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Interaction between coat protein and replication initiation protein of *Mung bean yellow mosaic India virus* might lead to control of viral DNA replication

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Abstract

In addition to their encapsidation function, viral coat proteins (CP) contribute to viral life cycle in many different ways. The CPs of the geminiviruses are responsible for intra- as well as inter-plant virus transmission and might determine the yield of viral DNA inside the infected tissues by either packaging the viral DNA or interfering with the viral replicative machinery. Since the cognate Rep largely controls the rolling circle replication of geminiviral DNA, the interaction between Rep and CP might be worthwhile to examine for elucidation of CP-mediated control of the viral DNA copy number. Here a reasonably strong interaction between Rep and CP of the geminivirus *Mung bean yellow mosaic India virus* is reported. The domain of interaction has been mapped to a central region of Rep. The replication initiation activity of Rep, i.e., its nicking and closing function, is down regulated by CP. This report highlights how CP could be important in controlling geminiviral DNA replication.

Keywords: MYMIV; DNA replication; Rolling circle replication (RCR); Rep; CP; Pull-down; Yeast two-hybrid; Oligomerization; Nicking and closing

Introduction

Geminiviruses form a diverse family of phytopathogens and are classified in four genera (*Begomovirus*, *Curtovirus*, *Topocuvirus* and *Mastrevirus*) depending on their genomes, insect mode of disease transmission and host ranges (Buck, 1999). These viruses have either monopartite or bipartite single-stranded DNA genome, each component being about 2.7 kb. The viral genome encodes only one type of coat protein (CP) that is responsible for forming the typical twinned (geminate) particle structure (Mullineaux et al., 1988; Townsend et al., 1985). Recently the geminate structure of the mastrivirus *Maize streak virus* (MSV) has been determined to 25 Å resolution by cryo-electron microscopy and three-dimensional image reconstitution (Zhang et al., 2001). Besides the encapsidation function, CP is also required for transmission of the virus both within and between the plants. The CP of the monopartite geminiviruses facilitates the transfer of infecting viral DNA into the host cell nucleus and is essential for systemic virus movement (Boulton et al., 1989; Lazarowitz et al., 1989; Liu et al., 1999; Woolston et al., 1989). In contrast, bipartite begomoviruses may not require coat protein for systemic transmission (Gardiner et al., 1988; Jeffrey et al., 1996; Pooma et al., 1996; Stanley and Townsend, 1986), although the disease symptoms are often attenuated and the onset of disease is delayed when plants are systemically infected with CP mutants (Hayes and Buck, 1989; Sanderfoot and Lazarowitz, 1996; Unseld et al., 2004). The CP also determines the vector specificity (Briddon et al., 1990; Hofer et al., 1997; Hohnle et al., 2001) and protects the viral ssDNA from degradation during transmission by the insect vector (Azzam et al., 1994) or mechanical inoculation (Frischmuth and Stanley, 1998). Sequences necessary for vector transmission have been located in the central part of the protein (Liu et al., 2001; Qin et al., 1998; Unseld et al., 2001).

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CP also participates in the process of multiplication of geminiviral DNA that occurs essentially by rolling circle replication (RCR) (Saunders et al., 1991; Stenger et al., 1991). The end products of RCR accumulate as both double-stranded (ds) and single-stranded (ss) circular viral DNA. The absence or inactivation of CP generally results in reduced levels of viral ssDNA without reduction in the level of dsDNA, as observed in plants and protoplasts infected with CP mutants (Briddon et al., 1989; Brough et al., 1988). However, CP mutations in *Tomato golden mosaic virus* (TGMV) had no effect on DNA accumulation in plants (Brough et al., 1988; Gardiner et al., 1988) but reduced ssDNA accumulation and increased dsDNA accumulation in protoplasts (Sunter et al., 1990). Significantly, disruption of CP synthesis of *Tomato leaf curl New Delhi virus from India* (ToLCNDV) resulted in drastic reduction in ssDNA accumulation and a 3- to 5-fold increase in dsDNA accumulation in infected protoplasts (Padidam et al., 1999). In the case of *Squash leaf curl virus* (SqLCV), mutations in the nuclear localization signal (NLS) of CP also reduced accumulation of ssDNA (Qin et al., 1998). Similarly for the mastrevirus *Bean yellow dwarf virus* (BeYDV), transexpression of the CP gene resulted in a marked increase in ssDNA accumulation (Hefferon and Dugdale, 2003). All of these studies clearly suggest a role for CP in shaping the nature of replicated products of the geminiviral DNA.

Reduction in ssDNA accumulation has been ascribed to the loss of nuclear localization of CP (Qin et al., 1998) and its inability to bind ssDNA (Padidam et al., 1999). Though the molecular events leading to regulation of ssDNA accumulation still need to be explored, there is little doubt that the expression of CP influences replication of the viral DNA. However, this inference is not very surprising since CP is expressed late in the geminivirus infection cycle and thus might be expected to influence (down-regulate) the early events including the DNA synthesis. In order to gain insight about the role of CP in viral DNA replication, we have studied the physical and functional interaction between CP and replication initiation protein (Rep), the latter being an essential protein for RCR. Both proteins were derived from the bipartite begomovirus, *Mung bean yellow mosaic India virus* (MYMIV) (previously referred to as IMYMV; Bagewadi et al., 2004). Here we report that the central region of MYMIV Rep, including its oligomerization domain, interacts with MYMIV CP. Following interaction, the site-specific nicking and closing but not the ATPase activities of Rep are inhibited. Thus, CP might have a role in controlling the copy number of the geminiviral DNA.

Results

Expression of recombinant coat proteins of MYMIV

The full-sized coat protein of MYMIV (i.e., CP1 1–230) did not express well in *Escherichia coli*. Hence, we resorted to expression of recombinant CP2 23–230 using the third methionine of CP1 as the initiation codon, which is located at amino acid position 23. The CP2 sequence retained most of the NLS, DNA binding and oligomerization domains of CP1 (Palanichelvam et al., 1998; Qin et al., 1998). Moreover, the amino acid sequences of CP2 are extremely conserved amongst the begomoviruses. The amount of expressed CP2 in the soluble protein fraction of *E. coli* was reasonably high. Fig. 1a shows the purified 50-kDa GST-tagged CP2 and the 70-kDa MBP-tagged CP2 in lanes 3 and 6, respectively. The MBP fusion protein was generally stable but a slow release of the GST tag from the fusion protein was observed on storage for several weeks at 4 °C.

