The EMBO Journal vol.13 no.22 pp.5383–5392, 1994

A novel DNA binding protein with homology to Myb oncoproteins containing only one repeat can function as a transcriptional activator

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Communicated by L.Willmitzer

A cDNA clone encoding a novel Myb-related protein, designated MybSt1, was isolated from a potato cDNA expression library by South Western screening using the CaMV 35S promoter domain A as a probe. Sequence comparison shows a small region with some homology to the highly conserved DNA binding domain of the c-*myb* proto-oncogene consisting of three imperfect repeats. The Myb motif of the MybSt1 protein is distinct from the plant Myb DNA binding domain described so far. In contrast to the known plant Myb proteins, with two repeats required for the DNA binding activity, the clone *mybSt1* contains only one such repeat. Nevertheless, the Myb-related protein MybSt1 is able to bind to DNA in a sequence-specific manner. In addition to the Myb-like region, the protein MybSt1 contains an acidic segment in its central region as well as a proline-rich region near the C-terminus. Applying the random binding site selection technique, high-affinity DNA binding sites for MybSt1 were identified, sharing the core motif GGATA. In transient expression assays using plant protoplasts, clear evidence was obtained for this *myb* clone functioning as a transcriptional activator.

Key words: c-*myb*/c-*myb* related *mybSt1* gene/DNA binding protein/MybSt1 binding sites/transcriptional activator

Introduction

The interaction of regulatory proteins with DNA sequences plays a critical role in the regulation of gene expression. Many DNA binding proteins identified to date can be grouped into distinct classes based on their conserved DNA binding domains, characterized by particular structural motifs (Mitchell and Tjian, 1989; Struhl, 1989; Pabo, 1992). One such strongly conserved domain is found in the c-Myb protein.

The cellular *myb* proto-oncogene (c-*myb*) is involved in the proliferation and/or differentiation of haematopoietic progenitor cells, while the viral oncogene (v-*myb*) is responsible for its transformation (reviewed in Lüscher and Eisenman, 1990; Mucenski *et al.*, 1991).

c-*myb*, as well as its truncated version, v-*myb*, encodes nuclear DNA binding proteins (c-Myb and v-Myb) that bind to DNA in a sequence-specific manner and function as transcriptional activators (Klempnauer *et al.*, 1983; Biedenkapp *et al.*, 1988; Weston and Bishop, 1989; Howe *et al.*, 1990; Kalkbrenner *et al.*, 1990). The specific Myb binding site, PyAACG/TG, was defined (Biedenkapp *et al.*, 1988) and subsequently confirmed by the identification of the cellular target gene, *mim*-1, directly regulated by the v-*myb* protein (Ness *et al.*, 1989).

Genes similar to c-*myb* have been isolated from a variety of different organisms, ranging from human (Nomura *et al.*, 1988), *Xenopus* (Amaravadi and King, 1994), *Drosophila* (Katzen *et al.*, 1985) and yeast (Tice-Baldwin *et al.*, 1989) to plant (Paz-Ares *et al.*, 1987; Marocco *et al.*, 1989; Jackson *et al.*, 1991; Oppenheimer *et al.*, 1991; Avila *et al.*, 1993; Leech *et al.*, 1993).

Common to all *myb* gene products is the strongly conserved DNA binding domain that is located at the N-terminal end (Frampton *et al.*, 1989, 1991; Howe *et al.*, 1990). In animals this Myb domain consists of three imperfect repeats of 50–53 amino acids each (R1, R2 and R3) with highly conserved, regularly spaced tryptophans, whereas in plants and yeast two repeats (R2 and R3) are present. An exception, however, is found in the REB1 from yeast and Adf1 from *Drosophila*, with one and a half or even less than one repeat respectively (Ju *et al.*, 1990; England *et al.*, 1992). Each repeat of the Myb domain has been proposed to fold into a helix–turn–helix motif similar to that found in bacterial repressors and eukaryotic homeodomains (Frampton *et al.*, 1989, 1991; Ogata *et al.*, 1992).

Furthermore, Myb and Myb-related proteins have characteristic transcriptional activation domains. Such activator domains can be divided into three classes according to their amino acid composition. They are either rich in acidic amino acids, prolines or glutamines (Ptashne, 1988; Latchman, 1991). A transactivation region, acidic in nature, has been mapped in the central part of the c-Myb protein (Sakura *et al.*, 1989; Kalkbrenner *et al.*, 1990). Myb-related proteins from *Petunia*, MybPh1 and MybPh3, contain, in addition to an acidic region, a domain rich in proline and in glutamine respectively (Avila *et al.*, 1993). For the Myb homologue C1 from maize this domain, a highly acidic region located in the C-terminus, has been shown to be functional (Goff *et al.*, 1991).

Such a modular structure with two separate active domains, a DNA binding and an activator region, is a common feature of transcriptional activators (Ptashne, 1988; Mitchell and Tjian, 1989; Latchman, 1991).

In this report we describe the isolation and characterization of a potato cDNA encoding a novel sequence-specific DNA binding protein, designated MybSt1, that contains a small region bearing substantial homology to the DNA binding domain of c-Myb. We determine the exact high-affinity binding sites of the MybSt1 protein and demonstrate that MybSt1 is able to activate transcription using transient expression systems.

Results

Isolation of potato cDNA clones specifically binding to the CaMV 35S promoter

A potato leaf cDNA expression library was screened using a multimerized, radiolabelled −90 to +2 35S promoter fragment in an attempt to isolate DNA binding proteins which might be responsible for the root-specific expression conferred by domain A of the CaMV 35S promoter (Benfey *et al.*, 1989). This fragment contains two copies of the TGACG pentamer motif between positions −83 and −63 (as-1 element) and has been shown to bind nuclear factors present in potato, tobacco and pea extracts (Lam *et al.*, 1989; Prat *et al.*, 1989).

Out of 120 000 phage plaques screened, four positive cDNA clones were isolated interacting specifically with the probe. Based on restriction analysis and cross-hybridization studies, these clones were divided into two classes. A first class revealed clone λd11 (Frohberg *et al.*, in preparation) encoding a protein that is 77% identical to the tobacco protein TGA1a (Katagiri *et al.*, 1989). A second class comprising clone λd13, encoding a protein with binding characteristics different from λd11, was selected for further characterization.

Sequence analysis of the 1207 bp λd13 insert showed that this clone did not represent a full-length cDNA. To obtain a complete cDNA clone, the insert sequence of λd13 was used as a probe to rescreen the potato cDNA library. DNA sequence analysis of the longest recombinant clone identified, designated λd13F, demonstrated that it was derived from the same mRNA species as λd13. Clone λd13F, representing an identical, but extended, version of λd13 contains an additional 258 bp at the 5′ end. The nucleotide and deduced amino acid sequences of clone λd13F, containing a cDNA insert of 1465 bp, are shown in Figure 1A. The longest open reading frame starts with an ATG codon at position +246 and encodes a 342 amino acid protein with a predicted molecular weight of 38 kDa. An ATG triplet immediately followed by a stop signal (TAA) is found at position +161, preceding the actual start codon. This feature is shared by the mRNA of a number of regulatory proteins and has been shown to be involved in the translational control of these genes (Lohmer *et al.*, 1993).

