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Repression of the CaMV 35S promoter by the octopine synthase enhancer element

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A 16 base-pair palindrome upstream of the *Agrobacterium tumefaciens* octopine synthase (*ocs*) gene functions as a positive regulatory element in plant cells (Ellis et al. (1987) EMBO J. 6, 3203–3208; Fromm et al. (1989) Plant Cell 1, 977–984). We have converted it into a negative element by locating two copies flanking the TATA-box of the constitutively expressed CaMV 35S promoter. The reduced promoter activity is very likely due to sterical hindrance of the *ocs* binding protein (OCSTF) × *ocs* complex with the transcription initiation complex. We propose that this type of constructs can be used for the identification of recognition sites for DNA-binding proteins which are labile in vitro as well as for determining the DNA-binding activity of a *trans*-acting factor in vivo.

CaMV 35S promoter; In vivo binding studies; Octopine synthase enhancer; Sterical hindrance

1. INTRODUCTION

One of the best characterized positive cis acting elements in plants is the octopine synthase (*ocs*) enhancer element, a 16 bp palindrome, first identified in the upstream of the *ocs* gene [1]. The *ocs* gene is located on the T-DNA of the tumor inducing (Ti) plasmid of *Agrobacterium tumefaciens* and is expressed only after transfer to plant cells [3]. Placement of the *ocs* palindrome to an enhancerless heterologous promoter results in an over 100-fold increase in promoter strength in a transient expression system using maize or tobacco protoplasts [1]. The association of two identical protein units (OCSTF) with the *ocs* element is necessary for maximal transcriptional activation [4].

In this study we show that the transcriptional activator(s) binding to the *ocs* sequences (OCSTF) can repress the normally constitutive CaMV 35S promoter in vivo. We have placed two *ocs* palindromes in the vicinity of the TATA box, so that sterical interference of the activator with the transcription initiation complex seemed likely. We propose that this type of analysis can generally be used to detect binding of *trans*-acting factor in vivo. It may also serve to test, whether a defined sequence as well as mutants thereof are recognized by a DNA binding protein.

2. MATERIALS AND METHODS

2.1. *Recombinant DNA techniques*

Standard procedures were used for recombinant DNA work [5].

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2.2. *Constructs*

To construct a suitable cassette promoter for cloning *cis*-regulatory sequences flanking the TATA box of the CaMV 35S promoter, five unique restriction sites were introduced between position −54 and +2 of the CaMV 35S promoter. A pair of synthetic oligonucleotides carrying the appropriate bp substitutions with cohesive *Hga*I- and *Bgl*II-ends was cloned into pTET7 using the same strategy as described earlier for pTET-14 [6]. Subsequently, a 23-bp synthetic oligonucleotide containing the 16-bp *ocs* palindrome was cloned into pIGF107 cut with *Spe*I and *Sna*BI, yielding pOCS-1. In a third step, a second *ocs* palindrome was cloned again as a synthetic oligonucleotide between the *Stu*I- and the *Xho*I site downstream of the TATA box of pOCS-1, yielding pOCS-2 (Fig. 1). pMOCS-1 and pMOCS-2 were gained by introducing a 51-bp synthetic oligonucleotide between the *Spe*I and the *Sna*BI-site of pOCS-2. Altered promoter regions were sequenced according to the dideoxy chain termination method [7] using synthetic primers located either upstream of the *Eco*RV or downstream of the *Bgl*II site.

2.3. *Binding studies*

Isolation of nuclei, nuclear protein extraction, and gel retardation assays were done as described [8].

2.4. *Transient expression analysis*

Preparation of protoplasts, DNA transfer using polyethylene glycol and incubation conditions were done as described [9]. For each experiment, 10 μg of plasmid pTET7, pIGF107, pOCS-2, pMOCS-1 or pMOCS-2 was mixed with 10 μg pAT1 [10] and 180 μg herring sperm DNA.

2.5. *Enzyme assays*

Assays for chloramphenicol acetyl transferase (Cat) and β-glucuronidase (Gus) activity were performed as described [11,12].