DNA binding of the recombinant CP2

As a southwestern assay to detect DNA binding did not work with the GST-tagged CP2, a pull-down approach was devised to detect the DNA–CP2 complex. About 10 ng of double-stranded or heat-denatured labeled DNA (specific activity = 0.8×10^7 cpm/μg) was allowed to bind to the indicated amounts of GST-CP2 protein (Fig. 2). The DNA–protein complex was pulled down using glutathione beads and the radioactivity associated with the precipitated complex was measured. As seen in Fig. 2, recombinant CP2 bound to ssDNA preferentially over dsDNA. The pulled-down radioactivity shown for each data point represents the average derived from six independent experiments. The observed fluctuations of each data point were minimal at high CP concentrations. For example, the

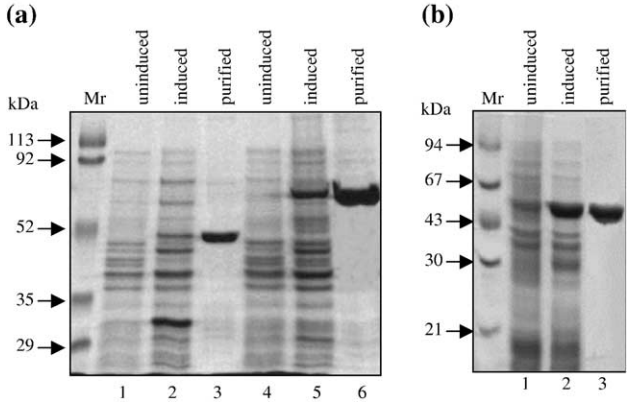


Fig. 1. Expression and purification of recombinant CP2 and Rep. (a) CP2 over-expression with GST and MBP fusion tags. The 10% SDS–PAGE profile of total extracts of *E. coli* BL21 (DE-3) containing plasmid pGEX4T1-CP2 is shown under uninduced (lane 1) and 0.25 mM IPTG-induced conditions (lane 2). Glutathione sepharose column purified GST-CP2 (lane 3), total protein extracts of *E. coli* TB1 cells containing plasmid pMAl-p2x-CP2 under uninduced (lane 4) and induced (lane 5) conditions and amylose column-purified MBP-CP2 (lane 6) are displayed. Arrows indicate standard molecular weight markers (Mr). (b) Rep over-expression in *E. coli* BL21 (DE3) cells as a 6× His-tagged protein from pET28a vector. Total *E. coli* proteins were resolved by 12% SDS–PAGE and visualized by staining with Coomassie blue. The uninduced (lane 1) and induced (lane 2) *E. coli* proteins and Ni-NTA column-purified His-Rep (lane 3) are shown.

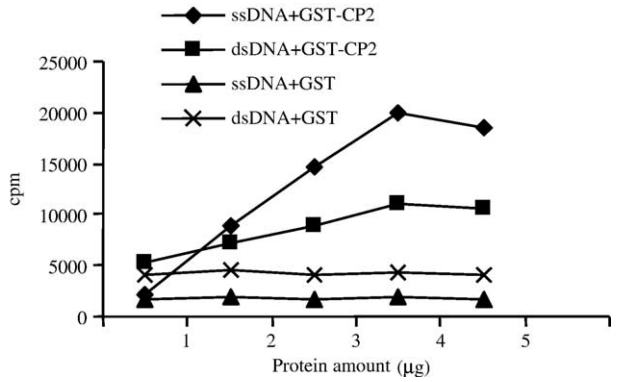


Fig. 2. DNA binding activity of CP2. Increasing amounts of GST-CP2 protein were incubated with linear double-stranded (ds) and single-stranded (ss)DNA probe (pUC-MYMV-A). GST-CP2–DNA complexes were pulled down using glutathione beads and radioactivity was measured.

variations recorded at 35 μg/ml of CP were in the range of 200–500 cpm. Although the CP binding differential between the ssDNA and dsDNA was not very large (within 10% of the input DNA or 50% of the bound ssDNA at 35 μg/ml of CP), it was reproducibly measurable. The binding was independent of the sequence of the template DNA used, and the CR (or replication origin) sequence of MYMIV failed to show any preferential binding over any other segment of the viral DNA (data not shown). The GST protein alone was used as a negative control for the binding experiments. Similar results were also obtained with MBP-tagged CP2.

Oligomerization of recombinant CP2

The CP is the only known structural geminivirus protein. The N-terminal one-third segment of CP has been found to be responsible for multimerization activity (Hallan and Gafni, 2001; Unseld et al., 2004). Hence, we were curious to know whether the recombinant CP2, which lacked the first 22 amino acids from the N-terminus of CP1, would also multimerize. A GST pull-down assay was carried out to examine multimerization. A mixture of MBP-CP2 and GST-CP2 was incubated with glutathione beads and the bead-bound proteins were separated from the supernatant. The bound proteins were eluted and resolved by SDS–PAGE. Fig. 3a shows that MBP-CP2 was retained in the beads along with GST-CP2 whereas a 5-fold higher molar concentration of MBP (compared to MBP-CP2) alone failed to attach to the beads in the presence of GST-CP2 (Fig. 3c). Additionally, Fig. 3b reveals that there was no interaction between GST and MBP. Hence, it appeared that the recombinant CP2 was competent for self-interaction and oligomerization. Oligomerization of CP2 was confirmed in a yeast two-hybrid assay as shown later in Fig. 5a.

CP2 interaction with MYMIV Rep in vitro

The recombinant CP2 showed properties characteristic of other geminivirus CPs, namely, DNA binding, oligomerization and harboring NLS(s). In order to explore the role played by CP in viral DNA replication, the interaction between CP2 and Rep was studied first. The purity of CP2 and 6× His-Rep has been assessed earlier (Figs. 1a and 1b, respectively) and Fig. 4a shows the purified recombinant full-length as well as truncated Rep with various N-terminal tags. Rep and CP2 interactions were analyzed using GST pull-down assays and SDS–PAGE. Fig. 4b shows that MBP-Rep and GST-CP2 could interact (lanes 1 and 2).

