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Relationship of Oligomerization to DNA Binding of Wheat Dwarf Virus RepA and Rep Proteins

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Members of the genus *Mastrevirus* (family *Geminiviridae*) produce a complementary-sense (c-sense) transcription unit with the potential to encode two proteins, RepA and Rep. In the present work, we have studied the DNA–protein complexes formed by the *Wheat dwarf virus* (WDV) RepA protein within the WDV large intergenic region. WDV RepA forms large nucleoprotein complexes near the TATA boxes of the viral complementary-sense and virion-sense (v-sense) promoters (the RepA C- and V-complexes, respectively), a location similar to those of WDV Rep–DNA complexes but with distinct DNase I footprints. We have also studied the relationship of oligomerization of WDV RepA and Rep proteins to DNA–protein complex formation. Using chemical cross-linking, we have determined that both WDV proteins can form oligomers in solution. Interestingly, the pH is critical for the monomer–oligomer equilibrium and small changes produce a displacement in such a way that at pH $\leq$ 7.0, the predominant species is an octamer while at pH $\geq$ 7.4 it is a monomer. Complex formation is also strongly affected by pH and occurs more efficiently at pH 7.0–7.4. We found that preformed oligomers interact very poorly with DNA. Thus, our data are consistent with a stepwise model for protein–DNA complex assembly in which monomers interact with DNA and then with other monomers to assemble an oligomeric structure on the DNA. These results may be relevant for studies on the DNA binding, replication, and transcription properties of geminivirus proteins.

Key Words: Oligomer; DNA binding; wheat dwarf geminivirus; DNA replication; transcription.

INTRODUCTION

The *Geminiviridae* family members, which are grouped into three different genera (*Mastre-*, *Curto-*, and *Begomovirus*), have a single-stranded DNA genome and replicate their DNA through double-stranded DNA intermediates by a rolling-circle mechanism (Saunders *et al.,* 1991; Stenger *et al.,* 1991). Considerable attention has been given in recent years to these viruses and different aspects of their molecular and cellular biology have been the subject of recent reviews (Bisaro, 1996; Palmer and Rybicki, 1998; Hanley-Bowdoin *et al.,* 1999; Gutierrez, 1999; Lazarowitz, 1999).

Transcription of viral genes, which occurs bidirectionally from the two divergent complementary-sense (c-sense) and virion-sense (v-sense) promoters, yields viral mRNAs which encode, among others, proteins involved in DNA replication and/or transcription. Replication-associated proteins are produced in two different ways depending on the genera. Thus, for mastreviruses, the single transcription unit produced from the c-sense promoter has the potential to encode two proteins, RepA (formerly C1) and Rep (formerly C1:C2), the latter after a splicing event (Accotto *et al.,* 1989; Schalk *et al.,* 1989; Mullineaux *et al.,* 1990; Dekker *et al.,* 1991; Wright *et al.,* 1997). As a consequence, they share ~200 N-terminal amino acids of their primary sequence. Begomoviruses and curtoviruses lack a homologue of the mastrevirus RepA protein and their Rep (also named AC1, AL1, or C1) protein is encoded by a distinct c-sense transcript. In all geminiviruses, Rep is a highly conserved multifunctional protein absolutely required for viral DNA replication and transcription (reviewed in Bisaro, 1996; Gutierrez, 1999; Hanley-Bowdoin *et al.,* 1999). Initiation of rolling-circle DNA replication has been mapped to the invariant 9-nt sequence TAATATT↓AC (↓ denotes the cleavage site) conserved in all geminiviruses (Stenger *et al.,* 1991; Heyraud *et al.,* 1993; Stanley, 1995; Heyraud-Nitschke *et al.,* 1995; Laufs *et al.,* 1995a). It depends on the interaction of the initiator protein Rep with specific *cis*-acting sequences (Fontes *et al.,* 1992; Lazarowitz *et al.,* 1992; Sanz-Burgos and Gutierrez, 1998; Castellano *et al.,* 1999).

In other viral systems, DNA–protein complex formation frequently depends on the ability of viral DNA-binding proteins to form oligomeric structures. Such complexes are maintained through extensive interactions, including both protein–DNA and protein–protein interactions, which can occur either before or upon DNA binding (Dean *et al.,* 1992; Aslani *et al.,* 2000; Sanders and Stenlund, 2000). One of our interests is to determine how the function of geminiviral proteins, in particular the replication-associated proteins, in DNA replication and transcription depends on the interaction with regulatory sequences within the viral genome through the formation of nucleoprotein complexes and on the oligomerization properties of the viral proteins.

The oligomerization properties of replication-associ-

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ated proteins in relation to DNA–protein complex formation are not known in most cases. For mastreviruses, *Maize streak virus* (MSV) Rep and RepA proteins have been shown to self-interact in two-hybrid experiments (Horvath *et al.,* 1998), pointing to the possibility that they can oligomerize. The visualization of large wheat dwarf virus (WDV) Rep–DNA complexes by electron microscopy (Sanz-Burgos and Gutierrez, 1998; Castellano *et al.,* 1999) and DNase I footprinting analysis (Castellano *et al.,* 1999) is fully consistent with the association of an oligomeric form of WDV Rep with DNA. For begomoviruses, *Tomato golden mosaic virus* (TGMV) Rep has been detected as an oligomer in solution (Orozco *et al.,* 1997). Based on DNA-binding studies, dimerization of TGMV Rep has been proposed to be a prerequisite for DNA binding (Orozco *et al.,* 1997; Orozco and Hanley-Bowdoin, 1998).

In the present work, we have determined the similarities and differences, at the nucleotide level, between the high-affinity DNA–protein complexes formed by WDV RepA and Rep around the TATA boxes for c- and v-sense transcription. We have also studied the oligomerization state in solution of both WDV RepA and Rep as well as the monomer–oligomer equilibrium. Our studies have revealed that the oligomeric form of both WDV proteins interacts very poorly with DNA and that the efficiency of DNA–protein complex assembly is strongly affected by pH. These results have led us to propose a stepwise model for assembly of an oligomeric protein complex based on the initial binding of a protein monomer followed by addition of other monomers instead of a direct association of the protein oligomer with DNA.

