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A DNA-Binding Protein Factor Recognizes Two Binding Domains within the Octopine Synthase Enhancer Element

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A protein that binds to the enhancing element of the octopine synthase gene has been identified in nuclear extracts from maize cell suspension cultures. Two protein-DNA complexes are distinguishable by electrophoretic mobility in gel retardation assays. Footprint analyses of these low and high molecular weight complexes show, respectively, half and complete protection of the ocs-element DNA from cleavage by methidiumpropyl-EDTA·FE(II). Two lines of evidence indicate that the element has two recognition sites, each of which can bind identical protein units. Elements that are mutated in one or the other half and form only the low molecular weight complex interfere with the formation of both the low and high molecular weight complexes by the wild-type element. Protein isolated from a complex with only one binding site occupied can bind to the wild-type ocs-element and generate complexes with protein occupying one or both binding sites. Occupation of both sites of the ocs-element is a prerequisite for transcriptional enhancement.

INTRODUCTION

Gene expression is modulated at the transcriptional level by the interaction of transcription factors with specific *cis*-regulatory DNA sequences (Schleif, 1988). Some of these *cis*-regulatory sequences that are transcriptional enhancers have been shown to be organized from combinations of DNA sequence motifs, each of which bind proteins to generate transcriptionally active complexes (Fromental et al., 1988; Ondek, Gloss, and Herr, 1988).

An upstream sequence of the octopine synthase gene (*OCS*) has been identified as a transcriptional enhancer (Ellis et al., 1987; Leisner and Gelvin, 1988). The *OCS* gene is transferred and integrated with the T-DNA of *Agrobacterium* into the genome of plant cells during initiation of the crown gall tumor. The gene is not expressed in *Agrobacterium* but is expressed in the plant (Otten et al., 1981). Although the infectivity of *Agrobacterium* is limited generally to dicotyledonous plants, the transcriptional enhancer of the *OCS* promoter functions in both monocots and dicots and does not require any factors supplied by other genes of the bacterium (Hooykaas-van Slogteren et al., 1984; Ellis et al., 1987).

A 16-bp palindrome sequence (ACGTAAGCGCTTACGT, the ocs-element) from the promoter region of the *OCS* gene, when inserted upstream of a reporter gene containing a truncated inactive promoter, enhances expression of the reporter gene in transient expression assays with protoplasts of tobacco and maize (Ellis et al., 1987). Mutations of the palindrome, such as multiple base substitutions, a 4-bp insertion in the center of the palindrome, or specific base changes at critical positions all greatly reduce enhancing activity, indicating a sequence-dependent activity (Ellis et al., 1987; Singh et al., 1989).

In this study we have investigated the protein binding properties of the ocs-element. Gel retardation and footprint analyses were used to characterize the specific interaction with proteins present in maize nuclear extracts. Mutated ocs-elements that exhibited little transcriptional enhancing activity in vivo, but formed protein-DNA complexes in vitro (Singh et al., 1989), were used to identify the protein binding sites within the ocs-element. A reconstruction experiment showed that the element has two binding sites for the same protein. These results have allowed us to establish a model of the protein-DNA interaction required for transcriptional enhancing activity.

RESULTS

Specificity of the Protein-ocs-Element Interaction

A crude extract was prepared from a nuclear fraction of cell suspension culture of maize and assayed, by gel retardation analysis, for DNA binding activity specific to

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the ocs-element (Garner and Revzin, 1981; Fried and Crothers, 1981). As shown in Figure 1A, the maize extract contained binding activity that retarded the mobility of ^{32}P-labeled DNA fragments (probe) containing the ocs-element. These probes had flanking sequences either from the polylinker of pUC118 or the *OCS* gene (see Table 1, wild type and synthetic EcoRI-HindIII). Both probes resolved a pattern of two discrete bands (Figure 1A, lanes 1 and 2). In contrast, probes that lacked the element did not produce this same pattern of retarded bands (lanes 3 and 4). Treatment of the extract with trypsin or proteinase K before the binding reaction eliminated the formation of retarded bands. A similar pattern of two bands was obtained with nuclear protein extracts from *Nicotiana plumbaginifolia* cell suspension cultures and from extracts prepared from etiolated maize root and shoot tissue samples (Singh et al., 1989; J.G. Tokuhisa, K. Singh, E.S. Dennis, and W.J. Peacock, unpublished results).

Specificity of the binding activity was confirmed with the use of unlabeled competitor DNA that either contained or lacked the 16-bp palindrome. Competitor DNA containing the palindrome (Table 1, synthetic BamHI-EcoRI), when mixed with the 67-bp EcoRI-HindIII ocs-element-containing probe before the binding reaction, competed for the binding activity generating both the lower and upper retarded bands. Figure 1B shows that a 50-fold molar excess competed almost all of the upper band and some of the lower band in the reactions (lane 3); a 500-fold molar excess competed all binding activity (lane 4). Similar concentrations of DNA lacking the palindrome were not effective competitors (lanes 5 to 7). Together these experiments showed that both retarded bands are formed by nuclear protein binding specifically to the ocs-element sequence.