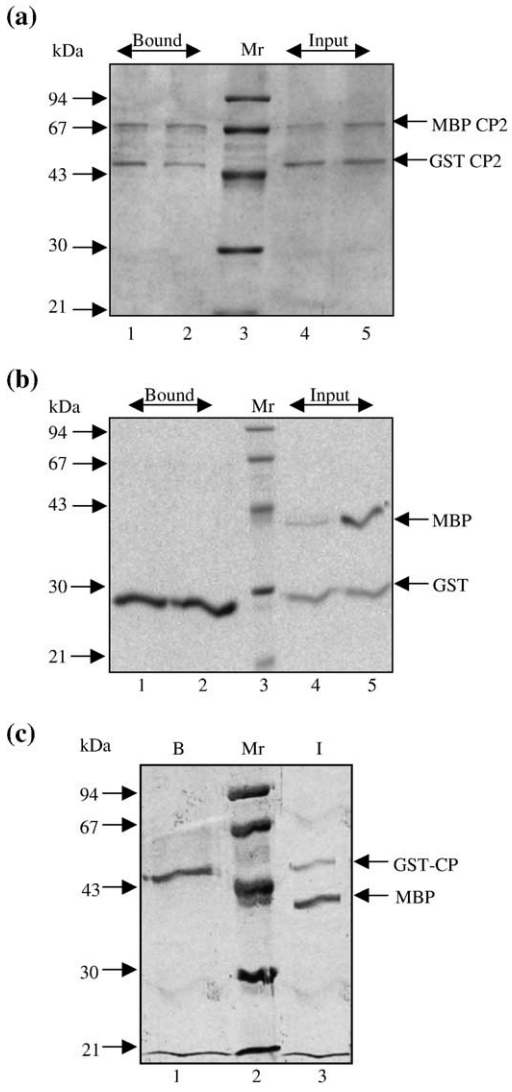
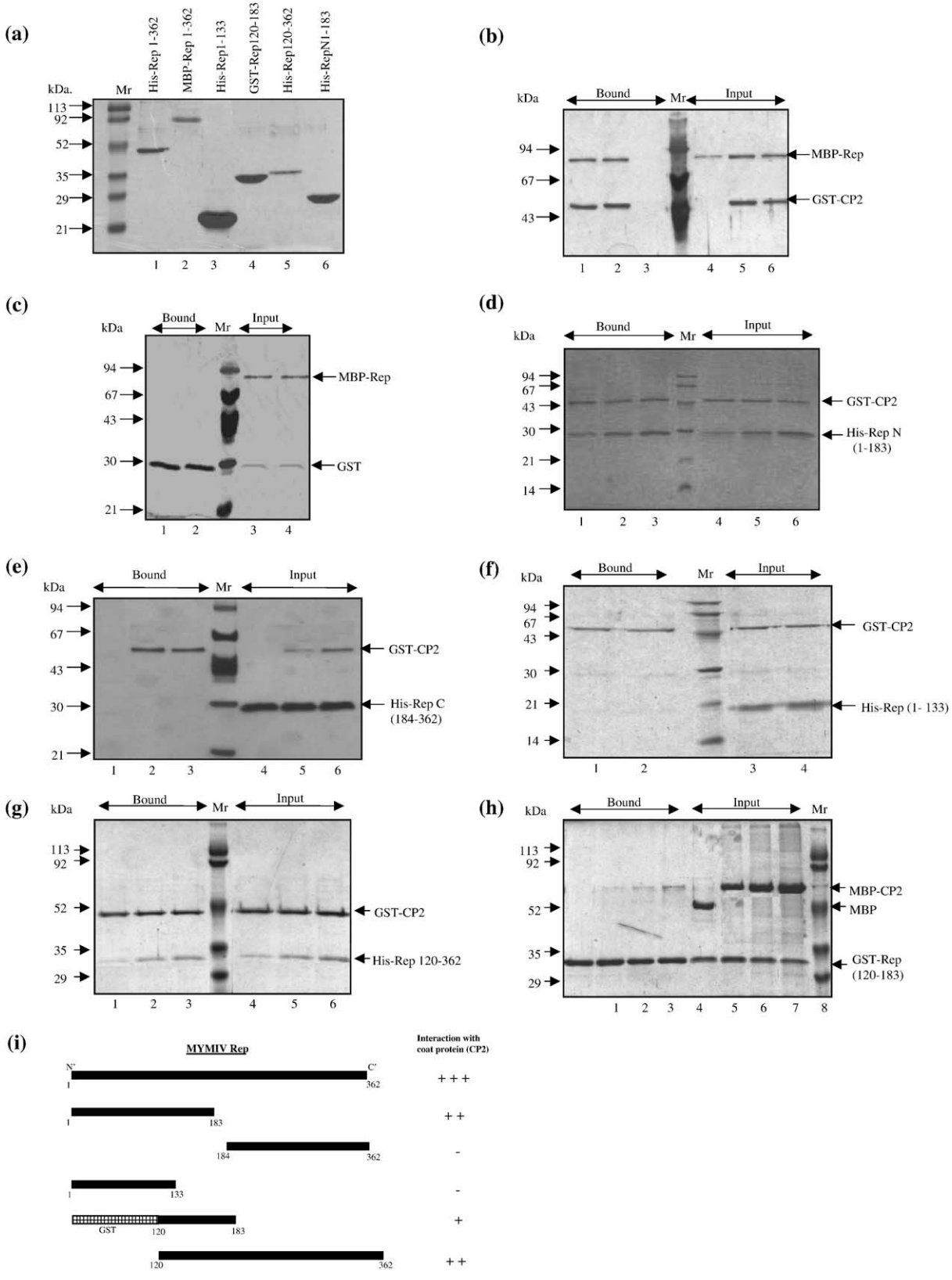


Fig. 3. Oligomer formation of CP2. (a) In vitro interaction of GST-CP2 and MBP-CP2 by GST pull-down assay. Purified MBP-CP2 protein was incubated with GST-CP2, bound to glutathione sepharose beads and eluted in SDS sample buffer. Bound (lanes 1 and 2) and input (lanes 4 and 5) proteins were resolved by 12% SDS–PAGE, followed by Coomassie staining. The molecular weight markers are shown in lane 3. (b) Interaction between GST and MBP proteins. Increasing concentrations (0.5 and 1.5 μg) of MBP were incubated with 1.5 μg GST protein (input lanes 4 and 5), and proteins bound to glutathione beads were resolved as above (lanes 1 and 2). (c) MBP binding to GST-CP2. 2.5 μg MBP was incubated with 1 μg GST-CP2 and the bead-bound proteins were analyzed as above. B and I stand for the bound and input fractions, respectively.

