Journal of General Virology (1997), **78**, 1795–1799. Printed in Great Britain SHORT COMMUNICATION

Nicking and joining activity of banana bunchy top virus replication protein *in vitro*

Gregory J. Hafner,[1] Mark R. Stafford,[2] Lindsay C. Wolter,[2] Robert M. Harding[1] and James L. Dale[1]

Centre for Molecular Biotechnology, School of Life Sciences[1], and Cooperative Research Centre for Diagnostic Technologies[2], Queensland University of Technology, GPO Box 2434, Brisbane, Queensland 4001, Australia

The major open reading frame of banana bunchy top virus (BBTV) DNA-1 encodes a putative replication initiation protein (Rep). *In vitro*, a fusion protein of BBTV Rep linked to a maltose-binding protein exhibited both site-specific nicking and joining activities. These activities were dependent on the presence of Mg^{2} or Mn^{2}, but did not require ATP. The fusion protein specifically cleaved ssDNA between bases 7 and 8 of a conserved nonanucleotide loop sequence which is present in the virion-strand of the stem–loop common region of each BBTV component. During this reaction, the fusion protein became covalently attached to the 5′ end of the 3′ cleavage product. After the nicking reactions, the fusion protein was also capable of catalysing the joining of two nicked ssDNA fragments in a site-specific manner. Based on these activities, BBTV Rep would appear to be very similar to the Rep proteins of the geminiviruses.

Banana bunchy top virus (BBTV) has 18–20 nm isometric virions and a genome comprising at least six circular ssDNA components, each of approximately 1 kb (Burns *et al.*, 1995). BBTV shares a number of characteristics with four other plant viruses [subterranean clover stunt virus (SCSV) (Boevink *et al.*, 1995), coconut foliar decay virus (CFDV) (Rohde *et al.*, 1990), faba bean necrotic yellows virus (FBNYV) (Katul *et al.*, 1995) and milk vetch dwarf virus (MDV) (Sano *et al.*, 1993)] which all have isometric virions and circular ssDNA genomes with components of approximately 1 kb.

All ssDNA components of BBTV have a similar organization, including two conserved regions which have been designated the major common region (CR-M) and the stem–loop common region (CR-SL) (Burns *et al.*, 1995). Located within the CR-SL is a putative stem–loop structure which includes a highly conserved nonanucleotide loop sequence (5′ TANTATTAC 3′). This sequence is not only conserved between BBTV components but also within the components of other BBTV-like viruses and geminiviruses. Within the geminiviruses this conserved nonanucleotide sequence has been shown to be part of the viral origin of replication (Heyraud *et al.*, 1993).

Author for correspondence: James L. Dale.
Fax +61 7 3864 1534. e-mail j.dale@qut.edu.au

The large ORF of BBTV DNA-1 was initially thought to encode a putative replicase protein based on the presence of an NTP-binding motif (Harding *et al.*, 1993). However, it is more likely that this gene encodes a replication initiator protein (Rep) based on the presence of other motifs conserved within this protein (Ilyina & Koonin, 1992; Koonin & Ilyina, 1992) and the lack of any similarity with known polymerases. Rep proteins have been found to be associated with a variety of plasmids of Gram-positive (Koepsel *et al.*, 1985; te Riele *et al.*, 1986; Gros *et al.*, 1987; Gruss & Ehrlich, 1989) and Gram-negative bacteria (Yasukawa *et al.*, 1991), ss and dsDNA phages (Baas & Jansz, 1988) and viruses, including the geminiviruses (Laufs *et al.*, 1995*a*), all of which replicate via a rolling-circle mechanism. These proteins generally have multifunctional roles and are involved in both initiation and termination of rolling-circle replication.