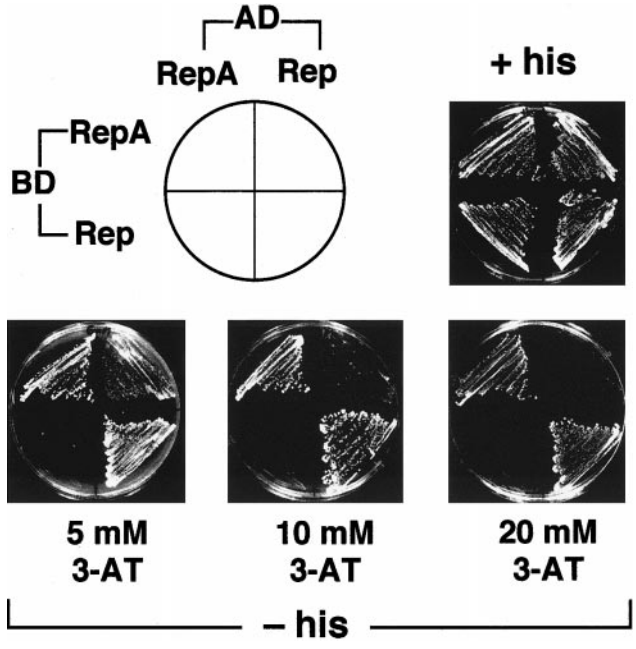

FIG. 1. Interaction of WDV RepA and Rep proteins assessed by yeast two-hybrid analysis. Yeast HF7c cells were cotransformed with plasmids expressing WDV RepA or Rep proteins fused to either the Gal4 DNA-binding domain (BD) or the Gal4 activation domain (AD). Cotransformants were allowed to grow on plates lacking tryptophan and leucine in the presence or absence of histidine (±his), and, in this case, in the presence of 5, 10, or 20 mM 3-amino-1,2,4-triazole (3-AT), to assess the strength of the protein interaction. Plasmids used in each cotransformation experiment are indicated (top left).

RESULTS

WDV RepA and Rep proteins self-interact in yeast

Previous experiments have shown that TGMV Rep oligomerizes (Orozco *et al.,* 1997; Orozco and Hanley-Bowdoin, 1998) and that the MSV replication-associated proteins can self-interact in yeast (Horvath *et al.,* 1998). To confirm whether this can be extended to other geminiviruses, e.g., WDV RepA and Rep proteins, we first used a yeast two-hybrid approach. Yeast cells were cotransformed with different combinations of plasmids expressing WDV RepA and Rep proteins fused to either the Gal4 DNA-binding or activation domains, as indicated in Fig. 1. Both RepA–RepA and Rep–Rep interactions were found to be strong, based on the ability of yeast cells to grow in selective medium under restrictive conditions (5–20 mM 3-aminotriazol (3-AT); Fig. 1). On the contrary, Rep–RepA interaction was comparatively less strong as it disappeared at a relatively low 3-AT concentration, irrespective of the plasmid from which the proteins were expressed (Fig. 1). These results, which are fully consistent with previous data for MSV RepA and Rep proteins (Horvath *et al.,* 1998), indicate that WDV proteins self-interact strongly, in particular in the case of RepA, suggesting that they can oligomerize.

An octameric form of WDV RepA occurs in solution

To determine directly the oligomeric state of WDV replication-associated proteins, we purified bacterially expressed RepA and Rep protein and eliminated the GST and the MBP tags enzymatically. Then, we used the purified proteins as substrates in chemical cross-linking reactions to stabilize protein–protein contacts between monomers. For this purpose, we found the oxidative cross-linking procedure mediated by glycine-glycine-histidine(GGH)–Ni(II) treatment useful (Brown *et al.,* 1995, 1998). Increasing amounts of the GGH–Ni(II) cross-linker produced a high-molecular-weight band which can be detected in SDS–PAGE gels, concomitantly with the disappearance of the monomeric species of WDV RepA (Fig. 2A). Running the gel under conditions that yielded a better separation of the high-molecular-weight material allowed us to detect a ~240-kDa band (Fig. 2B), consistent with the size for an octamer of WDV RepA (monomeric molecular mass of 31 kDa). Similar experiments were carried out with WDV Rep. In this case, however, the GGH–Ni(II) treatment was much less efficient and only at the highest possible concentration of the cross-linker could a faint band of an oligomeric form of WDV

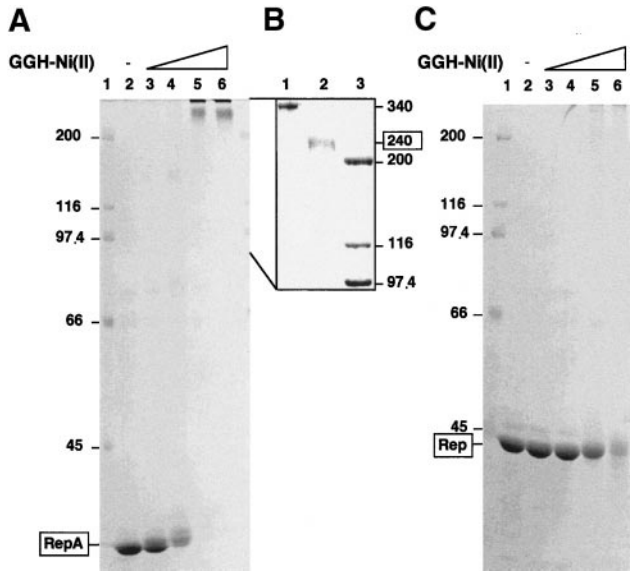

FIG. 2. Detection of WDV RepA and Rep oligomers by cross-linking with GGH–Ni(II). (A) Samples (500 ng) of purified WDV RepA protein were cross-linked using increasing amounts of the GGH–Ni(II) reagent (lane 2 contains a GGH–Ni(II) mock-treated sample and lanes 3, 4, 5, and 6 contain 15, 60, 250, and 1000 μM GGH–Ni(II), respectively) and fractionated by SDS–PAGE in 8% gels. Protein markers (kDa) are shown in lane 1. The position of purified WDV RepA monomers (apparent molecular mass 31 kDa) is indicated. (B) Lane 2 contains the same sample as in lane 6 of A, but the fractionation was carried out in a 6% gel. Protein markers are shown in lanes 1 and 3. The position of the WDV RepA oligomer (apparent molecular mass ~240 kDa) is indicated. (C) Same as in A, but using samples (650 ng) of purified WDV Rep protein.

