative product of ORF2 of BFDV and ORF2 of PCV (Meehan *et al.,* 1997). Apart from this, there was no significant similarity in the nt or amino acid sequences of ORFs 3–7 of BFDV with other ORFs of PCV (Meehan *et al.,* 1997) or with the ORFs of plant circoviruses, including the coat protein genes of BBTV (Wanitchakorn *et al.,* 1997), SCSV (Boevink *et al.,* 1995), and FBNYV (Katul *et al.,* 1995, 1997). There also was no significant similarity in the nt or amino acid sequence of BFDV and CAV.

DISCUSSION

The data presented here confirm previous research that demonstrated BFDV contained a circular ssDNA genome of <2 kb (Ritchie *et al.,* 1989). Based on the similarity of the sequence we describe with the reported sequences of PCV and plant cicoviruses (Table 1, Figs. 2C and 3B), it is likely that the nt sequence presented here represents the encapsidated ssDNA strand of the virus. A hairpin loop located upstream in relation to ORF1, the putative Rep protein gene of BFDV (Fig. 2), was similar to a loop structure found in the genomes of PCV, plant circoviruses, and geminiviruses. This and other features of the genome were most closely related to PCV. Like PCV (Meehan *et al.,* 1997), BFDV contained seven major ORFs and lacked a distinctive noncoding region, thus affording highly efficient use of genetic material in both of these viruses. Both viruses have three ORFs in the encapsidated strand and four ORFs in the complementary strand of the replicative form. The putative protein products of ORF2 or BFDV and PCV shared 29.1% amino acid sequence identity (Fig. 4), and in both viruses, ORF2 is located on the complementary strand and begins close to or within the hairpin stem (Fig. 2A) (Meehan *et al.,* 1997). These features indicate that the proteins coded for by ORF2 of BFDV and PCV may have similar functions. The ORF3s of both BFDV and PCV also are located in similar positions on the complementary strand, respectively, although like the remaining four smaller ORFs, they shared no amino acid sequence identity.

Previous research into the relationships among the proposed animal circoviruses did not detect antigenic cross-reactivity or DNA cross-hybridization among BFDV, CAV, and PCV (Todd *et al.,* 1991). Although genetic analysis of BFDV was not reported, it was suggested by Meehan *et al.* (1997) and Mankertz *et al.* (1997) that CAV was sufficiently different from BFDV, PCV, and plant cir-

A.

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  1 MPSKEGSGCR RWCFTLNNPT DGEIEFVRSL GPDEFYYAIV GREKGEQGTP
 51 HLQGYFHFKN KKRLSALKKL LPRAHFERAK GSDADNEKYC SKEGDVILTL
101 GIVARDGHRA FDGAVAAVMS GRKMKEVARE FPEVYVRHGR GLHNLSLLVG
151 SSPRDFKTEV DVIYGPPGCG KSRWANEQPG TKYYKMRGEW WDGYDGEDVV
201 VLDDFYGWLP YCEMLRLCDR YPHKVPVKGA FVEFTSKRII ITSNKPPETW
251 YKEDCDPKPL FRRFTRVWWY NVDKLEQVRP DFLAHPINY
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B.

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          I           II          III         IV
BFDV   --FTLNN------G.TPHLQGY-----YCSK-----G.PPGCGKS--
PCV    --FTLNN------GRTAHLQGF-----YCSK-----G.PPGCGKS--
BBTV   --FTLNN------G.QKHLQGY-----YCSK-----GPNGNEGKS--
FBNYV  --FTLNN------G.NIHFQGY-----YSMK-----GPQGGEGKT--
MSV    --FLTYP------G.SLHLHAL-----YILK-----G.PTRTGKS--
BGMW   --FLTYP------G.EPHLHVL-----YIEK-----G.DSRTGKT--
BCTV   --FLTYP------G.SLHLHAL-----YIEK-----G.DSRTGKT--
SCSV   --FTLNY------G.QKHLQGF-----YCCK-----GSDGGEGKT--
CFDV   --FTLNY------G.QRHLQGF-----YCSK-----GRDGGDGKS--
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FIG. 3. (A) Amino acid sequence of the putative product of ORF1 of BFDV derived from the nt sequence shown in Figure 1 using the MacVector 3.5 sequence analysis software. The sequence is similar to the Rep proteins of porcine and plant circoviruses (see Table 1). The position of four motifs involved in RCR is underlined. (B) Edited alignment of selected domains of the putative protein product of ORF1 of BFDV and the Rep proteins of PCV (accession No. U49186), BBTV (accession No. 12586), FBNYV (accession No. Y11405), bean golden mosaic virus (BGMV; accession No. M88686), beet curly top virus (BCTV; accession No. U56975), maize streak virus (MSV; Boevink *et al.,* 1995), CFDV (accession No. M29963), and SCSV (accession No. U16731) using the PILEUP program (Feng and Doolittle, 1987) through the service provided by ANGIS. Motifs indicated by I, II, and III are three typical domains (bold letters) involved in RCR, and bold letters in motif IV indicate the nt-binding site.

