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Characterization of *cis*-Acting Elements Regulating Transcription from the Promoter of a Constitutively Active Rice Actin Gene

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The promoter of the constitutively expressed rice (*Oryza sativa*) actin 1 gene (*Act1*) is highly active in transformed rice plants (W. Zhang, D. McElroy, and R. Wu, Plant Cell 3:1150–1160, 1991). A region 834 bp upstream of the *Act1* transcription initiation site contains all the regulatory elements necessary for maximal gene expression in transformed rice protoplasts (D. McElroy, W. Zhang, J. Cao, and R. Wu, Plant Cell 2:163–171, 1990). We have constructed a series of *Act1* promoter deletions fused to a bacterial β-glucuronidase reporter sequence (*Gus*). Transient expression assays in transformed rice protoplasts, as well as transformed maize cells and tissues, identified two distinct *cis*-acting regulatory elements in the *Act1* promoter. A 38-bp poly(dA-dT) region was found to be a positive regulator of *Act1* promoter activity. Deletion of the poly(dA-dT) element lowered *Gus* expression by at least threefold compared with expression produced by the full-length *Act1* promoter. By gel retardation and footprinting, we identified a ubiquitous rice protein which specifically recognizes this poly(dA-dT) element in the constitutively active *Act1* promoter. A CCCAA pentamer repeat-containing region was found to be a negative regulator of the *Act1* promoter in transformed rice protoplasts. Transient expression assays in different maize cells and tissues with use of the *Act1* deletion constructs suggested that the CCCAA pentamer repeat region functions in a complex tissue-specific manner. A CCCAA-binding protein was detected only in root extracts.

Actin is an essential component of the cytoskeleton in higher plants. Actin proteins are involved in many plant cell functions such as cytoplasmic streaming, cell shape determination, cell division, and organelle movement (26, 34, 35). Plant actin is encoded by a gene family in all plant species studied to date (35). Of the four previously characterized rice (*Oryza sativa*) actin genes, transcripts from the actin 1 gene (*Act1*) were found to be relatively abundant in all rice tissues and developmental stages examined (31, 32). *Act1* has a short 5′ noncoding exon, separated by a 447-bp intron (intron 1) from the first coding exon (32, 52). Transient assays of *Act1* 5′ region activity in transformed rice protoplasts have indicated that the *Act1* intron 1 is required for efficient gene expression from the *Act1* promoter (33). The mechanism of this intron-mediated stimulation of gene expression is thought to involve efficient in vivo RNA processing. Such transient assays also established that a region 834 bp upstream of the *Act1* transcription start site contains all of the *cis*-acting regulatory elements necessary for maximal *Act1* promoter activity in transformed rice protoplasts (33). It has been shown that the *Act1* 5′ region can stimulate high-level β-glucuronidase gene (*Gus*) expression in transient assays of transformed rice and maize cells (30, 33). Furthermore, in situ histochemical localization of *Act-Gus* expression in transgenic rice plants revealed that the *Act1* 5′ region is active in most, but not all, sporophytic cell types as well as in gametophytic pollen tissues. This pattern probably reflects a ubiquitous requirement for cytoskeletal components in plant cells (52). Therefore, it has been proposed that the 5′ region of the rice *Act1* gene represents an efficient, constitutively active regulator of foreign gene expression in transgenic cereal plants (6, 30). The existence of the ancient and divergent classes of plant actin genes suggests that these genes have unique patterns of gene regulation or encode proteins with unique functions (35). There is a possibility, however, that a single actin gene could have complex patterns of expression. A functional dissection of the rice *Act1* promoter could contribute to an understanding of plant actin gene families.

Two possible mechanisms by which specific DNA-protein interactions may regulate constitutive gene expression have been suggested. In the first model, *cis*-acting promoter elements are proposed to cause a sequence-specific alteration in chromatin structure or interact with ubiquitous *trans*-acting factors, thus mediating constitutive gene expression (47). Naturally occurring poly(dA-dT) sequences have been identified in constitutively expressed *Saccharomyces cerevisiae* genes (28, 43, 47). Another model has evolved from studies of the regulation of the nominally constitutive cauliflower mosaic virus 35S promoter in transgenic plants. In this model, it is proposed that constitutive promoters might contain multiple *cis*-acting elements, each of which interacts individually with cell- or tissue-specific *trans*-acting factors. The combined activities of a mosaic of individual *cis*-acting elements confer constitutive gene expression in most tissue types (4, 5, 50).

For some eukaryotic genes, optimal promoter activity can be achieved by the simultaneous binding of several proteins at neighboring sites (25). In other cases, gene expression can be regulated in both positive and negative manners by the binding of multiple factors to overlapping binding sites (2, 3). Studies on the regulation of animal actin gene expression have identified a variety of protein-binding sites in animal actin promoters. This finding suggests that the transcription of animal actin genes is regulated through complex interactions between multiple DNA-binding proteins (8, 10–13, 18). Furthermore, it has been suggested that cell- and tissue-

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specific gene expression is regulated through a specific combination of DNA-binding proteins (36, 37, 41, 45), with the observed level of gene expression reflecting changes in the concentrations and structures of individual DNA-binding proteins (17, 42).