MybSt1 is a novel Myb-related protein

Comparison of the D13F amino acid sequence with protein sequences available in the NBRF databank revealed some homology to Myb oncoproteins. Remarkably, this homology is limited to the DNA binding domain of Myb proteins. In contrast to the domain of animal Myb proteins, containing three imperfect repeats of 50–53 amino acids, each with strongly conserved tryptophans separated by 18–19 amino acids, Myb proteins isolated from plants so far include two such repeats (Lüscher and Eisenman, 1990, Avila *et al.*, 1993, Urao *et al.*, 1993). Interestingly, only a single putative Myb repeat is found in the D13F protein, which we therefore renamed MybSt1, for Myb *Solanum tuberosum* 1. The comparison of amino acid sequences of the DNA binding domain repeats of human c-Myb and selected Myb-related proteins from different organisms with that of MybSt1 is presented in Figure 1C. In order to optimize the alignment of the Myb-related region of MybSt1 with the domain repeats, gaps are introduced into the sequences. Accordingly, the putative Myb repeat of MybSt1 between amino acid residues 98 and 158 is most similar to the third repeat of human c-Myb, showing 43% amino acid identity, whereas it shares 33% identity with the third repeat of *Antirrhinum* Myb308.

It should be emphasized that most of the highly conserved amino acid residues identified as important for maintaining the Myb repeat structure are also present within the MybSt1 repeat (Figure 1C). In particular, the conserved tryptophans, characteristic of each Myb repeat, are found in the MybSt1 repeat, with isoleucine replacing the third tryptophan residue.

Outside the Myb repeats, no significant homology was found between the MybSt1 polypeptide and any proteins within the database.

Other interesting features associated with the MybSt1 protein include the presence of an acidic region (amino acid residues 165–222) located next to the Myb-like motif, as well as a proline-rich domain (amino acid residues 226–280) near the C-terminus, and are shown in Figure 1B. In addition, it is interesting to note that the protein MybSt1 contains several serine-rich regions.

Taken together, these results show that MybSt1 does not constitute a typical plant Myb protein, as unlike the known plant Myb homologues with two of the three N-terminal repeats, the *mybSt1* gene encodes only for one repeat of 61 amino acids.

Specific DNA binding activity of MybSt1

Based on the observation that MybSt1 bound to the CaMV 35S promoter domain A in filter binding assays, we were interested in investigating the DNA sequence recognized by the protein MybSt1 in greater detail. For the following DNA binding studies the MybSt1 protein was overexpressed in *Escherichia coli* using the T7 expression system (Studier *et al.*, 1990). Hence, the coding region of *mybSt1* was introduced into the expression vector pET-3b (see Materials and methods). Extracts from IPTG-induced *E.coli* cells overexpressing MybSt1 were partially purified and subsequently assayed for specific DNA binding activity in mobility shift experiments to confirm the specificity of the λ13F-encoded protein. As shown in Figure 2A, a specific retarded band was detected with the MybSt1 extract using the −90 to +2 35S promoter sequence as a probe. This complex formation was abolished by an excess of unlabelled homologous 35S promoter fragment, but not by an unrelated proteinase inhibitor II promoter fragment containing other protein binding sites (Sánchez-Serrano *et al.*, 1990) nor by pUC19 or salmon sperm DNA. These results show that MybSt1 is capable of interacting specifically with a distinct DNA motif present in the CaMV 35S promoter domain A.

In order to localize the sequences involved in protein−DNA interaction, various 35S promoter fragments were used in the binding assays. As shown in Figure 2B, fragments (a−c) extending from −90 to +2, −73 to +2 and −90 to −45 exhibited retarded bands, whereas the −48 to +2 promoter derivative (d) did not show any binding activity. This result indicates that the DNA motif recognized by the MybSt1 protein is localized

