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Identification of a Transcriptional Activator-Binding Element in the 27-Kilodalton Zein Promoter, the −300 Element

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By utilizing a homologous transient-expression system, we have shown that a 58-bp sequence from the γ-class 27-kDa zein promoter, spanning from −307 to −250 relative to the transcription start site, confers a high level of transcriptional activity on a truncated plant viral promoter. The transcriptional activity mediated by the 58-bp sequence is orientation independent, and it is further enhanced as a result of its multimerization. A similarly high level of transcriptional activity was also observed in protoplasts isolated from leaf tissue-derived maize suspension cells. In vitro binding and DNase I footprinting assays with nuclear proteins prepared from cultured endosperm cells revealed the sequence-specific binding of a nuclear factor(s) to a 16-nucleotide sequence present in the 58-bp region. The nuclear factor binding sequence includes the −300 element, a *cis*-acting element highly conserved among different zein genes and many other cereal storage protein genes. A 23-bp oligonucleotide sequence containing the nuclear factor binding site is sufficient for binding the nuclear factor in vitro. It also confers a high level of transcriptional activity in vivo, but in an orientation-dependent manner. Four nucleotide substitutions in the −300 element drastically reduced binding and transcriptional activation by the nuclear factor. The same nuclear factor is abundant in the developing kernel endosperm and binds to the −300 element region of the 27-kDa or the α-class zein promoter. These results suggest that the highly conserved −300 element is involved in the common regulatory mechanisms mediating the coordinated expression of the zein genes.

Genes encoding zeins, the major storage proteins of maize, are coordinately regulated in a tissue- and developmental stage-specific manner during seed development. Their expression is confined to endosperm tissue and starts 10 to 12 days after pollination (25). A large number of zein genes encode proteins of discrete molecular sizes, which are divided into four classes according to similarities in the primary protein structures (13, 19). Whereas the α-class 22- and 19-kDa zeins are encoded by a large gene family of 25 to 50 members (6, 16, 43), the β-class 15-kDa zein, γ-class 16- and 27-kDa zeins, and δ-class 10-kDa zein are encoded by genes present in only a few copies (2, 10, 19, 44). Expression of these different zein genes is regulated at both the transcriptional and the posttranscriptional levels (3, 8, 20). The coordinated expression of different zein genes during seed development may be attained through a common regulatory mechanism. However, recent studies on a regulatory mutation, *opaque-2*, have clearly demonstrated the presence of an additional level of regulation which controls gene member-specific expression of zein genes (33, 41). Thus, multiple complex regulatory mechanisms are likely to be involved in the transcriptional regulation of zein genes.

In the search for potential regulatory mechanisms controlling the coordinated expression of zein genes in developing endosperm tissue, 5′ flanking sequences of various zein genes have been examined for the presence of conserved *cis*-acting sequences. In addition to the general transcription elements such as TATA and CAAT sequences, a highly conserved 7-bp sequence motif, 5′-TGTAAAG-3′, generally located 330 bp upstream from the first ATG initiator codon, has been identified in most of the cloned zein genes (2). This sequence motif, commonly referred to as the −300 element (also called the endosperm box or prolamin box), shares homology to the simian virus 40 core enhancer sequence (45). Among the α-class zein genes, the conservation of the sequence further extends to a 15-bp stretch, 5′-CACATGTGTAAAGGT-3′, surrounding the −300 element (5). In addition to the zein genes, the −300 element is also conserved in the 5′ flanking regions of many other cereal storage protein genes (4, 7, 12, 28, 34, 37) as well as those of other endosperm-specifically expressed maize genes (42). Thus, the −300 element has been proposed to play a role in the endosperm-specific expression of zein genes. This hypothesis has been strengthened by the specific binding of protein factors present in the endosperm cell nuclei to the −300 element region of a 19-kDa zein gene (23). Recently, a 43-bp sequence from a 19-kDa zein promoter containing the −300 element has been shown to confer a high level of transcriptional activity in transiently transformed maize endosperm protoplasts (31). However, the highly multigenic α-class zein genes, of which a large fraction constitutes pseudogenes (18, 32), make functional analyses of their promoters difficult.

To identify *cis*-regulatory mechanisms involved in the coordinated expression of zein genes, we have performed promoter deletion analyses on the γ-class 27-kDa zein gene. The 27-kDa zein gene is present in one or two copies in the maize genome, and its expression has recently been shown to be regulated by *opaque-2* modifier genes which determine the Quality Protein Maize phenotype (14). We have shown that a 23-bp upstream region containing the −300 element confers a high level of transcriptional activity on a truncated cauliflower mosaic virus 35S (CaMV 35S) promoter. The sequence-specific binding of a nuclear protein factor(s) to the −300 element is demonstrated, and a new core motif for the nuclear factor binding region is defined by DNase I footprinting.

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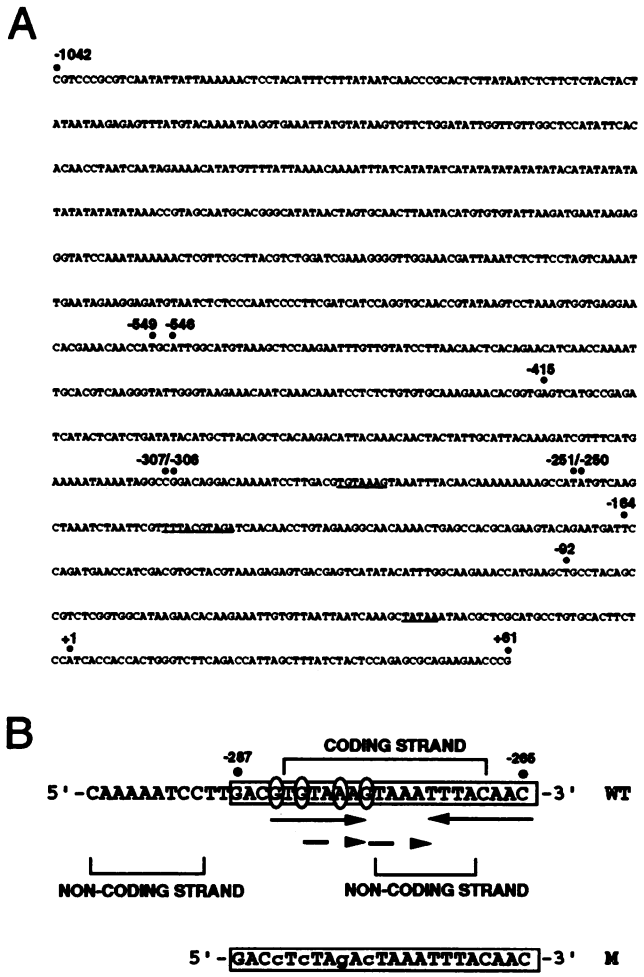

FIG. 1. 5′ flanking sequence of the 27-kDa zein gene. (A) Nucleotide sequence spanning from −1042 to +61 (relative to the transcription start site, +1). The endpoints of various deletion clones analyzed for their transcriptional activities are marked with filled circles, and their nucleotide positions are indicated above. The conserved elements, including the −300 element (TGTAAAG), Opaque-2-like target sequence (TTTACGTAGA), and TATA box, are underlined. (B) Summary of DNase I footprinting assays. The nucleotide sequence of the coding strand containing the nuclear factor binding site (WT) is shown. The sequences protected from DNase I digestion in the coding and noncoding strands are indicated by brackets. The unique direct repeat and imperfect inverted repeat are marked by broken and solid arrow lines, respectively. The synthetic 23-base oligonucleotide sequence (spanning from −287 to −265) is boxed. The four nucleotide substitutions made in the mutant oligonucleotide sequence (M) are circled in the WT sequence.

