Transcriptional Activation of the Rice Tungro Bacilliform Virus Gene Is Critically Dependent on an Activator Element Located Immediately Upstream of the TATA Box*

Xiaoyuan He‡, Thomas Hohn‡§, and Johannes Fütterer¶

From the ‡Friedrich Miescher Institute, P. O. Box 2543, CH-4002 Basel and the ¶Institute of Plant Sciences, Federal Institute of Technology, Zürich, Zürich CH-8092, Switzerland

To investigate the transcriptional mechanisms of rice tungro bacilliform virus, we have systematically analyzed an activator element located immediately upstream of the TATA box in the rice tungro bacilliform virus promoter and its cognate trans-acting factors. Using electrophoretic mobility shift assays, we showed that rice nuclear proteins bind to the activator element, forming multiple specific DNA-protein complexes via protein-protein interactions. Copper-phenanthroline footprinting and DNA methylation interference analysis indicated that multiple DNA-protein complexes share a common binding site located between positions −60 to −39, and the proteins contact the activator element in the major groove. DNA UV cross-linking assays further showed that two nuclear proteins (36 and 33 kDa), found in rice cell suspension and shoot nuclear extracts, and one (27 kDa), present in root nuclear extracts, bind to this activator element. In protoplasts derived from a rice (Oryza sativa) suspension culture, the activator element is a prerequisite for promoter activity and its function is critically dependent on its position relative to the TATA box. Thus, transcriptional activation may function via interactions with the basal transcriptional machinery, and we propose that this activation is mediated by protein-protein interactions in a position-dependent mechanism.

In eukaryotes, the transcription of protein-coding genes by RNA polymerase II is regulated via two distinct types of DNA sequences: core promoter elements, located near the transcription start site that are sufficient to direct the accurate initiation of transcription; and upstream promoter elements, which contain binding sites for sequence-specific transcriptional activator and/or repressor proteins (1, 2). Transcriptional activators stimulate transcription by recruiting the RNA polymerase II machinery to a core promoter and/or stabilizing the transcription-initiation complex (3–6). Activators have been proposed to directly or indirectly (through coactivators) interact with components of the basal transcription machinery in mammalian systems (3, 7, 8).

Although the basal transcriptional machinery, also referred to as the polymerase II transcription initiation complex, has not been studied in plants as extensively as in mammalian, Drosophila, and yeast systems, TATA-binding proteins (TBP)s, 1 TFIIID, TFIIA, and RNA polymerase II subunits have been isolated from plants (9–12). Moreover, a number of plant trans-acting factors have been identified (13). Little is known in plants about the molecular mechanisms of transcriptional activation. It has been shown that TGA1a, a transcription activator interacting with the activation sequence-1 element in the CaMV 35 S promoter (14), increases the rate of preinitiation complex formation (15, 16). The plant transcription factor GT-1 belongs to the class of trihelix DNA-binding proteins, and it binds to a promoter cis-element with the core DNA sequence 5′-GGTTAA found initially in light-regulated genes (17). Recently, it has been shown that Arabidopsis GT-1 can interact with and stabilize the TFIIA-TBP-TATA complex, suggesting that GT-1 may activate transcription through direct interaction with the transcription pre-initiation complex (18).

Rice tungro bacilliform virus (RTBV) is a plant pararetrovirus (19–22) and belongs to the “RTBV-like” genus of the caulimoviridae family (23). RTBV has a circular, double-stranded DNA genome of 8 kilobase pairs from which the terminally redundant pregenomic transcript is produced (19, 20, 24). A single promoter from the virus genome was isolated, and phloem-specific activity was observed in transgenic rice plants (25), suggesting that the promoter can account for the tissue-specific localization of this virus. The RTBV promoter has been analyzed in both transformed rice plants (26–28) and transfected rice protoplasts (29, 30). Multiple upstream elements have been identified as being required for phloem-specific gene expression in the context of the −164 to +45 promoter in transformed rice plants (26, 27). Moreover, the full-length RTBV promoter including the first 250 nucleotides downstream of the transcription start site is active in a wider range of rice cell types than one where these sequences are lacking (28). A promoter element required for gene expression in the vascular tissue was located to sequences between −165 and −100 to which proteins from rice nuclear extracts bind (28). In transfected protoplasts, the activity of the RTBV promoter also required downstream promoter elements located in the region from +1 to +90 (29, 30).