In the geminiviruses, the Rep protein is the only gene product essential for virus replication (reviewed by Laufs *et al.*, 1995*a*). Laufs *et al.* (1995*c*) have shown that the Rep protein of tomato yellow leaf curl geminivirus (TYLCV) initiates viral-strand DNA synthesis by introducing a nick within the conserved nonanucleotide loop sequence of the virion-sense strand. It is proposed that the 3′ end of the nicked strand is then available for extension by a host-encoded DNA polymerase, which uses the complementary strand as a template. Furthermore, the Rep protein has been shown to have a joining activity, catalysing the ligation of two nicked ssDNA molecules, which suggests that Rep functions *in vivo* to join nascent viral ssDNA into genomic units (Laufs *et al.*, 1995*c*). Both the nicking and joining activities are ATP-independent reactions.

Here we report that the putative Rep protein encoded by BBTV DNA-1 has enzymatic activities similar to other Rep proteins including site-specific nicking and joining activities.

Two oligonucleotides, BTRepEx2 (5′ ATGGCGCGATATGTGG 3′) and BTRepFD1 (5′GCGAGCTCTCAGCAAGA-

0001-4539 © 1997 SGM

Fig. 1. Sequence requirements for nicking activity of purified MBP–BTRep. Six oligonucleotides (A), representing various permutations of the conserved stem–loop sequence were 5′ end-labelled. Oligo-1 was incubated either alone (B, lane 1), with MBP–BTRep (B, lane 2) or with MBP (B, lane 3). Oligonucleotides CompSL, Oligo-2, Oligo-3, Oligo-4 and Oligo-5 were also incubated alone (B, lanes 4, 6, 8, 10 and 12, respectively), or with MBP–BTRep (B, lanes 5, 7, 9, 11 and 13, respectively). The reaction products were denatured, electrophoresed on 20% denaturing polyacrylamide gels and autoradiographed.

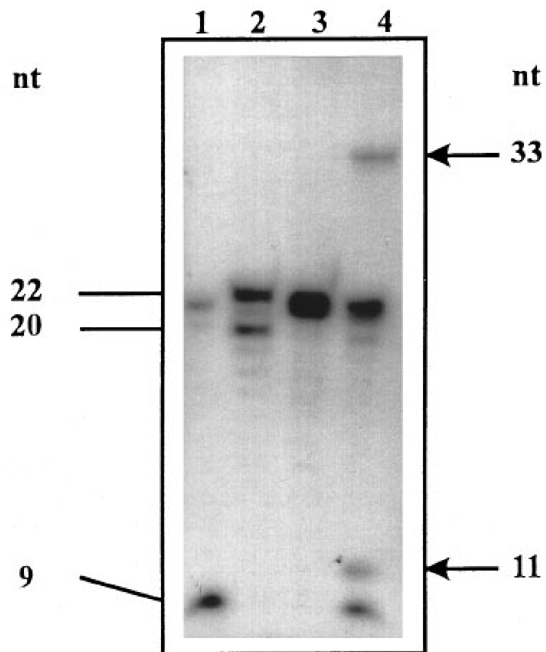

Fig. 2. Joining activity of MBP–BTRep. Oligo-4 and Oligo-5 were either incubated separately with MBP–BTRep (lanes 1 and 2, respectively), mixed together with MBP (lane 3) or mixed together with MBP–BTRep (lane 4). The reaction products were denatured, electrophoresed on 20% denaturing polyacrylamide gels and autoradiographed.

AACCAACT 3′), were designed and synthesized from the nucleotide sequence of BBTV DNA-1 (Harding *et al*., 1993) and used as primers in a PCR reaction with *Pfu* DNA polymerase (Stratagene) and purified BBTV genomic ssDNA to amplify the major BBTV DNA-1 ORF. The amplified 869 bp product was ligated into a *Xmn*I-digested pMAL-C2 expression vector (New England Biolabs) to produce the recombinant vector, pMAL-BTRep, capable of producing the maltose-binding–BBTV Rep fusion protein (MBP–BTRep). The recombinant vector was subsequently electroporated into *E. coli* DH5α. The fusion protein was expressed and purified according to the manufacturer's protocols.

