Sequence-specific and Methylation-dependent and -independent Binding of Rice Nuclear Proteins to a Rice Tungro Bacilliform Virus Vascular Bundle Expression Element*

Received for publication, July 25, 2000, and in revised form, October 11, 2000
Published, JBC Papers in Press, October 17, 2000, DOI 10.1074/jbc.M006653200

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Nuclear proteins from rice (Oryza sativa) were identified that bind specifically to a rice tungro bacilliform virus promoter region containing a vascular bundle expression element (VBE). One set of proteins of 29, 33, and 37 kDa, present in shoot and cell suspension extracts but hardly detectable in root extracts, bound to a site containing the sequence AGAAGGACCAGA within the VBE, which also contains two CpG and one CpNpG potential methylation motifs. Binding by these proteins was determined to be cytosine methylation-independent. However, a novel protein present in all analyzed extracts bound specifically to the methylated VBE. A region of at least 49 nucleotides overlapping the VBE and complete cytosine methylation of the three Cp(Np)G motifs was required for efficient binding of this methylated VBE-binding protein (MVBP).

Promoters for genes transcribed by RNA polymerase II are composed of different cis elements that provide binding sites for trans-acting factors (1–2). The interactions of these trans-acting factors with each other, with associated cofactors, and with the basal transcription machinery determine the expression characteristics of a promoter. Promoter activity is also influenced by epigenetic control mechanisms that may modulate the accessibility of promoter sequences for the transcription machinery in various ways. Plant promoter studies have elucidated several molecular components involved in tissue-specific gene expression (1–2). These include cis-acting DNA elements and trans-acting protein factors involved in regulating gene expression in vascular tissue (3–8).

The rice tungro bacilliform virus (RTBV)1 promoter (Fig. 1) has been analyzed in both transformed rice plants (9–11) and transfected rice protoplasts (12–15). It was found that sequences downstream of the transcription start site are required for promoter activity in protoplasts (13–15). In transgenic plants, the RTBV promoter containing the first 250 transcribed nucleotides showed activity in the epidermis and in all cells of the vascular bundle, not just the phloem (9). In the latter study, the most important promoter element for this vascular bundle activity was localized to the region between –100 and –165 (9), whereas previous analyses indicated sequences termed box II (–53 to –39), ASL box (–98 to –79), and GATA-like motif (–143 to –135) as phloem specificity elements (10, 11, 16). Proteins binding to all these regions were detected in nuclear extracts from rice. It was also found that the vascular bundle activity was suppressed in some transgenic rice lines with methylated promoter sequences,2 indicating that methylation directly or indirectly alters the assembly of transcription factors on the promoter.

In this report, we have further analyzed DNA-protein interactions of nuclear proteins with the vascular bundle expression element located in the region between –165 and –100 with a detailed mutagenesis analysis and have compared factor-binding to methylated and nonmethylated sequences. Our results show that independent of the methylation status, at least three rice nuclear proteins (29, 33, and 37 kDa) specifically interact with this element, and a motif of twelve residues (AGAAGGACCAGA) within this element is critical for protein binding. Binding activity is high in extracts from shoots and low in extracts from roots, correlating with the promoter activity in the respective tissues. It is also high but slightly different in extracts from cell suspensions, where the element has a negative effect on promoter activity. The methylated DNA sequence bound in addition or alternatively to a novel protein present in all extracts. This binding was highly sequence- and methylation-specific and may be related to the specific inactivation of the methylated promoter in vascular tissue.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Standard cloning techniques were used for all plasmid constructions (17). Plasmid R-218 contains RTBV promoter sequences up to position –218, the full-length RTBV leader sequences, and a chloramphenicol acetyltransferase (CAT) gene fused to RTBV open reading frame I (12).

To generate plasmid R-218Vd, a DNA fragment covering the region from –218 to –165 was amplified from the R-218 template by polymerase chain reaction with a 5′-end primer (P5) located at vector sequences upstream of the RTBV promoter and a primer corresponding to nucleotides –180 to –165 flanked with an EcoRV site. A second DNA fragment (from –100 to the first 20 nucleotides of the CAT open reading frame) was amplified with a primer corresponding to nucleotides –100 to –85 flanked with an EcoRV site and a 3′-end primer (P3) covering the first 20 nucleotides of the CAT open reading frame. These two polymerase chain reaction fragments were digested with EcoRV and then ligated. The resulting DNA fragments were digested with XbaI and XhoI and were cloned into the corresponding sites of R-218 to yield R-218Vd.