Relation of the Two Protein-DNA Complexes

The observation of two retarded bands raised the possibility that different proteins bind to the ocs-element and/or that the element has more than one protein binding site. The proportions of the two retarded bands were altered by varying the concentration of nuclear protein extract and carrier DNA in the binding reactions. As shown in Figure 2A, the lower retarded band predominates with low extract concentration or with high carrier DNA concentration (lane 1), whereas the upper retarded band is dominant at higher extract concentrations in the presence of low concentrations of carrier DNA (lane 6). The relation between the two protein-DNA complexes was further investigated with mutated ocs-elements as both probes and competitors in gel retardation assays. Singh et al. (1989) generated mutants of the ocs-element that contained multiple base substitutions confined to one or other half of the palindrome sequence. As shown in Figure 2B, mutants 3.3 and 6.1, which had base substitutions confined to the 5′ or 3′ palindrome halves, respectively (Table 2), showed only one retarded band under reaction conditions in which a wild-type element formed both bands (Figure 2B, lanes 2 and 3). Singh et al. (1989) had shown that these mutated elements had low transcriptional enhancing activity. The single retarded band formed by these two mutants comigrated with the lower of the two retarded bands of the wild-type probe. When these mutated elements were used as competitors in gel retardation assays, they competed both lower-band and upper-band complexes formed by wild-type and the lower-band complex of the mutated elements (Figure 2B, lanes 5 and 6; Table 2). An element with mutations in both halves was an ineffective competitor of the retarded bands (lane 7).

The ability of ocs-elements with base substitutions concentrated in either one of the halves of the palindrome to form lower but not upper band indicates that upper-band formation is sensitive to DNA sequence alterations and, therefore, is dependent on protein-DNA interactions. Fur-

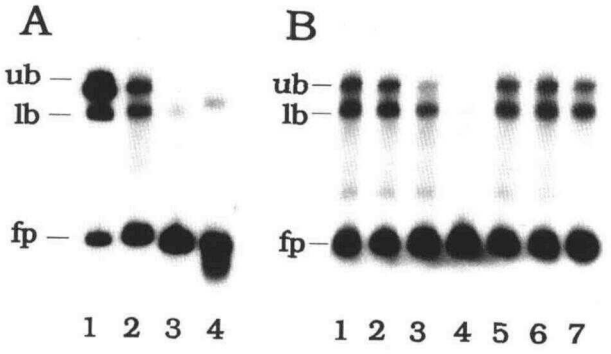

Figure 1. Gel Retardation Analysis for Binding Activity Specific to the ocs-Element.

(A) Binding reactions with different probes: Maize extract was mixed with poly(dI-dC)-poly(dI-dC) and incubated with various probes. Lane 1, a 67-bp EcoRI-Hind III fragment containing the ocs-element with flanking sequences from the pUC118 polylinker site (Table 1, EcoRI-HindIII); lane 2, a 58-bp XhoI-HpaI fragment containing the ocs-element and *OCS* gene flanking sequence (Table 1, wild type); lane 3, a 52-bp XhoI-SalI fragment from the promoter of the *OCS* gene lacking the element (see Methods); lane 4, a 54-bp PstI-SalI fragment from the maize alcohol dehydrogenase 1 gene containing the anaerobic regulatory element. The fractionated DNA is indicated as ub, upper band; lb, lower band; fp, free probe.

(B) Binding reactions using specific and nonspecific unlabeled DNA fragments as competitors of the binding activity. The probe, the 67-bp EcoRI-HindIII fragment containing the ocs-element and pUC118 polylinker sequence (Table 1), was incubated with extract and poly(dI-dC)-poly(dI-dC) after being mixed with no competitor (lane 1); specific competitor (the EcoRI-BamHI fragment, Table 1) at fivefold (lane 2), 50-fold (lane 3), and 500-fold (lane 4) molar excess; nonspecific competitor, the 80-bp HaeIII-HaeIII pUC18 fragment at fivefold (lane 5), 50-fold (lane 6), and 500-fold (lane 7) molar excess.

Table 1. Flanking Sequence Comparison of Wild-Type and Synthesized ocs-Elements

Sequence origin	Sequence	Citation
Wild type	+**ACCAAAAACGTAAGCGCTTACGTACATGGT**+[a]	De Greve et al. (1983)
Synthetic EcoRI-HindIII (67 bp)	+CCAGCTT**ACGTAAGCGCGGACGT**CTGCAGG+	Singh et al. (1989)
Synthetic BamHI-EcoRI	**CACGTAAGCGCTTACGTG**	Ellis et al. (1987)

[a] Boldface type indicates sequence matching the wild-type sequence; + indicates more sequence.

thermore, the ability of these mutated elements to compete both bands of the wild-type element indicates that the two binding sites in the ocs-element are identical. Co-migration of the lower bands generated by wild-type or mutated elements confirms that similar or identical proteins bind to each of the two sites.

The possibility that the same binding protein is involved in the formation of both the lower-band and upper-band complexes was tested directly in a gel retardation experiment in which protein isolated from a lower-band complex was shown to be sufficient to form both complexes in a reconstruction experiment. The mutated (Mut) element 6.1, in a 123-bp PvuII-SalI DNA fragment from pUC118 (Singh et al., 1989), was used as the initial probe for the isolation of the lower-band complex. This mutant sequence contains a HindIII restriction site (A/AGCTT, Table 2) that cuts 3 bp within the intact 5′ half of the element. Treatment of the probe with HindIII before a binding reaction abolished formation of the lower-band complex, showing that HindIII cleavage inactivated the binding site.