Rep, which almost did not enter the gel (even at lower polyacrylamide concentrations (data not shown), be detected (Fig. 2C). This behavior precluded the fractionation of the Rep oligomers. However, based on these data and on the similarities in the oligomerization domains proposed for MSV RepA and Rep (Horvath *et al.,* 1998), it is conceivable that WDV Rep may also exist in an octameric (or higher oligomeric) state in solution. To rule out the possibility that these results were an artifact of the GGH–Ni(II) treatment, we tried to reproduce the cross-linking results using a different chemical treatment. The use of glutaraldehyde yielded similar results (not shown here, although see below) but it should be noted that the cross-linking efficiency was lower than with GGH–Ni(II).

From the data shown above (Fig. 2A), it was also clear that using different cross-linker concentrations we mostly detected either monomers or octamers. Intermediate oligomeric species were only detectable in very low amounts after using an anti-RepA serum (R. Missich, M. B. Boniotti, and C. Gutierrez, unpublished results). This suggests that, in the monomer–oligomer equilibrium established under the present conditions, the more abundant species are the monomeric and the octameric states.

WDV RepA forms complexes with the WDV LIR DNA at or near the TATA boxes

Previous studies have established that WDV Rep is able to form multiple DNA–protein complexes within the WDV large intergenic region (LIR, Sanz-Burgos and Gutierrez, 1998; Castellano *et al.,* 1999). Two of them, named the C- and V-complexes, are high-affinity complexes located in close proximity to the TATA boxes controlling c-sense and v-sense transcription, and DNase I footprinting analysis has shown that ~75 bp is protected by a large Rep protein core (Castellano *et al.,* 1999). Since (i) WDV Rep and RepA share their 210 N-terminal amino acids where the DNA-binding domain is located (Heyraud-Nitschke *et al.,* 1995; Jupin *et al.,* 1995; Laufs *et al.,* 1995b) and (ii) RepA has been proposed to regulate v-sense transcription (Palmer and Rybicki, 1998; Collin *et al.,* 1996), we wanted to see whether we could detect nucleoprotein complexes of RepA within the LIR.

Preliminary gel-shift experiments using DNA fragments covering different regions of the WDV LIR revealed that purified WDV RepA protein was able to interact with DNA (data not shown). To map precisely the location of these RepA–DNA complexes and, eventually, compare them with that of Rep complexes, we carried out DNase I footprinting experiments. When a DNA fragment spanning positions −221 to −83 (position +1 being the A residue at the initiation site for rolling-circle DNA replication; Sanz-Burgos and Gutierrez, 1998) was incubated with increasing amounts of WDV RepA protein a DNA region of ~75 bp was protected from DNase I digestion (Fig. 3). The RepA footprint covered the region between positions −193 and −116 in the top DNA strand (Fig. 3A) and between positions −193 and −114 in the bottom DNA strand (Fig. 3B). It is noteworthy that the footprint was flanked by one strong DNase I hypersensitive site on each strand at the TATA box for c-sense transcription. A less strong hypersensitive site was also detected at the top strand toward the 5′ boundary of the complex. Based on this location and by analogy to the complex formed by WDV Rep in a similar location (Castellano *et al.,* 1999), we also called it the RepA C-complex.

A similar study was carried out but using a DNA fragment downstream from the replication initiation site, spanning positions +34 to +172 (Fig. 4). Again, a large DNA region between positions +74 and +135 (located just upstream of the TATA box for v-sense transcription) in both DNA strands was protected from DNase I digestion (Figs. 4A and 4B), indicative of formation of a RepA V-complex.

These studies led us to conclude that WDV RepA forms C- and V-complexes in locations similar to those of the Rep complexes but showing subtle differences in their boundaries and location of hypersensitive sites (see Discussion). In both cases, the large size of the

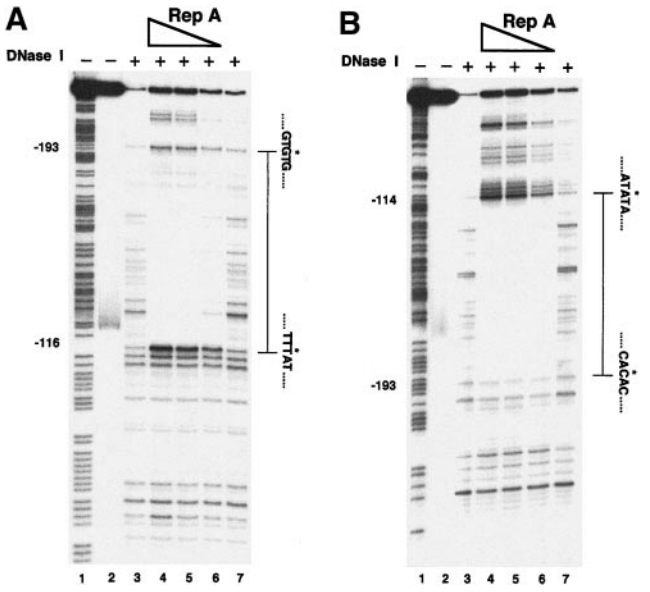

FIG. 3. DNase I footprinting analysis of the WDV RepA–DNA C-complex. A 138-bp DNA probe encompassing the region upstream from the initiation site of rolling-circle DNA replication (see Materials and Methods) was labeled at the 3′ end of the top (A) or bottom (B) strands and incubated with different amounts of purified WDV RepA (lanes 4, 5, and 6 contained 1000, 333, and 111 ng of RepA protein, respectively). After complex formation, the samples were subjected to DNase I footprinting assay. Lane 1 shows an A+G sequencing reaction of the same DNA fragment, lane 2, the labeled probe without DNase I digestion; and lanes 3 and 7 show the DNase I digestion pattern of the DNA probe in the absence of RepA protein. The region protected from DNase I digestion is shown at the right. The asterisks point to the boundaries of the protected region, in most cases coinciding with a DNase I hypersensitivity site.

complexes was fully consistent with the ability of RepA to form octamers.

Preformed oligomers of WDV RepA and Rep interact very poorly with DNA

Previous experiments as well as those shown above are consistent with the idea that both WDV RepA and Rep nucleoprotein complexes consist of a protein oligomer, most likely an octamer, bound to DNA. To assess the importance of oligomerization in the process of DNA–protein complex formation, we wanted to determine whether preformed oligomers were able to interact with DNA. Thus, we compared the DNase I footprinting pattern of RepA and Rep when a DNA probe is incubated with the purified proteins before and after chemical cross-linking with GGH–Ni(II), prior to digestion with DNase I. Surprisingly, both RepA and Rep oligomers were virtually unable to bind to DNA and produce the typical footprint (Fig. 5). These data also suggested that, although the capacity of RepA and Rep to oligomerize must be required for complex formation, the efficient assembly of a RepA and Rep complex must depend on the availability of protein monomers in the binding mixture.