1 This work was supported by the Novartis Research Foundation, a part of the Friedrich Miescher Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 A. Klöti, X. He, I. Potrykus, T. Hohn, and J. Fütterer, submitted manuscript.

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To obtain R-218Vm, two overlapping fragments, one amplified with primer P5 and a 3′-end primer of the antisense strand with mutations of nucleotides GTCTCTT (from positions −144 to −150) to CCAGGAA, and the other with a 5′-end primer corresponding to the sense strand with mutations of nucleotides AAGGACC (from positions −150 to −144) to TTTCTGG and primer P3, were used as templates to amplify a mutated promoter fragment using primer P5 and P3. The resulting products were digested with XbaI and Xhol and introduced between the corresponding sites of R-218.

To construct plasmids VR-218Vd(+), VR-218Vd(−), and VR-218Vd(−), double-stranded oligonucleotides corresponding to sequences from −165 to −100 were introduced into the XbaI site (filled in with Klenow fragment of DNA polymerase) of R-218Vd. The resulting constructs were designated VR-218Vd(+) and VR-218Vd(−), with the insertion in sense and antisense orientation, respectively.

Double-stranded oligonucleotides covering the sequences from −165 to −100 were phosphorylated and ligated. Dimers were purified from a 2% agarose gel. The dimers were cloned into the EcoRV site of R-218Vd to generate R-218Vd2 and into the XbaI site to yield 2VR-218d(+) with the insertion in sense orientation and 2VR-218d(−) with the insertion in antisense orientation.

To create plasmid mP165 for preparation of the DNA probe used in DNA UV cross-linking assays, double-stranded oligonucleotides corresponding to sequence from −165 to −100, flanked by an XbaI site at the 5′- and HindIII site at the 3′-end, were introduced between the corresponding sites of M13mp18.

All plasmids were confirmed by restriction digestions and DNA sequencing. Plasmids were isolated from Escherichia coli (strain DH5α) using a Qiagen plasmid kit.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from cell suspension cultures of Oryza sativa line Oc were prepared as described (13). Nuclear extracts from 2-week-old O. sativa plant shoots and roots were prepared as described previously (12).

Methylated or nonmethylated double-stranded oligonucleotides corresponding to sequences from −169 or −165 to −100 with a 5′-protruding end in the antisense strand, were labeled with [α-32P]dCTP using the Klenow fragment of DNA polymerase. The labeled probe was purified on a 5% native polyacrylamide gel. EMSAs were performed as described (12). Typical mixtures (15 μl) for in vitro binding reactions contained 5 μg of poly(dI·dC), 1× binding buffer (10 mM HEPES, pH 7.6, 8 mM MgCl2, 1 mM dithiothreitol, 4 mM spermidine, and 5% [v/v] glycerol), 10 μg of nuclear extracts, and 15,000 cpm of labeled DNA probe (around 0.04 pm DNA). For the competition experiments variable amounts of competitor as indicated were included. Binding reaction mixture was first preincubated for 10 min at room temperature before the addition of the labeled DNA probe and incubated for a further 20 min at room temperature after the addition of the labeled DNA probe and the competitors.

Protein clipping band shift assays were performed as described (18). One μl of protease was added to the binding reaction mixture at the amounts indicated 20 min after the addition of the labeled DNA probe and allowed to react for 5 min at room temperature. Upon incubation, the reaction products were separated on a 5% native polyacrylamide gel.

DNA UV Cross-linking Assays—Single-stranded mP165 DNA was prepared as described previously (17). A uniformly labeled DNA probe with thymidine residues substituted by bromodeoxyuridine (BrdUrd) was prepared with [α-32P]dCTP as described previously (19). The labeled DNA probe was digested with XhoI and HindIII and purified on a 5% native polyacrylamide gel. Binding reactions and UV cross-linking were performed as described previously (12).