The lower-band complex formed with the 6.1 probe was recovered from gels by electroelution. Gel retardation

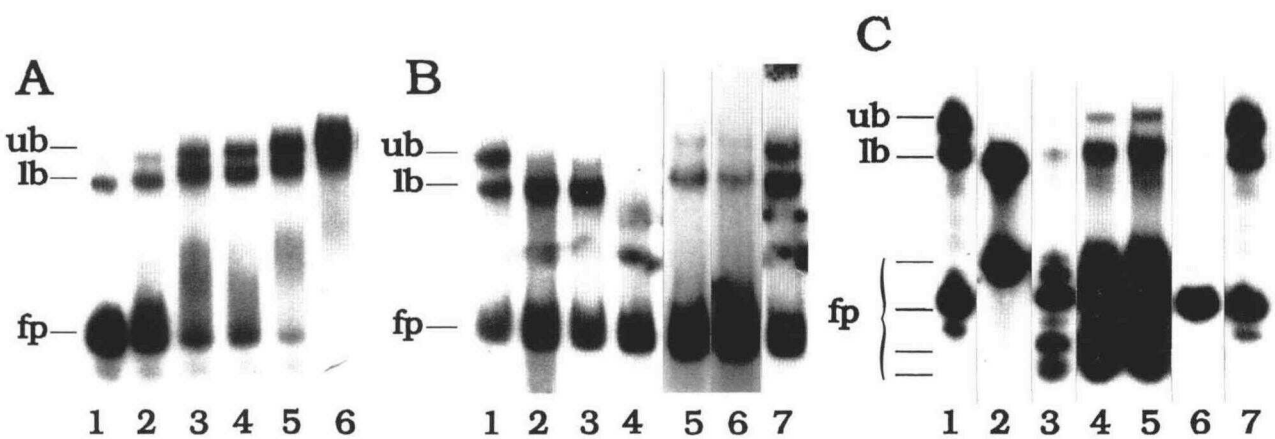

Figure 2. Gel Retardation Analysis of the Relationship of the Upper and Lower Bands.

(A) Titration of extract and carrier DNA in the binding reaction. Binding reactions contained maize nuclear extracts at 4 μg (lanes 1 to 3) and 8 μg (lanes 4 to 6) of protein; poly(dI-dC)-poly(dI-dC) as carrier DNA at 1.0 μg (lanes 1 and 4), 0.5 μg (lanes 2 and 5), and 0.3 μg (lanes 3 and 6); and the ocs-element probe used in Figure 1A, lane 1.

(B) The mutated ocs-elements as probes and competitors. Binding reactions contained maize nuclear extracts with 8 μg of protein, poly(dI-dC)-poly(dI-dC) at 1.0 μg, and the 123-bp PvuII-SalI fragments containing various ocs-elements as probes: lanes 1 and 5 to 7, wild type; lane 2, Mut 3.3; lane 3, Mut 6.1; lane 4, Mut 5.1. Competition of binding (lanes 5 to 7) was assayed as in Figure 1B with 100-fold molar excess of the unlabeled 123-bp fragments containing Mut 3.3 (lane 5), Mut 6.1 (lane 6), Mut 5.1 (lane 7).

(C) Reconstruction of upper-band complex from lower-band activity. The lower-band complex was generated by a binding reaction using the 123-bp PvuII-SalI probe containing the mutant 6.1 ocs-element followed by gel retardation of the reaction mixture. The lower-band complex was electroeluted from an excised gel piece, and a portion was refractionated (lane 2). The remaining eluate was treated with 1 M NaCl, HindIII, and the 67-bp EcoRI-HindIII DNA fragment containing the ocs-element as a probe, diluted to 0.1 M NaCl, incubated at 30°C for 30 min, and dialyzed into NBB. The mixture was refractionated at 0.1, 0.2, and 0.3 final volume to show, respectively, the various cut and uncut free probes (fp) (lane 3), and the lower-band and upper-band complexes (lanes 4 and 5). A gel piece corresponding to the lower-band complex was generated by the fractionation of a binding reaction done in the absence of probe. The electroeluted components were refractionated after treatment with HindIII and the 67-bp EcoRI-HindIII ocs-element containing probe (lane 6). Lanes 1 and 7 mark the upper (ub) and lower (lb) bands from the fractionation of a standard binding reaction with the wild-type ocs-element.

Table 2. Activity of ocs-Elements in Gel Retardation as Probes and as Competitors of Binding Activity

ocs-Element		Probe Bands Generated		Competitor	
		Lower	Upper	Lower	Upper
Wild type	ACGTAAGC|GCTTACGT	+	+	+	+
Mut 3.3	**AAGCAAGG**|GCTTACGT[a]	+	−	+	+
Mut 6.1	ACGTAAGC|**TTGCATGC**	+	−	+	+
Mut 5.1	**GCGACT**GC|GCTT**T**CGT	−	−	−	−

[a] Boldface type indicates base substitutions.

analysis of the eluate showed the lower-band complex and dissociated free probe (Figure 2C, lane 2). The restriction enzyme HindIII was added to the eluate to reduce the number of protein binding sites contributed by the mutated element. Labeled wild-type ocs-element as a 67-bp EcoRI-HindIII DNA fragment with additional sequences from the polylinker of pUC118 and that lacks a HindIII recognition sequence was added to provide an intact element for a binding reaction. The mixture was treated briefly with 1 M NaCl to dissociate existing protein-DNA interactions, and then salt concentrations were adjusted for restriction enzyme activity and for the binding reaction. Electrophoretic analysis of this mixture showed, at a low concentration, the cut and uncut mutated element and intact wild-type ocs-element (lane 3) and, at higher concentrations, two bands that co-migrated with the upper and lower bands of the wild-type ocs-element (lanes 4 and 5).