coviruses to warrant revision of its position within the *Circoviridae* family. There is similarity between PCV and BFDV in terms of both particle and genome sizes and morphology, but important physicochemical differences exist between these two viruses and CAV (Todd *et al.,* 1991). A comparison of nucleic and amino acid sequences of PCV (Mankertz *et al.,* 1990, Meehan *et al.,* 1997) and CAV (Todd *et al.,* 1993) has failed to demonstrate any significant similarity between these two viruses, and a noncoding region present in CAV (Classens *et al.,* 1991) is not present in PCV (Meehan *et al.,* 1997).

Sequence analysis has revealed similarity between the products of the Rep protein genes of PCV and the plant circoviruses BBTV, CFDV, and SCSV (Meehan *et al.,* 1997) that was not detectable with CAV (Meehan *et al.,* 1997; Mankertz *et al.,* 1997). The nt sequence analysis of FBNYV also showed significant similarity between the potential protein encoded by component 1 ORF with the ORF1 product of PCV and plant circoviruses (Katul *et al.,* 1997). Our results confirm the similarity of the genome of BFDV with that of PCV and the plant circoviruses and the differences between BFDV and CAV. This supports the suggestion that BFDV should be classified together with PCV and the plant circoviruses and separately from CAV (Noteborn and Koch, 1995; Meehan *et al.,* 1997).

The nature of the stem–loop structure we detected in BFDV is also evidence for a relationship among BFDV, PCV, the plant circoviruses, and geminiviruses. These viruses have similar stem–loop structures and an evolutionary conserved nonanucleotide motif at the apex of the stem–loop that is necessary for replication (Mankertz *et al.,* 1997; Dale *et al.,* 1997; Orozco *et al.,* 1996). In geminiviruses, the nonanucleotide motif is an essential *cis*-acting element that is required for DNA replication (Lararowitz *et al.,* 1992; Revington *et al.,* 1989). A nonanucleotide motif (TACTATTCC), with a 2-nt difference with BFDV, PCV, and the plant circoviruses (Fig. 2C), also is present in CAV but is not associated with a hairpin structure (Classens *et al.,* 1991; Noteborn and Koch, 1995).

A rolling-circle replication (RCR) mechanism with separate leading- and lagging-strand DNA synthesis steps is used for geminivirus replication, in which sites of initiation and termination of the replication of the plus strand have been mapped to a conserved nonanucleotide motif present in all geminiviruses (Heyraud *et al.,*

TABLE 1

Percentage Amino Acid Identity and Similarity of the Potential protein Product of ORF-1 of BFDV[a] and the Replicase-Associated Proteins (Reps) of Porcine Circovirus (PCV[b,c]), Banana Bunchy Top Virus ORF V1 (BBTV-ORF V1[d]), Australian Isolate Component 1 (BBTV-A.C1[e]), Taiwanese Isolates T1 (BBTV-T1[f]) and T2 (BBTV-T2[g]), Coconut Foliar Decay Virus (CFDV[h]), Subterranean Clover Stunt Virus Components 1 (SCSV-C2[i]) and 2 (SCSV-6[j]), Faba Bean Necrotic Yellows Virus Components 1 (FBNYV-C1[k]) and 2 (FBNYV-C2[l]) and three Representatives of Geminivirus Subgroups of I, II and III, respectively, Maize Streak Virus (MSV[m]), Beet Curly Top Virus (BCTV[n]), and Bean Golden Mosaic Virus (BGMV[o]) Using BestFit Method in the Wisconsin Analysis Package™ Provided by ANGIS

Virus	% Identity with BFDV	% Similarity with BFDV
BFDV	100	100
PCV	45.6	64.3
BBTV-ORF V1	34.9	57
BBTV-T1	31.7	54.3
BBTV-T2	28.5	53.7
BBTV-A-C1	25.4	47.1
CFDV	32.5	55.8
FBNYV-C1	22.1	51.3
FBNYV-C2	24.6	53.9
SCSV-C2	29.6	50.7
SCSV-C6	23.6	51.4
GV I: MSV	27.2	47
GV II: BCTV	20.4	45.5
GV III: BGMV	22.1	48.3

[a] Data from this article.
[b] Mankertz *et al.,* (1998).
[c] Meehan *et al.,* (1997) (GenBank accession No. U49186).
[d] Wu and You (1994) Unpublished (GenBank accession No. U12586).
[e] Harding *et al.,* (1993) (GenBank accession No. s56279).
[f,g] Wu *et al.,* (1994) (GenBank accession No. L32166, L32167).
[h] Rohde *et al.,* (1990) (GenBank accession No. M29963).
[i,j] Katul *et al.,* (1995) (GenBank accession No. U16731, U16735).
[k] Katul *et al.,* (1997) (GenBank accession No. X80879).
[l] Katul *et al.,* (1997) (GenBank accession No. Y11405).
[m] Boevink *et al.,* (1995).
[n] Stenger *et al.,* (1990) (GenBank accession No. U56975).
[o] Gilbertson *et al.,* (1991) (GenBank accession No. M88686).

