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A Novel AT-Rich DNA Binding Protein That Combines an HMG I-like DNA Binding Domain with a Putative Transcription Domain

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There is growing evidence that AT-rich promoter elements play a role in transcription of plant genes. For the promoter of the nuclear gene for chloroplast glutamine synthetase from pea (*GS2*), the deletion of a 33-bp AT-rich sequence (box 1 native) from the 5′ end of a *GS2* promoter–β-glucuronidase (*GUS*) fusion resulted in a 10-fold reduction in *GUS* activity. The box 1 native element was used in gel shift analysis and two distinct complexes were detected. One complex is related to the low-mobility complex reported previously for AT-rich elements from several other plant promoters. A multimer of the box 1 sequence was used to isolate a cDNA encoding an AT-rich DNA binding protein (ATBP-1). ATBP-1 is not a high-mobility group protein, but it is a novel protein that combines a high-mobility group I/Y-like DNA binding domain with a glutamine-rich putative transcriptional domain.

INTRODUCTION

Promoters of yeast and plant genes often contain stretches of AT-rich DNA sequences. These AT-rich DNA sequences have been reported to stimulate transcription in yeast (Russell et al., 1983; Struhl, 1985; Chen et al., 1987); however, their function in plant genes is less well understood. There are several reports that AT-rich plant promoter elements possess transcriptional activity; however, these reports are contradictory. An AT-rich element from the promoter of the French bean β-phaseolin gene fused to the −90 cauliflower mosaic virus 35S promoter resulted in increased expression of the β-glucuronidase (*GUS*) reporter gene in transgenic tobacco (Bustos et al., 1989). Additional evidence supporting a role for AT-rich sequences in transcriptional activation in plants was provided by studies of the pea small subunit of ribulose bisphosphate carboxylase (*rbcS-3A*) gene promoter (Lam et al., 1990). Recently, an AT-rich region of the soybean heat shock promoter, *Gmhsp17.5E*, has been reported to stimulate transcription when placed 5′ to a truncated heterologous promoter (Czarnecka et al., 1992). In contrast to these reports of transcriptional activation associated with AT-rich elements, Castresana et al. (1988) identified an AT-rich element of the *Nicotiana plumbaginofolia* chlorophyll *a/b* binding protein (*Cab-E*) gene promoter as a negative regulatory element.

Several groups have reported analyses of the interactions between AT-rich DNA sequences from plant gene promoters and binding activities present in nuclear extracts (reviewed in Schindler and Cashmore, 1990; Czarnecka et al., 1992). In these reports, generally two different DNA binding activities are detected; there are one or more high-mobility complexes (HMC) and a single low-mobility complex (LMC). The HMC proteins share many characteristics with the high-mobility group (HMG) proteins. HMG proteins are small proteins (<30 kD) with a high percentage of acidic and basic amino acids, are soluble in trichloroacetic acid, and are found in association with transcriptionally active chromatin (Johns, 1982; Goodwin and Bustin, 1988). The LMCs do not have these characteristics and contain proteins that are larger than known HMG proteins (Schindler and Cashmore, 1990; Pederson et al., 1991; Czarnecka et al., 1992). Taken together with the in vivo studies of AT-rich elements, these reports suggest that there are transcription factors that bind AT-rich elements in plant gene promoters.

Our interest in regulation of the nuclear gene encoding chloroplast glutamine synthetase from pea (*GS2*) has led us to study nuclear proteins that bind elements in the *GS2* promoter. Sequence analysis of the *GS2* promoter revealed a repeated AT-rich element (box 1). The results of *GS2–GUS* promoter fusion analysis suggest a role for the box 1 sequence in *GS2* gene regulation. In addition, when a box 1–containing fragment of the *GS2* promoter (box 1 native) was used in gel shift analysis, the complexes detected were similar to the HMCs and LMCs reported previously for other AT-rich promoter elements.

A multimer of the box 1 element was used in a southwestern screen to isolate a tobacco cDNA clone encoding a DNA binding protein. The protein encoded by the longest open reading frame was designated as ATBP-1 for AT-rich DNA binding protein-1. The DNA binding activity of ATBP-1 was very

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similar to the DNA binding activities detected in pea and tobacco extracts as judged by competition assays. Sequence analysis of ATBP-1 revealed two interesting features: (1) the amino-terminal domain is glutamine rich and therefore a potential transcriptional domain, and (2) the carboxy-terminal domain is similar to the DNA binding domain of the HMG class I/Y proteins. Unlike the other HMG proteins that show a preference for single-stranded DNA, HMG I/Y proteins have a higher affinity for double-stranded AT-rich sequences and do not bind single-stranded DNA (Solomon et al., 1986). The ATBP-1 protein is considerably larger than any reported HMG protein. Based on DNA binding activity and estimated size, we propose that ATBP-1 is involved in the LMC that has been reported for many plant genes and plant species. Furthermore, ATBP-1 is not an HMG protein, but a novel plant protein that combines an HMG I/Y-like DNA binding domain with a glutamine-rich putative transcriptional domain.

RESULTS

In Vivo Characterization of a Box 1-Containing Fragment of the Pea *GS2* Promoter

To identify the promoter elements important for regulation of expression of the pea *GS2* gene, in vivo promoter deletion analysis was performed using *GS2–GUS* fusion constructs in transgenic tobacco. The box 1 sequence was thought to be important because it is repeated at other positions within the *GS2* promoter sequence (G. Tjaden, J.W. Edwards, and G.M. Coruzzi, unpublished results). Deletion of the 33-bp box 1–containing sequence from the 5′ end of a transcriptional fusion resulted in a 10-fold reduction in *GUS* activity in the primary transformants, as shown in Figure 1A. The level of *GUS* activity in leaves of adult tobacco plants is shown in Figure 1B. Because the standard deviations for the two data sets were considerably different, we analyzed the data using the nonparametric Mann-Whitney test (Sprent, 1989). When this test was applied to the *GUS* activity data, the results were found to be highly significant (two-tailed P = 0.0052). The median *GUS* activity value for construct −840 *GS2* (833 pmol 4-methylumbelliferone per mg protein per min) is more than 10-fold greater than that of construct −807 *GS2* (67 pmol 4-methylumbelliferone per mg protein per min).

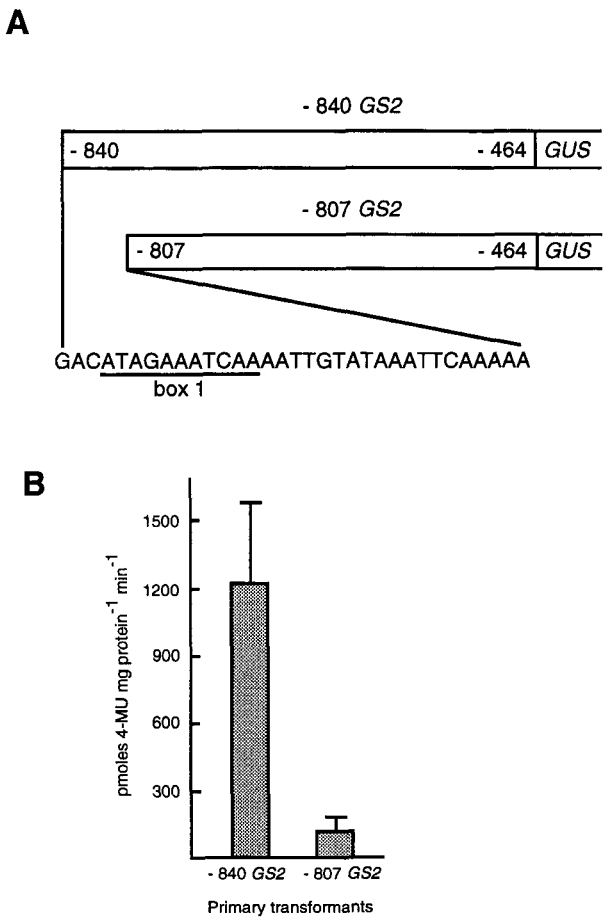

Figure 1. Deletion of 33 bp Reduces Transcription of the *GS2* Gene.