3. RESULTS

We have cloned two *ocs* palindromes into the immediate vicinity of the TATA-box of the CaMV 35S promoter in order to investigate whether the transcriptional activator OCSTF, which binds to the palindrome, can

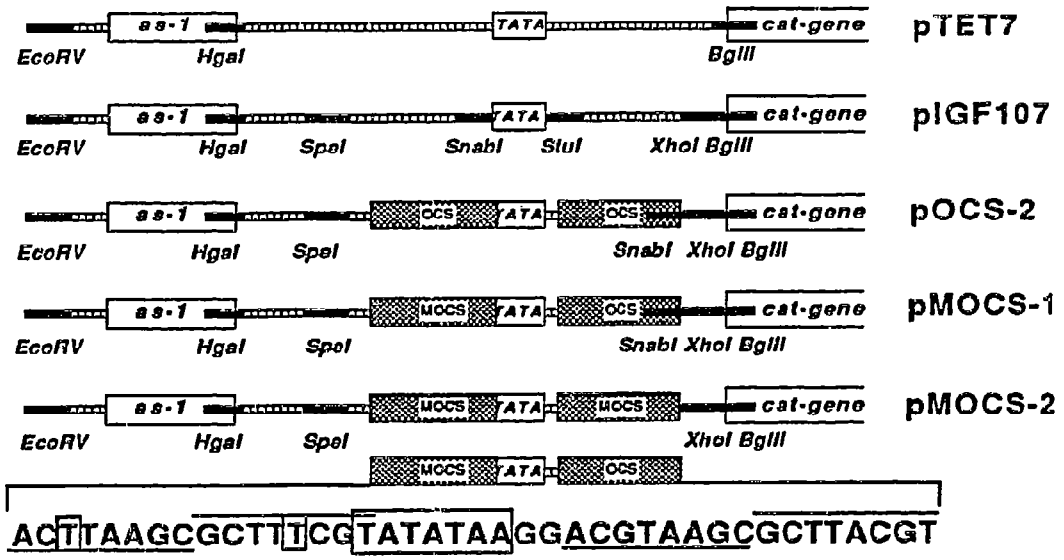

Fig. 1. Region −93 to +1 of different derivatives of the CaMV 35S promoter. Each square represents one bp. The sequence of *as-1* (TGACGTAAGGGATGACG) and of the TATA box (TATATAA) is boxed. The locations of restriction sites are indicated by black squares. The sequence and location of the *ocs*- and mutated *ocs* sequences are shown below. The palindrome is marked by bars, the TATA motif is boxed and altered bp within the mutated palindrome are indicated by squares.

also function as a repressor of transcription. In Fig. 1 the essential features of the different promoter derivatives are shown. The conserved sequence motifs TATA box and activating sequence 1 (*as-1*), which have previously been shown to be essential for promoter activity [13] are indicated by boxes. In all our constructs the spacing between the TATA box and *as-1* was maintained. pTET7 contains the wild-type CaMV 35S promoter, pIGF107 contains a modified version containing 13 single bp substitutions, which lead to the introduction of five unique restriction sites within the region −1 to −50, pOCS-2 contains two wild-type *ocs* palindromes, pMOCS-1 contains one wild-type *ocs* palindrome downstream and a mutant *ocs* palindrome upstream of the TATA box, pMOCS-2 contains two mutated palindromes. For constructing non-functional *ocs* palindromes we changed the bases in positions 3 and 13 of the *ocs* palindrome to T residues, because saturation mutagenesis of the *ocs* element had defined these positions as critical for enhancing activity and in vitro binding [14]. Except for the base pair substitutions within the region −1 to −50 of the CaMV 35S promoter, all plasmids were identical. The gene for the chloramphenicol acetyl transferase (*cat*), to which a nopaline synthase polyadenylation signal (*nos*) was fused [11], was used as a reporter gene.

We have compared the activity of the different CaMV 35S derivatives in a transient expression system using tobacco protoplasts because the *ocs* element was identified as an enhancer in this system [1]. To exclude that a reduced activity was due to the plasmid preparation we did the experiment with two independent preparations (upper and lower part of Fig. 2). As a second means to standardize our values we co-transformed the different derivatives with pAT1, which contains the β-glucuronidase (*gus*) gene behind a CaMV 35S promoter derivative [10]. 20% of the protein extract gained from the transformed protoplasts were tested for Gus activity and the amount of extract tested for Cat activity was normalized according to the Gus values.

The relative promoter activities calculated from the mean values of the triple points taken in each experiment are depicted in the diagram of Fig. 2. The introduction of the restriction sites led to a reduction of the promoter derivative down to 60%, though none of the conserved motifs had been affected by the sequence alterations. It could well be, that altering the sequences in the immediate vicinity of the TATA-box to generate the *Sna*BI- and the *Stu*I sites, led to reduced expression. Exchange of the sequences to introduce the mutated *ocs* palindrome did not lead to a further reduction of the promoter activity. However, restoring a functional palindrome downstream of the TATA-box, which was achieved by changing only 2 bp within the plasmid, led to a 4- to 5-fold drop in promoter activity, and restoration of both palindromes to a 20- to 40-fold reduction. We conclude that this repression is achieved by sterical interference of the transcriptional activator that binds to the *ocs* palindrome with the transcription initiation complex.