Two oligonucleotides (PB6.1, 5′ GCCTGCAGAGTTGT-GCTGTAATGTT 3′; PB6.2, 5′ GCGGATCCGCTTCTGCC-TTCCGCT 3′) were used in a PCR with purified BBTV DNA to amplify a 638 bp product representing the intergenic region of BBTV DNA-6. Initially, approximately 20 ng of this PCR product was used, either untreated or denatured, in assays with 150 ng MBP–BTRep at 25 °C for 60 min in a 10 μl reaction containing 10 mM Tris–HCl, 50 mM potassium acetate, 10 mM magnesium acetate, pH 7·5. The MBP–BTRep was only capable of cleaving the denatured PCR product (data not shown) resulting in the generation of 390 and 250 nucleotide products; this was consistent with the fusion protein nicking the template within the conserved nonanucleotide loop sequence. MBP alone was unable to cleave any of the templates.

Various oligonucleotides were synthesized to determine the precise sequence requirements essential for *in vitro* nicking activity (Fig. 1A). Oligo-1 and CompSL were both designed around the central conserved nonanucleotide loop sequence and represent the virion- and complementary-sense strand of the viral genome, respectively. Approximately 20 fmol of each oligonucleotide was 5′ end-labelled with [γ-^{32}P]ATP and assayed for nicking by incubation at 25 °C for 60 min with either 150 ng MBP–BTRep, MBP or without additional proteins in 10 μl reactions containing 10 mM Tris–HCl, 50 mM potassium acetate, 10 mM magnesium acetate, pH 7·5. Products were analysed by electrophoresis in 8 M urea–20% polyacrylamide gels alongside a DNA sequencing ladder and oligonucleotides of known sizes as standards and exposed to X-ray film or assayed quantitatively by direct analysis with the BAS1500 Phosphoimager using the MCID4 software package (Fuji). No nicking was observed when Oligo-1 was incubated with MBP (Fig. 1B, lane 3) and CompSL with the MBP–BTRep (Fig. 1B, lane 5). However, MBP–BTRep was found to nick approximately 60% of Oligo-1 in 1 h to produce a 20 nucleotide product (Fig. 1B, lane 2). These *in vitro* studies showed that the BBTV Rep portion of the fusion protein specifically cleaved ssDNA between bases +7 and +8 of the conserved nonamer loop sequence ^{1}TAnTATT↓^{8}AC, which is present in the virion-sense of the CR-SL of each component of BBTV (Burns *et al*., 1995) (Fig. 1A).

Four additional oligonucleotides were synthesized to determine the importance of the regions flanking the conserved loop in the nicking assays. The first two, Oligo-2 and Oligo-3, were similar to Oligo-1 except that the 3′ and 5′ stem sequences, respectively, were randomized to determine whether any specific sequences within these regions were required for nicking activity. Both oligonucleotides were incubated at 25 °C in the presence of MBP–BTRep and were nicked to produce 20 nucleotide products, while the untreated controls remained intact (Fig. 1B, lanes 6 and 8, respectively). Two further oligonucleotides, Oligo-4 and Oligo-5, were synthesized; these were also similar to Oligo-1, but without 5′ and 3′ stem sequences, respectively. When incubated with MBP–BTRep at 25 °C, both Oligo-4 and Oligo-5 were nicked to produce 9 and 20 nucleotide products, respectively (Fig. 1B, lanes 11 and 13, respectively). Both reactions were consistent with the nick site being located between nucleotides +7 and +8 of the conserved nonanucleotide loop (Fig. 1A). These results suggested that neither the sequence nor structural elements outside the loop sequence were essential for *in vitro* nicking activity and, in particular, that stem–loop formation was not a prerequisite for nicking activity.