MATERIALS AND METHODS

Plasmids and chimeric gene constructs. Cloning of the 1,103-bp 5′ flanking sequence of the 27-kDa zein gene, spanning from −1042 to +61 with respect to the transcription start site of the gene (Fig. 1A), was previously described (39). A series of deletion clones whose endpoints reside between −1042 and −92 was generated from this fragment by utilizing restriction sites present on it (Fig. 2A). They were fused 5′ to a truncated (spanning to −90 with respect to the transcription start site) cauliflower mosaic virus (CaMV) 35S promoter. The synthetic promoters were fused to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) reporter gene equipped

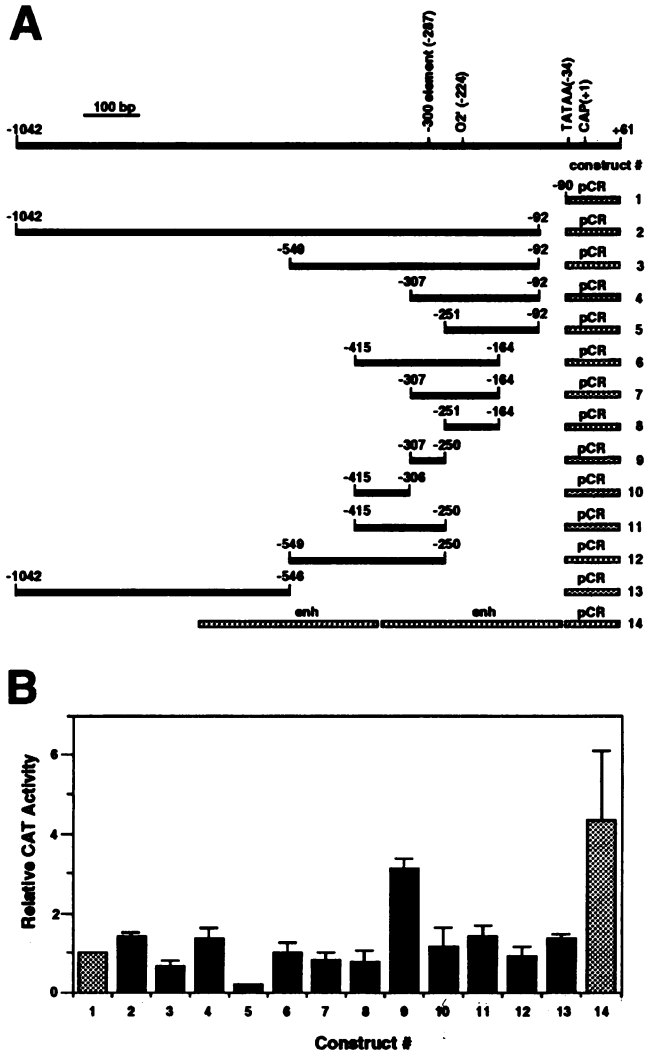

FIG. 2. Transcriptional activities of various 5′ flanking regions of the 27-kDa zein gene. (A) Schematic representation of synthetic promoters containing various 5′ flanking regions of the 27-kDa zein gene and a truncated −90 CaMV 35S promoter. The intact 1,103 bp of 5′ flanking region from the 27-kDa zein gene is shown at the top, and the relative locations of the −300 element, Opaque-2-like target sequence (O2′), TATA box, and the transcription start site (CAP) are indicated. The endpoints of the 5′ flanking regions with respect to the transcription start site are indicated (constructs 2 through 13). These 5′ flanking regions were fused 5′ to a truncated −90 CaMV 35S promoter (pCR). As control promoters, the truncated CaMV 35S promoter (construct 1) and the CaMV 35S promoter containing duplicated enhancer elements (enh) (construct 14) were used. All of these synthetic promoters were fused to the CAT reporter gene equipped with the CaMV 35S 3′ control sequence. (B) Transcriptional activities of the synthetic promoters in transiently transformed endosperm protoplasts. Relative CAT enzymatic activities derived from the CAT constructs are shown. The level of CAT activity derived from the promoterless CAT construct was subtracted from those obtained from the synthetic promoter-CAT constructs in order to correct for nonspecific CAT activity. For each construct, the level of CAT enzymatic activity was standardized to that of GUS enzymatic activity, derived from the coelectroporated pFFGUS plasmid containing the GUS reporter gene regulated by the CaMV 35S promoter (with duplicated enhancer elements) and its 3′ control sequence. These values were expressed as relative to that for pCRCAT (construct 1). The average value of data from five independent experiments is shown for each chimeric construct in the histogram, with the standard deviation from the mean indicated by an error bar.

with the 3′ control sequence of the CaMV 35S gene (41). In addition, a 58-bp 5′ flanking region spanning from −307 to −250 was multimerized in a head-to-tail orientation in the pUC119 plasmid vector. For this, a *Bgl*II linker was inserted at the *Pst*I site after it had been made blunt by T4 DNA polymerase. The multimerization was carried out by cutting out the *Bgl*II-*Bam*HI fragment from the plasmid and cloning it into the *Bam*HI site of another plasmid. The multimers of the 58-bp fragment were placed 5′ to the truncated (−90) CaMV 35S promoter in both correct and opposite orientations and were subsequently inserted into the CAT expression cassette. Similarly, 23-bp sequences containing the wild-type and the mutated −287 to −265 promoter sequence of the 27-kDa zein gene were multimerized in a head-to-tail orientation in a plasmid vector to generate hexamers. They were fused 5′ to the truncated CaMV 35S promoter spanning to −90 or −42 (generated by exonuclease III digestion) with respect to the transcription start site and subsequently inserted into the CAT expression cassette.

Electroporation of protoplasts. The establishment of a maize (*Zea mays* L., A636 inbred) endosperm cell suspension culture and isolation of protoplasts from the culture have been described previously (39). One million protoplasts isolated from the endosperm suspension cells were electroporated with 25 μg of plasmid DNA containing each chimeric CAT gene construct as previously described (39). For each electroporation, 10 μg of pFFGUS plasmid (38), containing the *E. coli* β-glucuronidase (GUS) reporter gene placed under the control of the CaMV 35S promoter (with duplicated enhancer elements) and 3′ control sequence, was coelectroporated to standardize the electroporation efficiency. As negative and positive controls for transient-expression experiments, ϕ-CAT, a promoterless CAT gene equipped with the CaMV 35S 3′ control sequence, and pFFCAT, the CAT gene placed under the control of the CaMV 35S promoter (with duplicated enhancer elements) and the 3′ control sequence (39), respectively, were used. To examine tissue specificity of transcriptional activity, some chimeric CAT gene constructs were also electroporated into protoplasts isolated from leaf tissue-derived suspension culture of maize Black Mexican Sweet (BMS), as previously described (41). Electroporated endosperm and BMS protoplasts were cultured as previously described (39, 41).

CAT and GUS enzyme assays. At the end of a 44- to 48-h culture period, cellular protein extracts were prepared from electroporated protoplasts and assayed for the CAT and GUS enzymatic activities as previously described (39). The CAT activity was quantitated by measuring in a scintillation counter the radioactivity of silica gel spots containing the ^{14}C-labeled chloramphenicol and its acetylated forms. GUS activity was determined fluorometrically, using 4-methylumbelliferyl glucuronide as a substrate. Fluorescence was measured with a fluorometer (model TKO100; Hoefer Scientific Instruments, San Francisco, Calif.) with excitation at 365 nm and emission at 460 nm.

Preparation of nuclear extracts. Intact nuclei were isolated from cultured maize cells and the endosperm tissue from developing kernels of maize A636 inbred plants at 13 days after pollination, as previously described (9). Partially purified (30% ammonium sulfate-cut) nuclear extract was prepared from the isolated nuclei as previously described (15). After the protein concentration was determined by the Bradford assay, nuclear extract was stored in aliquots at −80°C.

Oligonucleotide sequences. A 23-base oligonucleotide sequence, 5′-GACGTGTAAAGTAAATTTACAAC-3′, which includes the binding site of a nuclear factor in the 27-kDa zein promoter in the coding strand, and its complementary sequence, 5′-GTTGTAAATTTACTTTACACGTC-3′, were synthesized. Another set of 23-base oligonucleotides, 5′-GAC CTCTAGACTAAATTTACAAC-3′ and 5′-GTTGTAAATT TAGTCTAGAGGTC-3′, containing four nucleotide substitutions in the nuclear factor binding site was also synthesized. In addition, a set of complementary 15-base oligonucleotides, 5′-CACATGTGTAAAGGT-3′ and 5′-ACCTTTACACATG TG-3′, corresponding to the −300 element region highly conserved among all α-class zein promoters was synthesized. These complementary oligonucleotides were phosphorylated, annealed, and cloned into pUC119 plasmid vector at the *Hin*cII site. The sequences of the cloned oligonucleotides were confirmed by the dideoxy sequencing method.