MBP-Rep alone did not bind to the GST beads (lanes 3 and 4), and GST was not responsible for interaction with MBP-Rep (Fig. 4c). These results suggest that a specific interaction occurs between CP2 and Rep.

In an attempt to map the domain of interaction within Rep, various deletion derivatives of Rep were purified to apparent homogeneity, some of which are shown in Fig. 4a. Neither the C-terminal (184–362 aa) nor the N-terminal



region (1–133 aa) of Rep was able to bind GST-CP2 (Figs. 4e and 4f). On the other hand, a larger segment of the N-terminal region (Rep 1–183) showed binding competence to GST-CP2 in a similar pull-down assay (Fig. 4d). Thus, a region spanning amino acids 133 to 182 of Rep seemed to be important for binding to CP2. Hence, a C-terminal derivative of Rep including this domain, namely His-Rep 120–362, was used to study its interaction with CP2. As shown in Fig. 4g, His-Rep 120–362 efficiently bound to GST-CP2 in a dose-dependent fashion (lanes 1–3). Furthermore, Rep 120–183 was fused in-frame to a heterologous protein, namely GST, at its C-terminal end to examine the efficacy of binding of the fusion protein to CP2 using a similar pull-down assay. Fig. 4h shows that GST-Rep 120–183 bound to MBP-CP2 (lanes 2–4), albeit with reduced efficiency. In the control, MBP alone failed to bind to the fusion protein (lanes 1 and 5). These studies established that the middle portion of MYMIV Rep (Rep 120–183) is sufficient to interact with CP2. The results of the in vitro interaction have been summarized in Fig. 4i.

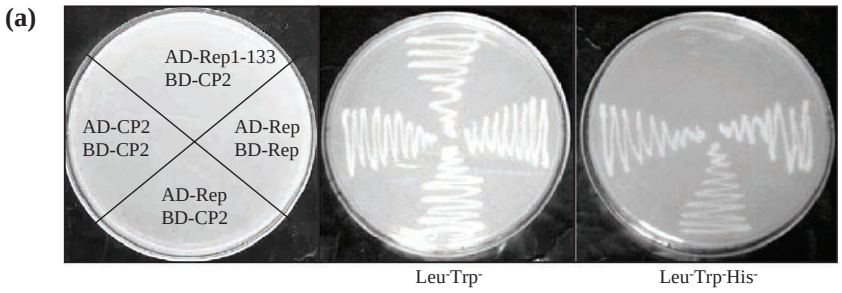
CP2 interaction with Rep in a yeast two-hybrid assay

The interaction between MYMIV Rep and CP2 was investigated by yeast two-hybrid analysis (Fig. 5). Rep and CP2 proteins were individually fused in-frame with the activation domain (AD) and DNA binding domain (BD) of Gal 4 protein. The yeast strain AH109 harboring combinations of fusion proteins, namely AD-Rep/BD-Rep or AD-CP2/BD-CP2, grew well in medium lacking histidine, indicating the presence of both Rep–Rep and CP2–CP2 interactions. Yeast transformed with constructs encoding the fusions AD-Rep 1–362 and BD-CP2 also grew in medium lacking histidine, but yeast failed to grow when transformed with the AD-Rep 1–133 and BD-CP2 constructs. Hence, Rep 1–362 interacted well with CP2 but the N-terminal fragment Rep 1–133 failed to interact in this assay. Thus, the yeast two-hybrid data corroborated well with the in vitro pull-down data. A β-galactosidase filter-lift experiment, using the colonies which grew well on Leu-Trp-double drop-out plates, was also carried out. β-Galactosidase expression occurred in each case except for yeast transformed with AD-Rep 1–133 and BD-CP2 (Fig. 5b). The strength of the protein–protein interactions was also assayed by restreaking the colonies from Leu-Trp-plates onto the triple drop-out plates (Leu-Trp-His-) containing different concentrations of 3-AT, an inhibitor of histidine biosynthesis. Inhibitory concentrations of 3-AT for Rep–Rep, CP2–CP2 and Rep–CP2 interactions were > 20, 15 and 5 mM, respectively. Hence, the Rep–CP2 interaction was of moderate strength.

Inhibition of Rep nicking-closing activity by CP2

Fig. 6a shows that the site-specific nicking activity of Rep was inhibited by both GST-CP2 and MBP-CP2 in a dose-dependent manner. Neither GST nor MBP alone influenced the nicking activity (data not shown). This inhibition might occur by two different mechanisms. CP2 might bind to the substrate oligonucleotide in such a manner that Rep might fail to gain access to the nicking site. Alternatively, the CP2–Rep complex might cause Rep to adopt an inefficient conformation for nicking. To distinguish between these two possibilities, we examined the site-specific nicking activity of Rep 1–133 in the presence of CP2. The DNA binding and the nicking activities of Rep 1–362 and Rep 1–133 were almost similar (Bagewadi et al., 2004; Fig. 6b, lanes 2 and 5). Therefore, if CP2 binding to the ssDNA substrate was important, the nicking activity of Rep 1–133 would have been similarly reduced in presence of CP2. However, the data of Fig. 6b indicate that CP2 failed to influence the nicking activity of Rep 1–133 (compare lanes 5 and 7). Hence, the failure of CP2 to interact with Rep 1–133 (Figs. 4e, 4h and 5) might explain why there was no loss of nicking activity of Rep 1–133. This indicates the importance of protein–protein interaction between CP2 and Rep in down-regulating the nicking activity of Rep. Similarly, the site-specific ligation activity of Rep (Fig. 6c, lane 3) was also repressed by GST-CP2 (lanes 4–7) whereas the ligation activity of Rep 1–133 did