A common feature of all rolling-circle initiator proteins is the requirement for Mg^{2+} or Mn^{2+} to catalyse the nicking reactions (Baas & Jansz, 1988). Various divalent cations including Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were tested in nicking assays with MBP–BTRep. Only Mg^{2+} and Mn^{2+} were found to efficiently nick Oligo-1, while Ca^{2+} resulted in a low

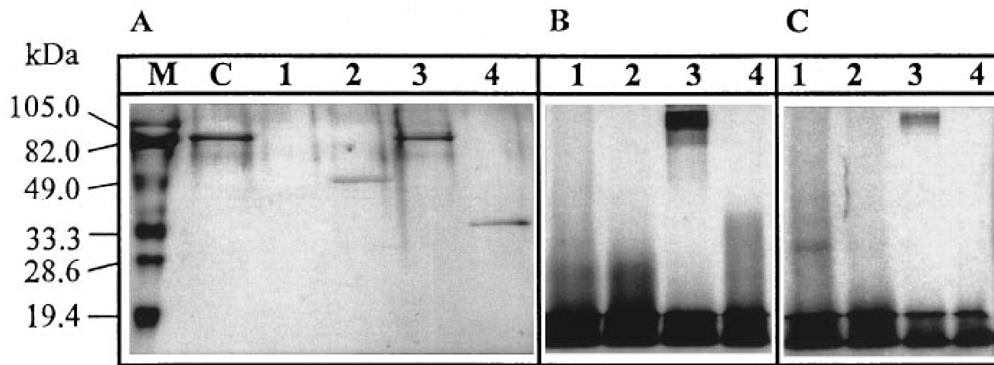

Fig. 3. Covalent attachment of MBP–BTRep to oligonucleotides. Unlabelled (A) or 3′ (B) or 5′ (C) end-labelled Oligo-1 was incubated either alone (lane 1), with MBP (lane 2), with MBP–BTRep (lane 3) or with MBP–BTRep followed by post-treatment with Proteinase K (lane 4). The reactions were analysed by SDS–PAGE using prestained Bio-Rad low-range protein standards (A, lane M) and purified MBP–BTRep (A, lane C) as markers. The gels were analysed by either silver staining (A) or autoradiography (B, C).

level of nicking. The nicking activity was abolished when divalent cations were removed from the reaction buffer (data not shown).

The MBP–BTRep was also assayed for its ability to join two cleaved oligonucleotides. When Oligo-4 and Oligo-5 were 5′ end-labelled and incubated with MBP–BTRep at 10 °C for 1 h, the expected 9 and 20 nucleotide products, respectively, were obtained (Fig. 2, lanes 1 and 2, respectively). These products were not visible when both Oligo-4 and Oligo-5 were incubated at 10 °C with MBP alone (Fig. 2, lane 3). However, when Oligo-4 and Oligo-5 were mixed, nicked with MBP–BTRep at 10 °C for 1 h and then incubated at 37 °C for a further 1 h, two new products of 33 and 11 nucleotides were visible in addition to the uncleaved substrates and expected nicked products. The 33 nucleotide product was presumably formed by joining of the 5′ nicked product of Oligo-5 with the 3′ nicked product of Oligo-4, while the 11 nucleotide product presumably resulted from the 3′ nicked product of Oligo-5 joining to the 5′ nicked product of Oligo-4. This result indicated that the joining reaction was sequence specific and that the hybridization of the sequences flanking the loop to form the stem conformation was not required to aid the joining process.

Covalently linked protein–DNA complexes are typical of other nicking-joining enzymes such as the Rep proteins of the geminivirus TYLCV and RC-plasmid pT181, the gene A product of ϕX174, the Tra protein of RP4 and the Mob protein of RSF1010 (Pansegrau *et al.*, 1990; Thomas *et al.*, 1990; Rasooly & Novick, 1993; Scherzinger *et al*, 1993; Laufs *et al.*, 1995 *b*). However, other Rep proteins such as Rep B of pMV158 (Moscoso *et al.*, 1995) and the gene II product of coliphage fd (Meyer & Geider, 1979) effect protein–DNA interactions through transient phosphodiester bonds or through non-covalent binding. To investigate the possibility that MBP–BTRep remained bound to one of its products during the nicking reactions, 150 ng of the fusion protein was incubated with 2 pmol of 5′[γ-^{32}P]ATP and 3′[α-^{32}P]dCTP end-labelled Oligo-1 at 25 °C for 1 h. The products of the reaction were denatured, analysed by discontinuous SDS–PAGE and either silver stained or dried and autoradiographed.