Gel retardation assays. The plasmid fragments containing the 58-bp promoter fragment spanning from −307 to −250 and the 23-bp sequences spanning from −287 to −265 were used as the probes for gel retardations. Both the 58-bp promoter fragment and oligonucleotide sequences were initially cloned in pUC119 plasmid vector at the *Hin*cII site. To label the probes, the plasmids were cut at the *Xba*I site in the polylinker and the 3′ ends were labeled with [α-^{32}P]dCTP by the Klenow fragment of DNA polymerase I. After extraction with phenol and chloroform and ethanol precipitation, the plasmid was digested with *Hin*dIII. The plasmid DNA digests were fractionated on 8% polyacrylamide gels, and the labeled probes were isolated from gel fragments. Gel retardation assays were carried out either at room temperature or on ice in a final volume of 10 to 15 μl containing 10 mM Tris, pH 8.0, and 1 mM EDTA. Nuclear protein extract prepared from cultured maize cells or the endosperm tissue from developing maize kernels was incubated with 5 μg of poly(dI-dC) for 5 min. Subsequently, 0.25 to 0.5 ng of DNA probe end labeled with ^{32}P was added and further incubated for 30 min. At the end of incubation, the reaction mixtures were fractionated on 8% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. After being dried, gels were subjected to autoradiography. For competition experiments, increasing amounts of the unlabeled probes or nonspecific DNA sequences were added to the binding reactions.

DNase I footprinting assays. The probe used in the DNase I footprinting assay was the plasmid fragment containing the 252-bp *Hin*fI fragment spanning from −415 to −164 of the 27-kDa zein 5′ flanking sequence (Fig. 1A). The fragment was initially cloned into pUC119 plasmid vector at the *Hin*cII site after its ends had been made blunt by the Klenow fragment of DNA polymerase I. To label the probe at the 3′ end of the coding strand, the plasmid was digested with *Xba*I and labeled with [α-^{32}P]dCTP by the Klenow fragment of DNA polymerase I. After extraction with phenol and chloroform and ethanol precipitation, the plasmid was further digested with *Hin*dIII. To label the probe at the 3′ end of the noncoding strand, the plasmid was digested with *Hin*dIII prior to a labeling with [α-^{32}P]dATP by the Klenow fragment of DNA polymerase I. After extraction with phenol and chloroform and ethanol precipitation, the plasmid was further digested with *Xba*I. The plasmid DNA digests were fractionated on 8% polyacrylamide gels, and the probes end labeled with ^{32}P were isolated from gel fragments. For DNase I footprinting assays, 30,000 cpm (about 2 fmol) of end-labeled probe was incubated with 0, 2, 4, or 8 μg of nuclear protein extracts. DNase I digestion products were fractionated on 6% polyacrylamide gels containing 8 M urea. The A + G and G sequencing reactions of the probes end labeled with ^{32}P done by a chemical modification method (27) were also run along with the DNase I digestion mixtures.

TABLE 1. Comparison of the −300 (prolamin) box sequences in different zein promoters

Class	Gene product size (kDa)	Sequence[a]	Location[b]	Reference
α	22 (*22Z-4*)	CACATGTGTAAAGGT	−229/−220	33
α	19 (*LZG99*)	CACATGTGTAAAGGT	−241/−232	30
β	15	TTGACATGTAAAGTT	−114/−105	29
γ	27	TTGACGTGTAAAGTA	−252/−243	11
δ	10	TAGAGGTGTAAATGG	−452/−443	19
Consensus sequence		ANGTGTAAAG		

[a] The −300 (prolamin) box sequences from various zein genes are aligned, and the conserved sequences are underlined.
[b] The location of the decanucleotide consensus sequence with respect to the TATA box of the gene is indicated.

RESULTS

The 58-bp −307 to −250 region of the 27-kDa zein promoter confers a high level of transcriptional activity. In our previous transient-expression studies, we have shown that the 1,103-bp 5′ flanking sequence of a 27-kDa zein gene (spanning from −1042 to +61 with respect to the transcription start site [Fig. 1A]) exhibits a low but significant level of transcriptional activity in protoplasts isolated from cultured endosperm cells (39, 41). To circumvent the low transcriptional activity of the 5′ flanking sequence, we have utilized a truncated −90 CaMV 35S promoter to examine the transcriptional activities of various upstream regions located between −1042 and −92, as shown in Fig. 2A. These synthetic promoters were fused to the bacterial CAT reporter gene equipped with the CaMV 35S 3′ control region (39). Transcriptional activities mediated by the upstream regions of the 27-kDa zein promoter were assessed by monitoring the levels of transiently expressed CAT enzymatic activity in endosperm protoplasts after electroporation with the chimeric CAT gene constructs (Fig. 2B).

Although most deletions had little effect, two upstream regions significantly affected the basal transcriptional activity of the truncated CaMV 35S promoter. The region spanning from −251 to −92 drastically suppressed the basal transcriptional activity of the truncated CaMV 35S promoter (construct 5 in Fig. 2), suggesting the presence of a negative *cis*-regulatory element(s) in this region. On the other hand, the region spanning from −307 to −250 conferred a high level (about threefold) of transcriptional activation on the truncated CaMV 35S promoter (construct 9 in Fig. 2). This 58-bp region includes the −300 element (Fig. 1A) conserved in almost all zein genes, as shown in Table 1, as well as in other cereal storage protein genes. However, when the sequence located immediately upstream or downstream of the 58-bp region was also present, the transcriptional activity of the 58-bp region was abolished (compare construct 9 with construct 6, 7, 11, or 12 [Fig. 2]). These adjacent regions had little effect on the basal transcriptional activity of the truncated CaMV 35S promoter when they were independently placed upstream of the truncated promoter (constructs 8 and 10 [Fig. 2]). These results suggest that multiple *cis*-acting elements present around the 58-bp −307 to −250 upstream region regulate its transcriptional activity.

Orientation- and tissue-independent transcriptional activity of the −307 to −250 region. We have further examined whether the high level of transcriptional activity mediated by the 58-bp −307 to −250 region is independent of its orientation by placing it upstream of the truncated −90 CaMV 35S promoter in the opposite orientation, as shown in Fig. 3A. We have also generated multimers of the 58-bp fragment, fused in a head-to-tail orientation, to examine whether they might further increase the transcriptional activity of the fragment (Fig. 3A). When a monomer of the 58-bp fragment was placed 5′ to the truncated CaMV 35S promoter in the opposite orientation, a high level of transcriptional activity was observed (pCR1XUR9oCAT in Fig. 3). This transcriptional activation level (about 2.5-fold) was slightly lower than that obtained with the monomer placed in the correct orientation (pCR1XUR9cCAT in Fig. 3). The transcriptional activity of the 58-bp fragment increased with its copy number; when placed in the correct orientation, three and five copies of the 58-bp fragment conferred about five- and sevenfold enhancement, respectively, of the basal transcriptional activity of the truncated CaMV 35S promoter (Fig. 3). Equally high levels of transcriptional activity were also observed when these multimers were fused 5′ to the truncated CaMV 35S promoter in the opposite orientation. These results have demonstrated that the transcriptional activity mediated by the 58-bp −307 to −250 upstream region is orientation independent and increases as the result of multimerization.

To examine the tissue specificity of the transcriptional activity mediated by the 58-bp −307 to −250 upstream region, the chimeric CAT constructs were electroporated into protoplasts isolated from leaf tissue-derived suspension culture cells of maize BMS. Unlike the cultured maize endosperm cells, the BMS cells do not express zein genes (39) or the gene encoding Opaque-2, the transcriptional regulator of the 22-kDa zein genes (41). A monomer and a trimer of the 58-bp fragment, when fused in the correct orientation to the truncated CaMV 35S promoter, conferred about 2- and 6.5-fold enhancement, respectively, over the basal transcriptional activity derived from the truncated promoter (Fig. 3C). The level of enhancement by the monomer was lower in BMS protoplasts than in endosperm protoplasts; however, that conferred by the trimer was significantly higher in BMS protoplasts. These results demonstrated that the high level of transcriptional activity mediated by the 58-bp −307 to −250 region is not restricted to the cultured endosperm cells.