Fig. 4. CP2–Rep interaction and mapping of Rep domain for interaction. (a) Purified full-length and truncated Rep. Recombinant proteins were purified by affinity matrix chromatography, resolved by 12% SDS–PAGE and visualized by Coomassie staining. The purified proteins have been marked on the top of the lanes (1–6). (b) Interaction of full-length Rep with CP2. The in vitro interaction of GST-CP2 and MBP-Rep was carried out by GST pull-down assay. The input proteins are shown in lanes 4–6, corresponding to the bound proteins in lanes 3–1, respectively. For lanes 4–6, the input reaction mixture contained 0.5, 1.5 and 1.0 μg MBP-Rep and 0, 2.5 and 1.5 μg GST-CP2, respectively. Proteins were visualized by silver staining. (c) Interaction between MBP-Rep and GST. MBP-Rep and GST were treated as above. The bound fractions (lanes 1 and 2) and input fractions (lanes 3 and 4) were visualized by silver staining. The input reaction mixtures contained 2.0 μg each of MBP-Rep and GST (lane 3), and 2.0 μg MBP-Rep along with 1.5 μg GST (lane 4), respectively. (d) Binding of His-Rep N 1–183 to GST-CP2. About 1 μg GST-CP2 was incubated separately with 0.5 (lane 4), 1.0 (lane 5) and 2.0 (lane 6) μg of His-Rep 1–183 and processed in a pull-down assay. The bound (lanes 1, 2, 3) and input proteins (lanes 4, 5, 6) were visualized by silver staining. (e) Binding of His-Rep 184–362. About 5 μg His-Rep 184–362 was treated with 0 (lane 4), 0.5 (lane 5) and 1.0 (lane 6) μg GST-CP2. The bound and input fractions were stained with silver nitrate. (f) Interaction between His-Rep 1–133 and GST-CP2. The components were processed as above. The bound (lanes1 and 2) and input fractions (lanes 3 and 4) are shown. Proteins were stained with silver nitrate. (g) Interaction of His-Rep 120–362 with GST-CP2. Aliquots of 0.8,1.25 and 2.5 μg of His-Rep 120–362 were added to a fixed amount (5 μg) of GST-CP2 (lanes 4–6, respectively). Bound His-Rep 120–362 is shown in lanes 1–3. (h) GST-Rep 120–183 binding to MBP-CP2. The bound proteins shown in lanes 1–4 correspond to the input proteins shown in lanes 5–8. The input reaction mixture contained 3.0, 5.0 and 7.5 μg MBP-CP2 to a fixed 2.0 μg GST-Rep 120–362 for lanes 6–8, respectively. Control interaction of 3.0 μg MBP with 2.0 μg GST-Rep 120–183 is also shown (bound lane: 1, input lane: 5). Proteins were stained with Coomassie blue. (i) Summary of Rep-coat protein (CP-2) interaction. The + and − represent the presence and absence of interaction, respectively. The +++ indicates the strongest interaction. The hatched box represents the in-frame GST fusion.



(c)

Interaction	3-AT concentration
Rep-Rep	> 20 mM
Rep-CP2	5 mM
CP2-CP2	15 mM

Fig. 5. Yeast two-hybrid analysis of Rep–CP2 interaction. (a) Growth of yeast strain AH109 harboring two-hybrid constructs in selective medium. At the extreme left, a sector diagram illustrates the combination of fusion constructs used. (b) β-Galactosidase expression by filter-lift assay using a replica of the double drop-out plate. (c) Concentration of 3-AT which was inhibitory for each interaction.

not change in the presence of GST-CP2 (data not shown). Since the initiation of RCR activity is essentially determined by the nicking-closing function of Rep, CP might down-regulate RCR activity to a large extent.

Another important biochemical activity of Rep is its ATPase function, which is also essential for RCR (Desbiez et al., 1995). Hence, we examined whether Rep-associated ATPase activity was also affected in the presence of CP2. The liberation of radioactive orthophosphate from [γ-^{32}P] ATP by Rep was monitored on a TLC plate. As shown in Fig. 6d, CP2 did not affect the ATPase function of Rep (compare lanes 2 and 4).

Discussion

At the final stage of biosynthesis of viral genomes, the termination or maturation factors are supposed to play their roles in limiting synthesis of viral nucleic acids. This category of proteins includes the late proteins of the viral life cycle. In many cases, the encapsidation process itself or some of its components actively inhibit synthesis of the viral nucleic acid. The leading strand synthesis of bacteriophage ϕX 174 DNA by RCR is known to be actively down-regulated by ϕX c protein, which is required for packaging of ϕX174 DNA into proheads (Goetz et al., 1988). The Rubella virus CP also down-regulates the replication activity of its RNA genome (Chen and Icenogle, 2004). The CP of alfalfa mosaic virus, a virus with a plus-strand ssRNA genome, plays a direct role in the regulation of plus- and minus-strand RNA synthesis (De Graff et al., 1995; Tenllado and Bol, 2000). We wanted to know whether the geminivirus CP, which is a late protein, would have a similar role in RCR of the viral genome. To address this question, the interaction between the coat protein of MYMIV CP and Rep, a protein essential for RCR (Gutierrez, 1999), was examined. The MYMIV CP, containing an N-terminal deletion of 22 amino acids (CP2), not only interacted with Rep but also inhibited one of the essential activities of viral replication, namely Rep nicking/closing activity. This result suggests that MYMIV CP might have a role in limiting viral DNA copy number by blocking initiation of RCR.