Transient Expression Assays—Protoplasts from O. sativa line Oc were isolated from cell suspension cultures and transfected by polyethylene glycol-mediated transfection (20, 21). Routinely, 10 μg of test plasmid DNA was used to transfect 0.6 × 106 protoplasts, and an internal control plasmid expressing β-glucuronidase under the control of the cauliflower mosaic virus 35S promoter was cotransfected in all experiments. Protein extracts were prepared after overnight incubation of protoplasts and were analyzed for CAT (CAT ELISA kit, Roche Diagnostics Ltd) and β-glucuronidase activities (22, 23). CAT activity was normalized to β-glucuronidase activity in the same extract in all cases. Relative expression levels did not vary more than ±15% with the given constructs in this study. All constructs were tested in at least three independent experiments with at least three independent colonies from each transformation.

RESULTS

Nuclear Proteins Interact with the Vascular Bundle Expression Element—In our previous study of the RTBV promoter in transgenic rice plants, a promoter element required for gene expression in the vascular tissue was identified between nucleotide positions −165 and −100 (9). Here, we refer to this region as the vascular bundle expression element (VBE).

To further characterize nuclear proteins interacting with the VBE, EMSAs were performed using labeled DNA probes from −165 to −100 of the RTBV promoter and nuclear extracts prepared from rice shoots (S), roots (R) and cell suspension cultures (C). Multiple DNA-protein complexes were detected upon incubation of each of these extracts with the labeled DNA probe (Fig. 2). However, these complexes were barely detected in root nuclear extracts even if the amount of nuclear extracts in the assay was increased, indicating that the DNA-binding proteins responsible for complex formation are much less abundant in roots.

DNA-protein complexes VBP1 and VBP2 show a slightly lower mobility in cell suspension nuclear extracts than in shoot nuclear extracts. Additional DNA-protein complexes (VBP3, VBP4, and VBP5) were seen in nuclear extracts from cell suspensions. The observed gel shift patterns were qualitatively reproducible; however, the intensity of individual bands varied with different batches of extracts and sometimes complexes were resolved in two bands (e.g. VBP1 in Fig. 2 and
VBP2 in Fig. 3). Whether this is caused by the presence of different proteins or by natural or artificial protein modifications will have to await protein characterization.

**Determination of Minimal Sequences Required for Protein Binding**—Initially, the specificity of DNA-protein interactions was analyzed using specific unlabeled DNA fragments as competitors in EMSA, and it was found that all observed complexes are equally well competed by DNA fragments covering the regions \(-165\) to \(-140\) or \(-150\) to \(-125\) (data not shown). Therefore, the sequence common to both fragments was analyzed in greater detail by introduction of mutations into a fragment covering the region from \(-165\) to \(-125\), which we designated as fragment V (Fig. 3A). These mutants (Vm1 to Vm10) were used as competitors in EMSA for protein binding to the labeled fragment V. From these, only the competitors Vm9 and Vm10 abolished the formation of complex VBP2 (Fig. 3A, lanes 23, 24 and C), whereas other competitors reduced complex formation only slightly (Fig. 3A, lanes 9, 10, 13–20 and C).

These data indicate that sequences from at least \(-149\) to \(-145\) are required for formation of complex VBP2, but binding was modulated by sequences closely upstream of them. Mutations of the same DNA region in some cases also abolished the formation of complexes VBP1; however, the binding requirements for VBP1 and VBP2 may be different because e.g. the mutations in Vm2 and Vm4 only affected VBP2 formation and Vm9 mainly VBP1 formation. These differences also suggest that the formation of the complexes occurs independently because abolishing one complex did not alter the gel shifts caused by the other. All fragments still efficiently competing for complex VBP1 formation contain two intact AGA motifs flanking the sequence important for VBP2 formation.

Mutation of the GATA motif, implicated by Yin et al. (11) in phloem specificity to AGCG in fragment Vm10, did not impair the competitivity (Fig. 3B, lanes 23, 24 and C) and thus excludes this sequence as a binding site for the factors observed in our assay.