Sequence-specific binding of a nuclear factor(s) to the 58-bp −307 to −250 region. To examine whether the high level of transcriptional activity of the 58-bp −307 to −250 region is mediated by its interaction with a *trans*-acting factor, binding of nuclear proteins extracted from cultured endosperm cells to the 58-bp fragment was analyzed by gel retardation assays. As shown in Fig. 4A, after incubation of the nuclear protein extract with the 58-bp fragment end labeled with ^{32}P and subsequent fractionation in a polyacrylamide gel, one retarded complex was observed. The amount of the protein-DNA complex increased proportionally with the increasing concentration of nuclear protein extract. Addition of a 50- or 100-fold molar excess of the unlabeled homologous competitor DNA reduced the binding of the nuclear protein(s) to the labeled fragment (Fig. 4B). In contrast, the addition of corresponding amounts of nonspecific competitor DNA, generated from

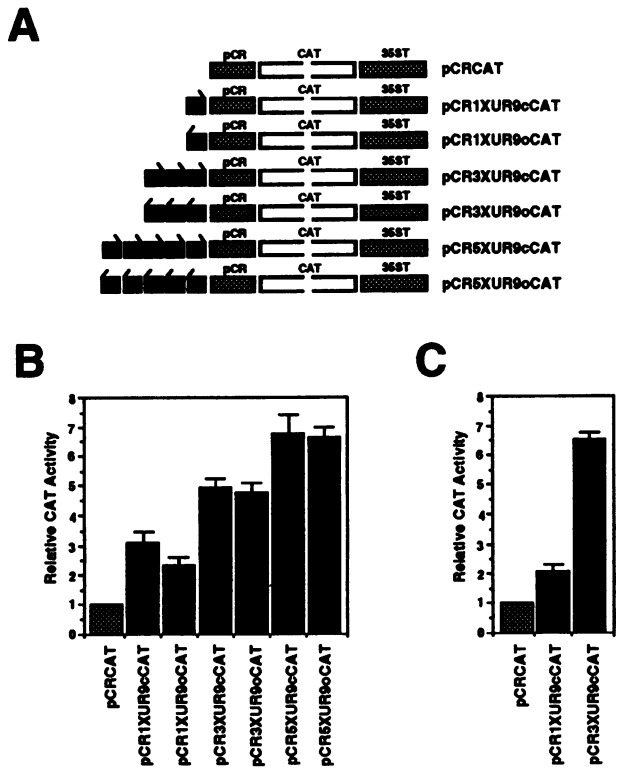

FIG. 3. Orientation- and tissue-independent transcriptional activity of the 58-bp −307 to −250 upstream region. (A) Schematic representation of chimeric CAT constructs consisting of the CaMV 35S 3′ control sequence (35ST) and the synthetic promoter composed of the truncated −90 CaMV 35S promoter (pCR) and the −307 to −250 5′ flanking sequence of the 27-kDa zein gene (solid boxes). The direction of the arrow on the solid box indicates the 5′→3′ orientation of the −307 to −250 sequence, and the number of solid boxes corresponds to the copy number of the −307 to −250 sequence fused to the truncated CaMV 35S promoter. (B) Transcriptional activity of the synthetic promoters in the transiently transformed endosperm protoplasts. (C) Transcriptional activity of the synthetic promoters in the transiently transformed BMS protoplasts. (B and C) The level of CAT activity derived from the promoterless CAT construct was subtracted from those obtained from the synthetic promoter-CAT constructs in order to correct for nonspecific CAT activity. For each construct, the level of CAT enzymatic activity was standardized to that of GUS enzymatic activity derived from the coelectroporated pFFGUS plasmid. These values were expressed relative to that for the pCRCAT construct. The average value of data obtained from five independent experiments is shown for each chimeric construct in the histogram, with the standard deviation from the mean indicated by an error bar.

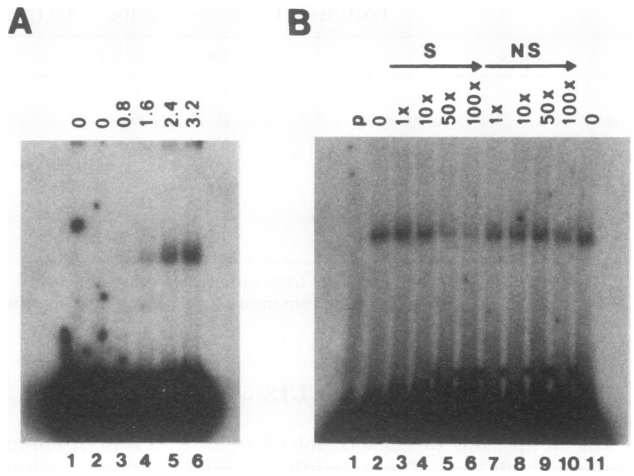

FIG. 4. Gel retardation and competition assays with the 58-bp −307 to −250 upstream fragment and the nuclear protein extract from cultured endosperm cells. (A) Gel retardation assay. The end-labeled plasmid fragment containing the 58-bp −307 to −250 upstream sequence was used as a probe. It was incubated in the binding buffer only (lane 1), the binding buffer containing 5 μg of poly(dI-dC) (lane 2), or the binding buffer containing 5 μg of poly(dI-dC) and an increasing amount of a partially purified nuclear protein extract from cultured endosperm cells (0.8, 1.6, 2.4, and 3.2 μg in lanes 3, 4, 5, and 6, respectively) before fractionation on a 6% polyacrylamide gel. (B) Competition assay. The formation of the nuclear protein-DNA complex was challenged with specific (S) or nonspecific (NS) competitor DNA composed of the unlabeled probe or a 136-bp sequence derived from the pUC119 plasmid, respectively. The end-labeled probe was incubated with 3.2 μg of the nuclear protein extract and increasing amounts of specific (lanes 3 to 6) or nonspecific (lanes 7 to 10) competitor DNA in the binding buffer containing 5 μg of poly(dI-dC). The samples were then fractionated on a 6% polyacrylamide gel. The molar ratios of the competitor DNA to the probe used in the assays are indicated at the top of the panel. For the control reaction mixtures, the probe alone (lane 1) or the probe incubated with the nuclear extract in the absence of the competitor DNA (lanes 2 and 11) was used.

pUC119 plasmid, did not have a pronounced inhibitory effect on the binding of the nuclear factor(s) to the labeled promoter fragment (Fig. 4B). These results indicated the sequence-specific binding of the nuclear protein factor(s) to the 58-bp upstream region.

To identify the nuclear factor binding site within the 58-bp −307 to −250 region, DNase I footprinting was performed. Nuclear protein extract was prepared from the cultured endosperm cells and incubated with a probe end labeled with ^{32}P spanning from −415 to −164 of the 27-kDa zein promoter (Fig. 1A). As shown in Fig. 5, a 16-base sequence, 5′-TGTA AAGTAAATTTAC-3′, present in the coding strand of the 58-bp upstream region was protected from digestion by DNase I. This protected region contains a direct repeat of a pentanucleotide, GTAAA, and an imperfect inverted repeat, both of which overlap with the −300 element, TGTAAAG (Fig. 1B). In the noncoding strand, 8 of the corresponding 16 nucleotides protected in the coding strand and another short nucleotide sequence, 5′-GGATTTTTG-3′, were also protected from DNase I digestion (Fig. 1B and 5).

The 23-bp oligonucleotide sequence containing the nuclear factor binding site mediates the factor binding and transcriptional activation. We have synthesized a 23-bp oligonucleotide, corresponding to the −287 to −265 sequence of the 27-kDa zein promoter (Fig. 1B) and containing the nuclear factor binding site, and examined whether it is sufficient for the binding of the nuclear factor in vitro. In addition, we have also synthesized a mutant 23-bp oligonucleotide sequence containing four nucleotide substitutions (Fig. 1B). Three of the four nucleotide substitutions were made in the −300 element present within the factor binding site, and the fourth substitution was in the nucleotide immediately adjacent to the −300 element. As shown in Fig. 6A, gel retardation assays demonstrated that the wild-type oligonucleotide sequence was sufficient for the binding of the nuclear factor(s) present in cultured endosperm cells. This binding was sequence specific, since the formation of the retarded complex was significantly reduced by the addition of a 50- or 100-fold molar excess of the unlabeled homologous oligonucleotide sequence. In contrast, little reduction in binding was observed when corresponding

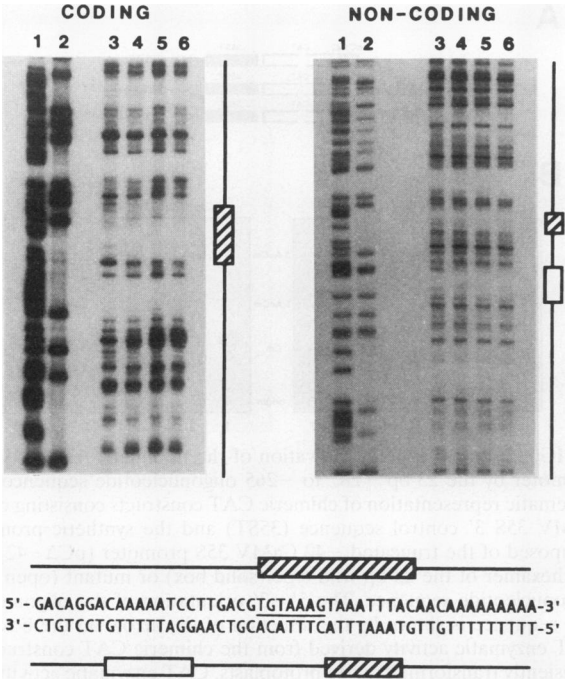

FIG. 5. In vitro DNase I footprinting analysis on the 27-kDa zein promoter. A pUC119 plasmid fragment end labeled with ^{32}P containing the 27-kDa zein promoter sequence, spanning from −415 to −164, was used as a probe for the DNase I footprinting assay. The probe was incubated with DNase I without (lane 3) or with prior incubation with an increasing amount of a partially purified nuclear protein extract prepared from cultured endosperm cells (2, 4, and 8 μg in lanes 4, 5, and 6, respectively). The left panel shows the footprint analysis performed on the coding strand; the right panel shows that on the noncoding strand. Lanes 1 and 2 in both panels show the Maxam-Gilbert sequencing reactions of the probes for A+G and G, respectively. The regions protected from DNase I digestion are shown in boxes in the right margins. The hatched boxes represent the region which was protected on both coding and noncoding strands, whereas the open box indicates an additional protected region on the noncoding strand. The nucleotide sequence spanning from −305 to −256 is shown at the bottom. The −300 element is underlined, and the nucleotides protected from DNase I digestion are indicated by the hatched and open boxes along each DNA strand.