In this report, we describe a detailed analysis of the rice *Act1* promoter. By transient expression analysis of transformed rice protoplasts, as well as suspension culture cells and leaf and root material from maize, we found that a poly(dA-dT) element is essential for high-level *Act1* promoter activity. Using gel retardation and footprinting analyses, we have identified a specific protein that binds to the poly(dA-dT) element of the *Act1* promoter. This protein is ubiquitously present in rice tissues, consistent with a role in the constitutive regulation of *Act1* promoter activity. Transient expression assays also showed that the activity of the *Act1* promoter is modulated by a set of imperfect pentamer repeats with the consensus sequence CCCAA. However, these CCCAA pentamer repeats appear to act in a tissue-specific manner. A specific protein that binds to this repeated element was observed only in rice root protein extracts.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from Boehringer Mannheim Biochemicals, Bethesda Research Laboratories, and Stratagene and were used according to the recommendations of the manufacturers. Synthetic oligonucleotides were obtained from the Cornell University Oligonucleotide Synthesis Facility. Polynucleotides were purchased from Boehringer Mannheim. Cell culture reagents and fine chemicals were obtained from Sigma Co. Radioactive isotopes were obtained from Amersham International.

Plasmid construction. Plasmids p*Act1*-F and p*Act1*-G (33), containing the *Act1* 5′ region, the *Gus* reporter gene, and the 3′ noncoding region of the nopaline synthase gene (*nos*), were used as controls in transient gene expression assays. These plasmids have 5′ deletions, generated by using convenient restriction sites (Fig. 1B), to nucleotides −834 and −459 (with the *Act1* transcription initiation site designated +1) in their respective *Act1* promoters. Plasmid p*Act1*-G was used as the starting material for the construction of *Act1* promoter 5′ deletion mutants by exonuclease III-mung bean nuclease digestion. Plasmid p*Act1*-G was cleaved with *Kpn*I and *Eco*RI, which have restriction sites in the polylinker region 5′ of the *Act1* promoter sequence. The linearized p*Act1*-G was treated with exonuclease III, which deletes only single-stranded DNA from the 3′ recessive end of the cleaved *Eco*RI restriction site. The deleted plasmids were blunted with mung bean nuclease and self-ligated. By stopping the exonuclease III treatment at different time points, we generated plasmids with 5′ deletions at nucleotides −378, −300, −260, −245, −152, and −37 in their respective *Act1* promoters. A plasmid with an internal deletion between nucleotides −459 to −152 was constructed by digesting the −152 deletion plasmid with *Kpn*I, blunting the ends with mung bean nuclease, and inserting a blunt-ended *Xho*I-*Eco*RI DNA fragment which spans nucleotides −834 and −459 of the *Act1* promoter (Fig. 1B). Plasmid structure was confirmed by double-stranded DNA sequencing (40).

Transient expression assay in rice protoplasts. Protoplasts were isolated from rice (*O. sativa* cv. Nipponbare) cell suspension cultures as previously described (1, 33). For transformation, 1 ml of protoplast suspension (between 10^6 and 10^7 protoplasts) was incubated with 10 to 20 μg of plasmid DNA, 50 μg of calf thymus carrier DNA, and an equal volume of 30% polyethylene glycol 4000. Transient assays of GUS specific activities were performed 7 days after protoplast transformation as previously described (21, 33). The activity of each test plasmid was assayed independently in transformed rice protoplasts on five different occasions, with each assay being done in triplicate, and the mean and standard error of the mean were determined.

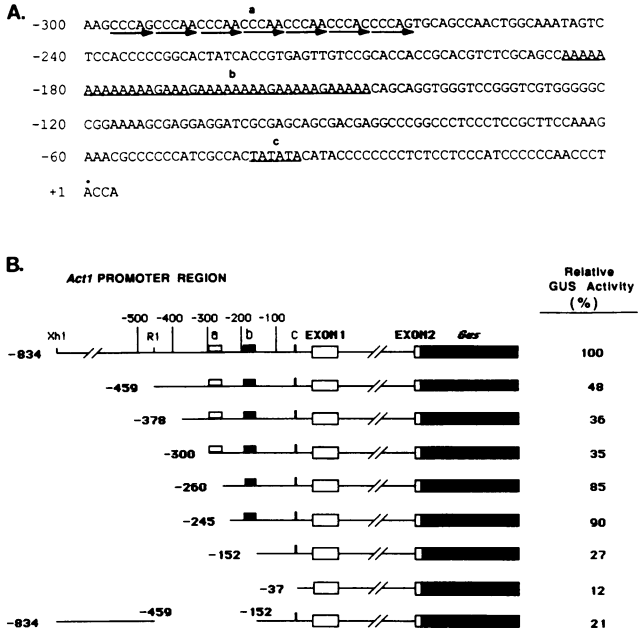

FIG. 1. Transient expression analysis of *Act1* promoter deletion constructs. A series of 5′ deletions to the *Act1* promoter region, fused to a *Gus* reporter sequence, was introduced into rice protoplasts by polyethylene glycol-mediated transformation. (A) Part of nucleotide sequence of the *Act1* promoter. The transcription initiation site is marked by an asterisk. Nucleotides are numbered with respect to the transcription start site at +1. The CCCAA pentamer repeats, the poly(dA-dT) element, and the TATA box are designated by lowercase letters and are underlined with arrows (for the repeats) or lines. (B) Maps of the various deletion constructs, shown next to their respective *Gus* expression levels. The endpoints of the deletions are indicated. Open boxes represent noncoding exons of the *Act1* gene; filled boxes represent the *Gus* coding region (not to scale). In the *Act1* promoter region, appropriate locations of potential regulatory elements are indicated. The open box represents the CCCAA pentamer repeats, the filled box represents the poly(dA-dT) element, and the filled bar represents the putative TATA box. Restriction enzyme sites: R1, *Eco*RI; Xh1, *Xho*I. The GUS specific activity of the 5′ deletion to nucleotide −834 (p*Act1*-F) was considered as full *Act1* promoter activity (33). The results of other *Act1* deletion constructs are given as the percentage of p*Act1*-F activity. Each number represents the mean of five independent transformation experiments.