Subtle changes in pH modulate the monomer–oligomer equilibrium

The possibility exists that GGH–Ni(II)-stabilized RepA and Rep protein oligomers did not interact with DNA because the cross-linking treatment chemically modified the proteins in such a way that it prevented their further interaction with DNA. Therefore, we searched for conditions which might affect the monomer–oligomer equilibrium and, hence, avoid the use of chemical treatment before DNA-binding studies.

We found that the solution pH is one factor that drastically affects the monomer–oligomer equilibrium. When the GGH–Ni(II) treatment of a RepA solution was carried out at different pH and the samples were fractionated by SDS–PAGE, we observed that within a relatively small pH range, the relative amount of monomers and oligomers changed significantly (Fig. 6A). Thus, between pH 6.2 and 7.0, most of the RepA protein can be detected in the octameric form while carrying out the GGH–Ni(II) cross-linking treatment at a slightly higher pH (7.4–7.8) rendered largely RepA monomers. This indicates that the monomer–oligomer equilibrium was displaced toward the monomeric state with this small pH increase.

The possibility that GGH–Ni(II) cross-linking might be affected by the solution pH within the range used is unlikely (Brown *et al.,* 1995, 1998), although it could not be ruled out completely. Therefore, we carried out similar experiments using another chemical cross-linking agent, e.g., glutaraldehyde, which acts by a different mecha-

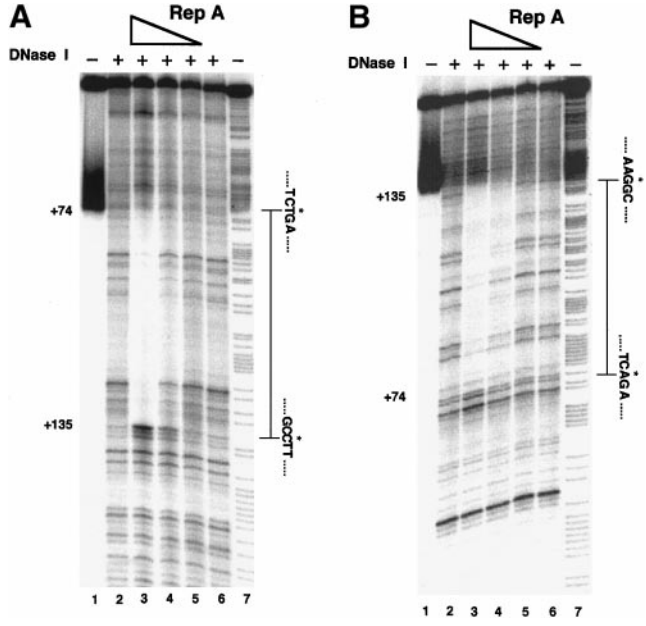

FIG. 4. DNase I footprinting analysis of the WDV RepA–DNA V-complex. Conditions were the same as in Fig. 3, except that a DNA probe encompassing the region downstream from the initiation site of rolling-circle DNA replication was used. Lane 1 shows the labeled probe without DNase I digestion, lanes 2 and 6 show the DNase I digestion pattern of the DNA probe in the absence of RepA protein, and lane 7 shows an A+G sequencing reaction of the same DNA fragment.

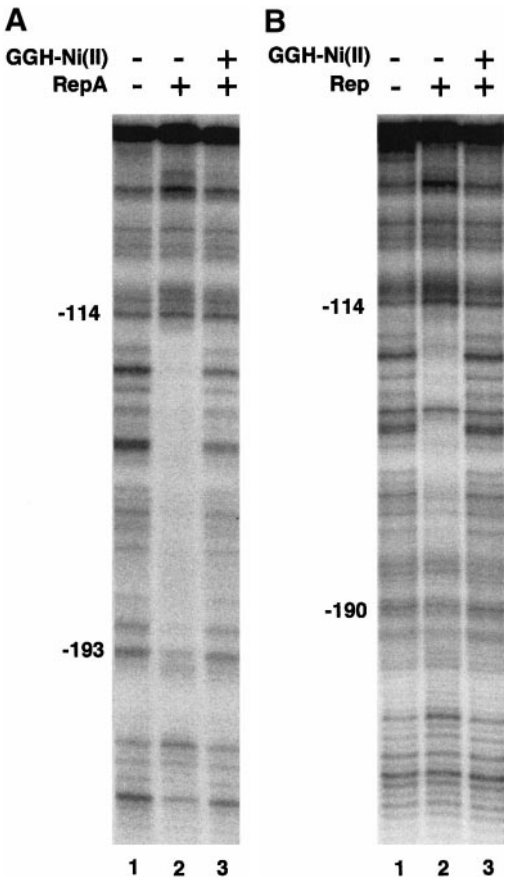

FIG. 5. Effect of cross-linking with GGH–Ni(II) on the DNase I footprinting pattern of WDV RepA and Rep C-complexes. The same DNA probe used in Fig. 3 was incubated with WDV RepA (500 ng, A) or Rep (650 ng, B) proteins untreated (lanes 2, both A and B) or treated (lanes 3, both A and B) with the GGH–Ni(II) cross-linking reagent. The DNase I digestion pattern of the DNA probe (bottom strand) in the absence of proteins is shown in lanes 1 (both A and B).

nism. In this case, again, a similar dependence of the solution pH was found (Fig. 6B). Based on these data, we conclude that at pH 6.2–6.6 most of the protein occurs as oligomers while at pH 7.4–7.8, it is in the monomeric state. Interestingly, the range of pH where this equilibrium is maintained is very narrow and close to what may represent physiological conditions.

WDV RepA– and Rep–DNA complex formation is largely dependent on pH

Since (i) preformed oligomers did not produce a clear DNA footprint and (ii) the solution pH significantly affects the monomer–oligomer ratio, it follows that pH might also have profound effects on RepA and Rep complex formation. We tested this prediction directly by carrying out gel-shift and DNase I footprinting assays at different pH.