Assays included Oligo-1 incubated (i) without additional proteins (Fig. 3 A, lane 1), or with (ii) MBP (Fig. 3 A, lane 2), (iii) MBP–BTRep (Fig. 3 A, lane 3) or (iv) MBP–BTRep followed by post-treatment with 200 ng Proteinase K (Boehringer Mannheim) at 37 °C for 1 h (Fig. 3 A, lane 4). MBP–BTRep was also used as a control (Fig. 3 A, lane C). When the gels with the respective 3′ (Fig. 3 B) and 5′ (Fig. 3 C) end-labelled oligonucleotides were autoradiographed, only the lanes with the end-labelled oligonucleotides and MBP–BTRep showed covalent linkage of the oligonucleotides and the fusion protein (Fig. 3 B, lane 3, Fig. 3 C, lane 3). Furthermore, the covalent linkage caused a retardation in the migration of MBP–BTRep due to the added molecular mass of the attached oligonucleotide. When the MBP–BTRep reactions were treated with Proteinase K, the protein covalently attached to the labelled oligonucleotide was digested. Quantitative analysis indicated that 23% of the 3′ end-labelled Oligo-1 was covalently attached to MBP–BTRep while only 3% of the 5′ end-labelled Oligo-1 was covalently attached to MBP–BTRep. Hence, as a consequence of the Rep-catalysed nicking reaction, the MBP–BTRep covalently binds to the 5′ end of the 3′ cleavage product. Low levels of covalent linkage were also observed with the 5′ end-labelled oligonucleotides. These molecules were likely to be intermediates of the reaction, with the MBP–BTRep forming covalent bonds with the entire oligonucleotide substrate, as the 5′ oligonucleotide cleavage products were easily resolved following the cleavage reactions. Based on these activities, the BBTV Rep protein is very similar in function to some of the other proteins associated with the initiation and termination of rolling-circle replication.

This work was supported by grants from the Australian Research

Council and Cooperative Research Centre for Diagnostic Technologies. Greg Hafner was supported by an Australian Postgraduate Research Award.

References

Baas, P. D. & Jansz, H. S. (1988). Single-stranded DNA phage origins. *Current Topics in Microbiology and Immunology* **136**, 31–70.

Boevink, P., Chu, P. W. G. & Keese, P. (1995). Sequence of subterranean clover stunt virus DNA: affinities with the geminiviruses. *Virology* **207**, 354–361.

Burns, T. M., Harding, R. M. & Dale, J. L. (1995). The genome organization of banana bunchy top virus: analysis of six ssDNA components. *Journal of General Virology* **76**, 1471–1482.

Gros, M. F., te Riele, H. & Ehrlich, S. D. (1987). Rolling circle replication of single-stranded DNA plasmid pC194. *EMBO Journal* **6**, 3863–3869.

Gruss, A. & Ehrlich, S. D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiology Reviews* **53**, 231–241.

Harding, R. M., Burns, T. M., Hafner, G. J., Dietzgen R. & Dale, J. L. (1993). Nucleotide sequence of one segment of the banana bunchy top genome contains a putative replicase gene. *Journal of General Virology* **74**, 323–328.

Heyraud, F., Matzeit, V., Kammann, M., Schaefer, S., Schell, J. & Gronenborn, B. (1993). Identification of the initiation sequence for viral strand DNA synthesis of wheat dwarf virus. *EMBO Journal* **12**, 4445–4452.