Microprojectile-mediated transformation of intact maize tissues. Maize (*Zea mays* cv. P3925) leaf, root, and suspension cells were prepared as previously described (7, 30) before being subjected to microprojectile-mediated transformation with 1.2-μm-diameter tungsten particles coated with a solution containing 10 μg of plasmid DNA (7). GUS specific activities were assayed 2 to 3 days after bombardment as previously described (21, 30).

Protein extract preparation. Fifty grams of 1- to 2-week-old rice (cultivar IR36) tissues was ground in liquid nitrogen and incubated with 500 ml of lysis buffer (15 mM *N*-2-

hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride (PMSF)] on ice for 1 h. The lysate was centrifuged for 20 min at 8,000 rpm, and the resulting supernatant was fractionated with solid ammonium sulfate. Proteins which precipitated at between 30 and 75% $(NH_4)_2SO_4$ saturation were resuspended in 20 ml of extraction buffer (25 mM HEPES [pH 7.6], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol) and dialyzed overnight at 4°C against the same buffer. Following dialysis, the extract was further fractionated through a 0.6-ml heparin-agarose column (12) and eluted with buffers containing 0.2, 0.4, 0.6, and 1.0 M KCl. The 0.4 M KCl fractions, which contained specific DNA-binding activity, were pooled and desalted by dialysis against binding buffer (20 mM HEPES [pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol) for 2 h. Protein concentrations were determined by the Bio-Rad dye-binding assay. The fractions were aliquoted and stored at −70°C.

Gel retardation assay. For probe production, blunt-ended DNA fragments from the *Act1* promoter, between nucleotides −245 and −136 and between nucleotides −300 and −136, were subcloned into the *Eco*RV site of pBluescriptII-KS vectors. Each of the subcloned DNA fragments was isolated following *Hin*dIII-*Pst*I digestion and labeled with [α-^{32}P]dATP, using DNA polymerase Klenow fragment (12). To construct the double-stranded oligonucleotide probe, single-stranded complementary oligonucleotides were denatured at 95°C for 3 min and allowed to anneal to each other by cooling to room temperature. The double-stranded oligonucleotide probe with sticky ends was end labeled as described above. The labeled fragments were purified from an 8% polyacryamide gel (acrylamide-bisacrylamide, 19:1). After autoradiography of the polyacrylamide gel, the DNA bands were excised from the gel and eluted overnight at 4°C in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) containing 0.5 M ammonium acetate. The resulting supernatants were precipitated with ethanol and resuspended in TE buffer (5,000 cpm/μl).

Binding reactions were performed as previously described (12) with poly(dI-dC) · poly(dI-dC) as the nonspecific competitor. Reactions were initiated by the addition of protein extracts. After incubation on ice for 10 min, the mixture was electrophoresed at 160 V through a 5% polyacrylamide gel (acrylamide-bisacrylamide, 80:1) in 45 mM Tris hydrochloride (pH 7.6)–45 mM boric acid–1 mM EDTA. The gel was then transferred to Whatman 3MM paper, dried, and autoradiographed. For competition experiments, the conditions were the same as described above except that unlabeled competitor DNA was added to the reaction mixture before addition of the protein extracts.

MPE footprinting. A DNA fragment between nucleotides −245 and −136 of the *Act1* promoter was chosen for methidiumpropyl-EDTA (MPE) footprinting analysis (20). Coding and noncoding strand probes were isolated by *Bam*HI-*Cla*I and *Hin*dIII-*Sst*I digestions of the subclone containing the test DNA fragment, respectively. Each probe was labeled by a 3'-end-filling reaction using DNA polymerase Klenow fragment and [α-^{32}P]dATP as previously described (12). Protein binding reactions with aliquots of the labeled DNA were performed as described for the gel retardation assays. After binding was completed, the reaction mixtures were treated with freshly made 1 mM MPE, 2 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 40 mM DTT at room temperature for 5 min. The samples were then extracted twice with phenol-chloroform (1:1) and precipitated with ethanol. The DNA pellets were resuspended in an 80% formamide–dye solution, denatured at 95°C for 3 min, and applied to a 10% polyacrylamide–urea sequencing gel. Chemical cleavage of aliquots of the labeled DNA at purine residues was performed as described previously (29). Samples of the cleaved products were run in parallel with the footprinting products. The gels were transferred to Whatman 3MM paper after electrophoresis, dried, and autoradiographed.