(A) The *GS2–GUS* transcriptional fusion constructs. The *GS2–GUS* transcriptional fusions are shown diagrammatically with the 33-bp sequence present in construct −840 *GS2* but deleted from construct −807 *GS2*, as indicated. The sequence is numbered relative to the start of translation of the *GS2* gene (G. Tjaden, J.W. Edwards, and G.M. Coruzzi, unpublished results).

(B) Fluorometric analysis of the *GS2–GUS* transcriptional fusions. Results are presented of fluorometric analysis of *GUS* activity (4-methylumbelliferone [4-MU] production) in leaves of primary transformants containing either construct −840 *GS2* (n = 11) or construct −807 *GS2* (n = 11). Error bars represent the standard error of the mean. These results were found to be highly significant when the Mann-Whitney nonparametric test was applied (P = 0.0052).

In Vitro Analysis of Box 1 DNA-Protein Interactions

This 33-bp AT-rich region of the *GS2* promoter, box 1 native as given in Figure 2A, was used as a probe in gel shift analysis. Incubation of the box 1 native probe with extract prepared from etiolated pea seedlings resulted in the formation of two complexes, an LMC and an HMC, as indicated in Figure 2B, lanes 1 to 3. Similar results were obtained with nuclear extracts prepared from light-grown and etiolated pea seedlings (data not shown) and with tobacco nuclear extracts (Figure 2C).

To further study the LMC, a probe containing four copies of the box 1 sequence was synthesized (box 1 multimer, Figure 2A). As predicted, the box 1 multimer probe formed complexes with the same mobility as those detected with the box 1 native probe (see Figure 2B, lanes 4 to 6). Because the binding reactions contain excess probe with equal specific

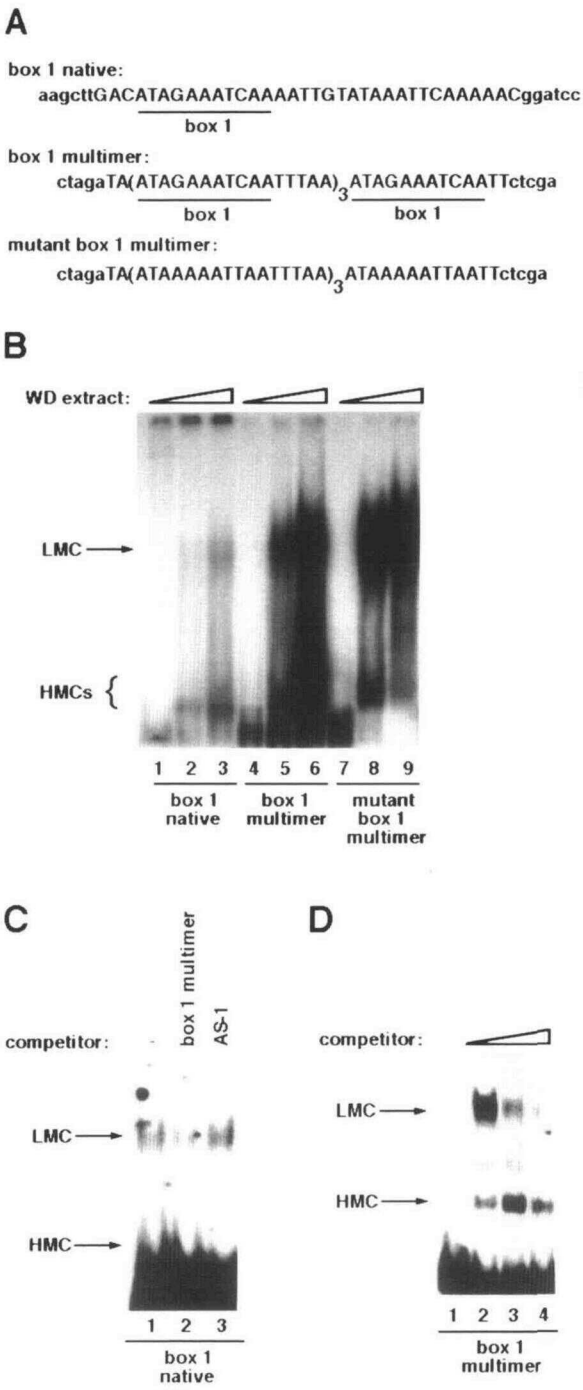

Figure 2. A Box 1–Containing Fragment of the *GS2* Promoter Forms Two Types of DNA–Protein Complexes, LMC and HMC.

(A) Nucleotide sequences of the *GS2* promoter fragment (box 1 native), the box 1 multimer, and the mutant box 1 multimer. Added restriction sites are shown in lower case letters.

(B) The box 1 native, box 1 multimer, and mutant box 1 multimer form LMCs with the same mobility but with different probe affinities. Gel shift analysis was performed with the box 1 native probe (lanes 1 to 3), no extract (lane 1), 40 μg of whole-cell extract prepared from etiolated pea seedlings (WD, lane 2), 120 μg of WD extract (lane 3); with the box 1 multimer probe (lanes 4 to 6), no extract (lane 4), 40 μg of WD extract (lane 5), 120 μg of WD extract (lane 6); and with the mutant box 1 multimer probe (lanes 7 to 9), no extract (lane 7), 40 μg of WD extract (lane 8), 120 μg of WD extract (lane 9). Note that the box 1 native free probe (lanes 1 to 3) was run off the gel.

(C) The box 1 multimer competes for LMC formation with the box 1 native DNA. Gel shift analysis was performed with the box 1 native probe and nuclear extract from dark-adapted tobacco leaves with no competitior (lane 1), a 50-fold molar excess of the box 1 multimer (lane 2), and, as a negative control, a 50-fold excess of AS-1 (lane 3). AS-1 contains four copies of the ASF-1 binding site (Lam et al., 1989; for the sequence, see Methods).

(D) The box 1 multimer LMC is competed more readily in self-competition assays than the HMC. The box 1 multimer probe was incubated with no extract (lane 1), WD extract and no competitor (lane 2), WD extract and the unlabeled box 1 multimer competitor at a 25-fold (lane 3) and a 50-fold molar excess (lane 4).

activities, the relative intensities of the complexes reflect the comparative affinities of the probes for the LMC protein(s). Therefore, these results indicate that multimerization of the box 1 sequence resulted in increased affinity for the LMC protein(s) (Figure 2B, lanes 2 and 5).