A control experiment eliminated the possibility that a protein not bound to the Mut 6.1 probe but co-migrating with the lower band had been extracted and bound to the wild-type probe to form the upper-band complex. Protein extract fractionated by gel electrophoresis in the absence of the probe was electroeluted and processed as described above. Fractionation of this mixture showed no protein-DNA complexes (Figure 2C, lane 6), indicating that the binding activity was derived from the protein-DNA complex.

Nucleotide Protection of the ocs-Element in the Lower-Band and Upper-Band Protein-DNA Complexes

Results obtained with the mutated ocs-elements and the data from the reconstruction experiment indicate that the lower-band protein-DNA complex is formed by one protein unit bound to one site of the ocs-element, whereas the upper-band complex is formed by two protein units bound one to each of the two sites of the ocs-element. This was confirmed by subjecting the DNA from the upper and lower bands, the 67-bp EcoRI-HindIII DNA fragment, to nucleotide protection analysis using methidiumpropyl-EDTA·Fe(II), MPE being a reagent causing single-strand cleavage of exposed DNA in solution (Van Dyke and Dervan, 1983). MPE was used in preference to DNase I because of its more random cleavage of the ocs-element. A preparative-scale binding reaction was treated briefly with MPE and fractionated under nondenaturing conditions. The DNA was then extracted from both retarded bands and refractionated under denaturing conditions on an acrylamide gel.

Bottom-strand DNA from the upper-band complex (Figure 3A, lane 6; Figure 3C) was protected from bases 1 to 22, with more complete protection from bases 1 to 19. A similar pattern of protection was obtained with the top-strand DNA from the upper-band complex (Figure 3B, lane 6; Figure 3C); in addition, there was one partially unprotected base, 9. These data indicate that protein binds to the entire element, but more avidly to the 5′ than to the 3′ half of the element. Preferential binding of protein to the 5′ half of the element is also indicated by the protection pattern of DNA from the lower-band complex. Bottom-strand DNA from the lower-band complex was protected only in the 5′ half of the element from bases 1 to 12 with more complete protection from bases 1 to 8 (Figure 3A, lane 5; Figure 3C). Top-strand DNA was also protected from bases 2 to 14 (Figure 3B, lane 5; Figure 3C).

The lower-band complex of the wild-type element consisted of protein bound to only the 5′ half of the element but, with the mutant 3.3, in which the 5′ binding site is altered by 3 base substitutions, footprint analysis of the lower-band complex revealed protection only of the 3′ half of the element. The bottom-strand DNA (Figure 4A, lane 4; Figure 4C) was protected from bases 11 to 21, with absolute protection of bases 14 to 19. The top-strand DNA (Figure 4B, lane 4; Figure 4C) was protected for bases 12 to 24, with absolute protection of bases 13 to 21. This result shows that the 3′ half of the element contains a protein binding site.

Protection of the upper band of the wild-type element extended beyond the palindrome for at least 3 bases in either direction. In the lower band, protection extended at least 2 bases 5′ of the palindrome sequence (Figure 3C), and 1 base beyond the internal boundary of the half palindrome was partially protected. In the 3.3 element, where binding was restricted to the 3′ half of the palin-