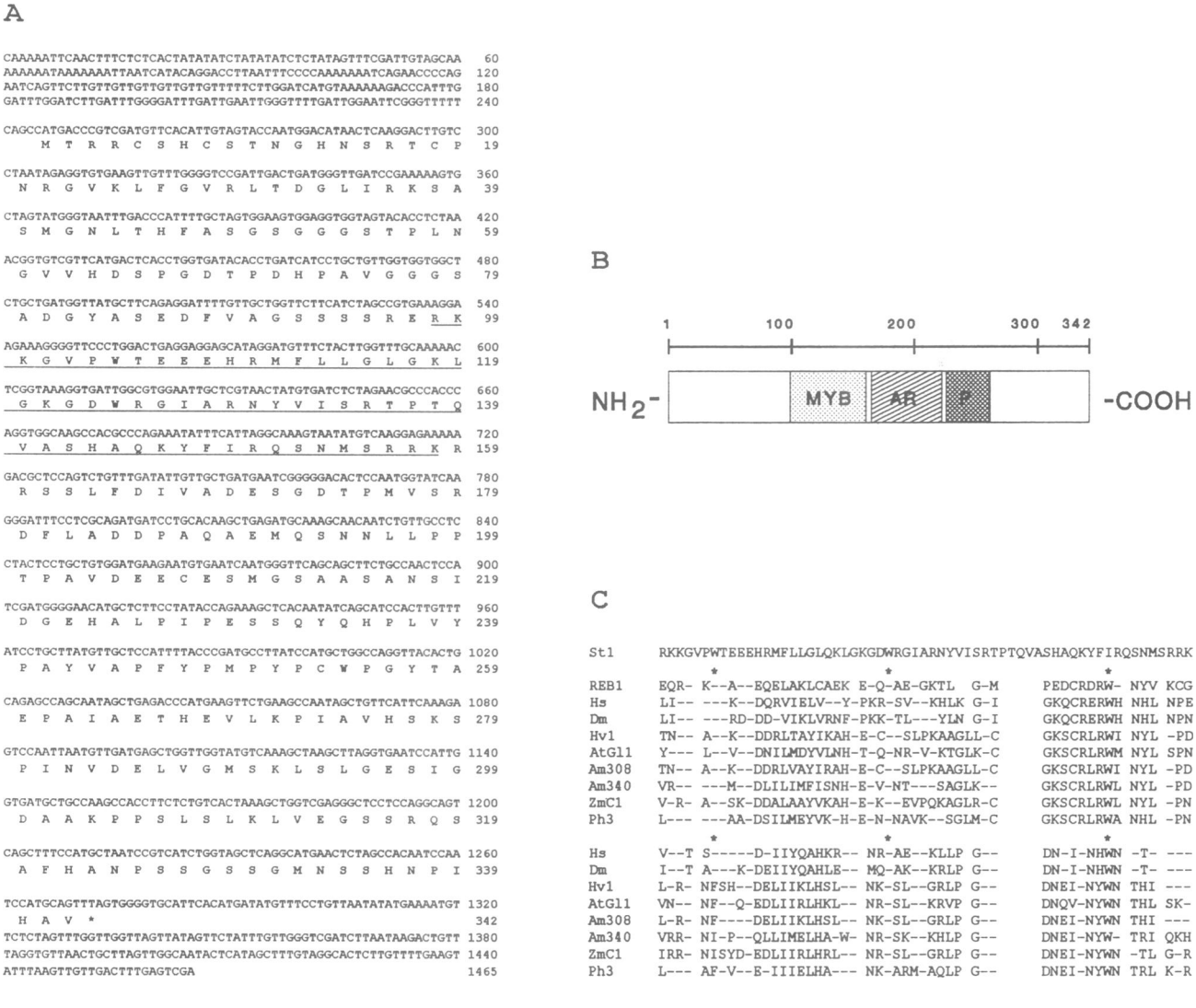

Fig. 1. Characterization of the d13F(mybSt1) cDNA sequence and its encoded D13F(MybSt1) protein. (**A**) Nucleotide sequence of the potato cDNA clone λd13F and its predicted amino acid sequence. Numbering begins with the first nucleotide and amino acid. The amino acid sequence is shown using single letters. The Myb-related sequence is underlined. The 5′ end of the λd13 insert is indicated (#). (**B**) Schematic representation of the 342 amino acid residues of the MybSt1 protein with the proline-rich (P) and the acidic-rich (AR) regions and the Myb-like motif (MYB). (**C**) Comparison of the amino acid sequence of MybSt1 with the repeats of the DNA binding domain from c-Myb and selected Myb-related proteins. The MybSt1 peptide sequence (residues 98–158) was aligned with the second (top) and third (bottom) Myb repeat from human c-Myb (Hs; Majello *et al.*, 1986), *Drosophila* (Dm; Katzen *et al.*, 1985), barley Hv1 (Hv1; Marocco *et al.*, 1989), *Arabidopsis* GL1 (AtGl1; Oppenheimer *et al.*, 1991), *Antirrhinum* Myb308 and Myb340 (Am308 and Am340; Jackson *et al.*, 1991), maize (ZmC1; Paz-Ares *et al.*, 1987), *Petunia* MYBPh3 (Ph3; Avila *et al.*, 1993) and with the Myb-related repeat from yeast REB1 (REB1; Ju *et al.*, 1990). Identical amino acids are designated by dashes. Gaps are introduced to maximize alignment. Asterisks mark the positions of the recurrent tryptophan residues. Note that the alignment constructed by the CLUSTAL program (Higgins and Sharp, 1988) was optimized by visual inspection.

within the sequence spanning positions −73 to −48 of the CaMV 35S promoter.

To determine the MybSt1 binding site, guanosine methylation interference experiments were performed. This analysis revealed an interference pattern centred over a GGATG pentamer sequence within the as-1 motif (Figure 2C). Methylated G residues at positions −73 and −72 strongly interfered with MybSt1 protein binding and were therefore under-represented in the bound DNA. The G residue at position −69, showing weak interference, seems to be less critical for MybSt1 binding. In contrast, no detectable binding interference could be seen on the lower strand (data not shown). Mutant oligonucleotides with substitution of the G residues in this pentamer motif were no longer recognized by the protein MybSt1, confirming the importance of the G residues within the potential MybSt1 binding site (data not shown).

The Myb-related motif of MybSt1 is required for DNA binding

To map the region of MybSt1 that is responsible for the specific DNA interaction, a series of deletions was made corresponding to the N- and C-terminal regions of the MybSt1 protein. Four different constructs were generated (Figure 3A): two C-terminal truncations, expressing amino acids 1–293 (MybSt1EH) and 1–219 (MybSt1EC) respectively, an internal fragment comprising amino acids 71–219 (MybSt1BC) and finally an N-terminal deletion, encompassing amino acids 220–342 (MybSt1CHc) but lacking the Myb-related domain. After expression in

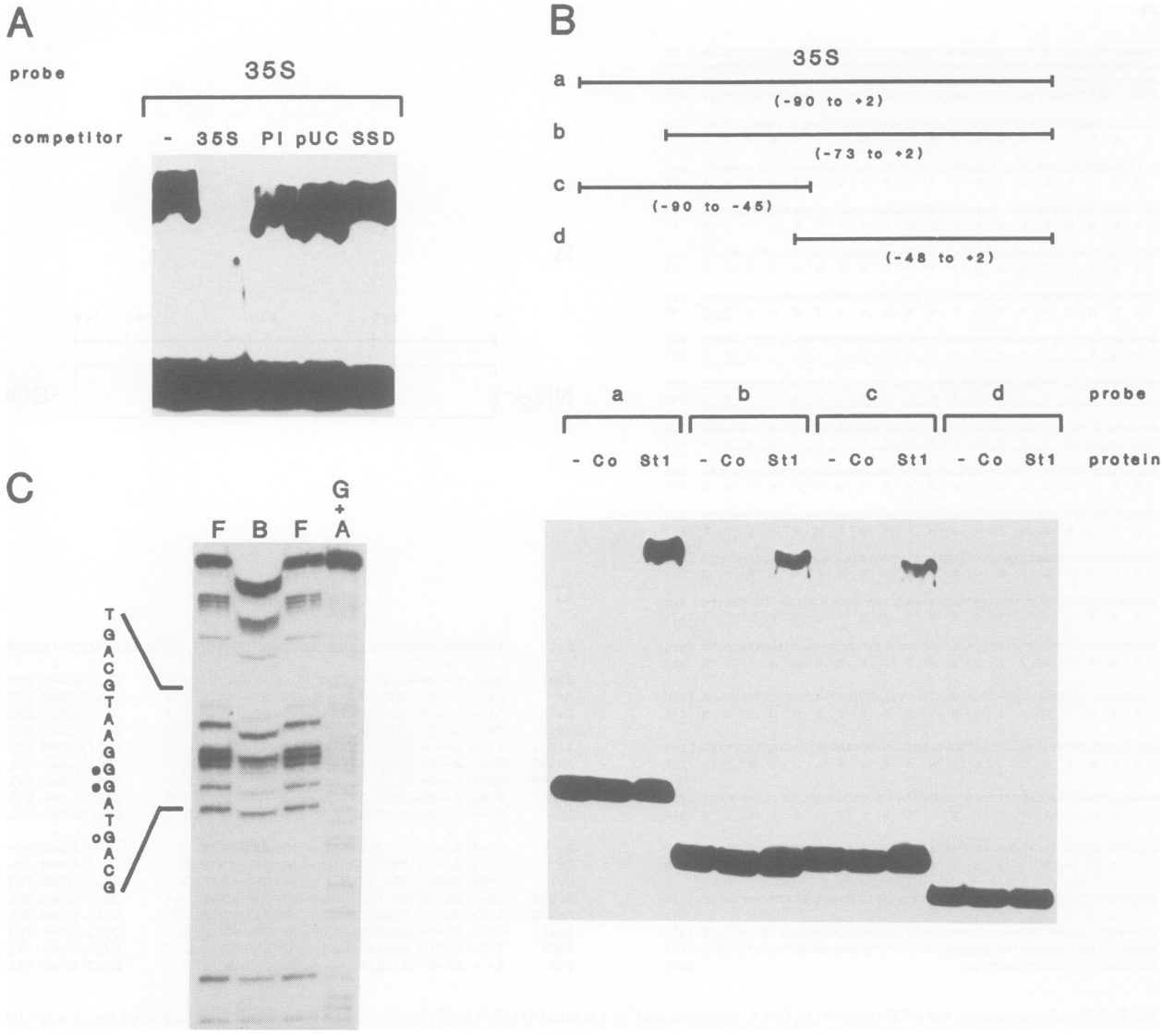

Fig. 2. Sequence-specific binding of MybSt1 to the CaMV 35S promoter domain A. (**A**) Competitive binding assay. The ^{32}P-labelled −90 to +2 CaMV 35S promoter fragment was incubated with 30 μg protein extract from *E.coli* overexpressing MybSt1 under the control of the T7 polymerase promoter. Where indicated, unlabelled homologous (35S) or non-homologous [pUC19 DNA (pUC), salmon sperm DNA (SSD), proteinase inhibitor II promoter (PI)] competitor DNAs were included in the binding reaction at a 500-fold molar excess. (**B**) Identification of the MybSt1 protein binding sequence on the CaMV domain A. Top: schematic representation of the 35S promoter (−90 to +2) and its subfragments used as DNA probes (a−d) in the binding studies. The positions of the fragments, relative to the transcription start site, are indicated. Bottom: the protein extract from *E.coli* overexpressing the MybSt1 protein (St1) and the control protein extract from *E.coli* harbouring the vector alone (Co) were incubated with the ^{32}P-labelled fragments (a−d) of the 35S promoter and subjected to gel retardation assay. −, radiolabelled promoter fragments in the absence of protein extracts. (**C**) Methylation interference analysis for critical G residues within the potential MybSt1 binding site of domain A. The partially methylated −90 to +2 fragment, which was ^{32}P-end-labelled on the upper strand, was incubated with MybSt1 protein extract and subjected to preparative gel retardation assay. Free (F) and bound (B) fractions were isolated, cleaved at methylated G residues and analysed on a polyacryamide−urea gel. G+A, marker. Methylated G residues strongly (●) or weakly (○) interferring with MybSt1 binding are indicated.