**Characterization of Nuclear Proteins Bound to the VBE by DNA UV Cross-linking Assays**—To identify nuclear proteins interacting with the VBE, we performed DNA UV cross-linking assays with a BrdUrd-substituted, uniformly radiolabeled DNA probe from \(-165\) to \(-100\) and nuclear extracts from rice shoots and cell suspension cultures. As shown in Fig. 4, three proteins with apparent sizes of 29, 33, and 37 kDa were cross-linked to the labeled probe in both shoot and cell suspension nuclear extracts (lanes 2 and 9), whereas other bands marked with asterisks (also present in reactions without added protein extract, lanes 1 and 8) resulted from incomplete digestion of the DNA probe. The 37-kDa protein predominates in both nuclear extracts, whereas the 29-kDa protein is much less abundant. We did not detect additional proteins in the suspension cell extract that could form the DNA-protein complexes VBP3,
VBP4, and VBP5 observed in EMSA. It might be that these proteins may function as accessory proteins that interact with other DNA-binding proteins via protein-protein interactions instead of directly binding to the DNA element. Competition experiments proved that the detected proteins bind specifically to the −165 to −125 DNA fragment and not to other DNA fragments (lanes 3, 4, 10, 11).

The VBE Acts as an Inhibitor Element in Transiently Transfected Rice Protoplasts—The VBE is very important for regulating gene expression in the vascular bundle (9) and interacts with nuclear proteins VBP1 and VBP2 prepared from all tissues tested (Fig. 2). Additional DNA-protein complexes (VBP3, VBP4, and VBP5) were detected predominantly with nuclear extracts from cell suspension cultures (Fig. 2). Therefore, it was of interest to assess the function of this element in a transient-expression system. To this end, we deleted or mutated the element in the context of the plasmid R-218 (Fig. 5) and tested the activity of the resulting plasmids in transgenic rice protoplasts. In repeated transfection assays, deletion of this element resulted in a 2-fold increase in promoter activity compared with wild-type R-218 (Fig. 5, R-218Vd). The negative effect of the VBE element was position independent and slightly increased by VBE duplication (Fig. 5). Mutation of nucleotides AAG-GACC (from nucleotide positions −150 to −144) to TTCCTGG abolished protein binding to VBE (Fig. 3, lanes 15, 16) and resulted in alleviation of the negative effect of VBE on expression in protoplasts (Fig. 5, R-218Vm).

An Additional Protein Binds to Methylated VBE—Previously, we observed in some transgenic rice lines harboring a β-glucuronidase gene under the control of the RTBV promoter, specific inactivation of β-glucuronidase expression in the vascular tissue.3 The resulting expression pattern was identical to that obtained with a VBE deletion and was correlated with the methylation of the promoter region including the VBE. We therefore investigated whether methylation of the VBE region interferes with protein binding by testing several methylated and unmethylated DNA fragments (Fig. 6) in the EMSA.

Methylation of fragment V did not interfere with binding of proteins from rice nuclear extracts that also bound to the unmethylated fragment. However, a novel, additional shift-band appeared (Fig. 7, MVBP). Formation of this novel complex required DNA methylation and consequently methylated but not unmethylated DNA fragments could compete for binding. All other VBE-binding proteins from rice cell suspensions, shoot, or root nuclear extracts bind to the DNA irrespective of the degree of VBE methylation. Chymotrypsin sensitivity proved that the novel complex was caused by a protein (MVBP), which we consequently named methylated VBE-binding protein (MVBP).

Simultaneous binding of MVBP and VBP4s is unlikely because no supershifts were observed. Rather, the different proteins may compete for the DNA fragments. This may be the reason why the VBP1 complexes appear less pronounced when DNA fragments are used that can also bind MVBP. VBP1 complexes are better detectable in cell-suspension extracts, which seem to contain a lower amount of active MVBP than the shoot extracts (Fig. 7).

MVBP Is a Sequence-specific DNA-binding Protein—The sequence specificity of MVBP binding was determined by competition with methylated DNA fragments (Fig. 6). All tested mutations between −164 and −116 abolished competitive binding of MVBP binding, whereas mutations outside of this region did not affect binding (Fig. 9). Even small changes of the sequence context of two methylated CpG motifs or a change of one CpNpG to a CpGpN were detrimental for binding. MVBP binding was also abolished by mutations of the region important for formation of a DNA-protein complex with the unmethylated V fragment (described above). These data indicate that the binding site(s) for MVBP is contained in a DNA region covering 49 base pairs, which overlaps the binding sites for the other VBE-associated proteins.