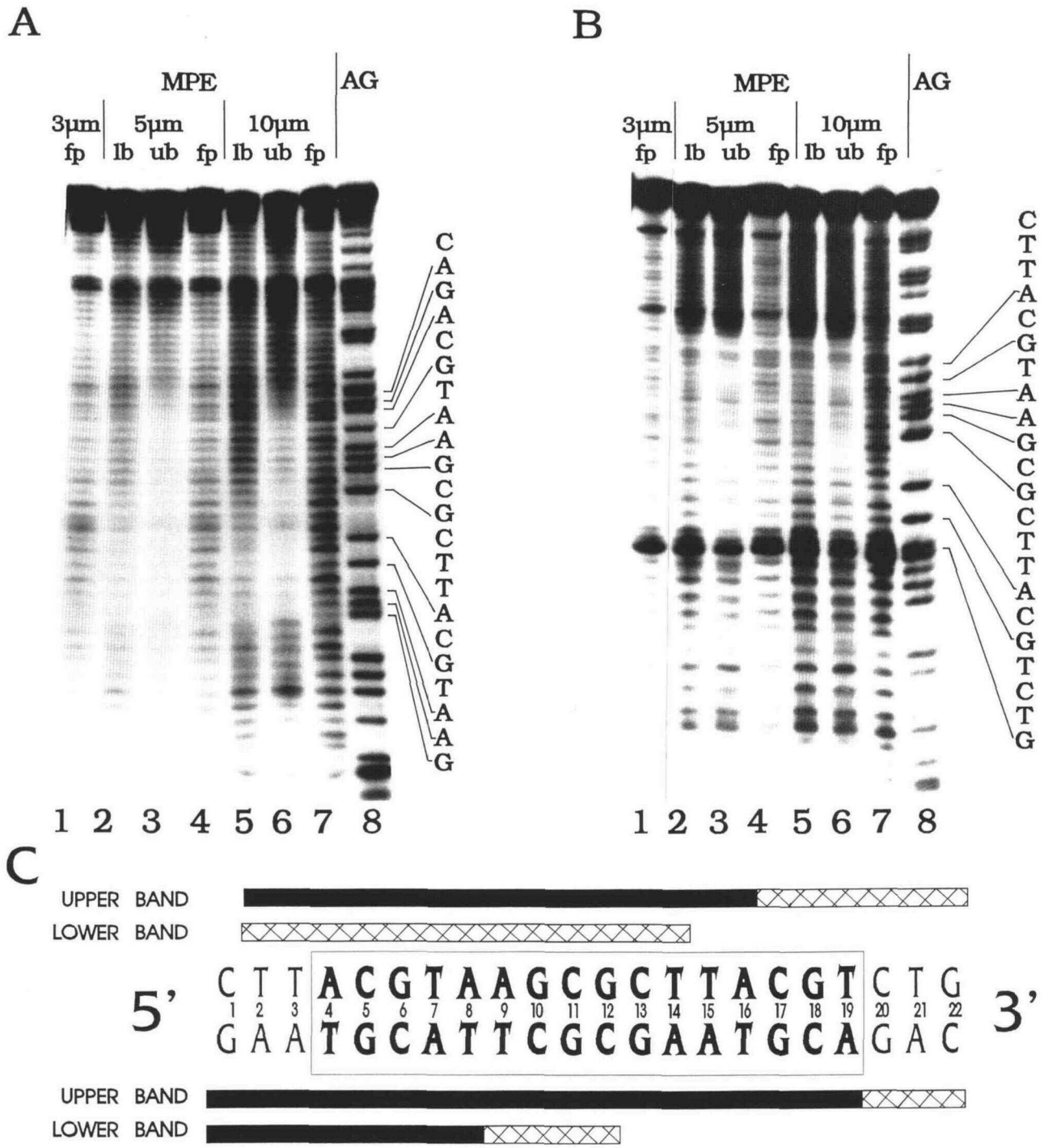

Figure 3. MPE Footprint of the Wild-Type ocs-Element as Free Probe, Lower-Band and Upper-Band Complex.

(A) Bottom strand.
(B) Top strand.
Protein-DNA complexes generated by the binding reaction were treated with 3 μM (free probe only, lane 1), 5 μM (lanes 2 to 4), and 10 μM MPE (lanes 5 to 7). Lanes 2 and 5, lower band; lane 3 and 6, upper band; lanes 1, 4, and 7, free probe; *lane 8, sequence marker*, A+G reaction.
(C) Summary of the footprint protection. Complete protection is indicated by a solid horizontal bar and partial protection by a hatched bar. The sequence is that of the 16-bp ocs-element (within box) in the pUC118 polylinker. Sequences identical to the native *OCS* gene are indicated in boldface.