In the current study, we have investigated the cis-acting element located immediately upstream of the TATA box in considerably more detail. We have compared the binding activity of different types of nuclear proteins and characterized the nuclear factors that bind to this element by DNA UV cross-linking assays. Furthermore, we have analyzed its function by

1 The abbreviations used are: TBP, TATA-binding protein; CaMV, cauliflower mosaic virus; RTBV, rice tungro bacilliform virus; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; ORF, open reading frame; EMSA, electrophoretic mobility shift assay; DOC, sodium deoxycholate; AE, activator element; TF, transcription factor; GUS, β-glucuronidase; PIPES, 1,4-piperazinediethanesulfonic acid.
deletion and mutation analysis in transfected protoplasts. The results reveal this element to be a prerequisite for promoter activity. Its function is critically dependent on its position relative to the TATA box. We propose that activation of the RTBV promoter by this element is mediated by a position-dependent transcriptional activation mechanism.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The basic plasmid used in this study, R-218.1-CAT (here called R-218), has been described previously (29, 30). The 5' deletions and point mutations were made by PCR with appropriate synthetic oligonucleotides. A common 5’ end oligonucleotide primer, designated P5, was located at vector sequences upstream of the Hin end and probes. The resulting plasmids were designated as pRP100(1)pBluescript II KS(2)HindIII site (filled in with Klenow polymerase) of M13mp18.

**Restriction Digestions and DNA Sequencing.** Plasmids were isolated from all the PCR reactions. All deletions and mutations were verified by appropriate synthetic oligonucleotides. A common 5’ Xba I and XhoI site were inserted into XbaI site of R-218 to yield R-218d.

**Electrophoretic Mobility Shift Assay (EMSA)**—A DNA probe was 5’ end-labeled with [α-32P]dCTP using the Klenow DNA polymerase with ClaI-digested pRP100(+), followed by a second restriction digestion at the EcoRV site. The resulting probe was purified on a 5% native polyacrylamide gel. Binding reactions were carried out essentially as described (30), except that 15,000 rpm of labeled DNA (around 1 μg of DNA) and 10–20 μg of nuclear extract proteins were used for each reaction. 5 μg of poly(dI-dC) (Amersham Pharmacia Biotech) was used as an unspecific competitor.

**Copper-Phenanthroline Footprinting Analysis**—The same 5’ end-labeled DNA probe was used as for EMSA. A 3’ end-labeled DNA probe was prepared in the same way as the 5’ end-labeled probe except using plasmid pRP100(+). The footprinting analysis was performed as described (32) with some modifications. Binding reactions were scaled up 10-fold as compared with the EMSA and fractionated by polyacylamide gel electrophoresis as with EMSA. In situ digestion of the DNA by the nuclease activity of 1,10-phenanthroline-cuprous complex was allowed to proceed for 5–6 min at 25 °C. DNA from free and bound fractions were visualized by autoradiography of the wet gel overnight.
Transcriptional Activation from the RTBV Promoter

Prepared as described (33). DNA probe that has had its thymidine residues substituted by bromodeoxyuridine was uniformly labeled with \( ^{32}\text{P} \)dCTP by extension of the M13 universal primer as described (35), and then digested with XhoI and HindIII, followed by purification on a 5% native polyacrylamide gel. The binding reactions were carried out as described for EMSA and included \( 1 \times 10^6 \) cpm of the uniformly labeled DNA probe. Following the 20-min incubation period, the reaction mixtures were irradiated with UV light (312 nm) for 30 min in a UV Stratalinker 1800 (Stratagene) at 4 °C. Samples were then digested with 10 units each of DNases I and micrococcal nuclease in the presence of 10 mM CaCl₂ for 30 min at 37 °C. The resulting mixtures were resolved on a 14% SDS-polyacrylamide gel.

**Transient Expression Assays**—Protoplasts derived from a rice Oc suspension culture were prepared and transfected by polyethylene glycol-mediated transfection as described previously (36, 37). 10 μg of plasmid DNA was used to transfect 0.6 × 10⁶ protoplasts, together with 3 μg of a plasmid expressing β-glucuronidase (GUS) under the control of the CaMV 35 S promoter to serve as an internal standard for transfection efficiency. Measurement of CAT and GUS activities in protoplast extracts prepared after overnight incubation was carried out by using a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics Ltd) and as described elsewhere (38), respectively. CAT activity was normalized to the GUS activity of the same extract. Relative expression levels did not vary more than ±10% with the given constructs in this study. All constructs were tested in at least three independent experiments with at least three independent clones of each transformation.

**RESULTS**

Identification of an Activator Element Located Immediately Upstream of the TATA Box—Our previous studies have shown that a version of the RTBV promoter truncated to position -50 relative to the transcription start site retained 40% activity of the full-length promoter in transient expression systems (29).