Increasing the pH of the binding mixture, which increases the relative amount of protein monomers relative to oligomers, produced an increase in the efficiency of complex formation, especially at low protein concentration; e.g., with 100 ng of RepA most of the DNA probe was retarded at pH 7.8 while at pH 6.2 it was almost unaffected (Fig. 7A). We also determined the extent of protection to DNase I digestion within the same pH range. RepA concentrations which produce a typical large footprint at pH 7.8 were not able to protect the DNA from DNase I digestion at pH 6.2, although the hypersensitive sites appeared, indicative of complex formation at a lower efficiency (Fig. 7B). It should be kept in mind that DNase I digestion was equally effective within the pH range used in these experiments as indicated by the digestion pattern obtained in the absence of protein (Fig. 7B). Similar results were obtained for WDV Rep both in the gel-shift assays and in the DNase I footprinting experiments (data not shown).

DISCUSSION

The aim of this study was to determine the oligomerization properties of WDV RepA and Rep proteins and to assess the relationship between oligomerization and DNA–protein complex formation. We have found that (i) WDV RepA produced a DNase I footprint similar to, but distinguishable from, that of WDV Rep (Castellano *et al.,* 1999) upon binding to regulatory sequences within the WDV LIR, (ii) Rep and RepA can exist as oligomers in solution, and (iii) the pH is a critical parameter in the monomer–oligomer equilibrium. Our results led us to propose that oligomerization is likely to be necessary to

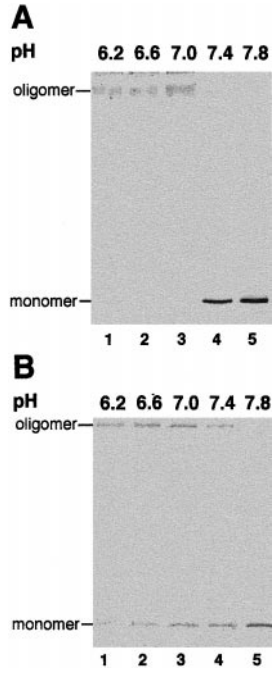

FIG. 6. Effect of pH on the oligomerization of WDV RepA. (A) Purified WDV RepA protein (500 ng) was treated with 1000 μM GGH–Ni(II) in 50 mM phosphate buffer, at the indicated pH, containing 100 mM NaCl, and the samples were fractionated by SDS–PAGE. (B) Same as in A, except that the buffer was 20 mM triethanolamine, at the indicated pH, and the protein was cross-linked with glutaraldehyde (250 μM).

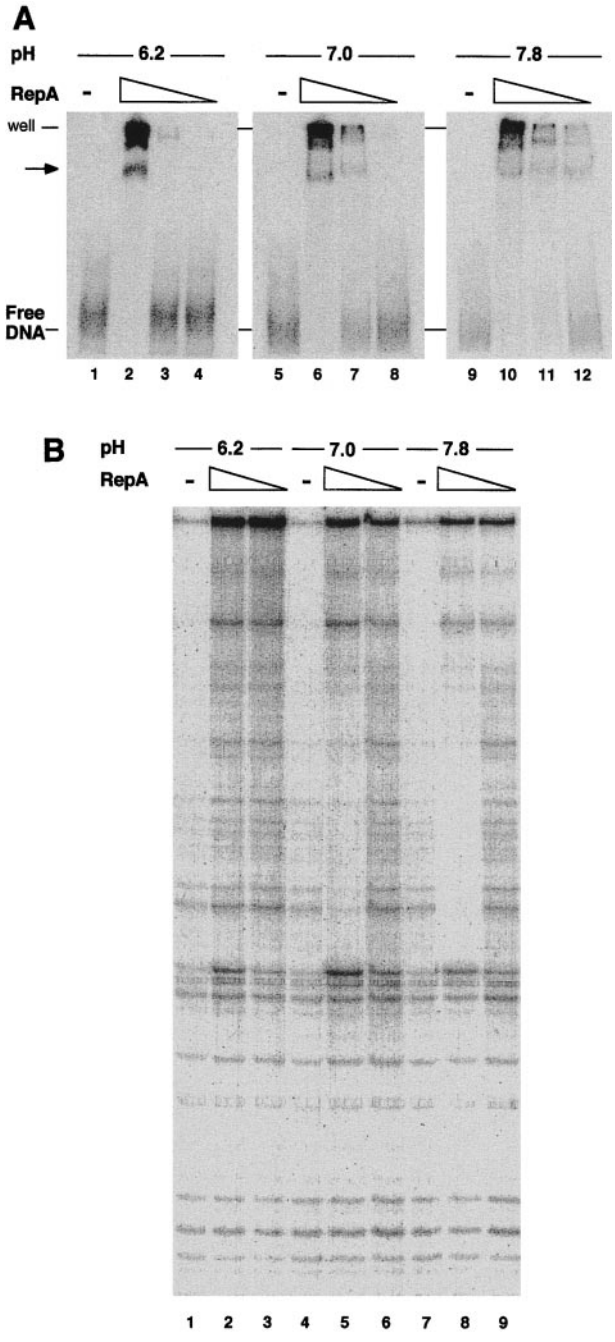

FIG. 7. Effect of pH on complex formation by WDV RepA protein. (A) Formation of RepA protein–DNA complexes on WDV LIR in 50 mM phosphate buffer at different pH, as indicated, analyzed by gel-shift assays. The amounts of RepA protein used were 500 (lanes 2, 6, and 10), 100 (lanes 3, 7, and 11), and 20 ng (lanes 4, 8, and 12). Nucleoprotein complexes were fractionated in 4% polyacrylamide gels. (B) Formation of RepA protein–DNA C-complexes in 50 mM phosphate buffer at different pH, as indicated, was analyzed by DNase I footprinting. The amounts of RepA used were 100 (lanes 2, 5, and 8) and 20 ng (lanes 3, 6, and 9). The DNA probe used was the same as that described in Fig. 3 (C-complex). The DNase I digestion pattern of the DNA probe, at different pH, in the absence of RepA protein is shown in lanes 1, 4, and 7.

assemble a RepA– or Rep–DNA complex but complex formation probably occurs by sequential addition of monomers rather than by direct association of a preformed oligomer with DNA. The relevance of these observations to interpret experiments on geminivirus DNA replication and transcription, where protein–protein and protein–DNA interactions are required, is discussed below.

RepA– and Rep–DNA complexes within the WDV LIR

For mastreviruses, the transcript produced from the c-sense promoter has the capacity to encode two proteins, namely RepA and Rep (formerly C1 and C1:C2), the latter after a splicing event (Accotto *et al.,* 1989; Schalk *et al.,* 1989; Wright *et al.,* 1997). As a consequence, RepA and Rep share a large N-terminal part of their primary sequence, e.g., 210 residues in the case of WDV proteins (Schalk *et al.,* 1989). This N-terminal region includes the DNA-binding domain which has been mapped in a number of geminivirus Rep proteins, including WDV (Choi and Stenger, 1995, 1996; Heyraud-Nitschke *et al.,* 1995; Jupin *et al.,* 1995; Orozco *et al.,* 1997; Orozco and Hanley-Bowdoin, 1998). Consequently, it was conceivable that RepA could form nucleoprotein complexes perhaps similar to those of Rep.