This conclusion is supported by the gel shift analysis shown in Fig. 3. A 200-bp restriction fragment between the *Spe*I-(−52) and the *Pvu*II site (+144) of pOCS-2 was end-labelled and used in binding studies with nuclear extracts from tobacco leaves. With limiting amounts of extract two bands with decreased electrophoretic mobility are observed. This banding pattern resembles the gel retardation assays performed by Singh et al., 1989

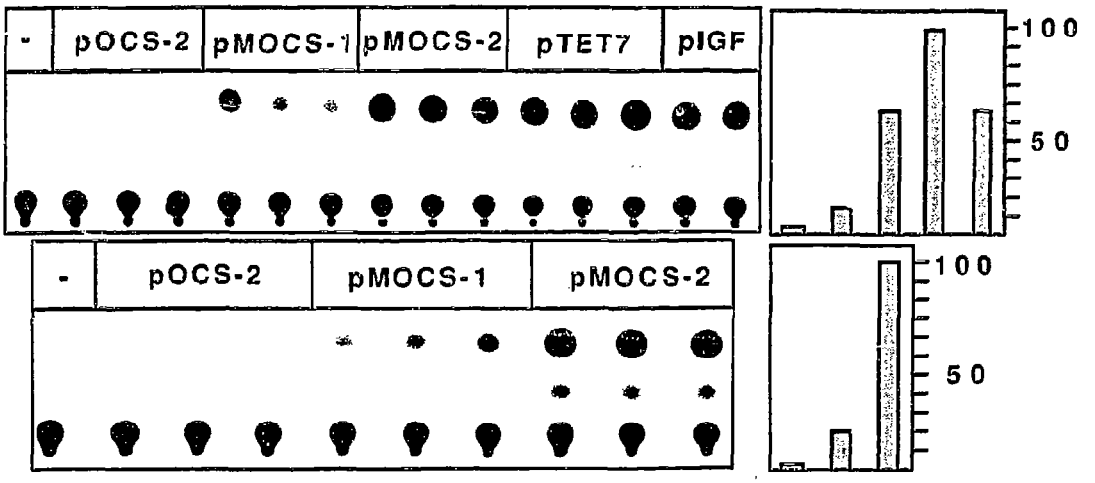

Fig. 2. Cat activity of *Nicotiana tabacum* protoplasts transiently transformed with the plasmids indicated. The analyses shown in the upper and lower panel were done with independent plasmid preparations. Relative amounts of Cat-activity are illustrated in the left panel.

[14]. The lower band was suggested to result from binding of a monomer and the upper band from binding of a dimer of the protein [4]. Increasing the amount of extract led to the occurrence of two further bands which suggests that simultaneous occupation of both *ocs* sites, which are spaced by only 8 bp in our construct, is possible in vitro. In contrast, an equivalent restriction fragment containing mutations in one of the two *ocs* elements led only to the formation of two complexes (Fig. 3B), indicating that this combination of point mutations, which was not included in the previous study [14] led to a reduced affinity of the factor down to undetectable levels. No specific binding to the equivalent restriction fragment of MOCS-2, where both *ocs* palindromes were mutated, could be observed, which correlates well with the complete derepression of this construct in our vivo assay system. Depending on the preparation of the extract, a second extract, a second complex with higher electrophoretic mobility is frequently observed (Fig. 3B), the formation of which is not affected by OCSTF.