E.coli, these truncated proteins were partially purified and used to identify the DNA binding domain of MybSt1 in mobility shift assays with the 35S promoter fragment as a probe. As summarized in Figure 3, DNA binding activity was observed with the full-length protein MybSt1, the C-terminal truncated derivates MybSt1EH and MybSt1EC and with the N- and C-terminal deleted protein MybSt1BC, but not with the N-terminal truncated MybSt1CHc, where the Myb motif is missing. These results define a 149 amino acid region (residues 71–219) responsible for MybSt1 binding to DNA. Indeed, this region contains the 61 amino acid motif bearing substantial homology to the Myb DNA binding domain.

Selection of high-affinity binding sites for MybSt1

In the filter binding assays, the MybSt1 protein bound poorly to the as-1 motif in comparison with the D11 protein (the D11 protein is homologous to the tobacco protein TGA1a, recognizing specifically the TGACG repeat within the as-1; Frohberg *et al.*, in preparation; Katagiri *et al.*, 1989). This observation might indicate that either (i) MybSt1, in contrast to D11/TGA1a, binds to DNA with low affinity or (ii) the GGATG sequence within the CaMV 35S promoter (as-1) is not the actual target of the MybSt1 protein. To identify DNA sequences with a higher affinity for MybSt1, we decided to use the random binding site selection method (Blackwell and Weintraub,

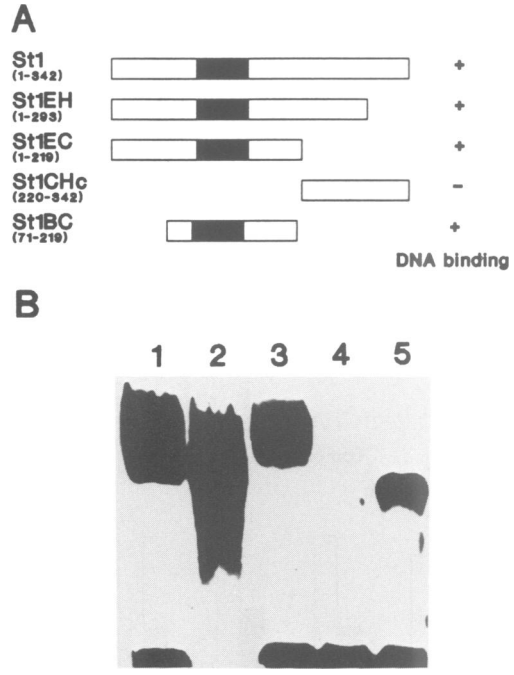

Fig. 3. DNA binding activity of full-length MybSt1 protein and truncated derivates. (**A**) Schematic representation of the constructs employed in the binding studies in (B) for localization of the DNA binding domain of MybSt1 protein. DNA binding activities and amino acid residues (in brackets) of various MybSt1 proteins are shown. The region (amino acids 98–158) bearing some homology to Myb proteins is shown in black. (**B**) The coding sequence of MybSt1 and of truncated versions were inserted behind the T7 promoter in the *E.coli* expression vectors pET-3c or pET-3a respectively and the resulting protein extracts were analysed by gel retardation assay. The ^{32}P-labelled −90 to +2 fragment of the 35S promoter was used as the probe for DNA binding activity. Lane 1, MybSt1EH (St1EH; amino acids 1–293); lane 2, MybSt1EC (St1EC; amino acids 1–219); lane 3, MybSt1, full-length protein (St1; amino acids 1–342); lane 4, MybSt1CHc (St1CHc; amino acids 220–342); lane 5, MybSt1BC (St1BC; amino acids 71–219).

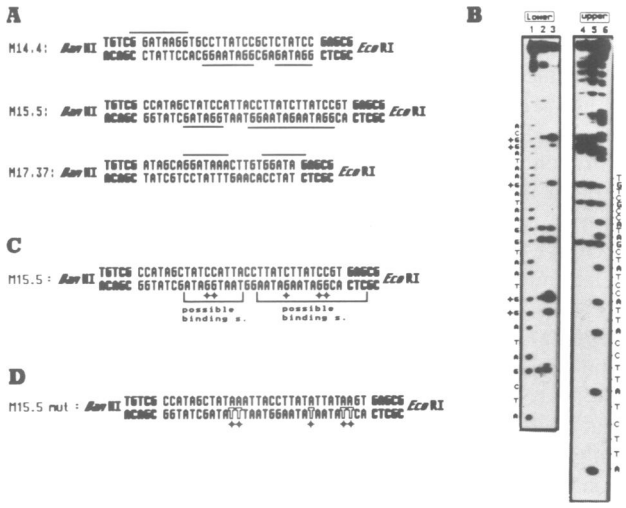

Fig. 4. (**A**) Sequences of the three best binding oligonucleotides selected by the random binding site selection method for the MybSt1 protein. Bold letters represent the fixed nucleotide positions flanking the randomized ones. Similar/identical stretches within the randomized positions are marked with bars. (**B**) Methylation interference analysis carried out with the M15.5 oligonucleotide using an extract of *E.coli* overexpressing the MybSt1 protein. Each group of three lanes corresponds to the cleavage products of one of the strands of the methylated DNA probe. 1: G+A reaction on the lower strand (marker), 2: probe bound by MybSt1, 3: free probe, 4: free probe, 5: probe bound by MybSt1, 6: G+A reaction on the upper strand (marker). Stars indicate the G residues which are critical for binding of the MybSt1 protein. (**C**) Sequence of the probe used in (A). Stars indicate the critical G residues for MybSt1 binding defined by the methylation interference assay. The potential binding sites of MybSt1 are marked with brackets. (**D**) Sequence of the mutagenized M15.5 oligonucleotide used for retardation and transient expression experiments. Critical G residues are changed into T residues.

1990; Thiesen and Bach, 1990; Ekker *et al.*, 1991; Sun and Baltimore, 1991; Kispert and Herrmann, 1993). This technique is based on the specific binding of the protein to its preferred target sites among a large pool of randomized DNA sequences and an enrichment of these in every cycle of the procedure. As a source of protein we used partially purified extracts of *E.coli* overexpressing the MybSt1 protein. After the normal mobility shift assay binding procedure, the protein−DNA complex was immunoprecipitated by adding an appropriate amount of anti-MybSt1 antiserum and protein A−Sepharose. The immunoprecipitated oligonucleotides were amplified via the polymerase chain reaction and used as the pool of DNA for the next round of selection. Seven cycles of selection were performed, including two mobility shift assays to separate bound and non-bound oligonucleotides. The relative enrichment in every cycle was controlled by calculating the ratio between the amount of offered and immunoprecipitated oligonucleotides. At the end of this selection procedure the pool of oligonucleotides was cloned and thirty subclones were individually assayed for protein binding in mobility shift assays. Oligonucleotides bound with the highest affinity by MybSt1 were sequenced and used for competition experiments using the as-1 element as a competitor.