MVBP Binding Requires Complete Cytosine Methylation—It has been shown that hemi-methylated DNA is a poor substrate for the binding of the mammalian methyl-CpG-binding protein 1 and 2 (MeCP1 and MeCP2; Refs. 24, 25). On the other hand, an Ac transposon-derived protein preferentially binds to hemimethylated DNA (26). To test whether MVBP can bind to hemi-methylated DNA, EMSA was performed using fully methylated DNA as substrate and hemi-methylated DNA as competitor (methylation on either the top or bottom strand as shown in Fig. 6, Hemi-U and Hemi-L). Methylation on only one of the strands resulted in only weak competition for MVBP (Fig. 10, lanes 7-10) when compared with symmetrically methylated DNA (lanes 3-4). This shows that hemi-methylated DNA is a less efficient substrate for MVBP.

The number of methylated CpGs required for binding of specific proteins can vary considerably from case to case. For example, mammalian MeCP1 requires at least 12 symmetrically methylated CpGs for binding (25), whereas MeCP2 can bind to DNA with a single methyl-CpG pair (24). Experiments were designed to assess the individual role of the two methylated CpG sites and the one CpNpG site in MVBP binding. For this purpose site-specific methylated oligonucleotides were synthesized (Fig. 6, Vm 11–15) and used as competitors in EMSA. The results show that cytosine methylation at only one of these sites (Vm11, Vm12, or Vm13) was incapable of competing the formation of MVBP (Fig. 9), suggesting that MVBP cannot bind to DNA containing a single methyl-CpG or CpNpG.

FIG. 4. Identification of DNA-binding proteins by DNA UV cross-linking assays. DNA UV cross-linking assays were performed using radiolabeled DNA substituted with BrdUrd in the absence (lanes 1 and 8) or in the presence of shoot (lanes 2–7) and cell suspension culture (lanes 9–14) nuclear extracts. Competitors indicated were used at a 100-fold molar excess. UV cross-linked proteins were separated in a 14% SDS-polyacrylamide gel. Molecular mass standards are indicated on the right. The UV cross-linked proteins are indicated by arrows on the left. Labeled DNA probe with incomplete digestion is marked with asterisks.
Further mutants were used to determine whether the binding of MVBP requires both CpG pairs (Vm14) and whether the CpApG pair could be replaced by a CpG pair (Vm15). None of these mutants competed with the wild-type element for MVBP binding.

As a control experiment, we used Vm8, Vm11, Vm12, Vm13, Vm14, and Vm15 as labeled probes in the EMSA. When tested with the nuclear extracts from shoots and roots, no binding of MVBP to any of the probes was observed (data not shown). These findings confirm the results obtained with the competition assays and taken together show the astonishing specificity of MVBP for sequence and methylation status of the target sequence.

DISCUSSION

Promoter specificity is determined by the complement of factors interacting on a given sequence, often in competition with other factors with similar or overlapping binding sites.

Methylated DNA-binding Protein in Plants
Protein binding can be modulated by epigenetic DNA modifications like chromatin condensation or cytosine methylation. In vertebrates, cytosine methylation can result in the attraction to the DNA of nonspecific, methyl cytosine-binding proteins like MeCP1 or MeCP2, which may suppress promoter activity directly (27–28) or tether corepressor complexes to the DNA that turn off promoters in a stable manner (29–34). In mammalian systems, there is evidence indicating that DNA methylation directly prevents the binding of some trans-acting factors to DNA (34–41). However, other transcription factors, such as Sp1, were found to be insensitive to DNA methylation (42) or affected at only some of their binding sites (Sp1, Ref. 43; E2F, Ref. 35). Therefore, DNA methylation could be involved not only in the general suppression of promoter activity but also in tissue- or development-specific regulation (35). The existence of a transcription factor family with sequence and methylation specificity, which is involved in the regulation of a number of mammalian genes, supports this possibility (44–46). In plants, DNA methylation as yet has been connected mainly to the stable transcriptional silencing of transgenes (47–48), but it is certainly also involved in developmental control of gene activity (49). Only a few methyl cytosine-binding proteins from plants are known (50), and only a few protein-DNA interactions have been investigated with respect to the influence of DNA methylation. Some CREB-like factors, present in nuclear extracts of several plant species, have been shown to be sensitive...
to cytosine methylation within their binding sites (51). In contrast, binding of the maize O₂ protein to its target sequence, TGACGTTG, was not impaired by CpG methylation in vitro (52), and the tobacco factor CG-1 could at least bind to hemi-methylated DNA (53).