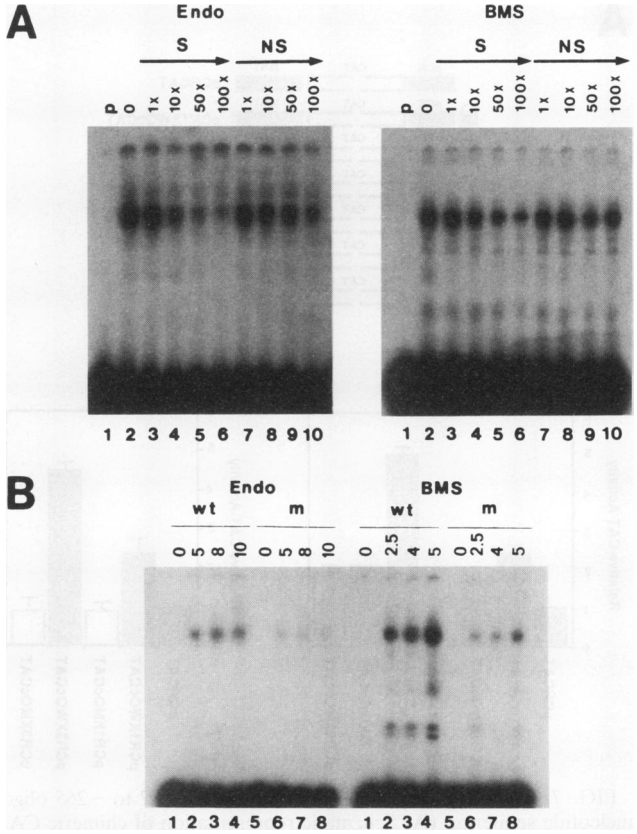

FIG. 6. Gel retardation and competition assays with the 23-bp oligonucleotide sequence and nuclear protein extracts from cultured endosperm and BMS cells. (A) Gel retardation and competition assays. The plasmid fragment end labeled with ^{32}P containing the 23-bp wild-type oligonucleotide was used as a probe. The formation of the nuclear protein-DNA complex was challenged with specific (S) (lanes 3 to 6) or nonspecific (NS) (lanes 7 to 10) competitor DNAs composed of the unlabeled probe or a mixture of 63- and 64-bp sequences derived from the pUC119 plasmid, respectively. Competition assays were carried out with 10 and 5 μg of partially purified nuclear protein extracts from the endosperm (Endo) (left) and BMS (right) cells, respectively. The molar ratios of the competitor DNA to the probe used in the assays are indicated at the top of the panel. Reaction mixtures with the probe alone (lane 1) or without any competitor DNA (lane 2) were used as controls. (B) Gel retardation assays with wild-type or mutant oligonucleotide sequences. The wild-type (wt) (lanes 2 to 4) and mutant (m) (lanes 6 to 8) oligonucleotide probes, ^{32}P end labeled, of equal specific activity were incubated with an increasing amount of nuclear protein extract from the endosperm (Endo) (left) or BMS (right) cells before fractionation on a 6% polyacrylamide gel. The amounts (in micrograms) of the protein extracts used are shown at the tops of the panels. The reaction mixture with the probe alone was used as a control (lanes 1 and 5).

amounts of nonspecific competitor sequence derived from pUC119 plasmid were added.

We have also tested the nuclear extract derived from BMS suspension cells in our gel retardation assays. One prominent retarded complex of a size similar to that observed with cultured endosperm cells was detected. Additionally, the binding of the BMS-derived nuclear factor(s) was also sequence specific, as inferred from competition assays (Fig. 6A). These results suggest the presence of a similar nuclear factor(s) in both endosperm and BMS cells. The nuclear factor is much more abundant in BMS cells than in endosperm cells, since much greater amounts of retarded DNA-protein complex were generated with smaller amounts of BMS nuclear protein extract (Fig. 6B). The four nucleotide substitutions in the mutant oligonucleotide dramatically reduced the binding of the nuclear factor present in both endosperm and BMS cells (Fig. 6B).

Finally, we have tested whether the 23-bp oligonucleotide sequence is sufficient to confer the transcriptional activity in vivo. A monomer and a multimer (trimer) of the 23-bp fragment were fused to the truncated −90 CaMV 35S promoter, and the synthetic promoters were inserted into the CAT expression cassette, as shown in Fig. 7A. In endosperm protoplasts, the monomer of the wild-type oligonucleotide, when placed in the correct orientation, conferred about a 2.5-fold increase in the basal transcriptional activity of the truncated CaMV 35S promoter (Fig. 7B). The trimer of the wild-type oligonucleotide increased the transcriptional activity by about fivefold (Fig. 7B), which is an increase similar to that previously achieved with the trimer of the 58-bp −307 to −250 fragment (Fig. 3B). However, unlike the situation for the 58-bp

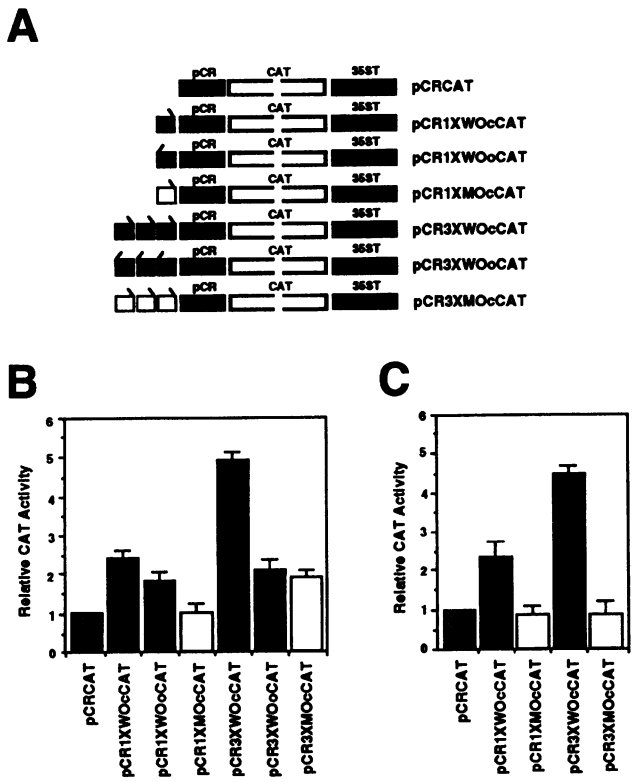

FIG. 7. Transcriptional activity of the 23-bp −287 to −265 oligonucleotide sequence. (A) Schematic representation of chimeric CAT constructs consisting of the CaMV 35S 3′ control sequence (35ST) and the synthetic promoter composed of the truncated −90 CaMV 35S promoter (pCR) and the 23-bp wild-type (solid box) or mutant (open box) oligonucleotide sequence. The 5′→3′ orientation of the oligonucleotide sequence is shown by the direction of the arrow on the box, and the number of boxes corresponds to the copy number of the oligonucleotide sequence fused to the truncated CaMV 35S promoter. (B) Transcriptional activity of the synthetic promoters in the transiently transformed endosperm protoplasts. (C) Transcriptional activity of the synthetic promoters in the transiently transformed BMS protoplasts. (B and C) The level of CAT activity derived from the promoterless CAT construct was subtracted from those obtained from the synthetic promoter-CAT constructs in order to correct for nonspecific CAT activity. For each construct, the level of CAT enzymatic activity was standardized to that of GUS enzymatic activity derived from the coelectroporated pFFGUS plasmid. These values were expressed relative to that for the pCRCAT construct. The average value of data from five independent experiments is shown for each chimeric construct in the histogram, with the standard deviation from the mean indicated by an error bar.