As estimated from competitive DNA binding studies, three oligonucleotides revealed a 20–30 times higher binding affinity for the MybSt1 protein compared with the as-1 element (data not shown) and contained the sequences shown in Figure 4A.

To define the MybSt1 binding sites within these oligonucleotides, one of them (M15.5) was used for G methylation interference assay (Figure 4B).

The methylation interference assay identified five clearly protected G residues on the lower strand in the randomized part of the M15.5 oligonucleotide. This could be due to two separate binding sites for the MybSt1 protein (Figure 4B), the first of them being a GGATAAG heptamer, the second resembling a truncated version of the first one and consisting of its first five nucleotides. This interpretation is supported by the fact that both of these sequences are present at least three times in the oligonucleotides displaying the highest binding affinities obtained in the random oligonucleotide selection assay (Figure 4A).

Mutations in this putative binding site were introduced, altering those G residues identified by methylation interference (Figure 4C), and their effect on binding to the MybSt1 protein was tested in mobility shift assays. Mutation of these G residues reduced binding of MybSt1 significantly (data not shown), confirming the results from the methylation interference experiment.

It should be pointed out that the MybSt1 binding site within the as-1 element, determined by methylation interference (Figure 2C), which differs in only one nucleo-

tide (GGATG instead of GGATA), possesses a 20–30 times lower binding affinity, estimated from competition experiments (data not shown), as compared with the oligonucleotide M17.37 (Figure 4A), containing two copies of the GGATA binding site.

Taken together, these results suggest that the MybSt1 protein appears to be a sequence-specific DNA binding protein, with the highest affinity *in vitro* exerted towards a GGATA motif, identified by the random oligonucleotide selection assay.

MybSt1 is a transcriptional activator

The presence of two potential activator domains within the MybSt1 protein (Figure 1B), together with the finding that MybSt1 is a sequence-specific DNA binding protein, suggested that MybSt1 might be able to activate transcription of a minimal promoter when fused to one of the three highest affinity MybSt1 binding sites described above. Each of the three high-affinity oligonucleotides were individually cloned in both directions [position −56 (*Spe*I) or −64 (*Hpa*I) relative to the transcriptional start site] into the pDoAHpaGUS plasmid containing the CaMV 35S TATA sequence in front of the *GUS* reporter gene (Frohberg *et al.*, 1991; Figure 5A). Two kinds of negative controls were used, either a plasmid containing a −64Δ35S minimal promoter in front of the *GUS* gene, designated pDoKGUS, or the M15.5 mut oligonucleotide fused in both orientations to a minimal promoter *GUS* construct (Figure 5A). For expression of the MybSt1 protein, its coding region was cloned into the pA7 plasmid between the CaMV 35S promoter and the OCS terminator (see Materials and methods). The resulting plasmid, designated pMybSt1, was co-transformed with the reporter constructs into potato leaf-derived protoplasts.

In the case of potato protoplast transformation, the use of constructs containing functional MybSt1 binding sites resulted in a 5–7 times higher GUS activity as compared with a construct containing no high-affinity binding sites (pDoKGUS; Figure 5B). Co-transformation of MybSt1 produced only a slight increase in GUS expression if large amounts of the effector plasmid (100 or 150 μg) were used, which might indicate that the MybSt1 protein is already present in saturating amounts in potato protoplasts. The orientation of the oligonucleotide elements relative to the minimal promoter had a minor influence on expression levels in potato (Figure 5B) as well as in tobacco protoplasts (data not shown). For further experiments the corresponding plasmid preparations were used as a 1:1 mixture.

To prove that the high promoter activity of the constructs containing the high-affinity MybSt1 binding sites was in fact due to this protein, leading to increased transcription of the *GUS* gene, we decided to change to a heterologous expression system, which should be devoid of this protein. Since we knew from Southern blot analysis that there is no closely related gene to mybSt1 present in tobacco (data not shown), tobacco leaf protoplasts were used as a source for the transformation experiments.

Figure 5C represents a typical result of a series of transient expression experiments carried out with tobacco protoplasts. The endogenous GUS activity of the reporter constructs containing the MybSt1 binding sites was approximately eight times lower as compared with potato

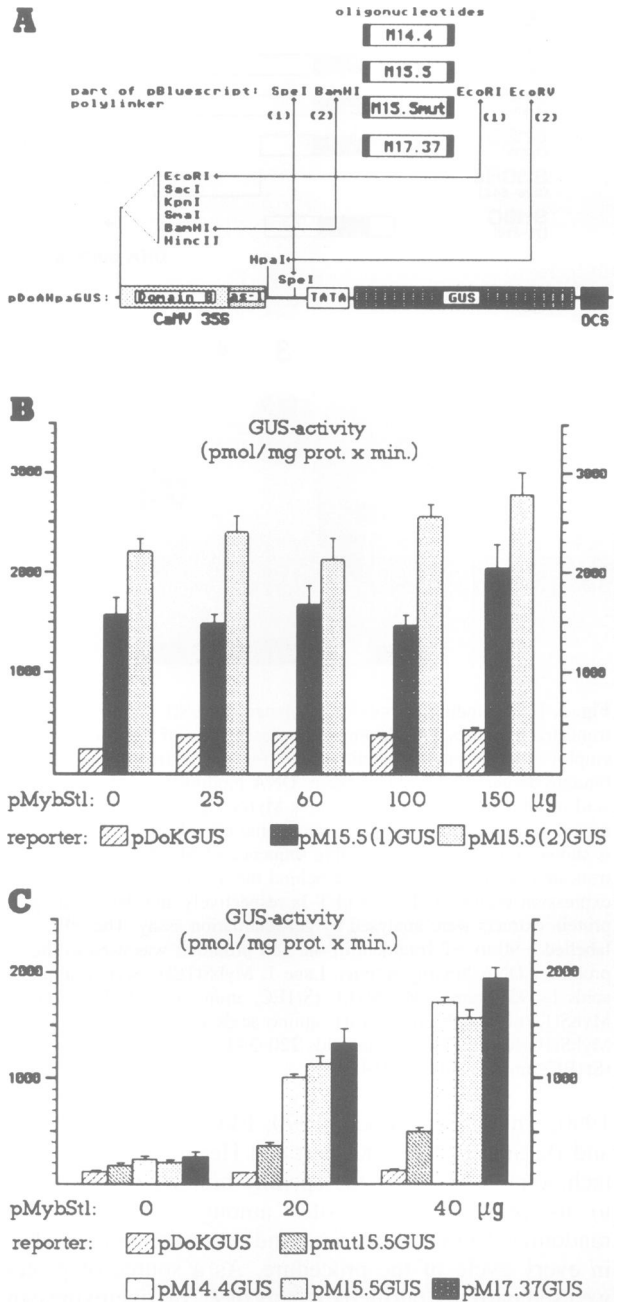

Fig. 5. (**A**) Scheme of the cloning procedure to create fusions of the three high-affinity oligonucleotides for MybSt1 and the mutagenized version of M15.5 to a CaMV 35S minimal promoter. High-affinity oligonucleotides were cloned as *Eco*RI/*Spe*I fragments [orientation (1)] or *Bam*HI/*Eco*RV fragments [orientation (2)] into pDoAHpaGUS. Resulting constructs were designated according to the inserted oligonucleotide and its orientation: pM14.4(1)GUS, pM14.4(2)GUS, pM15.5(1)GUS, pM15.5(2)GUS, pM15.5mut(1)GUS, pM15.5mut(2)GUS, pM17.37(1)GUS and pM17.37(2)GUS. The −64Δ35SGUS negative control, resulting from *Sma*I/*Hpa*I cleavage and religation of pDoAHpaGUS, was designated pDoKGUS. (**B**) Typical result of a transient expression experiment in potato leaf protoplasts. Forty micrograms of each reporter construct was transformed simultaneously with increasing amounts of the effector plasmid pMybSt. GUS measurements were done on transformed protoplasts after 48 h incubation. (**C**) Typical result of a series of transient expression experiments in tobacco protoplasts. Forty micrograms of each reporter construct (1:1 mixture of both orientations) were co-transformed with two different amounts of the effector plasmid pMybSt1. GUS measurements on protein extracts of transformed protoplasts were performed after 48 h incubation according to Jefferson (1987).