To further identify potential upstream cis-acting elements, a series of promoter 5′ deletion constructs was used to analyze RTBV promoter regulation in transfected rice protoplasts. RTBV promoter sequences to position -218 were used in these constructs, because the region between positions -618 to -218 does not influence transcription from the RTBV promoter in O. sativa protoplasts (29). All constructs included a full-length RTBV leader sequence and a chloramphenicol acetyltransferase (CAT) ORF fused to RTBV ORF I. As shown in Fig. 1, expression of the CAT reporter gene was not affected by deletion of upstream sequences to position -70 relative to the transcriptional start site. However, deletion to nucleotide -40, leaving only the TATA box and transcriptional start site, resulted in a significant loss of CAT activity to a level reflecting only basal transcription. Hence, these results clearly indicated that a cis-regulatory element conferring maximal promoter activity in transfected rice protoplasts is located between the sequences from -70 to -40 of the promoter. We refer to this region as activator element (AE).

**Multiple Nuclear Proteins Specifically Bind to Sequences from -60 to -39**—It has been shown that nuclear proteins from rice seedlings bind to DNA sequences from -53 to -39, called box II, of the RTBV promoter (26). To test whether nuclear proteins from rice cell suspension cultures can bind to this activator element, EMSA were performed with nuclear extracts from rice cell suspensions in comparison with rice shoot and root nuclear extracts. One or more DNA-protein complexes were detected upon incubation of each of these extracts with radiolabeled promoter sequences from -100 to -1 (Fig. 2). Four specific DNA-protein complexes, designated C1, C2, C3/C3', and C4, were repeatedly observed with nuclear extracts from rice shoots (S) and cell suspensions (C) (Fig. 2, lanes 2 and 3); C3 and C3' were observed in shoot nuclear extracts and cell suspension nuclear extracts, respectively. C1 and C2 are predominant complexes with high intensity, and C3' migrates slightly faster than C3. However, in root extracts (R), only one DNA-protein complex, named C, was observed with a slightly higher mobility than those in shoot or cell suspension nuclear extracts (Fig. 2, lane 4). Two additional DNA-protein complexes (C5 and C5') that migrate faster than any others were detected with nuclear extract prepared from cell suspension cultures (Fig. 2, lane 3).

To determine the specificity of protein binding to the DNA probe, competition experiments were performed with specific unlabeled DNA fragments. All complexes were completely competed with a 200-fold molar excess of unlabeled -100 to -1 probe DNA fragment (fl) (Fig. 3, B-D, lanes 2). A series of shorter double-stranded oligonucleotides covering subregions of this fragment (Fig. 3A) were then used as competitors. The presence of competitor m2 abolished the formation of all complexes (Fig. 3, B-D, lanes 4), while m1 and m3 showed no competition for complexes C1–C4 and C (Fig. 3, B–D, lanes 3 and 5). Complexes C5 and C5' can be competed completely by m2 and m3, but only partially by m1 (Fig. 3B, lanes 3, 4, and 5). These results indicate that nuclear factors bind to the m2 region from -70 to -35 to form multiple DNA-protein complexes, and that both m2 and m3 contribute to the formation of complexes C5 and C5'. To further determine the minimal sequence requirement for the formation of the major DNA-pro-
protein complexes, three overlapping double-stranded oligonucleotides covering different regions from −70 to −35 were used as competitors (Fig. 3A). It is evident that m5, which corresponds to the region from −60 to −39, is equally effective in competing complexes C1–C4 and C as m2 (Fig. 3, B–D, lanes 7). However, m4 and m6 were not able to compete those complexes (Fig. 3, B–D, lanes 6 and 8). M6, corresponding to the region from −70 to −54, competed for C5 and C5' efficiently (Fig. 3B, lane 8), while m5 showed a weak competition (Fig. 3B, lane 7) and m4 none (Fig. 3B, lane 6).

Taken together, these results indicate that the minimal sequence required for formation of DNA-protein complexes C1–C4 and C resides within the region from −60 to −39, while the binding sites for C5 and C5' complexes are located within the regions from −70 to −54 and −35 to −1. We did not detect the proteins that bind to the ASL box from −98 to −79 reported by Yin et al. (27). The differences in our results may be due to differences in the methods used to prepare nuclear extracts.

**Copper-Phenanthroline Footprinting Assays Show That Complexes C1 to C4 Share a Common Binding Site from −60 to −39**—Complexes C1 to C4 might bind to individual sites or share the same site within the region from −60 to −39. To address this question, we performed copper-phenanthroline footprinting assays. EMSAs were performed with nuclear extracts prepared from cell suspension cultures using the 100-bp probe utilized in Figs. 2 and 3 labeled on either the top or bottom strand. Partially methylated DNA bound to nuclear proteins was cleaved at G residues methylated at the N-7 position, which interferes with major groove DNA binding (39). Methylation of guanine nucleotides in the top strand and in regions from −60 to −54 and −50 to −43 in the bottom strand (Fig. 4C). The footprinting data are in precise agreement with the results of the binding assays performed with competitors and indicate that all complexes except C5 and C5' share a common binding site including nucleotides from −60 to −39. In all complexes, the A at position −96 showed an enhanced reactivity on the top strand in the presence of protein, while unbound DNA (F) is almost not cleaved at this residue under the conditions employed (Fig. 4A).