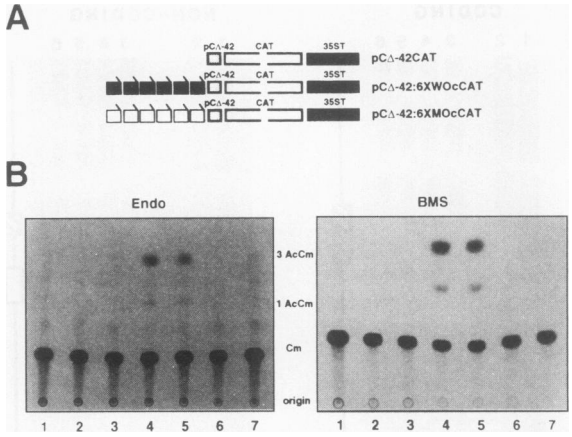

FIG. 8. Transcriptional activation of the minimal −42 CaMV 35S promoter by the 23-bp −287 to −265 oligonucleotide sequence. (A) Schematic representation of chimeric CAT constructs consisting of the CaMV 35S 3′ control sequence (35ST) and the synthetic promoter composed of the truncated −42 CaMV 35S promoter (pCΔ−42) and the hexamer of the 23-bp wild-type (solid box) or mutant (open box) oligonucleotide sequence. The 5′→3′ orientation of the oligonucleotide sequence is shown by the direction of the arrow on the box. (B) CAT enzymatic activity derived from the chimeric CAT constructs in transiently transformed maize protoplasts. CAT enzymatic activity was determined in endosperm (Endo) or BMS protoplasts which had been electroporated without plasmid construct (lane 1), with pCΔ−42CAT (lanes 2 and 3), with pCΔ−42:6XWOcCAT (lanes 4 and 5), or with pCΔ−42:6XMOcCAT (lanes 6 and 7). Fifty and 25 μg of protein extracts prepared from electroporated endosperm and BMS protoplasts, respectively, were used for CAT enzymatic assays. For each protoplast type, two independent sets of data are shown for each chimeric CAT construct. Abbreviations: Cm, unreacted substrate [^{14}C]chloramphenicol; 1 AcCm, 1-acetylchloramphenicol; 3 AcCm, 3-acetylchloramphenicol.

fragment, the transcriptional activity mediated by the 23-bp oligonucleotide sequence is orientation dependent. When the oligonucleotide sequence was fused in the opposite orientation to the truncated CaMV 35S promoter, the transcriptional activity did not increase with the copy number (Fig. 7B). In contrast to the wild type, the mutant oligonucleotide sequence containing the four nucleotide substitutions in the factor binding site did not confer a significant level of transcriptional activation on the truncated CaMV 35S promoter, and its multimerization had little effect on the level (Fig. 7B). Similarly, in the BMS protoplasts, the wild-type oligonucleotide sequence conferred a comparable level of transcriptional activity, whereas the mutant sequence lacked the transcriptional activity (Fig. 7C). Thus, the transcriptional activity mediated by the 23-bp −287 to −265 27-kDa zein promoter sequence is tissue independent. However, one may still argue that the tissue-independent transcriptional activity of this 27-kDa zein promoter sequence may be due to the synergistic effects of both the zein promoter element and the enhancer element present within the truncated −90 CaMV 35S promoter (1) utilized in our analyses. To rule out this possibility, we extended our functional analysis by utilizing an additional truncated CaMV 35S promoter in which the 5′ endpoint was extended to −42, thus eliminating its enhancer element. This truncated −42 CaMV 35S promoter by itself exhibits an insignificant level of transcriptional activity on the CAT reporter gene in both endosperm and BMS protoplasts (Fig. 8). When a hexamer of wild-type −287 to −265 27-kDa zein promoter sequence was placed 5′ to the truncated −42 CaMV 35S promoter in the correct orientation, the synthetic promoter mediated a high level of CAT expression in both endosperm and BMS protoplasts (Fig. 8). The level of transcriptional activity was higher in the BMS protoplasts than in the endosperm protoplasts. On the other hand, the synthetic promoter containing the hexamer of the mutant 27-kDa zein promoter sequence fails to exhibit a significant level of transcriptional activity in both types of protoplasts (Fig. 8). These observations are consistent with the previous results obtained with the truncated −90 CaMV 35S promoter. Thus, the high level of transcriptional activity mediated by the −287 to −265 27-kDa zein promoter sequence is independent of its synergistic effect with the enhancer element present in the −90 CaMV 35S promoter. These results are consistent with our previous

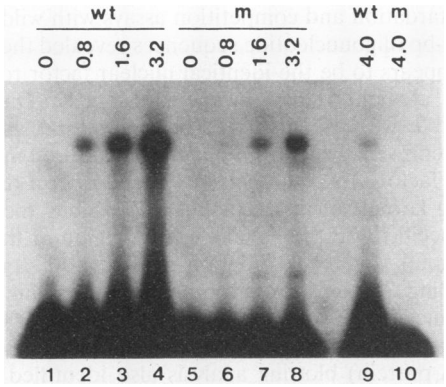

FIG. 9. Gel retardation assays with the nuclear protein extract from the maize kernel endosperm. Equal specific activities of the wild-type (wt) (lanes 2 to 4) and mutant (m) (lanes 6 to 8) 23-bp oligonucleotide probes, end labeled with ^{32}P, containing the 27-kDa zein −300 element were incubated with an increasing amount of the nuclear protein extract isolated from the maize kernel endosperm and fractionated on a 6% polyacrylamide gel. The amounts (in micrograms) of the protein extracts used are shown at the top of the panel. For comparison, 4.0 μg of nuclear proteins from cultured endosperm cells was also incubated with either the wild-type (lane 9) or the mutant (lane 10) oligonucleotide probe. The reaction mixtures with the wild-type (lane 1) or mutant probe (lane 5) alone were used as controls.

observation that the wild-type oligonucleotide sequence, but not the mutant, can mediate high-affinity binding of the nuclear factor.

The −300 element binding factor is abundant in the developing maize kernel endosperm nuclei. To confirm that the −300 element binding factor(s) we have identified in the cultured endosperm cells is also present in the kernel endosperm tissue, we have prepared the nuclear protein extracts from the developing endosperm tissue of maize (A636 inbred) kernels at 13 days after pollination. We performed gel retardation assays with the 23-bp oligonucleotide sequence end labeled with ^{32}P containing either the wild-type or the mutant factor binding site (Fig. 1B). As shown in Fig. 9, a prominent DNA-protein complex was formed with the wild-type oligonucleotide probe, whereas it was much reduced when the mutant oligonucleotide probe was used. These results are in agreement with those obtained with the nuclear protein extracts from cultured endosperm cells (Fig. 6B). However, the concentration of the nuclear factor(s) is much higher in the kernel endosperm tissue than in the cultured endosperm cells, since a very small amount of nuclear protein extract from the kernel endosperm tissue was sufficient to form the prominent DNA-protein complex. The molecular sizes of the DNA-protein complexes formed with the kernel and cultured endosperm nuclear protein extracts were identical (Fig. 9). Moreover, DNase I footprinting analysis revealed that the binding site of the nuclear factor(s) from the kernel endosperm around the −300 element region was essentially identical to that previously shown for the nuclear factor prepared from the cultured endosperm cells (data not shown). These results indicate that the −300 element binding factor(s) identified in the cultured endosperm nuclei is also present at a higher concentration in the kernel endosperm nuclei. This correlates with the much higher expression level of zein genes in the developing kernel endosperm than in the cultured endosperm cells (39).

The common nuclear factor(s) binds to the −300 element core sequences in different classes of zein promoters. Among all α-class 19- and 22-kDa zein genes, the conservation of the −300 element region extends to a 15-base stretch, 5′-CACAT GTGTAAAGGT-3′ (5). However, the sequence conservation in the −300 element region among different zein genes is limited only to a 10-base sequence, 5′-ANGTGTAAAG-3′ (Table 1), which constitutes the −300 core element. It is possible that there are multiple −300 element binding factors present in the endosperm nuclei and that the difference in the nucleotide sequence flanking the −300 core element might modulate the interaction of these factors to specific zein promoters. To examine these possibilities, we have performed in vitro gel retardation and competition experiments. We synthesized a 15-bp oligonucleotide sequence constituting the −300 element region of the α-class zein genes described above. By using it as a competitor, we challenged the binding of the nuclear factor in the kernel endosperm nuclear extract to the 23-bp oligonucleotide sequence probe containing the 27-kDa zein −300 element. As shown in Fig. 10, the addition of 10-, 50-, and 100-fold molar excesses of the α-class −300 element competitor DNA drastically reduced the formation of DNA-protein complex between the nuclear factor and the 27-kDa zein −300 element probe. Addition of nonspecific competitor derived from pUC119 plasmid at the corresponding amounts had a much-reduced inhibitory effect on the complex formation. These observations have demonstrated that a common nuclear factor(s) can bind to the highly conserved −300 element core regions of zein promoters belonging to different classes.