To verify the identity of the box 1 native and box 1 multimer LMCs, a competition analysis was performed. The results shown in Figure 2C indicate that the box 1 native and box 1 multimer probes compete for the same LMC protein(s) in gel shift assays. In contrast, the LMC formed with the box 1 native was not competed by non-AT-rich *cis*-acting DNA elements, such as GT-1 (Green et al., 1988) (data not shown) or AS-1 (Lam et al., 1989) (Figure 2C, lane 3). Self-competition analysis of the box 1 multimer LMC using unlabeled box 1 multimer DNA revealed different specificities of the probe for the LMC and the HMC protein complexes. The results shown in Figure 2D indicate that the LMC formed by the box 1 native probe was competed by a 25- and 50-fold molar excess of unlabeled box 1 native DNA (Figure 2D, lanes 3 and 4, respectively), whereas the HMC complex did not compete as readily.

A mutant box 1 multimer probe was synthesized such that the G and C residues within the box 1 sequence were changed to an A and a T, respectively (Figure 2A). This mutant box 1 native probe showed no reduction in LMC formation (Figure 2B, lanes 7 to 9), thereby indicating that the G and C residues in the box 1 sequence are not required for LMC formation. In fact, an increase in LMC formation was observed, indicating that the mutant box 1 multimer probe has a higher affinity for the LMC protein(s) (Figure 2B, compare lanes 5 and 6 with lanes 8 and 9). This may be due to the fact that the mutant is more AT-rich than the box 1 multimer. These results suggest that the box 1 multimer LMC did not result from sequence-specific binding to the box 1 sequence but rather from interactions with AT-rich sequences in general.

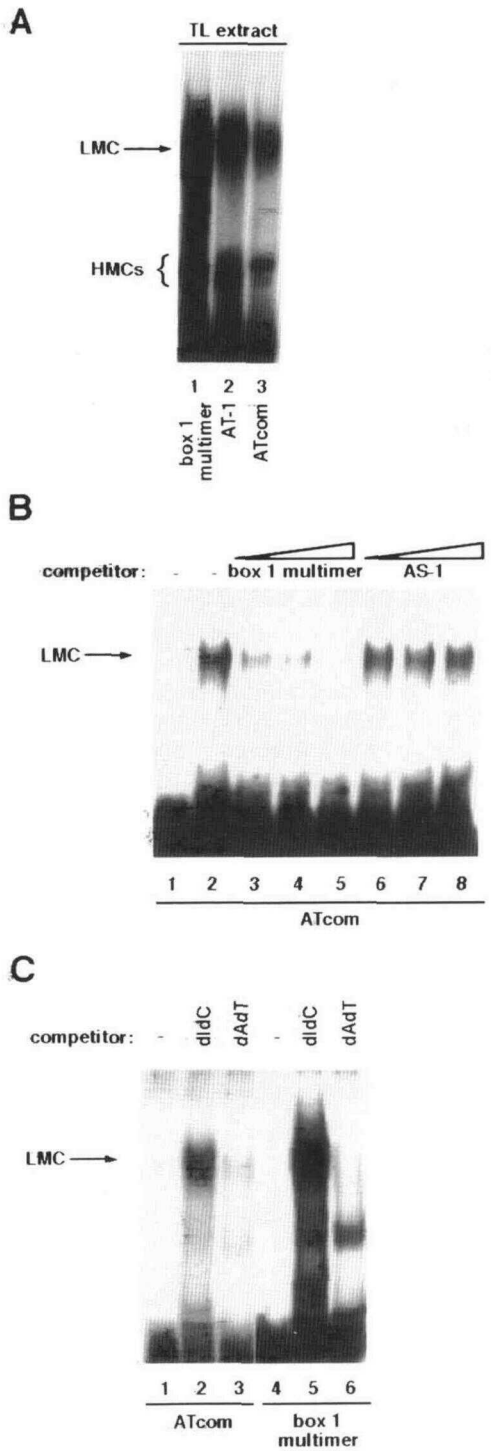

Figure 3. The Box 1 Multimer LMC Is Related to the LMC Detected with Other AT-Rich DNA Elements.

(A) The box 1 multimer, AT-1, and ATcom probes form LMCs with the same mobility in gel shift analyses. The box 1 multimer probe (lane 1), AT-1 probe (lane 2) (Datta and Cashmore, 1989), and ATcom probe (lane 3) (Czarnecka et al., 1992) were incubated with nuclear extract from leaves of light-grown tobacco plants (TL) and gel shift analysis was performed.

(B) The ATcom LMC is competed by the unlabeled box 1 multimer DNA in gel shift analysis. The ATcom probe was incubated with no extract (lane 1), or extract from light-grown tobacco plants (lanes 2 to 8), and lanes 3 to 5 also contain a 20-, 50-, and 75-fold molar excess of unlabeled box 1 multimer DNA. Lanes 6 to 8 also contain a 20-, 50-, and 75-fold molar excess of unlabeled AS-1.

(C) The box 1 multimer and ATcom LMCs are competed by poly(dA-dT) but not by poly(dI-dC). Gel shift analysis was performed with the ATcom (lanes 1 to 3; Czarnecka et al., 1992) and box 1 multimer (lanes 4 to 6) probes with TL extract and either poly(dI-dC) (lanes 2 and 5) or poly(dA-dT) (lanes 3 and 6) at a final concentration of 0.5 mg/mL. These binding reactions were run on the same gel, but different exposures are shown.

A comparison was made between the box 1 multimer complexes and complexes formed using AT-rich DNA fragments from other plant promoters such as AT-1, a negative regulatory element from the *N. plumbaginifolia CabE* promoter (Castresana et al., 1988; Datta and Cashmore, 1989) and ATcom, an AT-rich probe derived from the *Gmhsp17.5E* AT-rich regions (Czarnecka et al., 1992). The results shown in Figure 3A indicate that the complexes formed using the ATcom and AT-1 probes were similar in mobility to the box 1 multimer LMC. To determine whether the same protein(s) is involved in the formation of these DNA–protein complexes, a competition analysis was performed. The results shown in Figure 3B demonstrate that formation of the ATcom LMC was reduced by unlabeled box 1 multimer DNA. By contrast, the non-AT-rich AS-1 DNA (Lam et al., 1989) did not compete for the ATcom binding protein(s). Similar results were obtained for the AT-1 probe (data not shown). The box 1 multimer LMC could also be competed by a multimer of the AT-rich 3AF1 binding site shown to be involved in expression of the pea *rbcS* promoter (Lam et al., 1990) (data not shown). Figure 3C shows that both the box 1 multimer LMC and the ATcom LMC were specifically competed by poly(dA-dT) but not by the same concentration of poly(dI-dC). Based on the mobility of the complexes, the LMC formed with the box 1 multimer appeared to be related to the ATcom and the AT-1 LMCs. The competition experiments provide direct evidence that the LMCs formed by the box 1 multimer, ATcom, and AT-1 elements contained one or more of the same proteins.