protoplasts, but nearly reached the same activity upon co-transformation with pMybSt1, indicating that co-expression of the MybSt1 protein leads to an approximately 8-fold activation of these constructs. This activation is clearly due to high-affinity binding of the MybSt1 protein, because co-expression of pMybSt1 and pmut15.5 GUS resulted only in a 3-fold activation, probably due to the low remaining binding affinity of this oligonucleotide, observed in mobility shift assays (data not shown). This interpretation is supported by the finding, that the −64 CaMV 35S minimal promoter activity is not stimulated at all upon co-transformation with pMybSt1 (Figure 5C).

For a different series of transient expression studies the deletion MybSt1BC (149 amino acids), which lacks the complete proline-rich region but is still able to bind DNA (Figure 3), was cloned into the pA7 expression vector (see Materials and methods). Co-expression of this truncated MybSt1 protein with the reporter constructs in tobacco leaf protoplasts did not result in any increase in GUS activity. Furthermore, parallel expression of this truncated protein with the MybSt1 full-length protein, using the same amounts of both corresponding plasmids, resulted in a 2-fold reduction in activation compared with co-expression of the reporter constructs and MybSt1 alone (data not shown).

Genomic organization and expression pattern of the mybSt1 gene

The *mybSt1* gene was further characterized by Southern and Northern hybridizations. Southern blot analysis showed a simple hybridization pattern, suggesting that *mybSt1* is a single or low copy gene family (data not shown).

RNA blot analysis using total RNA from different tissues of the potato plant showed that *mybSt1* mRNA is expressed in all examined organs of the potato, an elevated amount of mRNA being detected in buds, flowers and roots grown in tissue culture (data not shown).

Discussion

mybSt1 encodes a novel Myb-related protein from potato

Myb-related proteins have been found in different species, including mammals, insects, yeast and plants (Katzen *et al.*, 1985; Nomura *et al.*, 1988; Tice-Baldwin *et al.*, 1989; Avila *et al.*, 1993). These proteins share a highly conserved region at the N-terminus consisting of two to three imperfect repeats that correspond to the DNA binding domain. Each repeat is characterized by three conserved tryptophan residues repeated every 18–19 amino acids, which were postulated as playing a critical role in the structure and function of the Myb DNA binding domain (Anton and Frampton, 1988; Saikumar *et al.*, 1990; Frampton *et al.*, 1991).

The MybSt1 protein described here represents no characteristic plant Myb homologue, because, in contrast to known plant Myb proteins with two repeats, the *mybSt1* gene product seems likely to contain only a single repeat, although the third tryptophan residue is replaced (Figure 1C). Furthermore, the Myb-like repeat of MybSt1 shows greater homology with the third repeat of human c-Myb than with the domain repeats of plant Myb-related proteins reported so far (Avila *et al.*, 1993; Leech *et al.*, 1993; Urao *et al.*, 1994). According to mutagenesis analyses, substitution of the last tryptophan in the third repeat of c-Myb by another aromatic or hydrophobic amino acid preserves the helical character of this repeat and therefore does not influence the c-Myb DNA binding activity (Saikumar *et al.*, 1990). Replacement of the third tryptophan in the last repeat is also observed in the $cdc5^+$ gene product from yeast (Ohi *et al.*, 1994), whereas in all plant Myb homologues described to date the first tryptophan of the last repeat is substituted (Avila *et al.*, 1993). Besides the tryptophans, other highly conserved residues are maintained in the proposed Myb repeat of MybSt1 (Figure 1C). These conserved positions, mostly hydrophobic in character, have been postulated to be important for the formation of a helix−turn−helix structure (Frampton *et al.*, 1989, 1991). Moreover, basic amino acids at the end of the third Myb repeat, suggested as being involved in direct DNA interaction, are also present in the MybSt1 repeat.

Each repeat of the Myb DNA binding domain includes three α-helices, whereby the second (α2) and third (α3) of these helices have been proposed to form a helix−turn−helix structure (Frampton *et al.*, 1989, 1991; Ogata *et al.*, 1992). Secondary structure predictions for the Myb-like domain (amino acids 98–158) of MybSt1 suggest the presence of two α-helices separated by a long sequence with helix breaking residues (Chou and Fasman, 1978). This turn region might be expected to tolerate mutations to a much higher extent than the helical regions.

In addition, the MybSt1 protein sequence contains an acidic central region and a proline-rich C-terminal domain. Such structural motifs are found in many regulatory proteins and have been implicated in transcriptional activation (Ptashne, 1988). The C-terminal acidic domain of the maize C1 protein has been shown to acivate transcription in plants (Goff *et al.*, 1991). The proline-rich domains of the mammalian factors CTF-1 and AP-2 have been demonstrated to be required for transcriptional activation (Mermod *et al.*, 1989; Williams and Tjian, 1991). Likewise, the proline-rich N-terminal domain of the plant bZIP protein GBF1 was able to stimulate transcription in plant protoplasts and mammalian cells when linked to the DNA binding domain of GAL4 (Schindler *et al.*, 1992). Indeed, we also show that the proline-rich region located in the C-terminus of the protein MybSt1 functions as a transcriptional activation domain in plant protoplasts. Furthermore, the high serine content of the MybSt1 protein is notable (13.45%), with serine-rich segments particularly in the N- and C-termini that could be sites for phosphorylation. Similarly to c-Myb (Lüscher *et al.*, 1990), phosphorylation at these serine residues could inhibit DNA binding activity of the protein and therefore provide a possible post-translational regulation of MybSt1 activity.

Taken together, these results suggest that although the *mybSt1* gene product is distantly related to plant and animal Myb proteins, it still shares a clear structural similarity with this group of regulatory proteins.

DNA binding properties of MybSt1

MybSt1 specifically recognizes a target sequence located between positions −73 and −48 of the CaMV 35S promoter, the single GGATG pentamer sequence motif, at

positions −73 to −69 (relative to the transcription start site) being critical for MybSt1 binding. Interestingly, this target sequence GGATG is located between the two TGACG pentamer repeats (as-1) bound by the tobacco protein TGA1a (Katagiri *et al.*, 1989), with the G residue at position −69 overlapping the second pentamer motif.

By testing a series of 5′ and 3′ protein deletions we showed that an N-terminal truncated protein, lacking the Myb-like motif, had lost its DNA binding capacity, whereas the 3′ and 5′ deletions containing the Myb-related region (amino acids 98–158) retained specific DNA binding. These results demonstrate that the Myb-like motif within the 149 amino acid domain (residues 71–219) is required for DNA binding.