**Methylation Interference Analysis Shows That Proteins Contact the Activator Element in the Major Groove**—To further identify the precise contacts made by nuclear proteins in binding to this region of DNA, methylation interference analysis was performed using the −100 to −1 probe labeled on either the top or bottom strand. Partially methylated DNA bound to nuclear proteins was cleaved at G residues methylated at the N-7 position, which interferes with major groove DNA binding (39). Methylation of guanine nucleotides in the top strand at positions −55 and −46 and in the bottom strand at positions −53, −45 to −42 strongly affected the specific DNA-protein interactions (Fig. 4B). Methylation of the guanine nucleotide in the bottom strand at position −52 resulted in a weak interference with the protein binding. Again, complexes C1, C2, C3', and C4 all involved the same G residue contacts in both top and bottom strands (Fig. 4C). The G at position −55 in the top strand was not involved in protein binding analyzed previously by DNase I footprinting (28). To verify the involvement of the G at position −55 in protein binding, a G to T mutation at this position (within the context of m2, −70 to −35, see Fig. 3A) was tested as a competitor in EMSA. The mutated sequence was not able to compete any of the complexes (data not shown), suggesting that the G at position −55 is crucial for formation of the DNA-protein complexes. Taken together, the results indicated that nuclear proteins directly contacted the activator element in the major groove.

**The Formation of Multiple DNA-Protein Complexes on the Activator Element Is Based on Protein-Protein Interactions**—Because multiple DNA-protein complexes are observed in EMSA and only a single protected area is detected in footprinting analysis from complexes C1, C2, C3', and C4, we hypothesized that these multiple complexes result from either protein-protein association of a common DNA-banding protein with non-DNA-banding proteins or the interaction of multiple DNA-binding proteins of different sizes to a common binding site. To test this hypothesis, we utilized the detergent DOC in EMSA, which is known to disrupt protein-protein interactions and has been widely used to characterize multi-protein complexes (40–43). When DOC was added in low concentration, the four DNA-protein complexes were converted into only two complexes that migrated faster than the initial complexes with nuclear extracts from cell suspension cultures and shoots (Fig. 5). Adding an excess of the nonionic detergent Nonidet P-40 reversed this effect (Fig. 5, lanes 5 and 10). This observation implies that protein-protein interactions were required for the formation of multiple DNA-protein complexes, and that there are two specific DNA-binding proteins, designated AEBP-1 and AEBP-2 (activator element-binding protein 1 and 2), respectively, directly recognizing the AE. The DOC treatment abolished the formation of complexes C5 and C5' even at very low concentration (Fig. 5A, lane 2), and complex formation could not be restored by Nonidet P-40 (Fig. 5A, lane 5), suggesting that proteins interact with each other and are unable to bind DNA alone.

**Identification of Proteins Bound to the AE by DNA UV Cross-
Our results demonstrate that formation of multiple DNA-protein complexes on the AE is based on protein-protein interactions, and only two proteins directly contact this element. To specifically identify these proteins, we performed DNA UV cross-linking assays with a bromo-dUTP-labeled probe. The bromo-dUTP-labeled probe formed the same specific DNA-protein complexes (data not shown) as the regular probe (Fig. 2). After UV cross-linking, DNA-protein complexes were analyzed by SDS-polyacrylamide gel electrophoresis. In addition to a nonspecific protein of 30 kDa (see below), which appeared in all extracts tested, two proteins with apparent molecular masses of 33 and 36 kDa were identified in nuclear extracts from cell suspension cultures and shoots, while only one protein of 26 kDa was detected in root nuclear extracts (Fig. 6). The 36-kDa protein predominates in shoot nuclear extracts, while the 33-kDa protein is more abundant than the 36-kDa protein in cell suspension culture nuclear extracts.

Competition experiments were performed in the presence of a 200-fold molar excess of three different unlabeled DNA competitors (m1, m2, and m3; see Fig. 3A). The 30-kDa band was not competed by any of these competitors; therefore, it was judged to be nonspecific. All the other cross-linked proteins were competed by competitor m2 (corresponding to region -70 to -35), but not by m1 and m3, demonstrating specific interactions between the AE and these cross-linked proteins (Fig. 6). These results are consistent with those determined by EMSA. Treatment of the UV-irradiated reaction mixture with proteinase K prior to gel electrophoresis abolished complex formation, indicating that nuclear proteins were indeed present in the bands detected (data not shown). We could not detect proteins that form complexes C5 and C5' with the nuclear extract prepared from cell suspension culture. Possibly, these might be present in the extract at levels below the limit of detection using this method.