Protein phosphorylation had been reported to inhibit binding by the *Gmhsp17.5E* AT-rich binding factor from soybean extract (Gurley et al., 1993) and by the pea extract AT-1 binding activity (Datta and Cashmore, 1989). To further compare the tobacco box 1 multimer LMC with these complexes, the effects of phosphorylation on tobacco LMC formation with the box 1 multimer probe were investigated. The results of this experiment are shown in Figure 4. Treatment of the tobacco nuclear extracts with sodium fluoride alone, which inhibits the activity of endogenous phosphatases, had little effect on LMC formation (Figure 4, lanes 3 and 8). However, when a protein

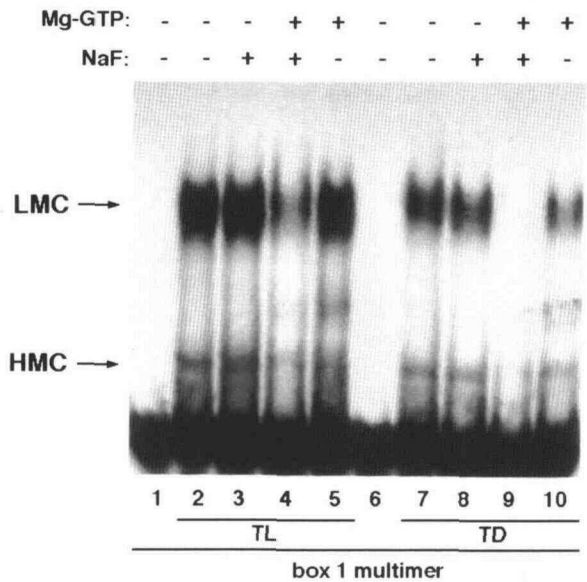

Figure 4. Phosphorylation Inhibits Formation of the Box 1 Multimer LMC.

Gel shift analysis was performed with the box 1 multimer probe and extract from either light-grown tobacco leaves (TL, lanes 2 to 5) or dark-adapted tobacco leaves (TD, lanes 7 to 10). Binding reactions contained the phosphatase inhibitor sodium fluoride (NaF, lanes 3 and 8), or the kinase activator Mg-GTP (lanes 5 and 10), or both (lanes 4 and 9), as indicated by the + and – signs.

kinase activator such as Mg-GTP was included, LMC formation was greatly reduced (Figure 4, lanes 4 and 9). Mg-GTP alone had little effect on complex formation (Figure 4, lanes 5 and 10). The same results were obtained whether light-grown or dark-adapted tobacco nuclear extracts were used (in Figure 4, compare lanes 2 to 5 with lanes 7 to 10). The fact that phosphorylation inhibited formation of the box 1 multimer LMC provides additional evidence that this LMC is related to the ATcom and AT-1 LMCs.

Isolation and Characterization of a cDNA Encoding an AT-Rich DNA Binding Protein

The box 1 multimer was used to screen a tobacco cDNA expression library by the method of Singh et al. (1988). Two different positive clones were identified. One of the positive phages (ATBP-1) had a considerably longer cDNA insert and was chosen for further study. Specific binding of the ATBP-1 protein to the box 1 multimer is shown in Figure 5A. ATBP-1 binds the box 1 multimer with high affinity but does not bind the ASF-1 binding site multimer AS-1 (Lam et al., 1989).

The ATBP-1 cDNA insert was found to be 1494 bp long with a 30-bp-long poly(A) tail. The length of the message and the absence of a methionine codon at the amino terminus suggest that the cDNA does not contain the entire coding sequence of *ATBP-1*. As shown in Figure 6, the longest open reading frame of this partial cDNA encodes a peptide (ATBP-1) of 380 amino acids, which can be divided into two putative functional domains, as shown in Figure 7. The amino-terminal domain consists of a 64–amino acid glutamine-rich domain (52%). ATBP-1 contains seven GRP (Gly-Arg-Pro) motifs distributed throughout the carboxy terminus. GRP motifs have been identified as DNA binding motifs with a high affinity for AT-rich double-stranded DNA (Lund et al., 1987; Reeves and Nissen, 1990). Figure 7 compares the seven ATBP-1 GRP motifs with the consensus sequences from human HMG I/Y proteins (Reeves and Nissen, 1990) and a plant GRP consensus derived from two soybean embryo HMG proteins (Laux et al., 1991). RNA gel blot analysis of tobacco RNA using an ATBP-1 probe revealed an mRNA approximately 2100 bases in length, as shown in Figure 8A.

To determine the relationship between the protein encoded by ATBP-1 and the LMC proteins detected in extracts, protein blots from the phage were probed with the box 1 multimer in the presence of either poly(dA-dT) or poly(dI-dC) nonspecific competitor. DNA binding with the control B9 protein (a nonsequence-specific DNA binding protein obtained from S.L. McKnight, Tularik Inc., S. San Francisco, CA) was effected equally by poly(dA-dT) and poly(dI-dC). However, DNA binding by ATBP-1 was competed by poly(dA-dT) but not by the same concentration of poly(dI-dC), as shown in Figure 5B. The competition of ATBP-1 created by poly(dA-dT) was similar to the results of poly(dA-dT) competition gel shift analysis with either the box 1 multimer or the ATcom probes and tobacco nuclear extract (Figure 3C). Together with the fact that ATBP-1 has a high affinity for the box 1 multimer, these results suggest that the protein encoded by *ATBP-1* is related to the LMC-forming proteins detected in the crude extracts.

Many of the genes with AT-rich promoter elements are light-regulated genes, e.g., *CabE*, *rbcS*, and *GS2* (reviewed in Tobin and Silverthorne, 1985; Tingey et al., 1988). Therefore, RNA gel blot analysis of *ATBP-1* mRNA was performed using RNA isolated from light-grown and dark-adapted tobacco plants. The results indicate that light did not affect steady state levels of the *ATBP-1* transcript in leaves of tobacco (Figure 8A).

To determine whether *ATBP-1* is encoded by a single-copy gene, we performed DNA gel blot analysis of tobacco genomic DNA using an 860-bp amino-terminal fragment of the ATBP-1 cDNA. The detection of multiple bands suggested that *ATBP-1* belongs to a small multigene family (Figure 8B). However, because *N. tabacum* is an amphidiploid hybrid, a single-copy gene might give two distinct hybridizing bands because each of the progenitors, *N. sylvestris* and *N. tomentosiformis*, contributes a genome to *N. tabacum*.

DISCUSSION

Studies of several different plant promoters provide evidence that AT-rich promoter regions can play either a positive (Bustos