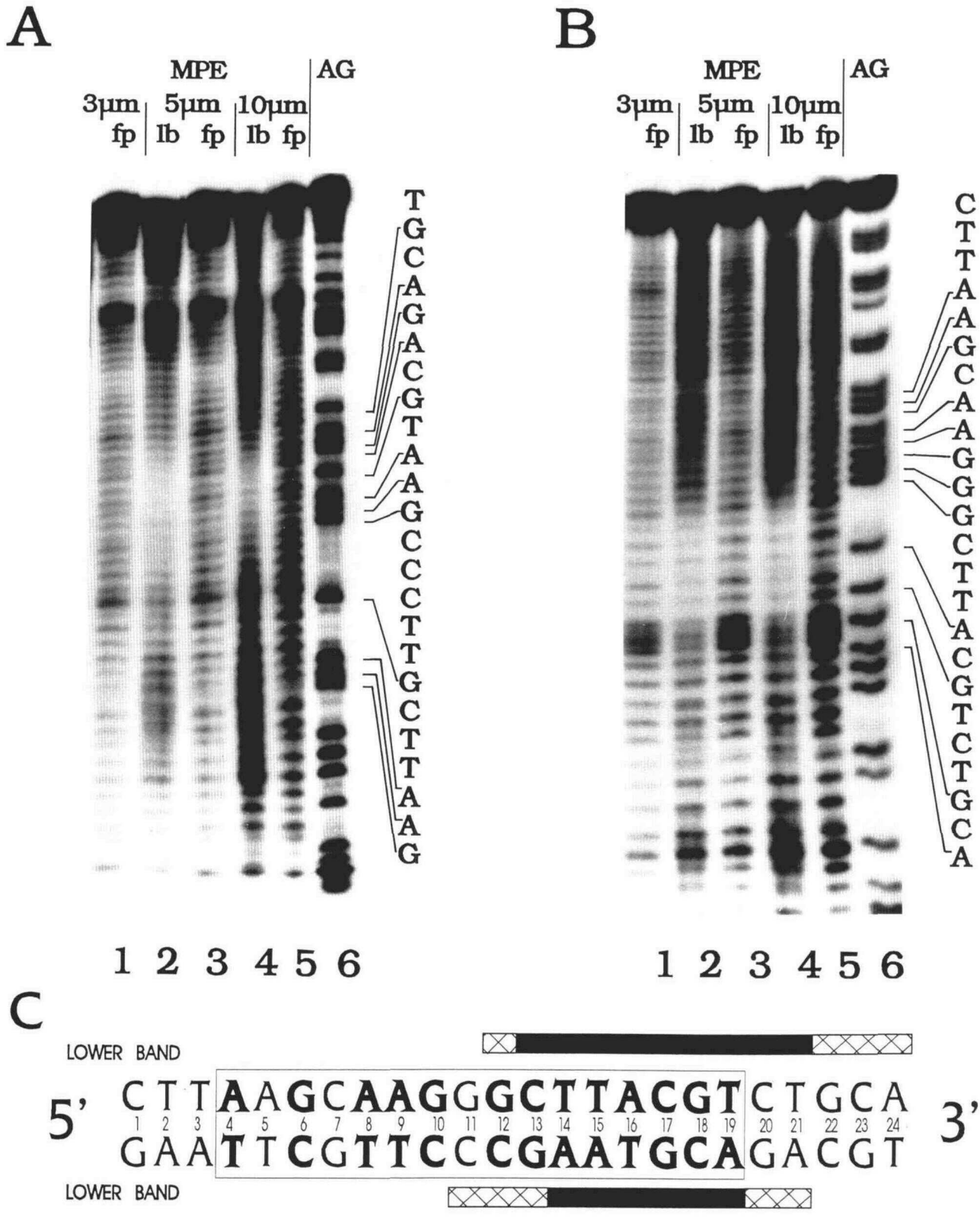

Figure 4. MPE Footprint of the Mut 3.3 ocs-Element as Free Probe, and Lower-Band Complex.

drome, protection again extended 2 to 5 bases beyond the palindrome boundary (Figure 4C).

DISCUSSION

The gel retardation assays showed that nuclear extracts of maize contained protein with high binding affinity for the ocs-element. The formation of two retarded bands raised the possibility that more than one protein interacts with the palindrome or that there are two binding sites in the ocs-element. Two independent binding sites were demonstrated by gel retardation and footprint analyses of mutated ocs-elements. Mutant elements with sequence alterations restricted to one half of the inverted repeat formed the lower molecular weight complex. Footprint analysis of the DNA from lower molecular weight complexes demonstrated partially protected elements, whereas the footprint of the DNA of the higher molecular weight complex showed complete protection. The ability of mutated ocs-elements that formed lower molecular weight complexes to compete binding activity associated with either the low or high molecular weight complexes confirmed that two directly comparable binding sites are distributed, each to one half of the palindrome. A reconstruction experiment demonstrated that the DNA-binding protein extracted from the lower molecular weight complex was sufficient for formation of the high molecular weight complex with the intact ocs-element probe.

The protein that binds to one site of the ocs-element has been identified as the *OCS* transcription factor (OCSTF) because Singh et al. (1989) have shown that transcriptional enhancement in vivo is correlated with the presence of upper bands in gel retardation assays. This correlation indicates that the two binding sites have to be occupied in order that the element-protein complex function as an enhancer. Although we have shown that an intact half element is recognized by OCSTF but is incapable of transcriptional activation, the prospect exists that, in a different sequence context, a half element may function. A motif corresponding to one half of the estrogen-responsive element, which is normally nonfunctional, does function as an estrogen-responsive element in the ovalbumin promoter at a specific position relative to the TATA box (Tora et al., 1988).

Two binding sites, one in each half of the palindrome, would be expected to have equal affinity for the transcription factor. The footprint analyses did not support this expectation in that the transcription factor was shown to be bound preferentially to the 5′ half of the element when only one site of the element was occupied (i.e., lower-band formation). In the mutation analysis of the ocs-element, Singh et al. (1989) had found unequal transcriptional enhancing effects of identical mutations in symmetric positions within the 5′ and 3′ halves of the element. These observations indicate that the ocs-element is larger than the 16-bp palindrome, encompassing adjacent nonpalindromic sequences that contribute different binding affinities to the two sites.

Consistent with this possibility, the footprint analyses show that DNA protection extends at least 3 bp on each side of the 16-bp palindrome, indicating protein-DNA interactions extending beyond the palindrome. Furthermore, Bouchez et al. (1989) have described a number of elements similar in sequence to the ocs-element that competed with the ocs-element for the same transcription factor. The sequences of this family of elements, when aligned with the 16-bp ocs-element, generate 20 bp of consensus sequence incorporating the sequence of the 16-bp ocs-element and 2 additional bases on either side (Table 3).

Thus, although the 16-bp palindrome is sufficient for interaction with two identical transcription factors through two repeated binding sites, it is clear that adjacent sequences affect the relative affinities of the two sites. We predict from the results of this present study that footprint analysis of a 20-bp perfect palindrome should demonstrate equal affinity for both halves of the element. Furthermore, mutations of a 20-bp perfect palindrome that are symmetric to the inverted repeat would be expected to have identical consequences for the relative affinities of the two sites.

Examination of the 20-bp wild-type ocs-element shows that both a near-direct repeat and an inverted repeat can be identified (Table 3). We have not, in the present experiments, identified the precise sequence of the binding site. If the binding site motif is a direct repeat, then the transcription factor may be a monomeric polypeptide. On the other hand, if each binding site is an 8-bp palindrome with a consensus sequence of TTACGTAA, then the transcription factor may actually be a dimer of polypeptides, each polypeptide recognizing a 4-bp sequence. The functional

Figure 4. (continued).

(A) Bottom strand.

(B) Top strand.