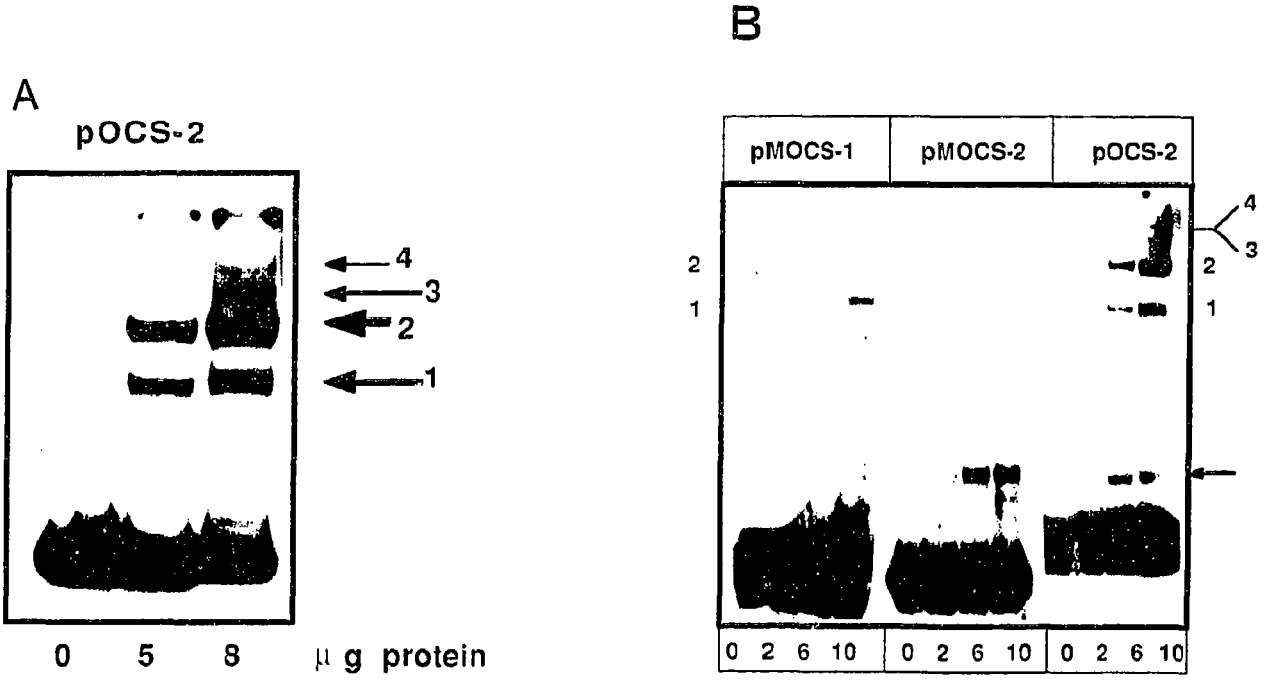

Fig. 3 (A) Gel retardation assay using a 200-bp fragment of pOCS-2 containing two functional *ocs* sites flanking the TATA box and 126 bp of the *cat* gene. The labelled fragment was incubated with increasing amounts of tobacco nuclear extract. The protein-DNA complexes were separated on a 4% native polyacrylamide gel as described [8]. (B) Gel retardation with the *SpeI/XhoI* restriction fragments of pMOCS-1 and pMOCS-2 and the *SpeI/BglII* fragment of pOCS-2 with increasing amounts of nuclear extracts. Numbers below the lanes mark the amount of nuclear extracts used (in μg), numbers at the side mark the four specific complexes. The arrow marks an additional complex, which is not due to binding of OCSTF.

4. DISCUSSION

We have cloned the positive acting *ocs* sequence flanking the TATA-box of the constitutive CaMV 35S promoter and observed a 20- to 40-fold reduction in promoter activity in a transient assay system. We conclude that this reduction is due to sterical interference of the transcriptional activator OCSTF, which binds to the *ocs* element, and the transcription initiation complex. This conclusion is supported by the fact, that mutating one of the two palindromes in positions that affect in vitro binding of the factor leads to a partial derepression of the promoter. Consistently, mutating both palindromes leads to a complete derepression.

Repression of a promoter by directing a transcriptional activator into a position within a promoter where it interferes with other components of the transcription complex has been shown for the octamer motif binding protein Oct1 [15] and for the chimeric adenovirus E1a/lexA protein [16]. In these experiments, the transcription factors were overexpressed in a transient expression system. Here we show, that the amount and affinity of the endogenous transcription factor binding to the *ocs* motif is sufficiently high to mediate detectable levels of repression by interfering with transcription initiation. This is even achieved in a transient expression system, where an artificially high copy number of binding sites is introduced into cells. It has to be determined, but seems likely, that repression is at least as pronounced in the transgenic situation, where less binding sites are introduced and a potential titration of the *trans*-acting factor is thus avoided.

We suggest that this type of analysis can be used as an additional tool for studying various aspects of gene expression: potential binding sites for proteins can be identified before establishing an in vitro binding assay. It can be applied for sequences identified as important by conventional promoter deletion analysis or linker scanning. In addition, it might be useful for the analysis of sequences showing homology to already published binding sites or of sequences which exhibit a palindromic structure. Sequences within a promoter, which seem to be non-functional when altered in their normal promoter context because of a redundancy of promoter elements can be identified as being occupied in vivo. Especially if a protien is labile in vitro, this system might facilitate testing sequence specificity. As shown in this paper, mutations within the palindrome lead to a derepression of a a suitably engineered promoter.

This system can also be used to address the question, whether a *trans*-acting factor is bound to its target sequence even under conditions where it does mediate activation. This could be a experimentally simple alternative to doing in vivo footprinting experiments.

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