CP2 shows the sequence motifs characteristic of nuclear localization, DNA binding and oligomerization, and thus probably retains most of the important functions of the full-length CP. There is one α-helix (WRKPRFY) at the N-terminus of CP2, and the rest of the protein contains potential β-sheets which may be required for various protein–protein interactions. About 17% of the protein is composed of basic amino acid residues and the estimated pI of the protein is 9.83, which may explain its sequence-independent DNA binding. We have shown that the CP2 binds preferentially to single-stranded DNA rather than dsDNA. Such ssDNA binding of CP has been documented

for several geminiviruses, including MSV, TYLCV, ACMV and SqLCV, and there are reports of cooperative as well as high-affinity ssDNA binding for CPs of SqLCV and TYLCV (Liu et al., 1997, 2001; Palanichelvam et al., 1998; Qin et al., 1998). However, for MYMIV CP2, high-affinity DNA binding may not occur since binding could not be detected either by gel-shift or southwestern assays (data not shown). We are not sure at the moment whether the loss of first 22 amino acids of the CP could be the reason for weak binding of CP2 to DNA. In the case of ACMV CP, small deletions within the N-terminal 20 amino acids did not alter the DNA binding capacity of the mutated derivatives (Unseld et al., 2004). Similarly, deletions of 50 amino acids from the N-terminus of the CP of *Tomato leaf curl Bangalaore virus* (ToLCBV) did not result in the loss of binding to DNA. The DNA binding motif of ToLCBV was mapped to a Zn-finger motif in the CP (Kirthi and Savithri, 2003). A putative C3H1 type of Zn-finger motif has also been located in the MYMIV CP in the region spanning amino acids 64 to 105, but its functionality is not yet known.

One hundred and ten CP monomers are arranged in 22 pentameric capsomeres to generate the overall geminate particle morphology (Bottcher et al., 2004; Zhang et al., 2001). Hence, the recombinant CPs are expected to embrace the homotypic interactions, which are mediated by the N-terminal part of the CP (Hallan and Gafni, 2001). We were curious to know whether the loss of 22 N-terminal amino acids in CP2 would result in the loss of its oligomerization property. Our results reveal that CP2 was capable of homotypic interaction, indicating that the first 22 amino acids are not essential for the process. However, the determination of the strength of the homotypic interaction for the full-length CP would be required to suggest a regulatory role for the missing amino acids.

The CP2 interacted with a specific domain of Rep required for oligomerization, which probably controls several protein–protein interactions. This domain is responsible for interaction with itself, the host factor PCNA (Bagewadi et al., 2004) and MYMIV REn (Vikash Kumar, unpublished), and similar domains of other begomovirus Reps have been implicated in interaction with several host factors. The determination of structure of this domain, which is probably conserved in view of the substantial degree of homology among the begomovirus Rep proteins, will be very important to shed light on the multiplicity of interactions. Some proteins are known to interact with several partners. For example, the *E. coli* single-stranded DNA binding protein (SSB) interacts with many factors through its C-terminal disordered structure (Savvides et al.,

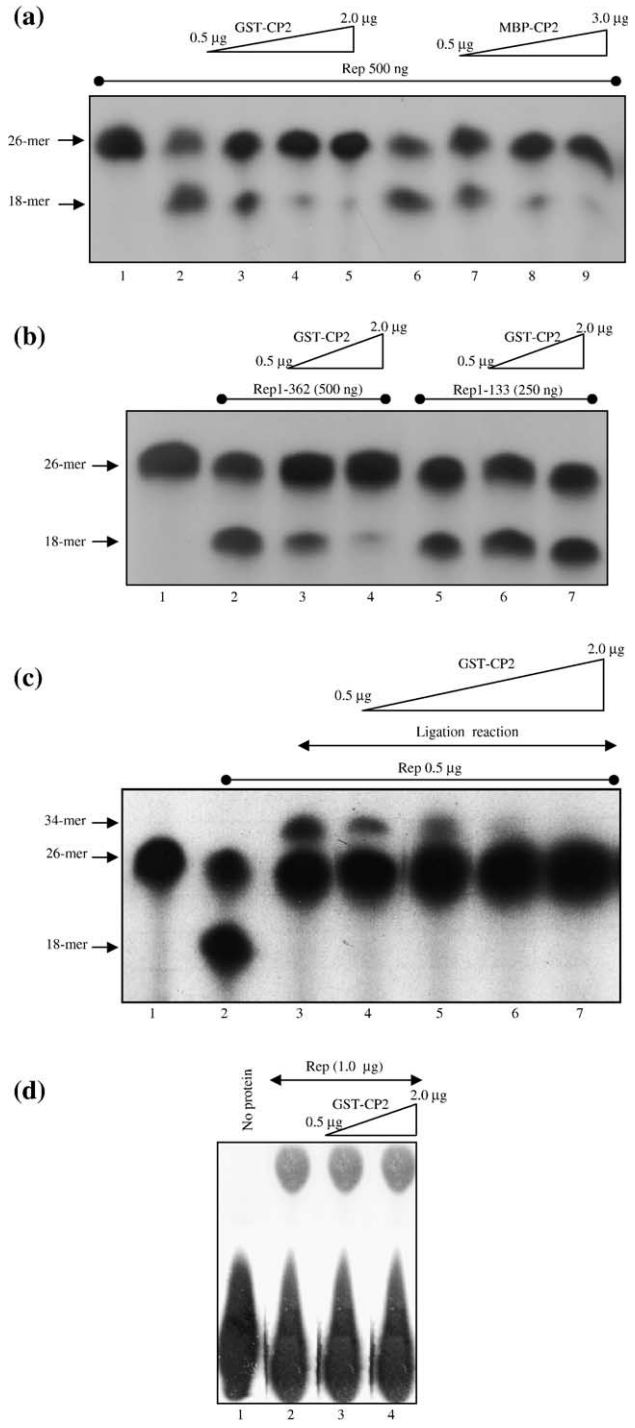


Fig. 6. Modulation of biochemical activities of Rep by CP2. (a) An autoradiogram of a 15% PAGE/urea gel showing the 5′ ^{32}P-labeled 26-mer substrate (lane 1) and the 18-mer cleaved products (lanes 2–9). The substrate was treated with 500 ng 6× His-Rep 1–362 alone (lanes 2 and 6) or in presence of 0.5,1.0 and 2.0 µg GST-CP2 (lanes 3–5, respectively) and MBP-CP2 (lanes 7–9, respectively). (b) Comparison of modulation of the nicking activities of full-length Rep 1–362 (lanes 2–4) and truncated Rep 1–133 (lanes 5–7) by CP2. Lane 1, uncut substrate 26-mer. Nicking activity is shown in the absence (lanes 2 and 5) and presence of 0.5 and 2.0 µg. GST-CP2 (lanes 3, 4 and 6, 7). (c) An autoradiogram of a 15% urea–PAGE gel showing Rep-mediated nicking (lane 2) and ligation (lanes 3–7). The ligated products (34-mer) in the presence of 0.5, 0.8, 1.5 and 2.0 µg GST-CP2 (lanes 4–7) are shown. (d) An autoradiogram showing the release of labeled phosphate from [γ-^{32}P] ATP using 1 µg of 6× His-Rep 1–362 alone (lane 2) and in the presence of increasing amounts of GST-CP2 (lanes 3 and 4). Lane 1 shows the untreated labeled ATP. Products were resolved by thin layer chromatography on PEI plates.