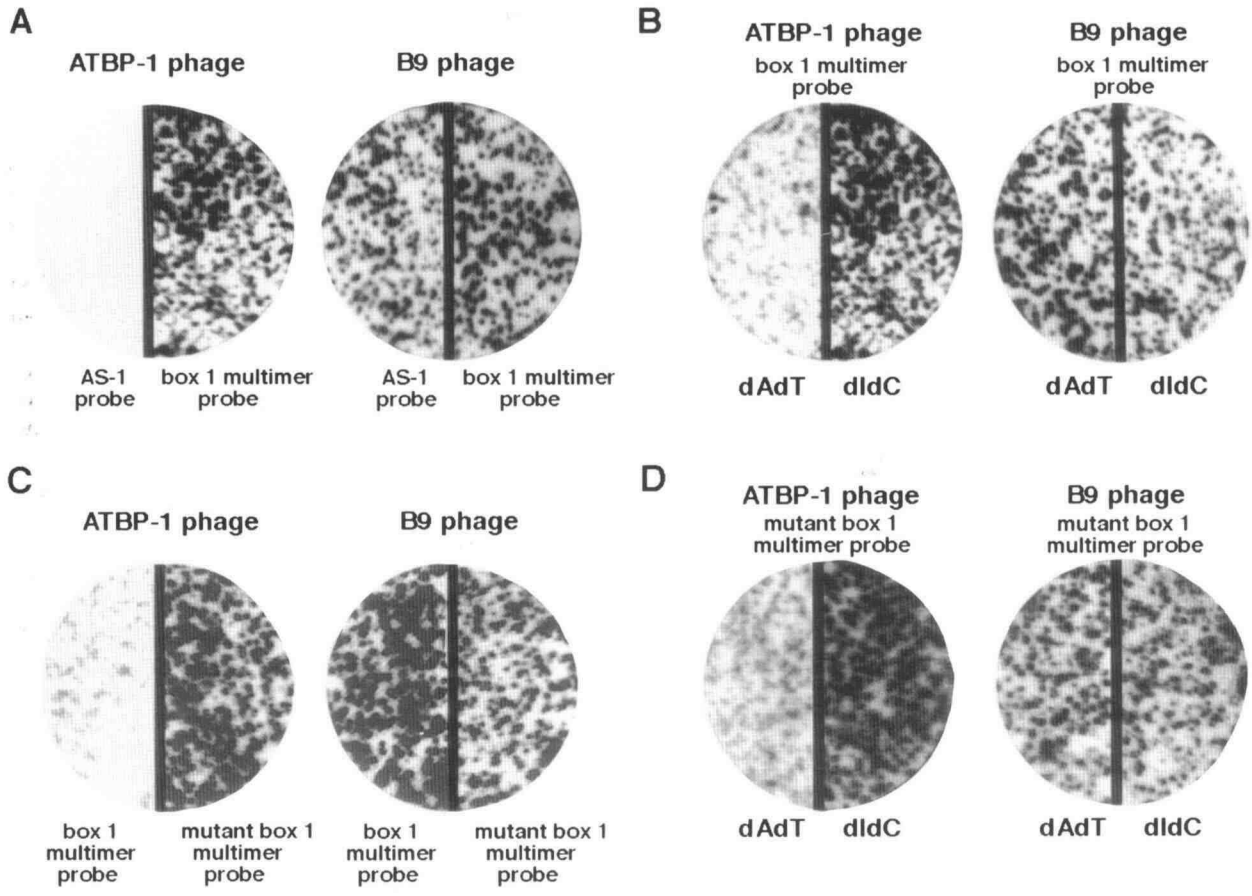

Figure 5. ATBP-1 Protein Binds the Box 1 Multimer and Mutant Box 1 Multimer.

(A) The protein encoded by *ATBP-1* binds to the box 1 multimer but not to AS-1. Phage-expressed proteins were blotted and probed with DNA under conditions that allow DNA–protein interactions. DNA binding activity of the ATBP-1 and positive control (B9) proteins was tested using the box 1 multimer and AS-1 (Lam et al., 1989) DNA probes. B9 encodes a nonsequence-specific DNA binding protein (see Methods).

(B) DNA binding activity of the protein encoded by *ATBP-1* is competed by poly(dA-dT). The DNA binding activity of the ATBP-1 and the B9 proteins was tested using the box 1 multimer DNA and either poly(dA-dT) or poly(dI-dC) as nonspecific competitor. Note that binding of the B9 phage to the box 1 multimer was effected equally by poly(dA-dT) and poly(dI-dC). Binding reactions were performed in parallel for the filters shown in **(A)** and **(B)**.

(C) The protein encoded by *ATBP-1* has a higher affinity for the more AT-rich mutant box 1 multimer DNA. DNA binding activity of the ATBP-1 and positive control (B9) proteins was tested using the box 1 multimer and mutant box 1 multimer.

(D) DNA binding activity of the protein encoded by *ATBP-1* is competed by poly (dA-dT). The DNA binding activity of the ATBP-1 and B9 proteins was tested using the mutant box 1 multimer DNA and either poly(dA-dT) or poly(dI-dC) as nonspecific competitor. Note that binding of the B9 phage to the mutant box 1 multimer was effected equally by poly(dA-dT) and poly(dI-dC). Binding reactions were performed in parallel for the filters shown in **(C)** and **(D)**.

et al., 1989; Lam et al., 1990; Czarnecka et al., 1992) or a negative (Castresana et al., 1988) role in transcription. Here, we report that in the case of the pea *GS2* promoter, deletion of a 33-bp AT-rich region resulted in a 10-fold reduction in *GUS* activity in transgenic tobacco. Whereas the reports of positive regulation by AT-rich elements are greater in number, the existence of data concerning their role as negative elements is provocative. Most of the evidence for positive regulation is provided by "gain-of-function" studies that analyze AT-rich sequences in conjunction with heterologous promoters. By contrast, Castresana et al. (1988) reported evidence for negative regulation using deletion analysis. Deletion analysis might be considered more reliable because the AT-rich element is analyzed in its native context. The *GS2* promoter deletion analysis results presented here support a positive role for AT-rich elements in transcription regulation. Perhaps the nature of the effects of AT-rich elements is determined by other *cis* elements in the context of the native promoter. Characterization of the

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CAAGACCAAT TCCAAGCTCA GCTTCAAGCC CAGCTTCAAG CCCAACTTCA   50
Q D Q F  Q A Q  L Q A  Q L Q A  Q L Q

AGCCCAACAG CAGCAAGCAG CCCAGTTTCA ACCTCAATTC CAACTCATCC  100
A Q Q  Q Q A A  Q F Q  P Q F  Q L I Q

AACAACAGCC CCAGTACTTA CCTCAACAAC AGTTCCAGCC CGACCCATTA  150
Q Q P  Q Y L  P Q Q Q  F Q P  D P L

CTCCAACCTC AGCAACAGTT CCAGACCCAG CCACAGACGC AGGCCTATGC  200
L Q P Q  Q Q F  Q T Q  P Q T Q  A Y A

TACTCCTGAA GGCCATAATT ATGCTGGCCT TGGCGCTGAA TCCGTGTTTG  250
 T P E  G H N Y  A G L  G A E  S V F V

TTTCTCTTGG GCTAGCTGAT GGGCCTGTTG GAGTTCAGAA TCCTGCTGTT  300
  S L G  L A D  G P V G  V Q N  P A V

GGGCTGGCTC CGGCACCGGG AGCTGAAGAG AGTACGGCAA AGAGACGACC  350
G L A P  A P G  A E E  S T A K  R R P

AGGTCGTCCC CGTAAGGATG GTTCCACTGT GGTTAAACCG GTGGAACCCA  400
G R P  R K D G  S T V  V K P  V E P K

AATTACCGGA CCAGAGCGGT GGTAGTAAGA GGAGACCTGG TCGTCCTCCT  450
  L P D  Q S G  G S K R  R P G R P P

AAGAGTGTGA CAGTTAATGC TGCTCCTGGA TCAGCTATGG GTTCTGGACG  500
K S V T  V N A  A P G  S A M G  S G R

ACGAGGTCGG CCCAGGAAAA ATTCTGTTCC TGGACGACGA GGTCGGCCCA  550
R G R P  R K N  S V P  G R R  G R P R

GGAAGAATGC GGCTGTTGCT GCTGCCAATG GCGGTGCCAA TGTCGCAAAT  600
  K N A  A V A  A A N G  G A N  V A N

ATTCCTTCTG TTGGTGCCAA TGTGACCAAT GTTCCAGCTG GTGGTGTCCC  650
I P S V  G A N  V T N  V P A G  G V P

GGGAGCCATA ACAACACCTA AAGGAAGGGG ACGGCCACCA AGGTCTAGTG  700
G A I  T T P K  G R G R P P  R S S G

GACCTCCTGC TGCTACTGTG GGTGTTACAG ATGTGCCTAT TGCTGCTGCT  750
  P P A  A T V  G V T D  V P I  A A A

TTTGATACGG AAAACTTGCC TAATGCTGTT GGTGGTGGCG GTGTCACAAA  800
F D T E  N L P  N A V  G G G G  V T N

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N G A  L P P L  G K R  R G R P  P K S