The AE Is a Prerequisite for Promoter Activity—To address the functional consequence of the DNA-protein interactions with the AE, deletions or mutations were made in the context of the whole leader and promoter sequences to -218. The activity of these constructs was assessed in transfected rice
protoplasts. Internal deletion from −70 to −35 resulted in a dramatic reduction in promoter activity to less than 10% of wild type (Fig. 7). Mutation of Gs identified as being directly contacted by nuclear factors to Ts had a similar effect. A double-stranded oligonucleotide from −70 to −35 bearing these mutations was also not able to compete any of the DNA-protein complexes when used as a competitor in EMSA (data not shown). These results highlight the crucial importance of the AE for efficient transcription from the promoter.

The Position of the AE Relative to the TATA Box Is Critical for Promoter Activity—To determine whether the AE could confer promoter activity from an upstream position, sequences from −70 to −35 were deleted in the wild-type position and one copy of this DNA fragment was placed at position −218 upstream of the transcription start site. As shown in Fig. 7, one copy of the AE positioned at nucleotide −218 in either forward or reverse orientation is not able to compensate at all for the deletion of sequences from −70 to −35. This finding indicated that the AE could not enhance promoter activity from a distance. Thus, activation by the AE is position-dependent.

Functional Association of the AE Is DNA Distance-dependent but Not Turn-dependent—Inspection of the sequences in the promoter-proximal region revealed that the binding sites for nuclear factors within the AE are very close to the TATA box, with only a very few base pairs separating the two elements. This close proximity, and the position-dependent characteristics of the AE raised the question whether AE-binding activator proteins would interact directly or indirectly with basal transcription factors to activate transcription from the promoter. If so, it would be predicted that altering the spacing between the AE and the TATA box could affect protein-protein interactions between these two sets of factors, and thereby influence transcription from the promoter. To test this prediction, a series of constructs was made in which the distance between the AE and the TATA box was varied from 5 to 20 bases (Fig. 8). The effect on promoter activity was tested in transfected rice protoplasts. As the distance between the AE and the TATA box increased, the promoter activity gradually decreased (Fig. 8). When five nucleotides, corresponding to half a DNA helical turn, were inserted between the AE and the TATA box, CAT activity was reduced to 73% of the wild-type construct, indicating a lack of strict dependence on stereospecific alignment between the AE and the TATA box for activation. A further reduction was observed with a 10-nucleotide insertion (a full DNA helical turn). Insertion of 15 or 20 nucleotides resulted in a significant loss of promoter activity (to 32% and 26% of wild-type, respectively). Thus, activation via the AE is DNA distance-dependent but not turn-dependent.

Loss of Promoter Activity Is Not Due to the Loss of Protein Binding to the AE—To determine whether these insertions affect the formation of DNA-protein complexes on the AE, EMSA was performed using DNA probe bearing the same insertions between −35 and −34 as in the above constructs. The DNA-protein complexes with these probes in nuclear ex-
tracts from both cell suspension cultures and shoots were identical to those obtained with wild-type probe, and the resulting DNA-protein complexes can be competed completely by wild-type sequences from 270 to 235 (data not shown). These results indicated that these insertions did not affect DNA-protein interactions on the AE, and therefore loss of promoter activity is not due to loss of protein binding to the AE.

**DISCUSSION**

To elucidate the molecular mechanisms involved in regulating transcription from the RTBV promoter, we have undertaken a systematic analysis of the AE and its cognate trans-acting factors. The current study provides a new insight into the transcriptional regulation mechanisms of RTBV. We have demonstrated that (i) nuclear proteins bind to the AE, forming multiple DNA-protein complexes through protein-protein interactions, (ii) AE is a position-dependent element whose function is critically dependent on its position relative to the TATA box, (iii) AE is a prerequisite for efficient promoter activity, and (iv) activators probably stimulate transcription via interactions with basal transcription factors.

**FIG. 5.** The multiple DNA-protein complexes are due to protein-protein interactions. EMSAs were performed with nuclear extracts as indicated. DNA-protein complexes were treated with different concentrations of the detergent DOC (%) as indicated. In addition, at 0.2% DOC, Nonidet P-40 was added to a final concentration of 1% as a control. DNA-binding proteins are indicated (AEBP-1 and -2).