Protein-DNA complexes generated by the binding reaction were treated with 3 μM (free probe only, lane 1), 5 μM (lanes 2 and 3), and 10 μM (lanes 4 and 5) MPE. Lanes 2 and 4, lower band; lanes 1, 3, and 5, free probe; lane 6, sequence marker, A+G reaction.

(C) Summary of the footprint protection. The sequence is that of the 16-bp Mut 3.3 ocs-element (within box) in pUC118 polylinker. Symbols are as in Figure 3.

Table 3. Sequence Comparison of the 20-bp Consensus Sequence with Wild-Type and Synthesized ocs-Elements

ocs-Element	Sequence	Comparison with Consensus	Comparison with Wild Type	Citation
Consensus	TGACG[T/C]AAG[CGA/GAC][T/T][G/C]ACGT[AA/CCC]	20/20	18/20	Bouchez et al. (1989)
Wild type XhoI-HpaI (58 bp)	AAACGTAAGCGCTTACGTAC	18/20	20/20	De Greve et al. (1983)
Synthetic EcoRI-HindIII (67 bp)	TTACGTAAGCGCTTACGTCT	18/20	16/20	Singh et al. (1989)
Synthetic BamHI-EcoRI (26 bp)	CCACGTAAGCGCTTACGTGA	17/20	16/20	Ellis et al. (1987)

transcription complex would then be a tetrameric polypeptide complex composed of two dimeric transcription factors binding to the 20-bp element.

Recent work with the ocs-element (Ellis et al., 1987; Singh et al., 1989) has been based on a 16-bp element. The data presented here and by Bouchez et al. (1989) indicate that the synthesized elements of these studies have only 16 out of 20 bp of the wild-type sequence; however, they do have 17 or 18 out of 20 bp of the consensus sequence (Table 3). Nevertheless, these studies have demonstrated that, in spite of these base changes, the 16-bp element with flanking sequences differing from those flanking the element in the *OCS* gene is sufficient both for transcriptional enhancement of transient expression in protoplasts and binding of the OCSTF.

The interaction of dimeric proteins with direct or inverted repeat sequences has been suggested as a mechanism to increase the selectivity of protein-DNA interactions through increased affinity (Schleif, 1988). However, such increased selectivity requires that the independent affinities of two separate protein-DNA interactions be combined either through a single dimeric binding factor with two binding sites properly aligned for the duplicated DNA sequence or a monomeric protein interacting cooperatively with a preexisting monomer-DNA complex. The protein-DNA interactions described here indicate neither a single transcription factor binding to the entire element nor strong cooperativity between two transcription factors with the two binding sites of the element. However, selectivity of transcriptional activation may be maintained with the ocs-element by requiring the exact position of two independent binding site motifs such that juxtaposition of two transcription factors allows formation of a structure necessary for the active transcriptional complex.

The similarity of the OCSTF-DNA interactions seen with maize and tobacco nuclear extracts implies that OCSTF is conserved between monocots and dicots. The presence of OCSTF in plants such as maize, which is not infected by *Agrobacterium*, suggests that it predates both the *Agrobacterium*-dicot association and the monocot-dicot divergence. This wide distribution indicates that the OCSTF may be an essential component of the transcriptional regulation of one or more key enzymes or of some other fundamental cellular process in plants.

METHODS

Preparation of DNA Fragments for Gel Retardation and Footprint Analyses

Two DNA fragments for gel retardation were from the promoter of the *OCS* gene (see Figure 3 of Ellis et al., 1987): a 58-bp XhoI-HpaI fragment containing the ocs-element and flanking sequence from the plasmid pΔ5′-206 corresponds to −206 to −143 relative to the transcription start site, and a 52-bp XhoI-SalI fragment from the plasmid pΔ5′-157 corresponds to −157 to −116. The 54-bp PstI-SalI DNA fragment from plasmid pLS-89/-80 spans −140 to −87 relative to the transcription start site of alcohol dehydrogenase 1 and contains the anaerobic regulatory element (see Figure 4 of Walker et al., 1987). The nonspecific competitor was an 80-bp HaeIII-HaeIII fragment from the plasmid pUC18. The PvuII-SalI fragment (123 bp) containing either the 16-bp wild type (Table 2, wild type) or various mutated ocs-elements (Table 2, Mut 3.3, Mut 6.1, Mut 5.1) was from plasmid gene constructs described in Figure 1 of Singh et al. (1989). The 67-bp EcoRI-HindIII fragment used for gel retardations and footprint analyses was generated from the HindIII-SalI fragment containing the wild type or Mut 3.3 ocs-elements (see Figure 1 of Singh et al., 1989), filled in at the HindIII site with the Klenow fragment of DNA polymerase I, and cloned into the SmaI-SalI polylinker site of pUC118. The 26-bp BamHI-EcoRI fragment from the plasmid pUC19pal16 (Ellis et al., 1987) contained the 16-bp ocs-element and flanking sequences corresponding to BamHI and EcoRI recognition sequences.