RESULTS

Transient expression analysis of *Act1* promoter deletion constructs in rice protoplasts. The nucleotide sequence of the *Act1* 5' region, which includes 834 bp upstream of the transcription start site, has been determined (33). To define those DNA sequences in the *Act1* promoter required for high-level gene expression, a series of *Act1* promoter 5' deletions fused to the *Gus* reporter gene was introduced into rice protoplasts by polyethylene glycol-mediated transformation (33, 53). The *Act1* promoter sequence deleted in some of these *Act1-Gus* fusion constructs is shown in Fig. 1A. Seven days after transformation, the GUS specific activities of the various deletion constructs were determined. The results in Fig. 1B show the GUS specific activities of the various *Act1* 5' deletion constructs relative to that of the full-length control plasmid p*Act1*-F (33), which contains 834 bp of the *Act1* promoter sequence. Plasmid p*Act1*-F has previously been shown to contain the minimal amount of the *Act1* promoter necessary for maximal *Act1-Gus* gene expression in transient assays of transformed rice protoplasts (33).

In repeated assays, an *Act1* 5' deletion to nucleotide −459 (with the *Act1* transcription initiation site designated +1) in construct p*Act1*-G displayed (on average) 48% of the GUS specific activity shown by the full-length control plasmid p*Act1*-F. This result is consistent with those of previously published experiments (33), indicating that potential positive regulatory elements are located within the sequence between nucleotides −834 and −459 of the *Act1* promoter. *Act1* promoter deletions to nucleotides −378 and −300 exhibited GUS specific activities that were not significantly different from that of p*Act1*-G.

Deletion of the region from nucleotides −300 to −260, or −245, resulted in a more than twofold increase in GUS specific activity (relative to that of the −300 deletion construct) to a level that was at least 85% of that observed for the full-length control plasmid p*Act1*-F. The sequence between −300 and −260 contains (almost exclusively) seven tandem copies of an imperfect pentamer repeat with the consensus sequence CCCAA (Fig. 1A). This result suggests that these repeated pentamers may be involved in the observed down regulation of *Act1* promoter activity associated with the deletion between nucleotides −300 and −260. An *Act1-Gus* fusion plasmid containing a further deletion from nucleotides −245 to −152, which removed the 38-bp poly(dA-dT) element (Fig. 1A), displayed a GUS specific activity that was only 27% of that observed for the full-length control plasmid p*Act1*-F. This result suggests that the region between nucleotides −245 and −152 contains a strong positive regulator of *Act1* promoter activity. Further deletion to nucleotide −37, which is in the middle of the putative TATA box of the *Act1* promoter (Fig. 1A), caused a further twofold drop in GUS specific activity to a level that is only 12% of that observed for the full-length control plasmid p*Act1*-F.

TABLE 1. Transient expression analysis of *Act1* promoter deletion constructs in maize cells and tissues

Construct[a]	*GUS sp act* (10^{-1} *nmol/h/mg*)[b] in:					
	Suspension cells		Leaf		Root	
	Mean	SE	Mean	SE	Mean	SE
−834	65.9	1.3	9.2	0.8	23.5	0.2
−300	9.5	0.6	5.7	0.5	19.8	0.4
−245	29.4	0.1	6.2	0.8	10.1	0.4
−152	3.7	0.1	0.8	0.1	4.0	0.5

[a] Endpoint of *Act1* promoter deletion relative to the site of transcription initiation at +1.

[b] Two independent particle bombardment experiments were carried out with each of plant materials. GUS activities were fluorometrically assayed 3 days after transformation. Values were calculated after correction for background.

To confirm the presence of the putative regulatory regions identified by the *Act1* promoter 5′ deletion analysis, we evaluated the activity of an internal deletion construct which lacks the region between nucleotides −459 and −152 (Fig. 1B). This plasmid showed a GUS specific activity that was 21% of that observed for the control plasmid p*Act1*-F, which further suggests that the region between nucleotides −459 and −152 contains at least one positive regulator of *Act1* promoter activity.

Transient expression analysis of *Act1* promoter deletion constructs in intact maize tissues. To further explore the results obtained from transient assays of transformed rice protoplasts, we performed microprojectile-mediated transformation of suspension culture cells, leaf, and root of maize (Table 1). Maize tissues generally have lower levels of background GUS specific activity than does rice material (7a). The fusion construct with a deletion to nucleotide −152 in its *Act1* promoter resulted in eight-, eight-, and threefold decreases in GUS specific activity, relative to the −245 deletion construct, in maize suspension cells, leaf, and root, respectively (Table 1). This result is consistent with that observed in transient expression assays of transformed rice protoplasts (Fig. 1B) and confirms that the deleted region contains a positive regulator of *Act1* promoter activity. However, the deletion to nucleotide −245 in the *Act1* promoter showed a more complex result than we had expected. As was observed in transformed rice protoplasts, the region between nucleotides −300 and −260 was also seen to function as a negative regulatory element in maize suspension culture cells. However, deletion of this region, while causing no significant effect on *Act1* promoter activity in maize leaves, resulted in a twofold decrease of *Act1* promoter activity in maize root tissue. The control plasmid p*Act1*-F was used to demonstrate the activities of the full-length promoter in the assays.