2004), and similarly PCNA also binds to several eukaryotic proteins using its looped zones and β-sheets (Fukuda et al., 1995). Hence, it is tempting to speculate that the structure of the oligomerization domain might also be disordered, consisting mostly of coils and β-sheets. Significantly, the CP2–Rep interaction affected the nicking activity but not the ATPase activity of Rep. For nicking, some part of the oligomerization domain is needed (Bagewadi et al., 2004) and thus the interaction might affect the nicking activity. However, the ATPase domain is downstream of the oligomerization domain, and it is possible that the activities of these two domains are quite independent of each other. This independence might allow the folding of the ATPase domain to remain unaffected even following the interaction between the Rep oligomerization domain and CP. This interpretation corroborates well with the prevailing notion of the modular structure of Rep.

RCR can be divided in three overlapping stages of DNA synthesis, namely ss→dsDNA, ds→dsDNA and ds→ssDNA (Gutierrez, 1999; Saunders et al., 1991; Stenger et al., 1991). It has been widely observed that the absence or inactivation of CP led to reduced accumulation of geminiviral ssDNA with concomitant increase in the level of dsDNA replicative forms (Azzam et al., 1994; Briddon et al., 1989; Padidam et al., 1996, 1999; Sunter et al., 1990). It has been hypothesized that the process of encapsidation removes the ssDNA from the pool of replicative intermediates so that it does not re-enter RCR. Although this hypothesis calls for a reduction in the accumulation of viral ssDNA, it does not indicate if the production of ssDNA is actively blocked. This consideration is important since, even after the removal of ssDNA, the dsDNA template for ssDNA synthesis will still be present to start fresh rounds of RCR and generate new ssDNA. Our finding that CP2 can inhibit Rep activity indicates that CP could actively block the production of ssDNA in the late stage of the virus life cycle. In this way, CP could be an important contributor to the control of viral DNA copy number.

Materials and methods

DNA constructs and purification of the recombinant proteins

The coat protein gene fragment (CP2) was PCR-amplified from the DNA-A component of MYMIV using the specific primers CP2 Sense and CP2 Antisense (Table 1). CP2 was cloned in the expression vectors pGEX-4T-1 and pMAL-p2x using the *Eco*RI–*Xho*I and *Eco*RI–*Hin*dIII sites, respectively. CP2 was expressed in *E. coli* BL-21 (DE3) from the plasmid vectors pGEX-4T-1 and pMAL-p2x as GST- and MBP-fusion proteins, respectively, and purified to near homogeneity following the manufacturers' protocols (Pharmacia Biotech, NEB, USA).

The recombinant clone pET28a-Rep (Pant et al., 2001) was used as template to PCR-amplify various deletion mutants of Rep, employing primers containing *Bam*HI and *Hin*dIII restriction sites at the N- and C-termini, respectively (Table 1). The amplified fragments were first cloned in pGEMT vector (Promega, USA) and subsequently recloned in pET28a (Novagen, Germany) using the same restriction sites. The *Bam*HI–*Hin*dIII full-length Rep fragment was cloned into pMAL-p2x for expressing the MBP-Rep fusion protein. Rep 120–183 was cloned in pGEX-4T-1 to express the peptide as a GST fusion. The full-length and deletion mutants of Rep were over-expressed in *E. coli* BL-21 (DE3) strain and purified to near homogeneity under non-denaturing conditions as described previously (Pant et al., 2001). The biochemical activities of the recombinant Rep proteins were checked in vitro for site-specific cleavage (Pant et al., 2001) and/or ATPase function (Bagewadi et al., 2004; see below).

DNA binding assay

MYMIV DNA A cloned in pUC 18 was labeled with [α-^{32}P]-dCTP using the nick translation method (GIBCO-

Table 1
PCR primers

Primer	Sequence	Usage
Rep sense	5′-ATG GAT CCA TGC CAA GGG AAG GTC GT-3′	Full Rep 1–362
Rep antisense	5′-TGA AAG CTT TCA ATT CGA GAT CGT CGA-3′	
Rep C 184–362 sense	5′-AAA GGA TCC TTT ACA TTG GAG TCA TTC GAC-3′	Rep C 184–362
Rep C 184–362 antisense	5′-TGA AAG CTT TCA ATT CGA GAT CGT CGA ATT GC-3′	
RepN 1–183 sense	5′-ATG GAT CCA TGC CAA GGG AAG GTC GT-3′	RepN 1–183
RepN 1–183 antisense	5′-CTA AGC TTA GGC GAC TCA TAT GCC TG-3′	
Rep 1–133 sense	5′-ATG GAT CCA TGC CAA GGG AAG GTC GT-3′	Rep 1–133
Rep 1–133 antisense	5′-AGA AGC TTC TAT GCG TCG TTG GCA GAT TG-3′	
Rep 120–362 sense	5′-CCG GAT CCG GCA GAT CAG CTA GAG GAG G-3′	Rep 120–362
Rep 120–362 antisense	5′-TGA AAG CTT TCA ATT CGA GAT CGT CGA ATT GC-3′	
Rep 120–183 sense	5′-CCG GAT CCG GCA GAT CAG CTA GAG GAG G-3′	Rep 120–183
Rep 120–183 antisense	5′-CTA AGC TTA GGC GAC TCA TAT GCC TG-3′	
CP2 23–230 sense	5′-GAA GAA TTC TTC GAT ACC CCG CTA ATG-3′	CP2
CP2 23–230 antisense	5′-TGA AAG CTT TCA ATA CAA TCT TTA TTA-3′	