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 Y G A  A A A  A P T V  K R P  R K L

TCTGGAAAAC CTCTGGGTCG ACCTAGAAAG AATGTGACAT CCCCTGCAGT  950
S G K P  L G R P  R K  N V T S  P A V

TTCGGACCCC AAGTTGGTGG TGGCCTATGA AGAACTAAAG GGGAAACTTG 1000
 S D P  K L V V  A Y E  E L K  G K L E

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  H M Q  S R I  K E A A  N A L  K P C

TTAAATGCTG AATCGCCAGC AATTGCTCTG GCAGCATTGC AAGAGTTAGA 1100
L N A E  S P A  I A L  A A L Q  E L E

AGAGTTAGCA GCAGCAGGGG GGAATCCAGT GCAGCAAAAT TGATAAAAGA 1150
 E L A  A A G G  N P V  Q Q N  . .

AGATGTCGCA GAGATTAGGA ATATGGAGGC AGTGCTTAAA CTCAGAGTGT 1200
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AGACTAGTTG TTTGTGACAG GACGAAGATG CGCTTAGACT TGGAGGCAGT 1300
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TGATTTCGTC ATTTGATAAT TACCTTATTC TGCTAATTTG GTTAATGACA 1450
ATTAAGGGGG AGACAAAAAA AAAAAAAAAA AAAAAAAAAA AAAA       1494
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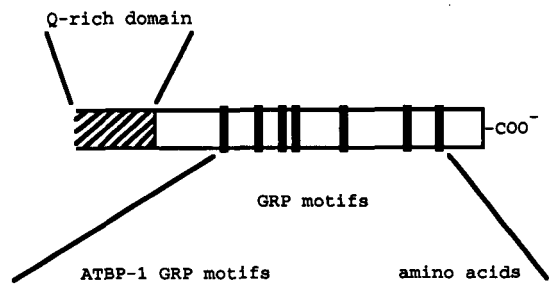
Figure 6. DNA Sequence of the ATBP-1 cDNA Clone and Predicted Protein Sequence.

The DNA sequence of the ATBP-1 cDNA showing translation of the longest open reading frame. The first amino acid of the longest open reading frame is designated as number 1. The glutamine-rich domain and GRP motifs are designated by underlining and underlined boldface letters, respectively. The GenBank accession number of the ATBP-1 DNA sequence is L26113.



ATBP-1 GRP motifs								amino acids
R	R	P	G	R	P	R	K	115-122
R	R	P	G	R	P	P	K	144-151
G	R	R	G	R	P	R	K	167-173
G	R	R	G	R	P	R	K	178-185
K	G	R	G	R	P	P	R	224-231
K	R	R	G	R	P	P	K	276-283
K	P	L	G	R	P	R	K	303-310
R	P	R	G	R	P	K	K	HMG-I consensus
R	P/G	R	G	R	P	P/K	K	Soybean embryo HMG consensus

Figure 7. Schematic Representation of ATBP-1.

The GRP motifs are designated as thick bars. "HMG I consensus" refers to the mammalian HMG I consensus peptide described in Reeves and Nissen (1990). "Soybean embryo HMG consensus" is derived from the GRP motifs reported by Laux et al. (1991).

trans-acting factors that bind AT-rich elements will lead to a better understanding of the role of AT-rich elements in transcription.

To detect nuclear proteins that interact with AT-rich elements, gel shift analyses have been performed for a variety of plant promoters. Interestingly, many researchers have obtained similar complexes using this technique. There are remarkable similarities between the complexes detected with the AT-rich elements of the pea *rbcS* promoter (Lam et al., 1990), *N. plumbaginafolia CabE* promoter (Datta and Cashmore, 1989), pea *ferredoxin-1* and wheat *emb* promoters (Pederson et al., 1991), and the soybean *Gmhsp17.5E* promoter (Czarnecka et al., 1992; Gurley et al., 1993) as well as others. Czarnecka et al. (1992) reported performing competition experiments that showed that AT-rich fragments from many genes competed for the same proteins. In all of these reports, primarily two complexes were detected: one or more HMCs and a single LMC. The HMCs were found to contain classically defined HMG proteins. This conclusion was based on gel shift analyses with purified HMG proteins (Pederson et al., 1991) and solubility of the HMC proteins in trichloroacetic acid (Czarnecka et al., 1992). In similar experiments, the LMC proteins were found not to be soluble in trichloroacetic acid and were found to be larger in size than the reported HMG proteins (Czarnecka et al., 1992). Gel shift analysis using an AT-rich element of the *GS2* promoter also revealed an HMC and an LMC.

The results presented herein for the box 1 element of the *GS2* promoter indicate that LMC formation was due to

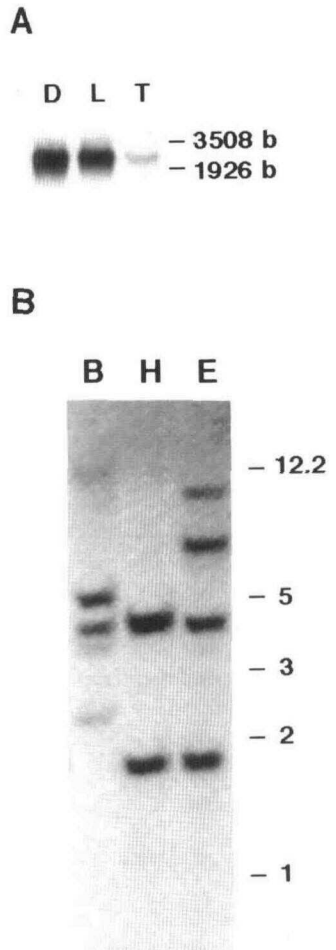


Figure 8. *DNA Gel Blot and RNA Gel Blot Analyses of ATBP-1.*

(A) RNA gel blot analysis using the ATBP-1 cDNA as probe. D, 5 μg of poly(A)$^+$ RNA isolated from leaves of 4-week-old dark-adapted tobacco; L, 5 μg of poly(A)$^+$ RNA isolated from leaves of light-grown tobacco; T, 20 μg of total RNA from light-grown tobacco. The probe was an 860-bp 5′ fragment of the ATBP-1 cDNA. b, bases.