Characterization of a DNA-binding factor which interacts with a poly(dA-dT) element in the *Act1* promoter. To investigate the relationship between *trans*-acting DNA-binding proteins that interact with *cis*-acting element(s) within the region from nucleotides −245 and −152, which had been shown to be important for efficient *Act1* promoter activity in vivo, we carried out gel retardation assays using rice leaf protein fractions prepared by heparin-agarose chromatography. A 120-bp DNA fragment spanning nucleotides −245 to −136 of the *Act1* promoter, containing the 38-bp poly(dA-dT) element (Fig. 1A), was used as a probe in these gel retardation assays (Fig. 2A). A retarded band was observed

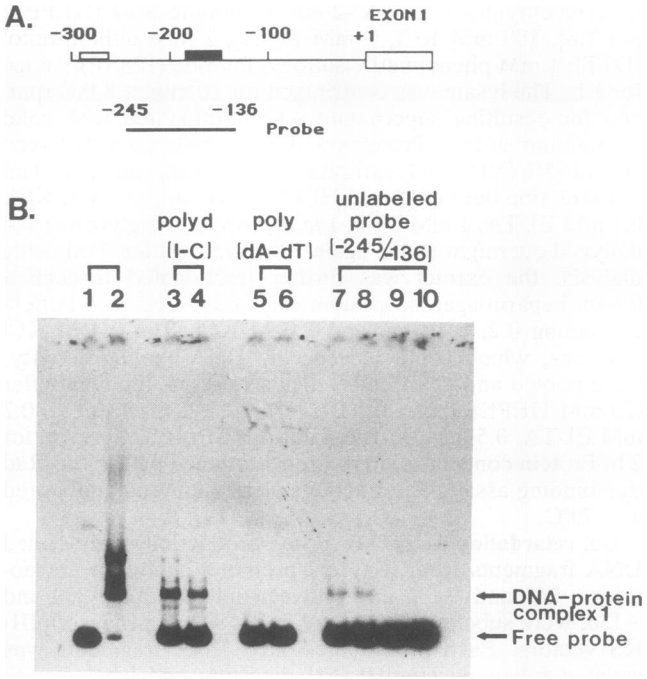

FIG. 2. Identification of a protein which binds to the poly(dA-dT)-containing region of the *Act1* promoter. (A) Structural map of part of the *Act1* 5′ region. In the *Act1* promoter, the filled box represents the poly(dA-dT) element and the open box represents the CCCAA pentamer repeat region. A 120-bp DNA fragment spanning nucleotides −245 to −136 of the *Act1* promoter was end labeled and used as a probe in the gel retardation assay. (B) Gel retardation assay using the poly(dA-dT)-containing probe from the *Act1* promoter. All binding reaction mixtures (except those in lanes 1 and 2) contained 1.5 μg of poly(dI-dC) as nonspecific competitor DNA. End-labeled probe (0.5 ng) was incubated without protein extracts (lane 1) or with 3 μg of protein from the 0.4 M KCl fraction of heparin-agarose-purified rice leaf extracts (lanes 2 to 10). The binding reaction mixtures were incubated with 10 and 100 ng of unlabeled poly(dA-dT) (lanes 5 and 6, respectively) and with 5, 10, 25, and 100 ng of unlabeled probe DNA (lanes 7 to 10, respectively). After incubation, reaction mixtures were fractionated through a 5% native polyacrylamide gel and the products were visualized by autoradiography.

in the presence of the end-labeled probe DNA and nonspecific competitor poly(dI-dC) DNA (Fig. 2B, lanes 3 and 4). This retarded band, designated DNA-protein complex 1, could be abolished with different amounts of the unlabeled probe DNA (Fig. 2B, lane 7 to 10), indicating that the observed binding activity is specific to that region of the *Act1* promoter. The protein fraction responsible for the specific DNA-binding activity was eluted between 0.2 to 0.4 M KCl from the heparin-agarose column. No specific binding activity was found in any other fractions. In a second set of gel retardation assays, competition reactions using synthetic poly(dA-dT) DNA as a competitor were carried out. The DNA-protein complex formed with the 120-bp probe was no longer observed when 10 or 100 ng of poly(dA-dT) DNA was included in the protein-binding assay (Fig. 2B, lanes 5 and 6). This result suggests that the 38-bp poly(dA-dT) region within the 120-bp probe is the likely site of protein binding.

To delineate the protein-binding region more precisely, MPE footprinting was performed on rice leaf protein extracts. Probes for both the coding and noncoding strands of the 120-bp DNA fragment were radiolabeled at either end,