We have found that WDV RepA forms two distinct DNA–protein complexes, the C- and V-complexes, similar to those described recently for WDV Rep (Castellano *et al.,* 1999). However, some differences can be established between the RepA–DNA and the Rep–DNA complexes on the basis of their size and the location of DNase I hypersensitivity sites (Fig. 8). The size of the C-complex is similar for both proteins (~75 bp) and, in both cases, the TATA box and the transcription initiation site are protected from DNase I digestion. The footprint of the RepA complex is displaced a few base pairs in the bottom strand, relative to the Rep complex in such a way that the first TA residues of the TATA box are not protected (Fig. 8). In addition, strong DNase I hypersensitivity sites flank the RepA C-complex both in the top and in the bottom strands, suggesting that a significant distortion in the DNA helix occurs upon RepA binding. The RepA V-complex has a boundary near the TATA box similar to that of the Rep complex. However, toward the 5′ side, it is ~10 bp smaller (Fig. 8). This places the direct repeats, whose consensus sequence is GTGTG-$AN_{22–23}$GTG(G)TC, asymmetrically located within the footprint. We believe that this sequence might act as a recognition signal for RepA– and Rep–DNA complex formation. As in the case of the RepA C-complex, strong hypersensitivity sites flank the footprint, in particular in both strands near the TATA box, but the V-complex lacks the hypersensitive site in between the direct repeats. Therefore, as a rule, RepA binding produced stronger DNase I hypersensitive sites flanking the complexes.

Different roles in transcriptional regulation of viral genes have been proposed for mastrevirus RepA and Rep proteins and begomovirus Rep proteins. Thus, RepA and Rep have been implicated in transcriptional regula-

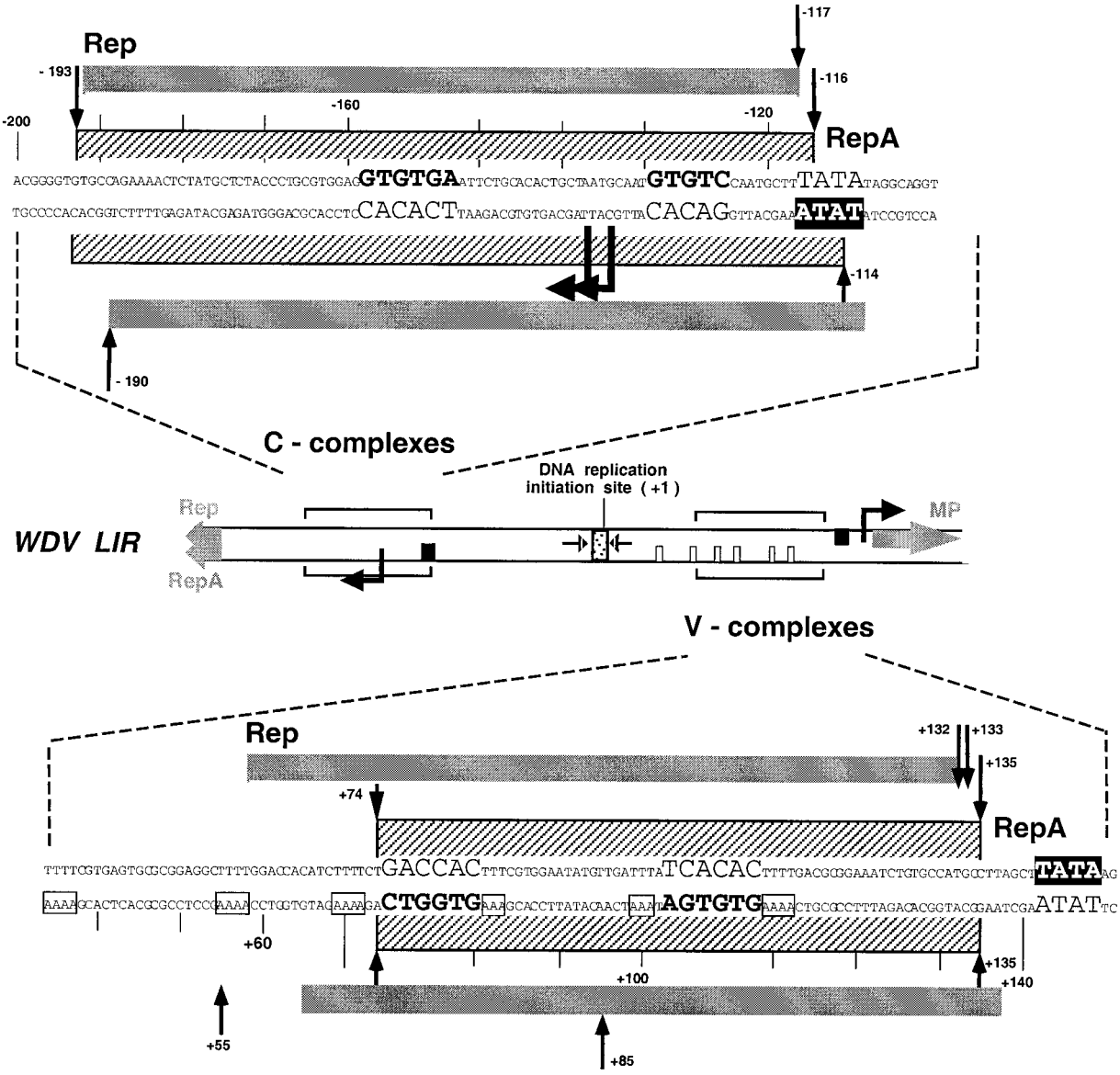

FIG. 8. A comparison of the WDV RepA and Rep protein–DNA complexes (C and V) formed on the WDV LIR as deduced from DNase I footprinting analysis. The WDV LIR is shown with its major landmarks including the invariant 9-nt sequence (dotted box), the initiation site of rolling-circle DNA replication (+1), flanked by the inverted repeats (small convergent arrows), the TATA boxes (black boxes), the transcription start sites (thick black arrows denote the direction of transcription), the beginning of the coding sequence of MP (movement protein), RepA and Rep proteins (thick gray arrows), the A/T tracts (small empty boxes), and the regions where WDV RepA and Rep proteins form the C- and V-complexes (brackets). These regions are enlarged to show the details for the C-complexes (top) and the V-complexes (bottom) of WDV RepA (dashed boxes) and Rep (gray boxes) proteins. The DNase I hypersensitive sites are indicated by arrows together with their positions.