(B) Genomic analysis of tobacco DNA using the ATBP-1 cDNA as probe. Genomic DNA (10 μg per lane) was digested with BamHI (B), HindIII (H), or EcoRI (E) and probed with an 860-bp 5′ fragment of the ATBP-1 cDNA. Molecular weight DNA size markers are shown in 10^{-3} kb.

interactions with AT-rich stretches of DNA and not to specific sequences of the box 1 element. Both the box 1 multimer and ATcom LMCs were competed by poly(dA-dT). Furthermore, mutations of the box 1 multimer, which increased the AT content of this DNA sequence, resulted in an increase in LMC formation. This DNA binding property is similar to that demonstrated for the mammalian HMG I/Y protein, which binds to any stretch of six or more A-T base pairs (Solomon et al., 1986). Whereas the similarity in mobility of the ATcom and box 1 multimer LMCs suggested that these LMCs might involve the same proteins, the competition analyses provided direct evidence for this conclusion. Overall, the results showed that the LMC proteins are shared between the box 1 native, box 1 multimer, ATcom, and AT-1 elements. These results suggest that there is a ubiquitous AT-rich DNA binding protein that binds different gene promoters from different plant species.

Inhibition of DNA binding by phosphorylation is a common mode of regulation (for a review, see Hunter and Karin, 1992). Two previous studies reported that phosphorylation inhibited LMC formation with AT-rich promoter elements. Datta and Cashmore (1989) reported inhibition with pea extract and the AT-1 binding site from the *N. plumbaginafolia CabE* promoter. Gurley et al. (1993) reported similar results with soybean extract and the ATcom probe from the soybean *Gmhsp17.5E* promoter. In both reports, the endogenous kinase was activated by Mg-GTP, which suggested that casein kinase II (CK II) was involved. A similar experiment was performed to determine whether phosphorylation would inhibit box 1 multimer LMC formation. The results indicated that activation of an endogenous kinase by Mg-GTP resulted in inhibition of box 1 multimer LMC formation. These results provide further evidence that the protein(s) making up the box 1 multimer LMC is related to the AT-1 (Datta and Cashmore, 1989) and ATcom binding proteins (Czarnecka et al., 1992; Gurley et al., 1993).

To further characterize the protein(s) that forms the tobacco LMC, a tobacco expression library was screened for DNA binding proteins. Using the box 1 multimer, the ATBP-1 phage was identified. The ATBP-1 protein can be divided into two putative functional domains. The amino-terminal domain consists of a 64–amino acid long glutamine-rich domain. To gain insight into the function of the glutamine-rich domain, the amino acid sequence of this domain was used to search the GenBank and EMBL data bases (Pearson and Lipman, 1988). This search revealed similarity with a variety of glutamine-rich proteins. Some examples of these are the wheat seed storage protein γ-gliadin (Sugiyama et al., 1986), the yeast transcription factors SNF5 (Laurent et al., 1990), cyc8 (ssn6; Schultz and Carlson, 1987), and Gal11 (Suzuki et al., 1988), and Drosophila transcription factors such as Hunchback (Tautz et al., 1987) and *zeste* (Pirotta et al., 1987). The best-characterized glutamine-rich domains belong to the mammalian transcription factor Sp1. In the case of Sp1, glutamine-rich domains function as protein–protein interaction domains that allow Sp1 to multimerize and to work with coactivator proteins (Pascal and Tjian, 1991; Hoey et al., 1993). Furthermore, the glutamine-rich domains are required for maximum Sp1 transcriptional activity (Courey and Tjian, 1988). It will be interesting to see whether the ATBP-1 glutamine-rich domain is important for ATBP-1 function. Perhaps protein–protein interactions via the glutamine-rich domain determine whether ATBP-1 has a negative or positive effect on transcription.

The carboxy terminus of ATBP-1 contains the putative DNA binding domain. In this region, there are seven GRP motifs or "AT-hooks" (Reeves and Nissen, 1990). These motifs have also been found in the mammalian HMG I/Y proteins (Reeves and Nissen, 1990). Studies have shown that HMG I/Y proteins

use AT-hooks to bind AT-rich DNA in the minor groove and alter the conformation and thermal stability of AT-rich regions of DNA (Lehn et al., 1988; Reeves and Nissen, 1990). The observation that ATBP-1 binds to poly(dA-dT) but not to poly(dI-dC) suggests that ATBP-1 does not interact solely with the minor groove because the minor groove of poly(dA-dT) is the same as the minor groove of poly(dI-dC). ATBP-1 has a higher number of GRP motifs than other GRP-containing proteins with the exception of the D1 satellite binding protein from Drosophila that has 10 GRP motifs (Ashley et al., 1989). A cDNA clone encoding a protein with two GRP motifs was isolated previously from tobacco. Lam et al. (1990) reported isolating the partial cDNA clone 3AF1 using an AT-rich element from the pea *rbcS* promoter to screen a cDNA expression library. However, there are no sequence similarities between ATBP-1 and 3AF1 outside of the GRP motifs.

With the exception of the GRP motifs, ATBP-1 does not fit the operational criteria for HMG proteins. HMG proteins are less than 30 kD, e.g., HMG I has 107 amino acids. HMG proteins are generally high in acidic and basic amino acids (~25% of each) and high in proline (at least 7%) (Johns, 1982). This can be compared with ATBP-1, which minimally encodes a 380–amino acid protein with 12% basic amino acids and 6% acidic amino acids. ATBP-1 is, however, extremely proline rich (12%) with 47 prolines. These numbers apply to the partial sequence and are subject to change when the full coding sequence is obtained. Interestingly, the estimated size of ATBP-1 correlates well with the UV cross-linking results of Czarnecka et al. (1992), who reported detecting proteins of 46 to 69 kD in the soybean ATcom LMC. In addition, Schindler and Cashmore (1990) found the proteins of the AT-1 LMC to be ~40 to 45 kD.

The only other known protein that, like ATBP-1, contains both a glutamine-rich domain and GRP motifs is encoded by the AAC11 cDNA previously isolated from Dictyostelium (Shaw et al., 1989). This protein contains a glutamine-rich domain at the amino terminus and four GRP motifs in addition to several long asparagine stretches. The function of the AAC11-encoded protein is not known, but it is interesting to note that levels of the transcript are developmentally regulated (Shaw et al., 1989).

The ATBP-1 protein expressed by the phage has DNA binding characteristics similar to the crude extract activity. DNA binding by ATBP-1 can be competed by poly(dA-dT). Similarly, the tobacco LMC detected with crude extract could be competed by poly(dA-dT). LMC-forming proteins that bind to the box 1 element are present in extracts prepared from both light-grown and dark-adapted tobacco leaves. Similarly, the ATBP-1 mRNA is present at the same level in light-grown or dark-adapted tobacco leaves. Overall, these results suggest that ATBP-1 may be involved in LMC formation.