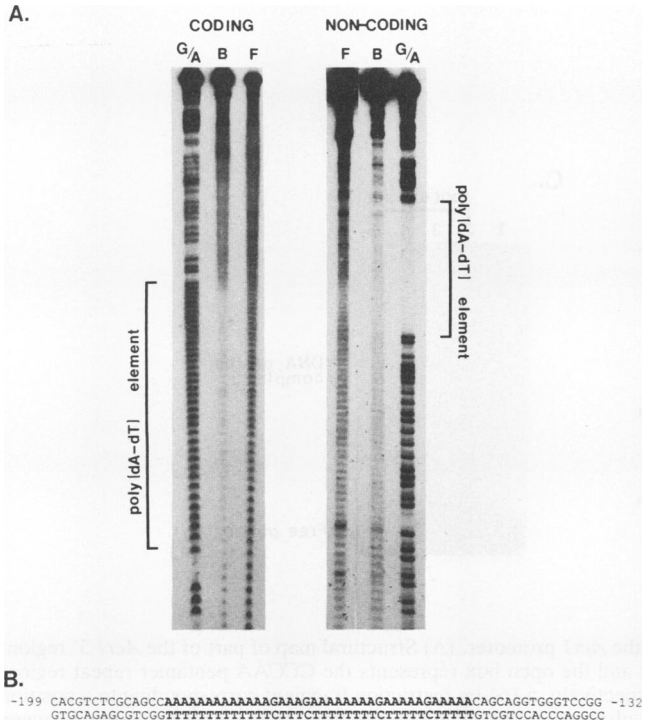

FIG. 3. MPE footprinting to delineate protein binding to the poly(dA-dT) region of the *Act1* promoter. (A) The autoradiograph of the sequencing gel shows the MPE footprinting ladder of both coding and noncoding strands from free DNA (F), protein-bound DNA (B), and a Maxam-Gilbert G+A sequencing reaction (29) as a marker (G/A). The correlations between the protected regions on both the coding and noncoding strands and the position of the poly(dA-dT) element are indicated. (B) The region of the *Act1* promoter sequence that is protected against MPE cleavage is indicated by boldface letters in the partial sequence of the *Act1* promoter.

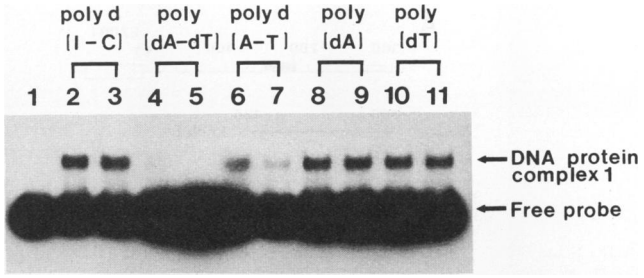

FIG. 4. Determination of poly(dA-dT)-binding protein specificity. The probe DNA used in this experiment was the same as that used for Fig. 2. All binding reaction mixtures contained 1.5 μg of poly(dI-dC) as nonspecific competitor DNA. The DNA probe was incubated without protein extract (lane 1) or with 3 μg of rice leaf proteins isolated as described in the legend to Fig. 2. The binding reaction mixtures were incubated with different amounts of potential competitor DNA as follows: additional 10 and 100 ng of unlabeled poly(dI-dC) (lanes 2 and 3, respectively); 10 and 100 ng of unlabeled double-stranded poly(dA-dT) [same as poly(dA) · poly(dT)] (lanes 4 and 5, respectively), 10 and 100 ng of unlabeled double-stranded poly[d(A-T)] (lanes 6 and 7, respectively), 10 and 100 ng of unlabeled single-stranded poly(dA) (lanes 8 and 9, respectively), and 10 and 100 ng of unlabeled single-stranded poly(dT) (lanes 10 and 11, respectively). After incubation, reaction mixtures were fractionated through a 5% native polyacrylamide gel and the products were visualized by autoradiography.

and DNA-protein complexes were formed. The ratio between the labeled DNA fragment and protein was determined by gel retardation assay. After treatment with MPE, the samples were denatured and fractionated through a 10% sequencing gel. A protected region spanning nucleotides −185 to −148, which exactly corresponds to the poly(dA-dT) element of the *Act1* promoter, was observed in such MPE footprinting gels (Fig. 3). The same results were obtained for protein extracts from rice callus and seed (data not shown). This result suggests that the poly(dA-dT)-binding protein(s), which is present in all rice material investigated, may act in vivo as a positive constitutive regulator of *Act1* promoter activity.

Determination of poly(dA-dT)-binding protein specificity. Several proteins, which preferentially bind to a variety of A+T-rich DNA sequences, have been identified in both animal and plant cells (16, 24, 46, 51). To test the specificity of the observed poly(dA-dT) binding, we carried out further gel retardation assays, using the same probe and protein fraction as described above, in competition with synthetic polynucleotides. Double-stranded poly(dA-dT) [same as poly(dA) · poly(dT)] has the same arrangements of nucleotides as occurs naturally within the poly(dA-dT)-rich region of the *Act1* promoter, with poly(dA) on one strand and poly(dT) on the opposite strand. Double-stranded poly[d(A-T)] contains alternating A's and T's on each strand. Poly(dA) and poly(dT) are single-stranded DNA molecules. Different amounts of each competitor DNA were used in the binding reactions (Fig. 4). The result of gel retardation assays showed that 10 ng of poly(dA-dT) or 100 ng of poly[d(A-T)] abolished DNA-protein complex 1 formation (Fig. 4). This result suggests that poly(dA-dT) competes for probe binding at least 10-fold more efficiently than does poly[d(A-T)]. Single-stranded poly(dA) or poly(dT) DNA did not compete for binding at all (Fig. 4). Our results suggest that the protein component of DNA-protein complex 1 is a specific double-stranded poly(dA-dT)-binding protein.