DISCUSSION

By utilizing a maize endosperm culture which continues to express zein genes (39), we have shown that the 58-bp sequence spanning positions −307 to −250 of the 27-kDa zein promoter is capable of conferring a high level of transcriptional activity on a truncated CaMV 35S promoter. The transcriptional activity mediated by the −307 to −250 region is independent of its orientation and is enhanced by increasing its copy number. We have demonstrated the sequence-specific binding of a nuclear protein factor(s) to an approximately 16-nucleotide sequence present in the −307 to −250 region. This nuclear factor binding region includes the −300 element, an element highly conserved in almost all zein genes and many other cereal storage protein genes (4, 7, 12, 28, 34, 37). The direct evidence that the −300 element present in the 58-bp promoter region is responsible for its transcriptional activity was obtained from the site-directed mutagenesis studies with a shorter 23-bp oligonucleotide sequence containing the nuclear factor binding site. This oligonucleotide sequence was sufficient for the high-affinity binding of the nuclear factor in vitro and to confer a high level of transcriptional activity, comparable to that observed for the 58-bp −307 to −250 region, in vivo. However, in contrast to the 58-bp −307 to −250 sequence, the increase in transcriptional activity mediated by the 23-bp oligonucleotide sequence was orientation dependent, as it was also observed for a short (43-bp) −300 element region from a 19-kDa zein promoter (31). Four nucleotide substitutions made in the −300 element in a mutant oligonucleotide sequence resulted in a drastic reduction in the nuclear factor binding in vitro as well as a decrease in the transcriptional activity of the oligonucleotide sequence in vivo. These observations corroborate the idea that the interaction between a transcriptional activator and the −300 element region modulates a high level of transcriptional activity.

The nuclear factor binding region identified in the 27-kDa zein gene shares homology over a 10-nt sequence, 5′-ANGT GTAAAG-3′ (Table 1), and this sequence constitutes the

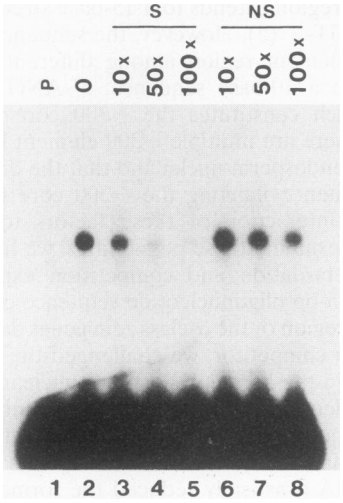

FIG. 10. Binding specificity of the nuclear factor to the −300 element sequence in the α-class zein genes. The formation of the protein-DNA complex with the nuclear protein extract from the maize kernel endosperm and the 23-bp oligonucleotide sequence containing the 27-kDa zein −300 element was challenged with the unlabeled oligonucleotide sequence containing the highly conserved 15-bp stretch (Table 1) of the −300 element region from the α-class zein genes. The 27-kDa zein −300 element probe end labeled with ^{32}P was incubated with 1.6 μg of the nuclear protein extract from the kernel endosperm together with increasing amounts of the α-class −300 element DNA (S) (lanes 3 to 5) or the nonspecific (NS) competitor DNA derived from the pUC119 plasmid (lanes 6 to 8) in the binding buffer containing 5 μg of poly(dI-dC). The samples were fractionated on a 6% polyacrylamide gel. The molar ratios of the competitor DNA to the probe used in the assays are indicated at the top of the panel. For the control reaction mixtures, the probe alone (lane 1) or the probe incubated with the nuclear extract in the absence of the competitor DNA (lane 2) was used.

−300 element motif conserved among different classes of zein genes. However, among all α-class 19- and 22-kDa zein genes, the conservation of nucleotide sequence around the −300 element extends further to 15 bp (5), 14 of which have been shown to constitute the nuclear factor binding site in a 19-kDa zein promoter (23). The functional significance of this extended sequence conservation in the α-class zein genes is not clear. It is possible that multiple nuclear factors are present in the nuclei and that they might differentially bind to the −300 element regions of different zein genes through the binding specificity determined by the nucleotide sequences flanking the −300 element core sequence. However, our in vitro binding and competition experiments have demonstrated that the same nuclear factor that binds to the −300 element in the 27-kDa zein promoter can also bind to that of the α-class zein promoters. Thus, it is likely that the −300 element binding factor is involved in the transcriptional regulation of different zein promoters. The sequences flanking the −300 core element may be involved in the formation of a secondary structure, which in turn may facilitate the binding of the nuclear factor, since the −300 core element constitutes, in part, an imperfect inverted repeat in both the 27- and the 19-kDa zein genes (23).

The high level of transcriptional activity mediated by either the 58-bp −307 to −250 or the 23-bp −287 to −265 upstream region was not restricted to the endosperm protoplasts; a comparable level of transcriptional activity was also observed in protoplasts isolated from leaf tissue-derived BMS suspension cells which do not express zein genes (39). Furthermore, the gel retardation and competition assays with wild-type and mutant 23-bp oligonucleotide sequences revealed the presence of what appears to be the identical nuclear factor recognizing the −300 element region in the BMS cells. This nuclear factor(s) that we have identified in the cultured maize cells does not represent a factor unique to the cultured maize cells. The same factor(s) is abundant in the endosperm tissue from developing kernels of maize plants and exhibits the identical binding specificity to the −300 element region of the 27-kDa zein promoter. By gel retardation analyses, different sets of DNA-binding proteins from both maize seedling and endosperm nuclei have been shown to bind to the −300 element region of a 19-kDa zein promoter (31). However, Southwestern (DNA-protein) blotting analysis also identified a prominent polypeptide of approximately 49 kDa in the nuclear extracts from both tissues which binds to the −300 element region (31). Thus, tissue specificity of the −300 element binding factor(s) in maize plants still remains to be investigated. The observations that a high level of transcriptional activity is mediated by the −300 element region in the dicot systems (21, 26, 36) further suggest the presence of a similar nuclear factor in heterologous plant species.

Multiple regulatory mechanisms are involved in the expression of zein genes. Member-specific expression of 22-kDa zein genes is regulated by an endosperm-specific transcription factor, Opaque-2, through its sequence-specific interaction with the DNA sequence that is unique to the 22-kDa zein promoter (33, 41). In addition to the member-specific regulation, common regulatory mechanisms seem to control the coordinated endosperm tissue- and developmental stage-specific expression of all classes of zein genes (40). It is likely that the highly conserved −300 element plays an essential role in such common regulatory mechanisms. Interestingly, the −300 element resides approximately 20 bp upstream from the Opaque-2 target site in the 22-kDa zein promoters (33). Because of the close proximity between the Opaque-2 target site and the −300 element, interaction between their corresponding transcription factors would be expected. Recently, developmentally coordinated binding of multiple factors to the −300 element and the neighboring GCN4 motif has been shown to correlate with the maximal expression of a wheat low-molecular-weight glutenin gene, *LMWG-1D1*, during grain ripening (17) and of a barley C-hordein gene in nitrogen response during endosperm development (28). Similarly, combinatorial binding of the −300 element binding factor and the Opaque-2 factor may provide synergistic transcriptional activation functions and elicit the maximal expression of the 22-kDa zein gene. For the 27-kDa zein promoter, the sequences present both upstream and downstream from the −300 element region influence its transcriptional activity. For example, we have identified a downstream region spanning from −251 to −92 which confers a negative effect on transcription. We predict that multiple *cis*-regulatory elements are localized in the −300 element region and that their interactions with various transcriptional repressors and activators including the −300 element binding factor(s) would elicit highly regulated expression of the 27-kDa zein genes. Binding of multiple endosperm tissue-specific and nonspecific nuclear proteins to the various promoter regions has been reported for other zein genes (22, 24, 35). Cloning the gene encoding the −300 element binding factor and analyzing the molecular interaction between the −300 element binding factor and other transcription factors will allow us to further define precise molecular mechanisms involved in the regulation of zein genes.