Because formation of the LMC is inhibited by phosphorylation, possibly catalyzed by CKII, it was of interest to determine whether ATBP-1 could be a substrate for CKII. However, a search of the ATBP-1 primary sequence for CKII phosphorylation consensus sites (Pinna, 1990) revealed none. Because inhibition of DNA binding by phosphorylation does not require phosphorylation within the DNA binding domain, ATBP-1 may be phosphorylated at a site within the missing amino terminus. Inhibition of c-Myb DNA binding results from CKII phosphorylation at a site ~50 residues from the DNA binding domain (Luscher et al., 1990). Of course there is also the possiblity that ATBP-1 is not itself phosphorylated, but that inhibition results from phosphorylation of an inhibitor which then prevents ATBP-1 from binding DNA. By contrast, CKII-catalyzed phosphorylation of the plant DNA binding protein GBF-1 results in an increase in its DNA binding activity (Klimczak et al., 1992).

In conclusion, we have isolated a cDNA encoding an AT-rich DNA binding protein from tobacco, ATBP-1, that has not been previously reported in plants. ATBP-1 has DNA binding properties that are similar to the AT-rich DNA binding activity forming LMCs. This similarity suggests that ATBP-1 might be involved in formation of the LMC detected with the box 1 multimer probe. The LMC detected with the box 1 multimer probe appears to be the same LMC as detected for several other AT-rich promoter elements in a variety of plant species. Although this complex is formed with AT-rich DNA sequences, it does not involve classically defined HMG proteins. ATBP-1 is not an HMG protein but a novel protein that combines an HMG I DNA binding domain with a glutamine-rich potential transcriptional domain. Recent studies have shown that HMG I/Y proteins can derepress transcription by displacing histone H1 (Zhao et al., 1993). Perhaps ATBP-1, with its many GRP motifs, displaces histone H1 and nucleates formation of active transcription complexes by interacting with other transcription factors via its glutamine-rich domain. Studies are underway to determine the role of this protein in transcriptional regulation of the *GS2* gene.

METHODS

In Vivo Analysis

Constructs used for transgenic analysis were produced by ligating polymerase chain reaction–generated promoter fragments into the pBI101.1 vector (Clontech, Palo Alto, CA). Constructs were sequenced as double-stranded DNA using the Sequenase method (U.S. Biochemical Corp., Cleveland, OH). These constructs were transformed into leaf discs of *Nicotiana tabacum* cv SR1 by *Agrobacterium tumefaciens*–mediated gene transfer (Horsch et al., 1985). β-Glucuronidase (*GUS*) activity of the resultant transformants was analyzed using fluorometry, essentially as described by Jefferson et al. (1987).

Statistical analyses were performed using Instat Mac Version 1.1 (GraphPad Software, Inc., San Diego, CA).

Transgenic tobacco plants were germinated on Murashige and Skoog (1962) medium with 3% sucrose (Sigma) containing kanamycin at 100 μg/mL and grown at 22°C for 16 hr at 100 μE m^{-2} sec^{-1} and 18°C for 8 hr with no light. Plants transferred to soil were grown on metromix 200 soil supplemented with osmocote 14-14-14 fertilizer (Hummert International, St. Louis, MO) at 26°C for 16 hr at 200 μE m^{-2} sec^{-1} and 24°C for 8 hr with no light.

Gel Shift Analysis

Nuclear and whole-cell extracts were prepared as described previously by Green et al. (1987, 1988). DNA probes were gel-purified restriction fragments labeled with α-^{32}P-deoxynucleotide triphosphates using the Klenow fragment of DNA polymerase I. The ATcom and AT-1 probes were prepared by annealing two complementary oligonucleotides producing 5′ overhangs and then labeled as above. Due to a low melting temperature, the labeled ATcom probe contained a significant proportion of single-stranded DNA. However, consistent with the observation of Czarnecka et al. (1992), only the double-stranded species shifted. DNA–protein binding reactions were performed for 15 min at room temperature in binding buffer (20 mM Hepes-KOH, pH 7.5, 40 mM KCl, 1 mM EDTA, 10% glycerol, 0.5 mM DTT) with 10,000 cpm of radioactive DNA probe (0.1 to 0.5 ng) and sonicated poly(dI-dC) or poly(dA-dT) (Pharmacia, Uppsala, Sweden) at a final concentration of 0.5 mg/mL. Each binding reaction contained 1 to 3 μg of nuclear extract protein or 20 to 40 μg of whole-cell extract protein in a final volume of 10 μL. The DNA–protein complexes were analyzed on a 0.7% agarose, 3.0% acrylamide gel buffered with 1 mM Tris-HCl, pH 8.0, 3 mM sodium acetate, 1 mM EDTA (final pH 7.95). Gels were dried on DEAE-cellulose paper (Schleicher & Schuell) and exposed with XAR-5 x-ray film at −80°C with an intensifying screen.

The sequence of the AT-1 probe was CTTAATATATTTTTAATTATTTTTATTCTCTTAA (Datta and Cashmore, 1989). The ATcom probe sequence was tcgacAAAAATAATATTAATATTATATTGAAAgtcga (lower case letters indicate restriction sites introduced into the oligonucleotides). The AS-1 probe consisted of a tetramer of the sequence CTGACGTAAGGGATGACGCACAATCCCAC with an added HindIII site at the 5′ end and an XhoI site at the 3′ end (Lam et al., 1989).

The phosphorylation experiment was performed essentially as described by Datta and Cashmore (1989). Tobacco nuclear extract was incubated for 5 min at room temperature in poly(dI-dC)-containing binding buffer (see above) with combinations of the following (final concentrations): 50 mM sodium fluoride, 20 mM $MgCl_2$, 2 mM GTP. After the 5-min incubation, 10,000 cpm of radioactive DNA probe was added, and the reaction was then incubated for 10 min at room temperature.

Analysis of cDNA Expression Library Proteins with DNA Binding Activity

Klenow fragment–labeled box 1 multimer probe was used to screen ~7 × 10^5 plaques of a λ ZAP *N. tabacum* cv SR1 library (Stratagene) essentially as described by Ausubel et al. (1988). The binding buffer was 20 mM Hepes-KOH, pH 7.5, 40 mM KCl, 1 mM EDTA, 1 mM DTT, 0.8 mM phenylmethylsulfonyl fluoride with 5 μg/mL sonicated poly(dI-dC). The B9 phage encodes a nonsequence-specific DNA binding protein (S.L. McKnight, Tularik Inc., S. San Francisco, CA). The pBluescript SKII+ plasmid was excised from the phage according to manufacturer's instructions and used for further analysis.

Characterization of the ATBP-1 cDNA

DNA sequence analysis was performed on both strands of the double-stranded DNA using the sequenase method (U. S. Biochemical Corp.).

For RNA gel blot analysis, total RNA was isolated from tobacco leaves according to the method of Jackson and Larkins (1976), separated on a 1% agarose–6% formaldehyde gel in Mops buffer, and transferred to a nitrocellulose filter as described previously by Ausubel et al. (1988). Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography (Aviv and Leder, 1972).

Genomic DNA was isolated from tobacco plants by the method of Dellaporta et al.(1983), separated on a 0.6% agarose gel in Tris-borate-EDTA (TBE) buffer, denatured, and transferred essentially as described previously (Southern, 1975; Ausubel et al., 1988).

For both DNA gel blot and RNA gel blot analyses, hybridization was performed as described previously (Ausubel et al., 1988). An 860-bp PstI 5′ restriction fragment of the AT-rich DNA binding protein (ATBP-1) cDNA was labeled with ^{32}P-dCTP using a random primer labeling kit (Boehringer Mannheim).

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