Binding of a root protein to a pentameric repeat region in the *Act1* promoter. To test whether there is a specific protein binding to the CCCAA pentamer repeats of the *Act1* promoter, we carried out gel retardation assay using rice leaf and root protein extracts. The probe used in these experiment (Fig. 5A) was a 164-bp DNA fragment from nucleotides −300 to −136 of the *Act1* promoter, which included both the CCCAA pentamer repeats and the poly(dA-dT) element (Fig. 1A). The binding activity of the poly(dA-dT) region was considered an internal control for the assay because, as shown above, this binding was found to be constitutively present in all rice tissues sampled. Gel retardation assays showed that an additional specific probe-binding activity was detected in root protein extracts but not in leaf protein extracts (data not shown). After fractionation of the protein extracts by heparin-agarose chromatography, we found that the root protein(s) responsible for the additional binding activity was eluted at between 0.2 to 0.4 M KCl. A gel retardation experiment using this fraction from either leaf or root protein extracts was performed (Fig. 5B). The result indicated that a specific binding activity, designated DNA-protein complex 2, was detected only in the root protein fraction (Fig. 5B, lanes 6, 7, 10, and 11), whereas the poly(dA-dT)-binding activity of DNA-protein complex 1 was observed with both leaf and root protein fractions (Fig. 5B). The difference in the amount of DNA-protein complex 1

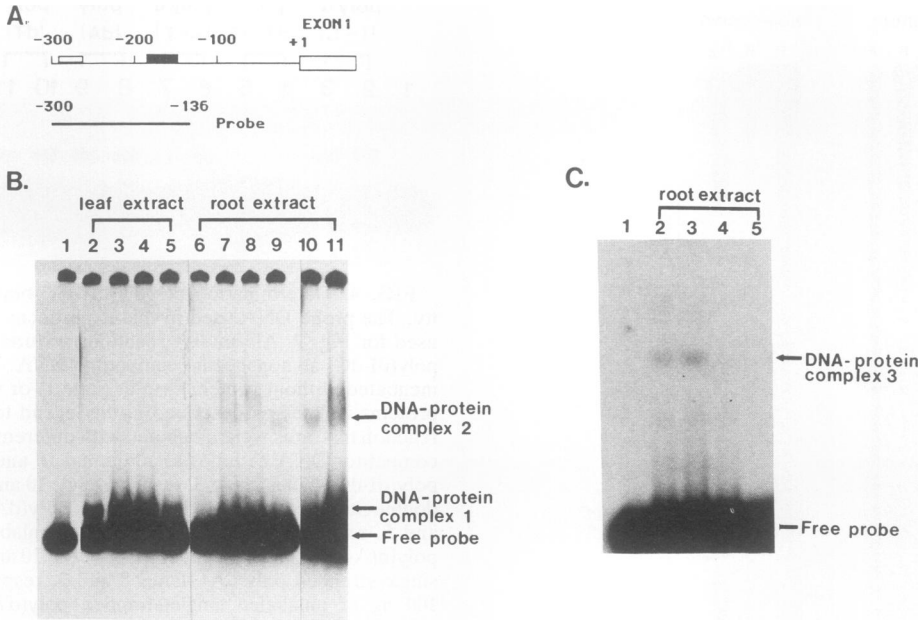

FIG. 5. Binding of a root protein to the CCCAA pentamer repeats of the *Act1* promoter. (A) Structural map of part of the *Act1* 5′ region. In the *Act1* promoter, the filled box represents the poly(dA-dT) element and the open box represents the CCCAA pentamer repeat region. The probes used in the gel retardation assays in panels B and C were, respectively, a 164-bp restriction fragment corresponding to a position in the *Act1* promoter between nucleotides −300 and −136 and a 20-bp oligonucleotide including positions in the *Act1* promoter between nucleotides −293 and −277. (B) Gel retardation assay using the 164-bp probe containing the CCCAA pentamer repeats and poly(dA-dT) element from the *Act1* promoter. The sources of rice protein extracts are indicated above the lanes. All binding reaction mixtures contained 1 μg of poly(dI-dC) as nonspecific competitor DNA. The probe was incubated without proteins (lane 1), with 3 μg (lane 2) or 6 μg (lanes 3 to 5) of rice leaf proteins, or with 3 μg (lanes 6 and 10) or 6 μg (lanes 7, 8, 9, and 11) of rice root protein extracts. Unlabeled CCCAA repeat-containing DNA, either 5 ng (lanes 4 and 8) or 20 ng (lanes 5 and 9), was added to the binding reaction mixtures. After incubation, reaction mixtures were fractionated through a 5% native polyacrylamide gel and the products were visualized by autoradiography. (C) Gel retardation assay using the 20-bp oligonucleotide probe containing three copies of the CCCAA pentamer from the *Act1* promoter. All binding reaction mixtures contained 1 μg of poly(dI-dC) as nonspecific competitor DNA. The probe was incubated without proteins (lane 1) or with 3 μg (lane 2) or 6 μg (lanes 3, 4 and 5) of rice root proteins. Unlabeled CCCAA repeat-containing DNA, 5 ng (lane 4) or 20 ng (lane 5), was added to the binding reaction mixtures. After incubation, reaction mixtures were fractionated through a 5% native polyacrylamide gel and the products were visualized by autoradiography.