Repeats 2 and 3 of the Myb domain have been shown to be necessary for DNA binding in animal as well as in plant Myb proteins described so far (Howe *et al.*, 1990; Urao *et al.*, 1993), with repeat 3 seeming to be responsible for the sequence specificity (Ogata *et al.*, 1992; Tanikawa *et al.*, 1993). DNA binding domains consisting of less than two repeats were also found in yeast and *Drosophila*. REB1, a yeast DNA binding protein, appears to have one and a half repeats, however, this Myb-related region alone does not show binding activity. Only together with another separated second region, which has a faint resemblance to the Myb domain, a functional bipartite DNA binding domain can be formed (Ju *et al.*, 1990; Morrow *et al.*, 1993). The *Drosophila* transcription factor Adf1 has some homology to a portion of the Myb repeat, though its DNA binding domain remains to be defined (England *et al.*, 1992).

It should be pointed out that although MybSt1 contains only a single conserved Myb repeat, it exhibits specific DNA binding activity.

MybSt1 requires a GGATA-containing sequence for high-affinity binding

Using the random binding site selection assay we determined DNA sequences that were preferentially bound by MybSt1. The identified high-affinity binding sites, all containing a GGATA motif, differ by only one nucleotide substitution from the target site GGATG in the CaMV 35S promoter.

The results from the random binding site selection assay clearly establish the requirement for a GGATA motif for high-affinity binding of MybSt1. It is noteworthy that this DNA sequence is largely different from the PyAAC-(G/T)G recognition sequence bound by animal c-Myb proteins (Biedenkapp *et al.*, 1988; Howe *et al.*, 1990), but shows some similarity to consensus sequences identified for two plant Myb proteins (Grotewold *et al.*, 1994; Sablowski *et al.*, 1994). Such divergent recognition sequences may be expected if we compare the relatively dissimilar structure of the DNA binding domain of these proteins. Actually, whereas the functional DNA binding domain of animal as well as plant Myb proteins requires repeats 2 and 3 (Howe *et al.*, 1990; Tanikawa *et al.*, 1993; Urao *et al.*, 1993) only a single Myb repeat appears to be involved in sequence-specific DNA binding by the potato factor MybSt1.

MybSt1 acts as a transcriptional activator

In transient expression experiments using plant protoplasts we could demonstrate that the potato factor MybSt1 activates transcription of the minimal CaMV 35S promoter fused to the identified high-affinity binding sites, and that MybSt1 does not stimulate transcription of the minimal promoter alone.

More interestingly, transient expression studies with a MybSt1 deletion (MybSt1BC) lacking the proline-rich region, but still containing the Myb-related domain and most of the acidic region, did not result in any stimulation of transcription, thus indicating that this proline-rich region is required for transcriptional activation. Such proline-rich sequences have been shown to act as transcriptional activator domains in mammalian cells and plant protoplasts (Mermod *et al.*, 1989; Williams and Tjian, 1991; Schindler *et al.*, 1992).

Taken together, these results point to a modular structure of the MybSt1 protein, which is shared with many other transcriptional activators isolated so far (Mitchell and Tjian, 1989; Latchman, 1991). Whereas the Myb-like motif in the central part of the protein MybSt1 seems to be involved in specific DNA binding, the C-terminal proline-rich segment of MybSt1 functions as a transcriptional activation domain.

Possible role for MybSt1

The fact that the MybSt1 protein factor was isolated using the −90 to +2 CaMV 35S promoter as a probe might raise the question of a possible functional role for MybSt1 in the tissue-specific expression conferred by this promoter fragment.

Functional analysis of the 35S promoter has identified a TGACG pentamer repeat in the −83 to −63 region (as-1) as being responsible for preferential expression in roots (Lam *et al.*, 1989). A tobacco protein, TGA1a, has been shown to bind specifically to the as-1 motif of the CaMV 35S promoter (Katagiri *et al.*, 1989).

On the other hand, methylation interference analysis showed that MybSt1 binds to a GGATG target sequence within the as-1 element, which clearly overlaps with the second TGACG motif. This superposition of the different binding sites would be expected to result in mutually exclusive binding of both proteins and therefore in competition for DNA binding. In gel retardation analyses, where protein extracts containing either MybSt1 or D11 (a TGA1a homologue; Frohberg *et al.*, in preparation) were mixed and incubated with the CaMV 35S probe, no further retarded complex was detected (data not shown). Consequently, the MybSt1 and D11/TGA1a proteins were not able to bind simultaneously to the as-1 motif. This overlap of binding sites might be functionally significant, as occupancy of both TGACG repeats is a prerequisite for as-1 activity. We could therefore think of MybSt1 as being a negative regulator of D11/TGA1a. From South Western screening it was evident that, in contrast to the D11 protein, MybSt1 binds with very low affinity to the potential target sequence within the CaMV 35S promoter, indicating D11/TGA1a to be the better candidate for 35S promoter activity.

In plants there is evidence for the existance of extensive *myb*-related gene families. Transcription factors containing a Myb domain have been reported in a variety of plants, such as maize (Paz-Ares *et al.*, 1987; Grotewold *et al.*, 1991), barley (Marocco *et al.*, 1989), *Antirrhinum* (Jackson *et al.*, 1991), *Arabidopsis* (Oppenheimer *et al.*, 1991;

Shinozaki *et al.*, 1992; Urao *et al.*, 1993), *Petunia* (Avila *et al.*, 1993) and *Physcomitrella* (Leech *et al.*, 1993). However, aside from the maize genes *C1*, *Pl* and *P*, which are involved in anthocyanin and phlobaphene biosynthesis (Paz-Ares *et al.*, 1987; Grotewold *et al.*, 1991, 1994) and the *Arabidopsis* gene *GL1*, which is required for leaf trichome differentiation (Oppenheimer *et al.*, 1991), the function of other plant *myb*-related genes remains to be determined.

We are currently investigating the regulatory role of the *myb*-related gene *mybSt1* containing only a single repeat by studying its expression in transgenic plants which overexpress *mybSt1*.

Materials and methods

South Western screening

A potato leaf-specific λgt11 cDNA library was screened with the multimerized −90 to +2 fragment of the CaMV 35S promoter as a probe using the procedure of Singh *et al.* (1988). From 120 000 recombinant phages, four potential positive clones were obtained. To confirm the specificity of the obtained putative positive plaques, the second screen was repeated with another multimerized, non-homologous promoter fragment (PI-II, data not shown). The multimers (containing three copies of the −90 to +2 promoter fragment) used as probes were gel-purified and afterwards radiolabelled using a random primed DNA labelling kit (Amersham).

Among these positive clones, clone λd13 was selected, its cDNA insert subcloned as a *Bam*HI fragment into the *Bam*HI site of pGEM4 (Promega) and sequenced by the dideoxy chain terminating method using T7 polymerase (Pharmacia). The full-length cDNA clone λd13F was isolated by plaque hybridization of the same library using the partial λd13 insert as a probe. The obtained full-size d13F cDNA insert was also subcloned as a *Bam*HI fragment into the *Bam*HI site of pGEM4, resulting in pG13F (pGSt1), and sequenced as above.