**FIG. 6.** Identification of the AE-binding proteins by UV cross-linking. Radiolabeled DNA probe substituted with bromodeoxyuridine was incubated without (lane 1) or with nuclear extracts (shoot (S), lanes 2–5; cell suspension (C), lanes 6–9; root (R), lanes 10–13) in the absence (−) or presence of a 200-fold molar excess of the competitor indicated. The reaction mixtures were irradiated with UV light, treated with DNase I and micrococcal nuclease, and analyzed on a 14% SDS-polyacrylamide gel. Molecular size standards are indicated on the right. The UV cross-linked proteins are indicated by arrows on the left. A nonspecific cross-linked protein appearing in all three extracts is marked with an asterisk.

Formation of multiple DNA-protein complexes on the AE is based on protein-protein interactions, and these interactions correlate with the functional activity of the promoter. EMSA analyses with different nuclear extracts have led to the identification of different DNA-binding proteins, which may be involved in regulating cell type-specific transcription from the promoter. Our EMSA results showed that the protein binding patterns are not exactly the same in cell suspension, shoot, and root nuclear extracts, indicating that different proteins from different types of nuclear extracts directly or indirectly bind to the same element. DNA UV cross-linking studies revealed two proteins of 36 and 33 kDa binding to this element, suggesting that formation of protein-protein interactions in the RTBV promoter requires the binding of both the 36- and 33-kDa proteins to this element. Based on the protein profile and the mobility of each complex in native gels, it is most likely that AEBP-1 is the 33-kDa protein, while AEBP-2 is the 36-kDa protein. Complex C1 contains two DNA-binding proteins of 33 and 36 kDa, and complexes C2, C3, C3', and C4 may represent heterodimers of the 33- or 36-kDa proteins with unknown
proteins, which do not directly bind to DNA. However, a 26-kDa DNA-binding protein was found in root nuclear extracts with which no protein-protein interaction was observed. Taken together, we may conclude that the direct or indirect binding of different nuclear factors to the same element may contribute to alternative transcriptional regulation mechanisms from the promoter in different cell types, and that protein-protein interactions play a critical role in transcriptional activation from the promoter.

The importance of the AE for transcription from the RTBV promoter in vivo is apparent from the effects of internal deletions and mutations in this element on the activity of the promoter tested in a transient expression system (Fig. 7). Moreover, when the DNA fragment from −70 to −35 was placed upstream of the RTBV promoter in a transient expression system (Fig. 7), the promoter activity was reduced to about 50% of the wild-type promoter activity. These results indicate that the AE is a position-dependent element, and that there is a complete correlation between nuclear protein binding in vitro and transcription activation in vivo. Interestingly, deletion or mutation of this element has a more drastic effect than deletion of upstream sequences to −40 (cf. Figs. 1 and 7). This is most likely due to an inhibitor element located upstream at sequences from −165 to −100 identified in transfected rice protoplasts. Studies on the RTBV promoter in transgenic rice plants have shown that three upstream elements, box II (−53 to −39), the ASL box (−98 to −79), and the GATA motif (−143 to −135), act combinatorially to confer phloem-specific gene expression in the context of the −164 to +45 promoter (27), and that a promoter element located to sequences from −164 to −100 is very important for gene expression in the vascular tissue (28). The results presented here show that, for full promoter activity, upstream sequences to −70 containing the activator element in the context of complete leader are required in transfected rice protoplasts. The differences in our results may reflect differences between stable and transient expression systems (28, 30), as demonstrated by EMSA analysis in this study.

In the α-amylase gene promoter regulated by gibberellin (GA) during germination, a cis-acting element called box T is located three bases upstream of the TATA box. Box T is a

---

2 X. He, J. Fütterer, and T. Hohn, unpublished results.
polypyrimidine sequence element (−50 GATCACATCCCCCT−36). It was suggested that box T may be bound by basal transcription factors (44). The FP56 element in the parsley 4CL promoter is also a pyrimidine-rich element (5′-TC-CCCATTTACCCCT-3′) on which multiple DNA-protein complexes form (45). This element consists of a perfect indirect repeat of the octanucleotide TCCCCATT, but its functional significance remains to be clarified. The AE is also pyrimidine-rich and is apparently not bound directly by basal transcription factors. Inspection of the AE nucleotide sequence revealed the presence of two motifs: a TGACG-like motif (−50 TGACC−51) and an octamer-like motif (−49 GTGCCCCC−41). This octamer sequence is found in the C4-type phosphoenolpyruvate carboxylase gene in maize and binds a light-inducible DNA-binding factor (MUF1) (46). The TGACG motif and variations have been identified in the promoters of a variety of plant genes, such as the auxin-regulated tobacco glutathione transferase gene in maize and binds a light-inducible DNA-binding sequence is found in the C4-type phosphoenolpyruvate carboxylase genes located between nucleotides (54). This factor was found to interact with RTBV promoter RF2a that is critical for vascular development was isolated (50). The factor can interact with and stabilize the TFIIA-TBP-TATA complex to the core promoter. On the promoter, the activator RTBV interacts with P36 and P33 through protein-protein interactions. For transcriptional activation, activators recruit holoenzyme containing TFIIID to the core promoter. On the promoter, the activators and TFIIID are brought into proximity, enabling proteins that directly or indirectly bind to the activator element to interact with TFIIID. The interactions between activators and TFIIID can facilitate and stabilize the transcriptional pre-initiation complex, and thereby stimulate transcription. As the distance between the bound activators and bound TFIIID is increased, this interactive effect is diminished, leading to a decrease in transcription. In the roots, no protein-protein interactions were observed. The low transcription level detected in roots of transgenic rice plants (26–28) may be due to the fact that the activator is not able to interact with basal transcription factors in this tissue. Isolation of cDNAs expressing these DNA-binding proteins, either by screening an expression library using the minimal binding sequence or by protein purification, will help us to verify the above model and further elucidate transcriptional regulation mechanisms from RTBV promoter.