BRL) and following the manufacturer's instructions. The labeled DNA was purified from the unincorporated radioactivity by Sephadex G-50 column chromatography. The indicated amounts of GST-CP2 protein were incubated with labeled DNA in binding buffer [10 mM Tris–HCl (pH 8.0), 300 mM NaCl, 5 mM $MgCl_2$, 2.5 mM DTT, 1 mM EDTA and 3% glycerol] for 30 min at room temperature. For conversion to ssDNA, the labeled DNA was boiled for 10 min and immediately kept on ice for 10 min. The GST and/or MBP were used in similar reactions as negative controls. The reaction was mixed with pre-washed glutathione beads with slow stirring for 30 min at room temperature. The final reaction mix of about 100 μl was subjected to centrifugation at 10000 rpm for 2 min, the supernatant was removed and matrix was washed three times with 1 ml of binding buffer. The Cerenkov radiation emitted by the protein-bound DNA was estimated using a multi-purpose scintillation counter (Beckman, LS 6500).

GST pull-down assay

Purified GST-tagged protein (GST-CP2) was incubated with indicated amounts of wild-type or mutant 6× His- or MBP-Rep fusion proteins in binding buffer B [25 mM Tris–HCl (pH 8.0), 75 mM NaCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 2.5 mM DTT, 1% NP-40] at 37 °C for 30 min, following which 10 μl of pre-washed and buffer B-equilibrated glutathione sepharose resin (Pharmacia, USA) was added. The mixture was slowly stirred for 30 min at room temperature. The unbound protein fraction was separated from the resin by centrifugation at 3000×*g* for 3 min. The resin containing the bound proteins was washed (3000×*g*, 3 min) with increasing concentrations of NaCl (100 mM to 300 mM) in binding buffer B. An equal volume of 2× sample buffer was then added to the resin, boiled for 5 min, centrifuged at 3000×*g* for 3 min and the supernatant was analyzed by SDS–PAGE. The protein bands were visualized by staining either with silver nitrate solution or Coomassie blue. Each experiment was repeated three times. For oligomerization studies, CP2 was purified with two different N-terminal tags (GST and MBP), and interaction between the two fusion proteins was analyzed by pull-down assays with glutathione and/or amylose resins (NEB, USA).

Yeast two-hybrid analysis

The DNA fragments encoding MYMIV Rep 1–362 and Rep 1–133 were excised from the *Bam*HI and *Xho*I sites of pET28a-Rep 1–362 and pET28a-Rep 1–133, respectively, re-cloned individually in pGADC1 (for activation domain fusion) and/or pGBDC1 (for DNA binding domain fusion) (Bagewadi et al., 2004). The DNA fragment encoding CP2, obtained by digestion of plasmid pGEX-4T-1-CP2 with *Eco*RI and *Xho*I, was cloned into *Eco*RI/*Sal*I-digested pGADC1 and/or pGBDC1. All the constructs were verified by restriction digestion and sequencing. DNA manipulations were carried out as described by Sambrook et al. (1989).

The yeast two-hybrid assay was performed using the AH109 yeast strain, which was transformed with the appropriate plasmid and grown on SD plates in the absence of Trp and Leu to score co-transformants. Protein interaction analysis was carried out on SD plates without Leu, Trp and His (SD Leu-Trp-His-). After 3 days at 30 °C, individual colonies were streaked out and tested for growth in the presence of 3-amino-1, 2, 3-triazole (3-AT). The suitable double transformants were also examined independently for β-galactosidase activity in a filter-lift assay (Raghavan et al., 2004). Using forceps, a filter (90 mm disc of 3 MM Whatman paper) was placed over the surface of the plate containing colonies to be tested. The filter was carefully lifted and completely submerged in liquid nitrogen for 10 s, and then allowed to thaw at room temperature. This freeze/thaw step was repeated 3–5 times, after which the filter was carefully placed on filter paper presoaked in 5 ml buffer Z [0.1 M Na-phosphate buffer (pH 7.0), 0.01 M KCl, 0.001 M $MgSO_4$], 20 μl 20% X-gal and 8 μl β-mercaptoethanol. After this, the filter was wrapped in parafilm and kept at 30 °C in the dark. The blue color developed within 2–3 h.

Cleavage and ligation activity of Rep

The oligonucleotide T1 (5′ CGACTCAGCTATAATATTACCTGAGT 3′) (Pant et al., 2001), used for the cleavage test, was 5′ end-labeled with T4 polynucleotide kinase. The oligonucleotide T2 (5′ CTATAATATTACCTGAGTGCCCCGCG 3′), employed for the ligation test, was unlabelled and used in 50-fold higher molar excess over the T1 oligonucleotide. Approximately 1 ng T1 (specific activity ~1.0 × 0^9 c.p.m./μg) was incubated with 500 ng purified Rep in 20 μl cleavage buffer (25 mM Tris–HCl [pH 8.0], 75 mM NaCl, 2.5 mM EDTA, 5.0 mM $MgCl_2$, 2.5 mM DTT) at 37 °C for 30 min. The cleavage reaction was terminated by adding 6 μl loading buffer (1% SDS, 25 mM EDTA, 10% glycerol) and heating to 90 °C for 2 min. For the ligation reaction, about 50 ng of T2 oligonucleotide was added to the cleavage reaction. The products were resolved on a 15% acrylamide-urea gel and visualized by autoradiography (Pant et al., 2001).

ATPase assay

The protocol was adapted from Fukuda et al. (1995). The buffer used for the ATPase reaction contained 20 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 100 mM KCl, 8 mM DTT and 80 μg/ml BSA. Briefly, 1 μl (10 μCi) of [γ-^{32}P]-ATP (6000 Ci/mmol) was diluted 50-fold with 5 mM ATP. Dilute radiolabeled ATP (1 μl) was mixed with the desired amounts of protein and incubated at 37 °C for 30 min. After the reaction, 1 μl of the reaction mix was spotted onto a TLC plate (PEI, Aldrich), air-dried and chromatography was

performed using 0.5 M LiCl and 1 M HCOOH as the solvent. Following completion of chromatography, the TLC plate was dried and autoradiographed (Bagewadi et al., 2004).

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