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REFERENCES

1. **Benfey, P. N., L. Ren, and N.-H. Chua.** 1990. Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J. **9**:1677–1684.
2. **Boronat, A., M. C. Martínez, M. Reina, P. Puigdomènech, and J. Palau.** 1986. Isolation and sequencing of a 28-kDa glutelin-2 gene from maize. Common elements in the 5′ flanking regions among zein and glutelin genes. Plant Sci. **47**:95–102.
3. **Boston, R. S., R. Kodrzycki, and B. A. Larkins.** 1986. Transcriptional and post-transcriptional regulation of maize zein genes, p. 117–126. *In* L. M. Shannon and M. J. Chrispeels (ed.), Molecular biology of seed storage proteins and lectins. University of California, Riverside.
4. **Brandt, A., A. Montembault, V. Camero-Mills, and S. K. Rasmussen.** 1985. Primary structure of a B1 hordein gene from barley. Carlsberg Res. Commun. **50**:333–345.
5. **Brown, J. W. S., C. Wandelt, G. Feix, G. Neuhaus, and H. G. Schweiger.** 1986. The upstream regions of zein genes—sequence analysis and expression in the unicellular green alga *Acetabularia*. Eur. J. Cell Biol. **42**:161–170.
6. **Burr, B., F. A. Burr, T. P. St. John, M. Thomas, and R. W. Davis.** 1982. Zein storage protein gene family of maize. J. Mol. Biol. **154**:33–49.
7. **Colot, V., L. S. Robert, T. A. Kavanagh, M. W. Bevan, and R. D. Thompson.** 1987. Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. EMBO J. **6**:3559–3564.
8. **Cruz-Alvarez, M., J. A. Kirihara, and J. Messing.** 1991. Post-transcriptional regulation of methionine content in maize kernels. Mol. Gen. Genet. **225**:331–339.
9. **Das, O. P., M. Cruz-Alvarez, S. Chaudhuri, and J. Messing.** 1990. Molecular methods for genetic analysis of maize. Methods Mol. Cell Biol. **1**:213–222.
10. **Das, O. P., and J. W. Messing.** 1987. Allelic variation and differential expression at the 27-kilodalton zein locus in maize. Mol. Cell. Biol. **7**:4490–4497.
11. **Das, O. P., E. Poliak, K. Ward, and J. Messing.** 1991. A new allele of the duplicated 27 kD zein locus of maize generated by homologous recombination. Nucleic Acids Res. **19**:3325–3330.
12. **DeRose, R. T., D.-P. Ma, I.-S. Kwon, S. E. Hasnain, R. C. Klassy, and T. C. Hall.** 1989. Characterization of the Kafirin gene family from sorghum reveals extensive homology with zein from maize. Plant Mol. Biol. **12**:245–256.
13. **Esen, A.** 1986. Separation of alcohol-soluble proteins (zeins) from maize (*Zea mays*) into three fractions by differential solubility. Plant Physiol. **80**:623–627.
14. **Geetha, K. B., C. R. Lending, M. A. Lopes, J. C. Wallace, and B. A. Larkins.** 1991. *opaque-2 modifiers* increase γ-zein synthesis and alter its spatial distribution in maize endosperm. Plant Cell **3**:1207–1219.
15. **Green, P. J., S. A. Kay, E. Lam, and N.-H. Chua.** 1989. In vitro DNA footprinting. Plant Mol. Biol. Manual **B11**:1–22.
16. **Hagen, G., and I. Rubenstein.** 1981. Complex organization of zein genes in maize. Gene **13**:239–249.
17. **Hammond-Kosack, M. C. U., M. J. Holdsworth, and M. W. Bevan.** 1993. In vivo footprinting of a low molecular weight glutenin gene (LMWG-1D1) in wheat endosperm. EMBO J. **12**:545–554.
18. **Heidecker, G., and J. Messing.** 1986. Structural analysis of plant genes. Annu. Rev. Plant Physiol. **37**:439–466.
19. **Kirihara, J. A., J. B. Petri, and J. Messing.** 1988. Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize. Gene **71**:359–370.
20. **Kodrzycki, R., R. S. Boston, and B. A. Larkins.** 1989. The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell **1**:105–114.
21. **Kriz, A. L., R. S. Boston, and B. A. Larkins.** 1987. Structural and transcriptional analysis of DNA sequences flanking genes that encode 19 kilodalton zeins. Mol. Gen. Genet. **207**:90–98.
22. **Maier, U.-G., J. W. S. Brown, L. M. Schmitz, M. Schwall, G. Dietrich, and G. Feix.** 1988. Mapping of tissue-dependent and independent protein binding sites to the 5′ upstream region of a zein gene. Mol. Gen. Genet. **212**:241–245.
23. **Maier, U.-G., J. W. S. Brown, C. Toloczyki, and G. Feix.** 1987. Binding of a nuclear factor to a consensus sequence in the 5′ flanking region of zein genes from maize. EMBO J. **6**:17–22.
24. **Maier, U.-G., K. D. Grasser, M. M. Haaß, and G. Feix.** 1990. Multiple proteins bind to the P2 promoter region of the zein gene pMS1 of maize. Mol. Gen. Genet. **221**:164–170.
25. **Marks, M. D., J. S. Lindell, and B. A. Larkins.** 1985. Quantitative analysis of the accumulation of zein mRNA during maize endosperm development. J. Biol. Chem. **260**:16445–16450.
26. **Matzke, A. J. M., E. M. Stöger, J. P. Schernthaner, and M. A. Matzke.** 1990. Deletion analysis of a zein gene promoter in transgenic tobacco plants. Plant Mol. Biol. **14**:323–332.
27. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
28. **Müller, M., and S. Knudsen.** 1993. The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J. **4**:343–355.
29. **Pedersen, K., P. Argos, S. V. L. Naravana, and B. A. Larkins.** 1986. Sequence analysis and characterization of a mize gene encoding a high-sulfur zein protein of Mr 15,000. J. Biol. Chem. **216**:6279–6284.
30. **Pedersen, K., J. Devereux, D. R. Wilson, E. Sheldon, and B. A. Larkins.** 1982. Cloning and sequence analysis reveal structural variation among related zein genes in maize. Cell **29**:1015–1026.
31. **Quayle, T., and G. Feix.** 1992. Functional analysis of the −300 region of maize zein genes. Mol. Gen. Genet. **231**:369–374.
32. **Rubenstein, I., and D. F. Geraghty.** 1986. The genetic organization of zein. Adv. Cereal Sci. Technol. **8**:297–315.
33. **Schmidt, R. J., M. Ketudat, M. J. Aukerman, and G. Hoschek.** 1992. Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kDa zein genes. Plant Cell **4**:689–700.
34. **Shotwell, M. A., S. K. Boyer, R. S. Chesnut, and B. A. Larkins.** 1990. Analysis of seed storage protein genes of oats. J. Biol. Chem. **265**:9652–9658.
35. **So, J.-S., and B. A. Larkins.** 1991. Binding of an endosperm-specific nuclear protein to a maize beta-zein gene correlates with zein transcriptional activity. Plant Mol. Biol. **17**:309–319.
36. **Thompson, G. A., R. S. Boston, L. A. Lyznik, T. K. Hodges, and B. A. Larkins.** 1990. Analysis of promoter activity from an α-zein gene 5′ flanking sequence in transient expression assays. Plant Mol. Biol. **15**:755–764.
37. **Thompson, G. A., and B. A. Larkins.** 1989. Structural elements regulating zein gene expression. Bioessays **10**:108–113.
38. **Timmermans, M. C. P., P. Maliga, J. Vieira, and J. Messing.** 1990. The pFF plasmids: cassettes utilizing CaMV sequences for expression of foreign genes in plants. J. Biotechnol. **14**:333–344.
39. **Ueda, T., and J. Messing.** 1991. A homologous expression system for cloned zein genes. Theor. Appl. Genet. **82**:93–100.
40. **Ueda, T., and J. Messing.** 1993. Manipulation of amino acid balance in maize seeds. Genet. Eng. **15**:109–130.
41. **Ueda, T., W. Waverczak, K. Ward, N. Sher, M. Ketudat, R. J. Schmidt, and J. Messing.** 1992. Mutations of the 22- and 27-kDa zein promoters affect transactivation by the Opaque-2 protein. Plant Cell **4**:701–709.
42. **Werr, W., W.-B. Frommer, C. Maas, and P. Starlinger.** 1985. Structure of the sucrose synthase gene on chromosome 9 of *Zea mays* L. EMBO J. **4**:1373–1380.
43. **Wienand, U., and G. Feix.** 1980. Zein specific restriction enzyme fragments of maize DNA. FEBS Lett. **116**:14–16.
44. **Wilson, D. R., and B. A. Larkins.** 1984. Zein gene organization in maize and related grasses. J. Mol. Evol. **20**:330–340.
45. **Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.