Acknowledgments—We especially thank Drs. Helen Rothnie and Patrick Matthias for critical reading of the manuscript. We highly acknowledge the expert technical assistance of Matthias Müller, Sandra Corsten, and David Kirk. We also thank Dr. Helen Rothnie for helpful discussions.

REFERENCES
1. Tjian, R., and Maniatis, T. (1994) Cell 77, 5–8
2. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) Genes Dev. 10, 2657–2693
3. Carey, M. (1998) Cell 92, 5–8
4. Singh, K. B. (1998) Plant Physiol. 118, 1111–1120
5. Hahn, S. (1998) Cell 95, 579–583
6. Ptashne, M., and Gann, A. (1997) Nature 386, 569–577
7. Querste, L. (1995) Trends Biochem. Sci. 20, 517–521
8. Halazoneto, E., and Reinberg, D. (1995) Curr. Opin. Cell Biol. 7, 352–361
9. Katagiri, F., Tamaki, T., Sumita, K., and Ishikawa, M. (1998) Plant Mol. Biol. 19, 867–872
10. Li, Y. F., Le Gourierrec, J., Torki, M., Kim, Y. J., Guerineau, F., and Zhou, D. X. (1999) Plant Mol. Biol. 39, 515–525
11. Guilfoyle, T. J. (1995) in Genetic Engineering (Setlow, J. K., ed) pp. 15–47, Plenum Press, New York
12. Larkin, R. M., and Guilfoyle, T. J. (1998) J. Biol. Chem. 273, 5631–5637
13. Meisel, L., and Lam, E. (1997) in Genetic Engineering (Setlow, J. K., ed) pp. 183–199, Plenum Press, New York
14. Katagiri, F., Lam, E., and Chua, N. H. (1989) Nature 340, 727–730
15. Yamazaki, K., Katagiri, F., Imaseki, H., and Chua, N. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7035–7039
16. Katagiri, F., Yamazaki, K., Horikoshi, M., Roeder, R. G., and Chua, N. H. (1990) Genes Dev. 4, 1899–1909
17. Kawata, T., Minami, M., Tamura, T., Sumita, K., and Iwabuchi, M. (1992) J. Gen. Virol. 73, 354–364
18. Bhattacharyya-Pakrasi, M., Peng, J., Elmer, J. S., Laco, G., Shen, P., Kaniewska, M. B., Elmer, J. S., Rochester, D. E., Smith, C. E., and Beachy, R. N. (1991) Virology 185, 354–364
19. Rothnie, H. M., Chapdelaine, Y., and Hohn, T. (1994) Adv. Virus Res. 44, 1–67
20. Hohn, T., and Tutterer, J. (1997) Crit. Rev. Plant Sci. 16, 153–161
21. Maye, M. A., and Pringle, C. R. (1996) J. Gen. Virol. 79, 649–657
22. Bao, Y., and Hull, R. (1994) Virology 204, 626–633
23. Bhattacharya-Pakrasi, M., Peng, J., Elmer, J. S., Laco, G., Shen, P., Kaniewska, M. B., Kononovich, H., Ven, F., Hodges, T. K., and Beachy, R. N. (1993) Plant J. 4, 71–79
24. Yin, Y., and Beachy, R. N. (1995) Plant J. 7, 969–980
25. Yin, Y., Chen, L., and Beachy, R. (1997) Plant J. 12, 1179–1188
26. Klotz, A., Heinrich, C., Riet, S., He, X. Chen, G., Burkhardt, P. K., Wuna, J.,
Transcriptional Activation from the RTBV Promoter

Lucca, P., Hohn, T., Potrykus, I., and Futterer, J. (1999) *Plant Mol. Biol.* 40, 249–266