tion from the v-sense promoter (Dekker *et al.,* 1991; Hofer *et al.,* 1992; Collin *et al.,* 1996), from which both the movement protein and the capsid protein are produced. MSV RepA has been shown to act as a trans-activator in yeast (Horvath *et al.,* 1998). In addition, a negative role for WDV RepA in viral DNA replication has also been proposed (Collin *et al.,* 1996). Based on the location and the footprinting pattern of the WDV Rep C-complex, a role in regulation of the c-sense promoter is conceivable (Castellano *et al.,* 1999). A virus-specific repression of the c-sense promoter by begomovirus Rep has been demonstrated in several cases (Sunter *et al.,* 1993; Eagle *et al.,* 1994; Eagle and Hanley-Bowdoin, 1997; Gladfelter *et al.,* 1997). Our direct demonstration that both WDV RepA (this work) and Rep (Castellano *et al.,* 1999) can form nucleoprotein complexes in the proximity of both the c-sense and the v-sense promoters makes it necessary to address in the future the question as to whether both, or only one of them, are implicated in transcriptional regulation of the two promoters. It will also be important to determine whether the RepA and Rep proteins from other mastreviruses can form complexes in similar locations. The fine structural differences between the C- and V-complexes formed by WDV RepA and Rep proteins

provided by our *in vitro* DNA binding studies should help in future studies to understand their functional significance.

Oligomerization of WDV RepA and Rep proteins in relation to DNA–protein complex formation

Detailed deletion studies have served to map the region containing amino acids critical for protein–protein interactions both in TGMV Rep (Orozco *et al.,* 1997; Orozco and Hanley-Bowdoin, 1998) and in MSV RepA and Rep (Horvath *et al.,* 1998) proteins. Based on amino acid sequence homology between MSV and WDV proteins, it is conceivable that residues similar to those in MSV proteins are important for WDV RepA and Rep protein–protein interactions. The ability of these proteins to form oligomers in solution, which we have shown directly in this study, is fully consistent with the large size of the complexes observed previously by electron microscopy and DNase I footprinting (Sanz-Burgos and Gutierrez, 1998; Castellano *et al.,* 1999).

One aspect which, to our knowledge, has not been addressed so far for these geminivirus proteins is the study of conditions that affect the monomer–oligomer equilibrium. We have found that small pH changes are crucial for producing a displacement of this equilibrium. Thus, at pH 7.4–7.8, most of the RepA and Rep protein exist as monomers while at pH 6.6–7.0, the oligomers are the predominant species. It is noteworthy that the displacement occurs within a narrow physiological pH range. We believe that the fact that the pH values could be considered within a physiological range might have important consequences on the ability of RepA and Rep proteins to interact with DNA and assemble functional complexes. Similar effects of the solution conditions on protein–protein interactions have been observed for other DNA-binding proteins. Thus, pH affects oligomerization and DNA–protein complex formation of polyomavirus large T-antigen (T-ag; Peng and Acheson, 1998) and oligomers of the SV40 T-ag disassemble into monomers in the presence of chelating agents (Montenarh and Henning, 1983). Such effects have also been detected with DNA nonbinding proteins, e.g., the antibody single-chain fragment (Arndt *et al.,* 1998).

Our observations are relevant to the current view of the formation of Rep and RepA complexes with DNA. Interaction between TGMV Rep monomers has been proposed to be a prerequisite for DNA binding but not for DNA cleavage (Orozco *et al.,* 1997). TGMV Rep binding requires both a DNA binding domain and an oligomerization domain (Orozco and Hanley-Bowdoin, 1998). For WDV Rep and RepA proteins, we have found that preformed oligomers interact very poorly, if at all, with DNA. Furthermore, using pH changes to manipulate the monomer–oligomer equilibrium, we have observed that at pH $\leq$ 6.6, at which the solution is enriched for oligomers, Rep and RepA do not produce the typical, large DNase I footprint, as occurs at pH $\geq$ 7.0. This pH effect might be due to the presence of titratable groups at the protein–protein interface. Based on these results, we think that it is necessary to distinguish between DNA binding or interaction and assembly of a full DNA–protein complex, the latter requiring oligomerization in addition to DNA binding.

Furthermore, based on our data we can propose a model in which complex assembly is a stepwise process consisting of at least two stages. The first stage is interaction of a protein monomer with DNA, a process mediated by the protein DNA-binding domain which recognizes specific DNA signals, e.g., the directly repeated sequence. The second stage is sequential addition of monomers which will lead to stabilization of the oligomer assembled on the DNA. At this stage, the protein–protein interaction domain would be required, although the DNA-binding domain may help in further stabilizing the oligomer–DNA interaction. Stepwise formation of oligomeric structures in DNA has previously been proposed for other proteins. For example, binding of polyomavirus T-ag to the origin DNA sequences is substantially increased at pH 6.0–7.0, at which protein–DNA complexes are more stable and cooperative and sequential addition of monomers is favored, relative to pH 7.4–7.8 (Peng and Acheson, 1998). Furthermore, SV40 T-ag can exist in equilibrium between monomers, tetramers, and hexamers (Montenarh and Henning, 1983; Runzler *et al.,* 1987) and proper oligomerization and assembly of functional higher order complexes is crucial for the many different activities of SV40 T-ag forms (Tack *et al.,* 1989; Gutierrez *et al.,* 1990; Simmons *et al.,* 1993; Weisshart *et al.,* 1996), consistent with the DNA-binding properties of different oligomeric forms of SV40 T-ag (Runzler *et al.,* 1987; Dean *et al.,* 1992).

Finally, the question of whether our observations can be extrapolated to other geminiviruses remains. Based on sequence homology and functional similarities, one would expect this to be the case. However, this should be addressed experimentally. In any case, we believe that the results presented here have significant implications for future studies, in particular *in vitro,* on DNA binding, DNA replication, and transcription where geminivirus proteins might have a different behavior depending on whether the monomeric or the oligomeric forms are the predominant species.