Ilyina, T. V. & Koonin, E. V. (1992). Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Research* **20**, 3279–3285.

Katul, L., Maiss, E. & Vetten, H. J. (1995). Sequence analysis of a faba bean necrotic yellows virus DNA component containing a putative replicase gene. *Journal of General Virology* **76**, 475–479.

Koepsel, R. R., Murray, R. W., Rosenblum, W. D. & Khan, S. A. (1985). The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase activities. *Proceedings of the National Academy of Sciences, USA* **82**, 6045–6049.

Koonin, E. V. & Ilyina, T. V. (1992). Geminivirus replication proteins are related to prokaryotic plasmid rolling circle DNA replication initiator proteins. *Journal of General Virology* **73**, 2763–2766.

Laufs, J., Jupin, I., David, C., Schumacher, S., Heyraud-Nitschke, F. & Gronenborn, B. (1995*a*). Geminivirus replication: genetic and biochemical characterization of Rep protein function, a review. *Biochimie* **77**, 765–773.

Laufs, J., Schumacher, S., Geisler, N., Jupin, I. & Gronenborn, B. (1995*b*). Identification of the nicking tyrosine of geminivirus Rep protein. *FEBS Letters* **18**, 258–262.

Laufs, J., Traut, W., Heyraud, F., Matzeit, V., Rogers, S. G., Schell, J. & Gronenborn, B. (1995*c*). *In vitro* cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proceedings of the National Academy of Sciences, USA* **92**, 3879–3883.

Meyer, T. F. & Geider, K. (1979). Bacteriophage fd gene II-protein. Specific cleavage and relaxation of supercoiled RF from filamentous phages. *Journal of Biological Chemistry* **254**, 12642–12646.

Moscoso, M., Del Solar, G. & Espinosa, M. (1995). Specific nicking-closing activity of the initiator of replication protein RepB plasmid pMV158 on supercoiled or single-stranded DNA. *Journal of Biological Chemistry* **270**, 3772–3779.

Pansegrau, W., Ziegelin, G. & Lanka, E. (1990). Covalent association of the traI gene product of the plasmid RP4 with the 5′-terminal nucleotide at the relaxation nick site. *Journal of Biological Chemistry* **265**, 10637–10644.

Rasooly, A. & Novick, R. P. (1993). Replication-specific inactivation of the pT181 plasmid initiator protein. *Science* **262**, 1048–1050.

Rohde, W., Randles, J. W., Langridge, P. & Hanold, D. (1990). Nucleotide sequence of a circular single-stranded DNA associated with coconut foliar decay virus. *Virology* **176**, 648–651.

Sano, Y., Isogai, M., Satoh, S. & Kojima, M. (1993). Small virus-like particles containing single-stranded DNAs associated with milk vetch dwarf disease in Japan. *6th International Congress of Plant Pathology*, Montreal, Canada, 1993. Abstract 17.1.27, pp. 305.

Scherzinger, E., Kruft, V. & Otto, S. (1993). Purification of the large mobilization protein of plasmid RSF1010 and characterisation of its site-specific DNA-cleaving/DNA-joining activity. *European Journal of Biochemistry* **217**, 929–938.

te Riele, H., Michel, B. & Ehrlich, S. D. (1986). Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences, USA* **83**, 2541–2545.

Thomas, C. D., Balson, D. F. & Shaw, W. V. (1990). *In vitro* studies of the initiation of staphylococcal plasmid replication. Specificity of RepD protein for its origin (*ori*D), and characterisation of the Rep-*ori* tyrosyl ester intermediate. *Journal of Biological Chemistry* **265**, 5519–5530.

Yasukawa, H., Hase, T., Sakai, A. & Masamune, Y. (1991). Rolling-circle replication of plasmid pKYM isolated from a Gram-negative bacterium. *Proceedings of the National Academy of Sciences, USA* **88**, 10282–10286.

Received 14 November 1996; Accepted 4 March 1997