29. Chen, G., Müller, M., Potrykus, I., Hohn, T., and Futterer, J. (1994) *Virology* 204, 91–100

30. Chen, G., Rothnie, H. M., He, X., Hohn, T., and Futterer, J. (1996) *J. Virol.* 70, 8411–8421

31. Martino-Catt, S. J., and Kay, S. A. (1994) in *Plant Molecular Biology Manual* (Gelvin, B., and Schilperoort, R. A., eds) pp. 1–13, Kluwer Academic Publishers, Dordrecht, The Netherlands

32. Garabedian, M. J., Labaer, J., Liu, W. H., and Thomas, J. (1993) in *Gene Transcription* (Hames, B. D., and Higgins, S. J., eds) pp. 243–293, Oxford University Press, Oxford

33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

34. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York

35. Jackson, S. P. (1993) in *Gene Transcription* (Hames, B. D., and Higgins, S. J., eds) pp. 189–242, Oxford University Press, Oxford

36. Datta, S. K., Peterhans, A., Datta, K., and Potrykus, I. (1990) *Bio/Technology* 8, 736–740

37. Shillito, R. D., Saul, M. W., Paszkowski, J., Müller, M., and Potrykus, I. (1985) *Bio/Technology* 3, 1099–1103

38. Futterer, J., and Hohn, T. (1991) *EMBO J.* 10, 3887–3896

39. Thanos, D., and Maniatis, T. (1992) *Cell* 71, 777–789

40. Baeuerle, P. A., and Baltimore, D. (1988) *Cell* 53, 211–217

41. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991) *Cell* 65, 1053–1061

42. Kremer, A., and Knebel-Morsdorf, D. (1998) *Virology* 249, 336–351

43. Després, C., Subramaniam, R., Matton, D. P., and Brisson, N. (1995) *Plant Cell* 7, 589–598

44. Willmott, R. L., Rushton, P. J., Hooley, R., and Lazarus, C. M. (1998) *Plant Mol. Biol.* 38, 817–825

45. Neustaeder, D. A., Lee, S. P., and Douglas, C. J. (1999) *Plant J.* 18, 77–88

46. Morishima, A. (1998) *Plant Mol. Biol.* 38, 633–646

47. Droog, F., Spek, A., van der Kooi, A., de Ruyter, A., Hoge, H., Libbenga, K., Hooykaas, P., and van der Zaal, B. (1995) *Plant Mol Biol* 29, 413–429

48. Lam, E., Benfey, P. N., Gilmarzini, P. M., Fang, R. X., and Chua, N. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 86, 7890–7894

49. Benfey, P. N., and Chua, N. H. (1990) *Science* 250, 959–966

50. Fromm, H., Katagiri, F., and Chua, N. H. (1989) *Plant Cell* 1, 977–984

51. Schindler, U., Beckmann, H., and Cashmore, A. R. (1992) *Plant Cell* 4, 1309–1319

52. Jin, G., Davey, M. C., Ertl, J. R., Chen, R., Yu, Z. T., Daniel, S. G., Becker, W. M., and Chen, C. M. (1998) *Plant Mol. Biol.* 38, 713–723

53. Strompen, G., Gruner, R., and Pfitzner, U. M. (1998) *Plant Mol. Biol.* 37, 871–883

54. Yin, Y., Zhu, Q., Dai, S., Lamb, C., and Beachy, R. N. (1997) *EMBO J.* 16, 5247–5259

55. Roberts, S. G., Choy, B., Walker, S. S., Lin, Y. S., and Green, M. R. (1995) *Curr. Biol.* 5, 508–516

56. Vilen, B. J., Ceggewell, J. P., and Ting, J. P. (1991) *Mol. Cell. Biol.* 11, 2406–2415

57. Li, H., and Capetanaki, Y. (1994) *EMBO J.* 13, 3580–3589

58. Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M., and Champon, P. (1986) *Nature* 319, 121–126

59. Inokuchi, K., and Nakayama, A. (1991) *Nucleic Acids Res.* 19, 3099–3103
Transcriptional Activation of the Rice Tungro Bacilliform Virus Gene Is Critically Dependent on an Activator Element Located Immediately Upstream of the TATA Box

Xiaoyuan He, Thomas Hohn and Johannes Fütterer

*J. Biol. Chem.* 2000, 275:11799-11808.
doi: 10.1074/jbc.275.16.11799

Access the most updated version of this article at [http://www.jbc.org/content/275/16/11799](http://www.jbc.org/content/275/16/11799)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/275/16/11799.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 52 references, 13 of which can be accessed free at [http://www.jbc.org/content/275/16/11799.full.html#ref-list-1](http://www.jbc.org/content/275/16/11799.full.html#ref-list-1)