MATERIALS AND METHODS

Yeast two-hybrid analysis

The yeast strain HF7c (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 URA3::GAL4*$_{17mers(x3)}$*-CyC1*$_{TATA}$*-LacZ;* Feilotter *et al.,* 1994), which contains the two reporter genes *LacZ* and *HIS3,* was used in the two-hybrid experiments (Fields and Song, 1989). Yeasts were first transformed, as described (Schiestl and Gietz, 1989), with plas-

mids expressing WDV RepA or Rep proteins fused to the Gal4 DNA binding (BD; *TRP1* marker) or activation (AD; *LEU2* marker) domains, as indicated. To corroborate the interaction between the fusion proteins, β-galactosidase activity was measured by a replica filter assay, as described (Breeden and Nasmyth, 1985). Plasmid pBWRepA has been described (Xie *et al.,* 1995). To generate plasmid pBWRep, we first eliminated the intron present in the WDV c-sense transcription unit by PCR and the intron-less transcription unit was cloned into pWori (Xie *et al.,* 1995) to generate pWoriRep. Then, pBWRep was constructed by cloning a 1.25-kbp *Nco*I–*Ssp*I fragment of pWoriRep into the pGBT8 vector digested with *Nco*I and *Nde*I. Plasmid pGAD-RepA was obtained by cloning a 845-bp *Bam*HI (made blunt with S1 nuclease)–*Sal*I fragment into the corresponding sites of the pGADGH vector. Plasmid pGADRep was generated by cloning the 1.22-kbp *Bam*HI (made blunt with nuclease S1)–*Sal*I fragment of pBWRep into the corresponding sites of the pGADGH vector.

DNA probes

WDV LIR DNA probes were prepared as follows. Two 138-bp PCR fragments containing the WDV LIR DNA upstream or downstream from the initiation site (Castellano *et al.,* 1999) were independently produced, using oligonucleotides ("left-up"; CGGCAGGTCCTTAGCG) and ("left down"; GCCCTGTTCTCCGCC) or ("right up"; TTTCGTGAGTGCGCGG) and ("right down"; ACCGAGATGGGCTACC). Plasmids pLLIR and pRLIR were generated by cloning the PCR DNA fragments covering the LIR DNA regions upstream or downstream from the initiation site, respectively, into the pCR2.1 vector (Invitrogen). DNA fragments were gel-purified after restriction with appropriate enzymes. Every strand was 3′ end-labeled by filling in with *E. coli* DNA polymerase I (Klenow fragment) in the presence of [α-^{32}P]dCTP. The labeled probes were used in gel-shift and DNase I footprinting assays.

Standard DNA manipulation techniques were carried out according to published protocols (Sambrook *et al.,* 1989). DNA sequencing was performed using the Amplicycle kit (Perkin–Elmer) and a 377 DNA sequencer (Applied Biosystem).

Purification of WDV RepA and Rep proteins

Overexpression and purification of GST-RepA was carried out according to Xie *et al.* (Xie *et al.,* 1999). The GST moiety was cleaved with thrombin (Boehringer Mannheim), according to the manufacturer's instructions and the RepA protein (31 kDa) purified as the flow-through of a glutathione–Sepharose column (Pharmacia). MBP-Rep was expressed and purified as described (Sanz-Burgos and Gutierrez, 1998; Castellano *et al.,* 1999). The MBP moiety was eliminated by treatment with factor Xa (New England Biolabs), as described by the manufacturer and the Rep protein (40 kDa) recovered as the flow-through of an amylose column (New England Biolabs). All proteins were kept in 50 mM phosphate buffer, pH 7.0.

Chemical cross-linking

Proteins cross-linking with glycine-glycine-hystidine complexed with nickel acetate (GGH–Ni(II)) was carried out essentially as described (Brown *et al.,* 1995, 1998). Cross-linking reactions were carried out in a total volume of 5 μl and, unless otherwise stated, the final concentrations were 50 mM phosphate buffer, pH 7.0, 100 mM NaCl. Protein and cross-linking reagents concentrations are indicated in the figure legends. The GGH–Ni(II) complex was formed by mixing a 1:1 molar ratio of nickel acetate and GGH in distilled H_2O. Following a 15-min equilibration time, the solution was diluted to the desired concentration with 50 mM phosphate buffer, at the desired pH, and added to the reaction mix. The reaction was then initiated by the addition of magnesium monoperoxyphthalic acid hexahydrate at the same final concentration of the GGH–Ni(II) complex. After 2 min of incubation at room temperature, the reaction was quenched by the addition of 1.7 μl of 4× gel loading buffer (0.24 M Tris, 8% SDS, 2.88 M β-mercaptoethanol, 40% glycerol, 0.4% bromphenol blue, 0.4% xylene cyanol). Samples were heated at 100°C for 5 min and then fractionated by electrophoresis using a 8% Tris–glycine SDS polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue.

Cross-linking reactions with glutaraldehyde (Serva, 250 μM) were carried out in a total volume of 5 μl of 20 mM triethanolamine, adjusted at the indicated pH, and 50 mM NaCl for 30 min at room temperature. After cross-linking, the products were analyzed as described above for the GGH–Ni(II) treatment.

Gel-shift assays

Gel-shift assays were carried out as described (Castellano *et al.,* 1999) using as probe the *Xho*I–*Bam*HI fragment from pLLIR (~0.5 ng of labeled DNA per assay).

DNase I footprinting analysis

DNase I footprinting reactions were performed with the end-labeled fragments described above. Unless otherwise stated, in a total volume of 24 μl, the DNA fragment (~1–3 ng) was incubated in a buffer containing 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, and 1 μg poly (dI-dC) as nonspecific competitor DNA, with different amounts of RepA and Rep proteins for 5 min at room temperature. The DNase I footprinting reaction was started by the addition of 1 μl of DNase I (Promega) solution (0.1 U/μl) and then allowed to proceed for 5 min at room temperature and finally stopped by the addition of 50 mM EDTA and 0.3 M sodium acetate in the presence of 10 μg of yeast tRNA. Precipitated DNA was recovered by centrifugation and resuspended in denaturing formamide loading buffer and fractionated in a 8% denaturing polyacryl-

amide gel. When needed, a G + A sequencing reaction (Belikov and Wieslander, 1995) of the same fragments was run in parallel as size standards.

When DNase I digestion was carried out after cross-linking with GGH–Ni(II), the treated protein sample (5 μl) was directly used in the binding reaction before DNase I treatment. The relative amount of cross-linked and uncross-linked proteins present in the reaction was assessed by fractionation in SDS–PAGE of parallel cross-linking reactions.

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