1993; Stenger *et al.,* 1991). During RCR, the virion sense DNA is nicked between nt 7 and 8 of the nonanucleotide (TAATATT-AC) motif (Heyraud-Nitschke *et al.,* 1995; Laufs *et al.,* 1995; Stanley, 1995). A similar mechanism of replication probably occurs in the plant and animal circovirus group (Mankertz *et al.,* 1997; Meehan *et al.,* 1997). Demonstration of specific binding of the Rep protein of BBTV to the loop sequence of the virus and its ability to nick and ligate within the nonanucleotide loop sequence also is strong evidence for an RCR mechanism in BBTV replication (Dale *et al.,* 1997). In this regard, comparison of the Rep proteins of PCV, SCSV, CFDV, FBNYV and BBTV, and TGMV and maize streak virus (MSV) (two representatives of subgroup I and II geminiviruses) has revealed significant homology around a conserved nt binding motif (Boevink *et al.,* 1995; Mankertz *et al.,* 1997) and three motifs typically found associated with Rep proteins involved in RCR of geminiviruses (Koonin and Ilynia, 1993). Our results (Fig. 3B) demonstrate that the putative Rep protein of BFDV also contains such motifs. The presence of similar structures and motifs in the genome of BFDV and PCV, plant circoviruses, and geminiviruses suggests that BFDV replicates by a similar RCR mechanism to that used by these viruses.

The mechanism of CAV replication (Noteborn and Koch, 1995) is different from that proposed for PCV and the other related viruses (Mankertz *et al.,* 1997). There are differences in the location of the hairpin structure and the nonanucleotide motif of CAV and other circoviruses: the hairpin structure in CAV is located 4 nt downstream of the stop codon for a major ORF (Classens *et al.,* 1991); in contrast to BFDV, PCV, and plant circoviruses, the top of this structure lacks a nonanucleotide motif. Instead, the nonanucleotide motif of CAV (Noteborn and Koch, 1995) is located 100 nt downstream of the hairpin structure (Classens *et al.,* 1991). CAV replicates via a circular double-stranded replicative form and an unspliced polycistronic mRNA molecule that contains three partially overlapping genes, each with its own start and stop codons (Noteborn and Koch, 1995). This is different from the pattern of transcription of the plant circoviruses, geminiviruses and PCV, and attempts to demonstrate RCR in CAV were unsuccessful (Todd *et al.,* 1996).

MATERIALS AND METHODS

BFDV DNA isolation and purification

BFDV was purified from a pool of feathers from five PBFD-affected cockatoos as described by Ritchie *et al.* (1989) and had a titer of 1:327,680 by haemagglutination assay (Raidal *et al.,* 1993). The purified BFDV suspension was treated with RQ1 RNase-Free DNase (Promega); sodium dodecyl sulfate was added to a final volume of 1%; and DNA was extracted once with phenol, pH 8.0 (Sigma), and twice with chloroform–isoamyl alcohol (24:1) (Sigma) and precipitated with ethanol and treated with RNase A (Promega). When this product was electrophoresed through PhastGel gradient media (PhastSystem; Pharmacia Biotech) and silver stain (PhastGel DNA Silver Staining Kit; Pharmacia Biotech), it produced a single band, indicating minimal genomic contamination.

Conversion of BFDV ssDNA to double-stranded DNA and cloning

Purified BFDV ssDNA was converted to double-stranded DNA (dsDNA) using Prime-a-Gene Labelling System (Promega) and was detected as a smear of $<$600 bp when electrophoresed onto an agarose gel. The dsDNA was digested with mung bean nuclease (Promega) to create blunt-ended dsDNA fragments, which were cloned into *Sma*I-digested pUC18 plasmid (Ready-to-go pUC18 SmaI/BAP+ Ligase Kit; Pharmacia Biotech) and transformed into *Escherichia coli,* strain XL1-Blue