formation in leaf and root proteins is a function of the relatively low protein content of the rice root extracts. DNA-protein complex 2 was abolished by unlabeled probe DNA (Fig. 5B, lanes 8 and 9) but not by other unrelated DNA fragments which have sizes similar to that of the probe (data not shown). An interesting feature of the observed DNA-protein interaction was that the retarded band became broader and more intense with increasing amounts of root protein extracts (Fig. 5B, lanes 7 and 11). This feature suggests that the repetitive CCCAA pentamers in the probe might be the site of protein binding because they provide potential multiple binding sites for the DNA-binding protein. To test this possibility, we carried out a gel retardation assay using a labeled 20-mer oligonucleotide probe which included three repeats of the CCCAA pentamer. A specific activity binding to the oligonucleotide probe was observed with the 0.4 M KCl fraction from the root protein extracts (Fig. 5C, lanes 2 and 3). Addition of unlabeled oligonucleotide prevented the labeled probe from complexing with the root protein(s), confirming the specificity of the binding activity (Fig. 5C, lanes 4 and 5). After examining rice protein extracts from callus, leaf, seed, and root, we observed this specific CCCAA pentamer binding activity only in the root protein extracts (data not shown). Since the results suggest that the CCCAA pentamers act as a positive regulator of *Act1* promoter activity only in transformed maize roots, the observed root-specific DNA-protein binding activity may be related to this function. In other rice tissues, the CCCAA pentamer repeat region may act as a negative regulator of *Act1* promoter activity.

DISCUSSION

The rice *Act1* promoter directs high-level constitutive expression of foreign genes in transformed monocot plant cells and tissues (6, 30, 33, 52). Previous results indicated that the region 834 bp upstream of the transcription start site of *Act1* contains of all the regulatory elements necessary for maximal gene expression in transformed rice protoplasts (33). By testing a series of 5′ deletion mutants, we have been able to identify two distinct elements that appear to regulate *Act1* promoter activity. One regulatory element, containing a repeating CCCAA pentamer, functions differently in different tissues. We propose that the *cis*-acting CCCAA pentamer repeat region might act to modulate *Act1* promoter activity through interaction with *trans*-acting regulatory factors which are expressed in a tissue-specific fashion. The isolation of such *trans*-acting factors will help to elucidate the role of the CCCAA pentamer repeats in the regulation of *Act1* promoter activity.

The presence of polypurine/polypyrimidine sequences within the promoters of eukaryotic genes has been noted by

many groups (19, 23, 24, 28, 43, 46–48). These elements have also been found in the 5′ regions of several plant actin genes (38, 39). It has been proposed that polypurine/polypyrimidine elements affect local chromatin structure by changing nucleosome arrangement, thus facilitating access of transcription factors to the promoter DNA (15, 22). Consistent with this hypothesis, it has been suggested that poly(dA-dT) elements, for example, found in several constitutively active yeast promoters act to stimulate transcription and that longer poly(dA-dT) tracts are more effective enhancers of transcription than are shorter ones (9). These elements are localized in vivo at nuclease-sensitive chromatin regions (27) and oligo(dA-dT) duplexes form a conformation significantly different from that of B-form DNA (14). Extensive studies in yeast cells have led to the identification of a protein that specifically binds to poly(dA-dT) elements. This protein, named datin, has been purified, and its corresponding gene has been cloned (51). It has been suggested that datin may function as a repressor of transcription (51). More recently, another yeast poly(dA-dT)-binding protein, named T-rich binding factor, has been characterized. It stimulates transcription from templates containing poly(dA-dT) elements as much as 30-fold (24, 28). Our results suggest that the *Act1* poly(dA-dT)-binding protein, which appears to be associated with the positive regulation of *Act1* promoter activity, may be functionally similar to the T-rich binding factor. Poly(dA-dT) and other polypurine/polypyrimidine elements have also been found to be a component of recombination initiation sites in *S. cerevisiae* and to be associated with the nuclear scaffold in *Drosophila melanogaster* (44, 49). These findings suggest that different elements, and the proteins that they interact with, can exert diverse effects on the regulation of gene expression.

In addition to the negative and positive regulatory regions described above, there are several other potential regulatory elements within the *Act1* promoter. Deletion between nucleotides −843 and −459 resulted in a twofold decrease in *Gus* expression in transformed rice protoplasts, suggesting that this deleted sequence also contains a positive regulator of *Act1* promoter activity that has not been further characterized. Deletion between nucleotides −152 and −37 also decreased GUS specific activity about twofold. Within this region, there is a putative TATA box. In addition, several sequences with interesting structural characteristics have previously been identified in the region upstream of the CCCAA pentamer repeat region (33). Further analysis of these sequences might provide information on their roles in the regulation of *Act1* promoter activity.

In conclusion, our results suggest that the *Act1* promoter contains multiple *cis*-acting elements, each of which interacts with either ubiquitous or tissue-specific *trans*-acting factors to confer the observed constitutive pattern of *Act1* promoter activity. This finding is similar to that found for the nominally constitutive cauliflower mosaic virus 35S promoter (4, 5). Analysis of transgenic rice plants transformed with an *Act1-Gus* fusion gene containing a full-length *Act1* promoter has showed that *Gus* expression in root tissue is about 50% of the level found in leaf tissue (52). Although transcripts from the rice *Act1* gene are present in all rice tissues (31), the difference in the level of *Act1* promoter activity in the roots and leaves of transgenic rice plants (52) may reflect the effects of combinations of tissue-specific and constitutively active regulatory elements. The functional analysis of the *Act1* promoter described in this report should contribute to an understanding of constitutive gene expression in general and of plant actin gene expression in particular. Furthermore, a specific understanding of *Act1* promoter regulation should facilitate the development of this promoter as a mediator of foreign gene expression in transgenic cereal plants.

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