Preparation of ^{32}P-Labeled DNA Fragments and Markers for Gel Retardation and Footprint Analyses

Fragments for gel retardation were isolated from polyacrylamide gels and incubated with the Klenow fragment of DNA polymerase I and nucleotide triphosphates in which an incorporated nucleotide was ^{32}P-labeled. The reaction was terminated by the addition of

ammonium acetate to a final concentration of 2 M and the DNA recovered by ethanol precipitation. The DNA concentration was adjusted to 5000 cpm (Cerenkov)/μL (0.1 to 0.5 ng/μL). For footprint analyses, DNA fragments were labeled on either the top or bottom strand as follows. The plasmids were restricted with either HindIII or EcoRI followed by heat inactivation. The linearized plasmids were incubated with the Klenow fragment of DNA polymerase I and nucleotide triphosphates with one of the incorporated nucleotides ^{32}P-labeled. The labeled plasmids were heat treated and incubated with the complementary restriction enzyme to generate DNA fragments. The fragments were isolated from polyacrylamide gels followed by ethanol precipitation. The DNA concentration was adjusted to 100,000 cpm (Cerenkov)/μL. The adenine-guanine positions in the DNA fragments were identified by specific base reactions described by Maxam and Gilbert (1980).

Generation of Nuclear Protein Extracts from Maize Cell Suspension Cultures

Protoplasts were prepared as described (Potrykus, Harms, and Lörz, 1979) from a *Zea mays* cv Black Mexican Sweet XII-II suspension cell line (Chourey and Zurawski, 1981) (generously provided by P. Chourey, University of Florida). Nuclei were isolated essentially by method II of Luthe and Quatrano (1980). Nuclear protein extracts were prepared as described by Parker and Topol (1984). In brief, nuclei (approximately 250 μL packed volume from approximately 230 mL packed volume of cell suspension culture) were resuspended to 8 mL with nuclei resuspension buffer (15 mM Hepes, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol). The nuclei were lysed after adjusting the nuclei resuspension buffer to 380 mM ammonium sulfate (3.3 M ammonium sulfate, 0.05 M Tris, pH 7.8). The lysate was clarified at 200,000*g* for 60 min (Beckman Ty65 rotor). The supernatant was concentrated by ammonium sulfate precipitation (0.25 g/mL), resuspended, and dialyzed into nuclear binding buffer (NBB, 10% glycerol, 25 mM Hepes, pH 7.8, 50 mM KCl, 1 mM EDTA, and 5 mM 2-mercaptoethanol). The final protein concentration was 1 to 3 μg/μL as determined by Bradford (1976) using BSA as a standard. The extract was stored at −80°C.

Fractionation of Protein-DNA Mixtures

Gel retardation assays were as described previously (Fried and Crothers, 1981; Garner and Revzin, 1981). For analytical assays, 3 to 10 μg of nuclear protein extract (stored at 1 to 3 μg/μL at −80°C) was incubated with 0.1 to 1.5 μg of poly(dI-dC)-poly(dI-dC) and DNA at 5000 cpm per reaction, adjusted to a final volume of 10 to 20 μL with NBB, and incubated at 30°C for 30 min. The mixtures were fractionated on 4% acrylamide gels containing 22 mM Tris-borate, pH 8.0, and 0.5 mM EDTA with water cooling at 4°C and buffer recirculation for 1.5 hr.

MPE Reactions

Reaction conditions were established according to Van Dyke and Dervan (1983). Preparative binding reactions (30 μL) containing 25 μg of nuclear protein extract, 4 μg of poly(dI-dC)-poly(dI-dC), and 400,000 cpm (Cerenkov) ^{32}P-labeled DNA fragments were incubated for 30 min at 30°C, cooled to 25°C, adjusted to a final concentration of dithiothreitol at 10 mM and MPE (the MPE generously provided by P. Dervan, California Institute of Technology, Pasadena, CA) at 0.001 to 0.01 mM, and incubated for 15 min at 25°C to effect DNA cleavage. The mixture was incubated at 4°C to inhibit the cleavage reaction and subjected to gel retardation as above with water cooling at −10°C. Radioactive regions of the gel, identified by autoradiography of the wet gel for 2 hr at 4°C, were excised and the DNA eluted with 500 mM ammonium acetate, 1 mM EDTA, and 10 mM magnesium acetate. The DNA fragments in the eluate were mixed with 7 μg of tRNA, recovered by ethanol precipitation, and fractionated on an 8% sequencing gel.

Reconstruction of the Upper-Band Protein-DNA Complex from the Lower-Band Complex

Preparative binding reactions (20 μL) containing 16 μg of nuclear protein extract, 7.5 μg of poly(dI-dC)-poly(dI-dC), and 300,000 cpm (Cerenkov, 6 ng DNA) PvuII-SalI fragment containing the mutated ocs-element 6.1 were prepared and fractionated as described above for gel retardation. The protein-DNA complex that co-migrated with the lower-band complex of the wild-type element probe was identified by wet gel autoradiography as described above and excised as a gel piece, and the protein-DNA complex was electroeluted into 22 mM Tris-borate, pH 8.0, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 5 μg/mL BSA at 4°C. The eluate was concentrated by centrifugal microconcentrators (30-kD cutoff, Centricon-30, Amicon Scientific, Sydney, Australia) and adjusted to a final concentration of 1 M NaCl. The eluate was diluted 10-fold with 10 mM Tris, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol after the addition of 10 units (Pharmacia LKB Biotechnology Inc) of HindIII and 30,000 cpm (Cerenkov) EcoRI-HindIII DNA fragment containing the wild-type ocs-element. The eluate was incubated for 1 hr at 30°C, concentrated by centrifugal microconcentration into NBB. Gel retardation of the concentrate was as above.

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