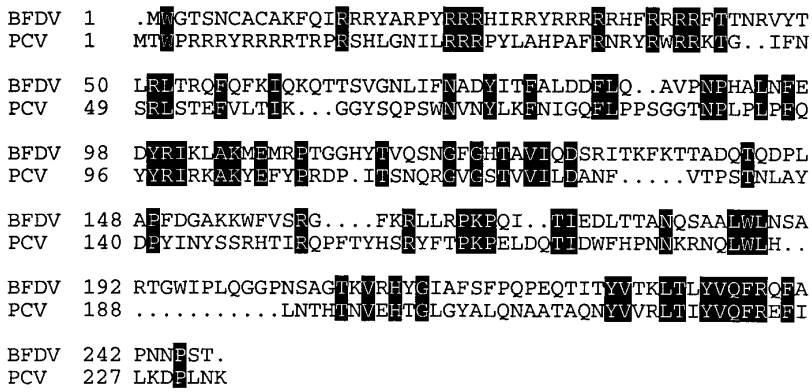

FIG. 4. Comparison of the deduced amino acid sequences of ORF2 of BFDV and PCV (Meehan *et al,* 1997) demonstrating 29.1% identity (reversed type) using the GAP method provided by ANGIS. In both viruses, ORF2 is located on the complementary strand beginning close to or within the hairpin stem (see Fig. 1A). These features indicate that the proteins coded for by ORF2 of BFDV and PCV may have similar functions.

competent cells (Inoue *et al.,* 1990). Transformed clones were selected on ampicillin (500 μg/ml) and X-Gal plates (Sambrook *et al.,* 1989), and potential recombinant clones were screened by polymerase chain reaction (PCR) using M13 forward and reverse primers (Perkin-Elmer). Twenty clones containing inserts were amplified in LB liquid culture (Sambrook *et al.,* 1989), and recombinant plasmids were isolated using the Wizard Plus Miniprep DNA Purification System (Promega). With M13 forward and reverse primers, the inserts were sequenced with the use of ABI PRISM DYE Terminator cycle sequencing kits (Perkin-Elmer) on the Applied Biosystems 373A DNA sequencing system (Perkin-Elmer) and ranged from 39 to 193 bp in length. Sequence data were checked for similarity with other viruses, vectors, and eucaryotes in GenBank and EMBL databases provided by the Australian National Genomic Information Service (ANGIS).

Preparation of BFDV replicative form from PBMCs

A 3-ml blood sample that was placed into EDTA was collected from a PBFD-affected sulfur crested cockatoo (*Cacatua galerita*), a normal sulfur crested cockatoo, six normal galahs (*Eolophus roseicapillus*), a chicken (*Gallus gallus*), and a pigeon (*Columba livia*). PBMCs were separated by centrifugation on Histopaque (Sigma) and washed three times in PBS, pH 7.6. The cells were resuspended at 4×10^6 cells/ml in PCR lysing buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40, and 250 μg/ml Proteinase K) and incubated at 45°C for 1 h, followed by enzyme inactivation at 95°C for 10 min (Manak, 1993).

PCR amplification, cloning, and sequencing of BFDV replicative form from PBMCs

Based on sequence data obtained from the blunt-end cloning described above, various PCR primers were designed to amplify by PCR (Definitive Tth Plus PCR Kit; Biotech International) longer segments of the replicative form present in the PBMC extracts. One set of these primers amplified a 495-bp product that was cloned in pCR 2.1 plasmid using a TA Cloning Vector kit (InVitrogen). Potential recombinant clones were selected as mentioned above, screened by PCR using M13 forward and reverse primers (Perkin-Elmer), and sequenced as described above. Based on the sequence of this 495-bp product, two sets of PCR primers (A′-TGGTACAAGGAGGACTGTGAC, nt 878–898/A′′-CCAGCACTTAATAAACACTCAG, nt 1224–1245; and B′-GTCTTTATTAAGTGCTGGGA, nt 1228–1247/B′′-GTCACAGTCCTGGTTGTACC, nt 879–898) were designed to amplify expected products of 368 bp and the remainder of the circular BFDV genome, respectively. Direct PCR sequencing was performed in duplicate in both directions on PCR products generated. PCR products also were cloned into pCR 2.1 using a TA Cloning Vector kit (InVitrogen), and recombinant clones were identified and similarly sequenced in duplicate in both directions.

Computer analysis of sequence data

The nt sequence of the BFDV genome was analyzed using the sequence editor program SeqEd Version 1.0.3 (Applied Biosystems). Database searches were performed in nonredundant nucleic (NR Nucleic) and protein (NR proteins) databases at ANGIS using BLAST (Altschul *et al.,* 1990) and FASTA (Pearson and Lipman, 1988) programs. Putative BFDV ORFs were identified using MacVector (MacVector 3.5 of ABI sequence analysis software) and numbered according to their size. Sequences and accession numbers of other viruses retrieved for comparison for GenBank and EMBL are included in Table 1 and Figures 2 and 3.

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