The promoter of RTBV contains within about 280 base pairs around the transcription initiation site the information for activity in all cells of the vascular bundle and the epidermis, and several protein binding sites in this region have been detected by EMSA. An element important for activity in the vascular bundle has been localized in the region between −100 and −165. This region probably interacts with others, and there may be some redundancy with elements closer to the TATA box (11). However, deletion of this region abolished expression in the vascular bundle (9), and the same expression pattern was obtained consistent with specific promoter methylation.² Protein binding sites in the region from −125 to −165 have been detected (9, 11) and particularly a GATA motif has been implicated in protein binding (11). We show here that several proteins bind to an AGAAGGACCAGA motif upstream of the GATA sequence designated here as VBE, but that GATA is not required for this binding. The binding activity was abundant in extracts of rice shoots but not in root extracts, correlating with the promoter activity in the respective tissue (9–10). In cell suspensions, a similar binding activity was found. However additional shift-bands were observed, indicating association with other proteins most likely by protein-protein interaction. In cell suspensions, the VBE has no positive effect on expression but rather reduces promoter activity, suggesting that the detected different protein interaction is repressive. Negative elements have been found in several promoters with differential regulation of expression in the different cell types of the vascular bundle (5–6). VBE duplication further represses promoter activity in protoplasts. How the VBE represses transcription from the RTBV promoter remains unknown. Possibly, VBE interacts with other regulatory elements located between −70 and −35 (12) or the basal transcriptional machinery via protein-protein interactions that will exert a negative effect in the transient expression system.

The VBE lacks C- or CA-rich elements that have been associated with the binding of regulatory factors to other vascular bundle-specific promoters (16). However, the VBE shows some homology to the VSF-1 binding site in the xylem-specific promoter of the bean grp1.8 gene (Fig. 11 and Ref. 4). This factor has a strong sequence similarity to the rice bZIP protein RF2a, which is expressed in the same cells where the RTBV promoter is active (16). RF2a has been characterized by its binding to another RTBV promoter region termed box II, which overlaps (or is identical to) the sequence identified by us as an activator element (12). The binding of RF2a to box II was not very efficient, and it therefore is of interest to determine whether RF2a alone or as part of a heterodimer can also bind to VBE.

DNA methylation did not have a negative effect on the binding of proteins to VBE or to other promoter regions (data not shown). The only alteration in the interaction pattern was the appearance of a novel binding activity, MVBP. This activity was present in all extracts tested and unlike most previously characterized, methylated DNA-binding proteins displayed a high specificity for the DNA sequence and the degree of methylation. Sequence alterations over a region covering 49 nucleotides abolished binding, and when the two CpG and one CpNpG sites were only partially methylated, binding was strongly reduced. To our knowledge, no protein with a comparable binding requirement has as yet been characterized for any organism. The closest would be the MDPB/RFX protein family whose members recognize a variety of 14-base pair motifs, some of which have to be methylated at specific sites for recognition by the factor (44–46, 54). On the other hand, MeCP1 requires at least 12 methylated CpG pairs, i.e. it most likely interacts with an extended DNA region, although in a sequence-nonspecific manner (25). It is tempting to associate MVBP binding with the tissue-specific promoter inactivation observed in plant lines with a methylated promoter sequence. This could be caused by competition between MVBP and one or the other VBP for DNA binding. Replacement of transcription factors by methyl cytosine-binding proteins has been observed for some methylated animal promoters (36, 55). Evidence for competition could be deduced from our experiments with extracts from suspension cells with apparently lower MVBP activity in which formation of the VBP1 complex with methylated oligonucleotides appeared to be efficient, whereas in extracts from rice shoots with higher MVBP activity, it was reduced. Alternatively, MVBP may be directly involved in promoter repression. The presence of such a protein in rice raises the question of its normal function. Isolation and further characterization of the protein will be required to address this question.

Acknowledgments—We thank Matthias Muller for expert technical assistance, Ingo Potrykus for excellent support, Andreas Klötter for interesting discussions, and Jean-Pierre Joost and Helen Rothnie for thorough critical reading of the manuscript.

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FIG. 11. Sequence comparison of RTBV VBE and a bean GRP1.8 enhancer region. Methylation signals are in bold and homologies are underlined. Footprints identified with GRP1.8 sequences (46) are indicated.