Plasmid constructs and their expression in E.coli

We used the T7 RNA polymerase expression system (Studier *et al.*, 1990) to produce the D13F protein, renamed MybSt1, and its truncations. To construct the plasmid pETSt1 (overexpressing the full-length MybSt1 protein) the *Eco*RI/*Hin*cII fragment of pGSt1, containing the entire MybSt1 coding region, was filled in with Klenow fragment and cloned behind the T7 promoter into the *E.coli* expression vector pET-3c at the *Bam*HI site, previously blunted. The plasmid pETSt1EH (overexpressing the C-terminal truncated protein MybSt1EH) was constructed by inserting the blunted *Eco*RI/*Hin*dIII fragment of pGSt1 into the blunted *Bam*HI site of pET-3c. The plasmid pETSt1EC (overexpressing the C-terminal truncated protein MybSt1EC) was constructed by inserting the blunted *Eco*RI/*Cla*I fragment of pGSt1 into the blunted *Bam*HI site of pET-3c. The plasmid pETSt1BC (overexpressing the N- and C-terminal truncated protein MybSt1BC) was constructed by inserting the blunted *Bcl*I/*Cla*I fragment of pGSt1 into the blunted *Bam*HI site of pET-3c. The plasmid pETSt1CHc (overexpressing the N-terminal truncated protein MybSt1CHc) was constructed by inserting the blunted *Cla*I/*Hin*cII fragment of pGSt1 into the blunted *Bam*HI site of pET-3a.

For expression and partial purification of the recombinant proteins, the *E.coli* strain BL21(DE3) was transformed with the expression plasmids described above and, as a control, with the pET vector alone. Cells containing the different plasmids were grown to an OD_{600} of 0.5 and then incubated with IPTG (final concentration 2 mM) for an additional 2 h. After harvesting, the induced cells were washed and resuspended in an extraction buffer (volume 1/100) containing 50 mM Tris−HCl, pH 8, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.1% Nonidet P-40. The suspension was frozen in liquid nitrogen and thawed slowly on ice. Lysozyme was added to a final concentration of 0.5 mg/ml. After incubation for an additional 15 min on ice, the solution was sonicated twice for 15 s to ensure complete lysis. Potassium chloride was added to a final concentration of 0.5 M and the mixture was stirred for 15 min at 4°C, followed by centrifugation for 30 min (15 000 *g*, 4°C), ammonium sulfate was slowly added (to 60% saturation) to the supernatant for protein precipitation. After centrifugation for 1 h (15 000 *g*, 4°C), the pellet was resuspended in 1 ml dialysis buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 15% glycerol) with 2 μg/ml each of antipain, leupeptin, pepstatin A, chymostatin and aprotinin and dialyzed against this buffer for 2–3 h. The extracts were centrifuged in a microcentrifuge for 5 min. The supernatants were aliquoted, frozen in liquid nitrogen and stored at −70°C. After analysis by SDS−PAGE and Western blotting (data not shown) these partially purified protein extracts were then directly used for binding studies.

DNA binding assays

Gel retardation assays were performed essentially according to Prat *et al.* (1989) with minor modifications. Protein extracts (30 μg) were incubated with ^{32}P-end-labelled DNA fragments (about 1 ng) in the presence of 4 μg poly(dI−dC) in a final volume of 30 μl. The binding mixtures were separated in native polyacrylamide gels (19:1, 10% glycerol, 0.05 mM DTT, 0.5 mM $MgCl_2$) at 4°C for ~3–4 h.

The different DNA fragments of the CaMV 35S promoter used in these binding assays as probes were excised from distinct plasmids with appropriate restriction enzymes, end-labelled by fill-in reaction using T4 DNA polymerase and purified by polyacrylamide gel electrophoresis.

Methylation interference assay

Methylation interference was performed according to Baldwin (1988).

Protoplast transformation

Reporter constructs for protoplast transformation (Figure 5) were based on pDoAHpaGUS, which contains a *Spe*I restriction site (at position −56) and a *Hpa*I restriction site (at position −64) between the TATA box and the as-1 element of the CaMV 35S promoter (Frohberg *et al.*, 1991) fused to the *GUS* reporter gene and the octopine synthase terminator. Upstream of the CaMV 35S promoter a truncated pUC19 polylinker (from *Eco*RI to *Sal*I) is located, which was also used for insertion of the oligonucleotides. The reporter construct, pDoKGUS, containing the −64 CaMV 35S minimal promoter, was constructed by digestion of pDoAHpaGUS with *Sma*I/*Hpa*I and religation. The effector plasmid contained the complete MybSt1 coding region, inserted as a *Eco*RI(blunted)/*Bam*HI fragment into the polylinker of pA7, which is a pUC18-derived vector and contains the CaMV 35S/OCS cassette. The truncated MybSt1 coding region (MybSt1BC) was cloned as a *Nde*I-(blunted)/*Bam*HI fragment from pETSt1BC into the *Sma*I/*Bam*HI restriction sites of the pA7 polylinker.

Protoplasts from *Solanum tuberosum*, cv. Désirée, and *Nicotiana tabacum*, cv. Samsun NN., were prepared and transformed based on the protocol of Damm and Willmitzer (1989). Forty micrograms of respective reporter plasmids and 10–150 μg effector plasmid were introduced into 500 000 protoplasts. In addition, an appropriate amount of pBluescript-KS(+) was used as a carrier, to adjust the total amount of plasmid DNA used for each transformation within one experiment. After 48 h incubation in B5 medium (Gamborg *et al.*, 1968), pH 5.7, including 72.6 g/l glucose at 25°C in the dark, the transformed protoplasts were lysed by three freezing and thawing cycles in GUS extraction buffer (Jefferson, 1987). GUS activity was assayed fluorimetrically in the supernatant as described by Jefferson (1987).

Random binding site selection

To select for MybSt1 binding sites, a mixture of randomized 52 base oligonucleotides was synthesized, which contained a randomized centre composed of 26 bp (5′ GTGGATCCTGTCG−26 random positions−GAGCGGAATTCGT 3′). Double strands were generated from primer-1 [5′ ACGAATTCCGCTC 3′ (13 bases)], which was annealed to the 3′ end of the 52 base oligonucleotide using T4 DNA polymerase and [α-^{32}P]dCTP. Twelve micrograms of the double-stranded DNA pool were incubated with MybSt1-containing protein extract, as described for the mobility shift assay. Protein−DNA complexes were immunoprecipitated by adding 40 μl anti-MybSt1 antiserum and protein A−Sepharose. After three washings with 100 mM TBS, the immunoprecipitated oligonucleotides were dissolved and denatured by shaking in 50 μl TE at 95°C for 5 min and 25 μl supernatant were used for PCR amplification. Two hundred nanograms of primer-1 and primer-2 (5′ GTGGATCCTGTCG 3′) were added to the PCR reaction mix and [α-^{32}P]dCTP was incorporated into the PCR product during the last PCR cycle. Half of the resulting DNA pool was used for the next cycle of selection; the cycle was repeated a total of seven times, including two mobility shift assays to seperate bound and non-bound oligonucleotides. The wet gel was then autoradiographed and the DNA of the retarded complex was eluted electrophoretically, using a Biotrap apparatus. The eluted DNA was recovered by ethanol precipitation. The PCR products obtained after the seventh cycle were digested with *Bam*HI and *Eco*RI

and ligated into the *Bam*HI and *Eco*RI sites of Bluescript-SK(+). The inserts of positive clones were excised, radiolabelled with T4 DNA polymerase using [α-^{32}P]dCTP and dGTP and assayed for protein binding in mobility shift assays.

Acknowledgements

We thank Antje Voigt for the photographic work. This work was supported by the Sonderforschungsbereich, SFB 344, 'Regulationsstrukturen von Nukleinsäuren und Proteinen'.

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Received on July 15, 1994; revised on August 18, 1994