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FUNCTION OF THE HEXAMERIC SEQUENCE IN THE CAULIFLOWER MOSAIC VIRUS 35S RNA PROMOTER REGION

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SUMMARY: The hexameric sequence ACGTCA functions in transcriptional regulation of wheat histone genes. The cauliflower mosaic virus (CaMV) 35S RNA promoter has the same hexameric sequence, and mutation analyses confirmed that the hexamer contributed greatly to transcription from the 35S promoter when a test gene with this promoter was introduced into sunflower cells. Electrophoretic mobility shift assays revealed the existence of a nuclear protein(s) in sunflower cells which is homologous to the HBP-1b that has been identified as binding to the 35S promoter in wheat. These results provide evidence of the involvement of the hexameric sequence and the HBP-1b-like DNA binding protein(s) in transcription from the 35S promoter.

We studied the regulatory mechanism for the transcription of wheat histone genes, by focusing on cis-acting elements and trans-acting protein factors (1-8) and found the wheat DNA-binding proteins HBP-1a and HBP-1b that interact specifically with the hexameric sequence ACGTCA which serves as a cis-element in the wheat H3 and H4 genes (3,7).

The same hexameric sequence has been found in dicot plants, nopaline synthase (NOS) (9), and cauliflower mosaic virus 35S RNA (CaMV 35S) genes (10). In addition, wheat HBP-1b has been shown to bind to this sequence in these promoters (Mikami et al., unpublished). Some cis-acting elements in the 35S promoter required for maximal transcription activity have been identified

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Abbreviations: CaMV, cauliflower mosaic virus; HBP-1, histone DNA-binding protein(s)-1; NOS, nopaline synthase; SV 40, simian virus 40; GUS, β-glucuronidase; NPT-II, neomycin phosphotransferase-II; SSC, sodium-sodium citrate.

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(11-14); CCAAT box-like sequences, inverted repeat structures, and a sequence homologous to the SV 40 core enhancer (15). Interestingly, the hexameric sequence is included in the reversed orientation in a regulatory region of the 35S promoter (12). Moreover, two hexamer-like sequences are located upstream of the region which also has been defined as a cis-element of the promoter (12). Therefore, questions arise as to whether the hexameric sequence also serves as a cis-element of the 35S promoter and whether there are any DNA-binding protein(s) specific to this sequence. To obtain answers, we constructed the promoter mutants for the hexameric, hexamer-like sequence, and an inverted repeat structure (11,12) in the fusion gene that consists of the 35S promoter and coding regions of the E. coli β-glucuronidase gene (GUS) as a reporter (16) and examined their promoter activities. We also searched for nuclear DNA-binding proteins capable of interacting with the hexameric motif. We here report that the hexameric sequence is necessary for maximal transcription from the 35S promoter and that a nuclear DNA-binding protein(s) specific to the hexameric sequence exists in sunflower as well as in wheat.

MATERIALS AND METHODS

Site-Directed Mutagenesis of CaMV 35S Promoter.

To construct the mutated 35S promoter, a BamHI-HindIII fragment of the 35S promoter region in pBI121 (16) was recloned into M13mp19 RF to make pM13-35S. Site-directed mutagenesis was done by the method of Kunkel (17) with synthetic oligonucleotides as the primers and uracil-containing single-stranded M13 as the template. Two mutated 35S promoters, pM13-35S MI and pM13-35S MII, were constructed. In the case of MIII, pM13-35S was digested at an EcoRV site and ligated with the SnaI linker to produce pM13-35S MIII. Each of the BamHI-HindIII fragment containing the mutated 35S promoters then was recloned into pBI101 (16) to make pBI121 MI, pBI121 MII and pBI121 MIII (Figure 1). A series of pBI121 mutants were digested with EcoRI and ligated with HindIII-linker, then the 3.1 kbp fragments (CaMV 35S/GUS/NOS) were isolated after HindIII digestion. These HindIII fragments were inserted into the HindIII site of the intermediate vector pBN (7), to make pBN-35S (WT), pBN-35S MI, pBN-35S MII and pBN-35S MIII (Fig. 1).

RNA Isolation and Northern Blot Analysis.

The procedures for triparental mating to prepare transconjugant cells of Agrobacterium tumefaciens which have the recombinant Ti plasmid that contains the 35S/GUS/NOS fusion gene and the transformation of sunflower (Helianthus annuus) cells by infection of these transconjugant cells are described elsewhere (2).

RNA was isolated from the transformed cells by the procedure of Tabata et al. (2). The RNA samples (50 μg) were electrophoresed in 1.5% agarose gels containing formaldehyde, then blotted onto nylon membranes (Hybond-N, Amersham). The blotted samples were hybridized with random-primed ^{32}P-labeled SacI/BamHI GUS gene fragment (2.0 kbp) and with the ^{32}P-labeled HindIII/BamHI fragment (1.2 kbp) containing the NOS/NPT-II gene which had been isolated from pMON200 (18) in 50% formamide, 5% Irish-cream liqueur, 5 X SSC, 0.5% SDS and 50 mM Na-phosphate, pH6.5, for 36 hours at 42°C. The filters were washed successively with 1 X, 0.5 X, 0.2 X, and 0.15 X SSC in the presence of 0.5% SDS, for 30 min each, at 65°C with constant agitation, then they were exposed to X-ray film at RT for 3 to 6 days. The autoradiograms obtained were scanned with a densitometer (TEFCO IMAGE ANALYSIS TIAS-100) and the relative promoter activities measured.

Preparation of the Nuclear Extract and the Mobility Shift Assay.

A crude nuclear extract was prepared from 50 g (fresh wt) of sunflower seedlings, as described previously (3). The mobility shift assay was done with a ^{32}P-labeled XbaI/HaeIII CaMV 35S promoter fragment (-209/+1) or one of its derivative mutants as the probe by the procedure as described elsewhere (3). Competitor DNA was isolated from pUC-H3(-184/-130) (19) after digestion with EcoRI and HindIII.

RESULTS

Hexameric Sequence in the CaMV 35S Promoter As the Cis-Element in Sunflower Cells.

To establish whether the hexameric and hexamer-like sequences present in the CaMV 35S promoter function in promoter activity, we constructed three mutants (MI, MII, and MIII [Fig. 1a]) of the 35S promoter. MI has double point mutations within the hexameric sequence at -80. MII also has double

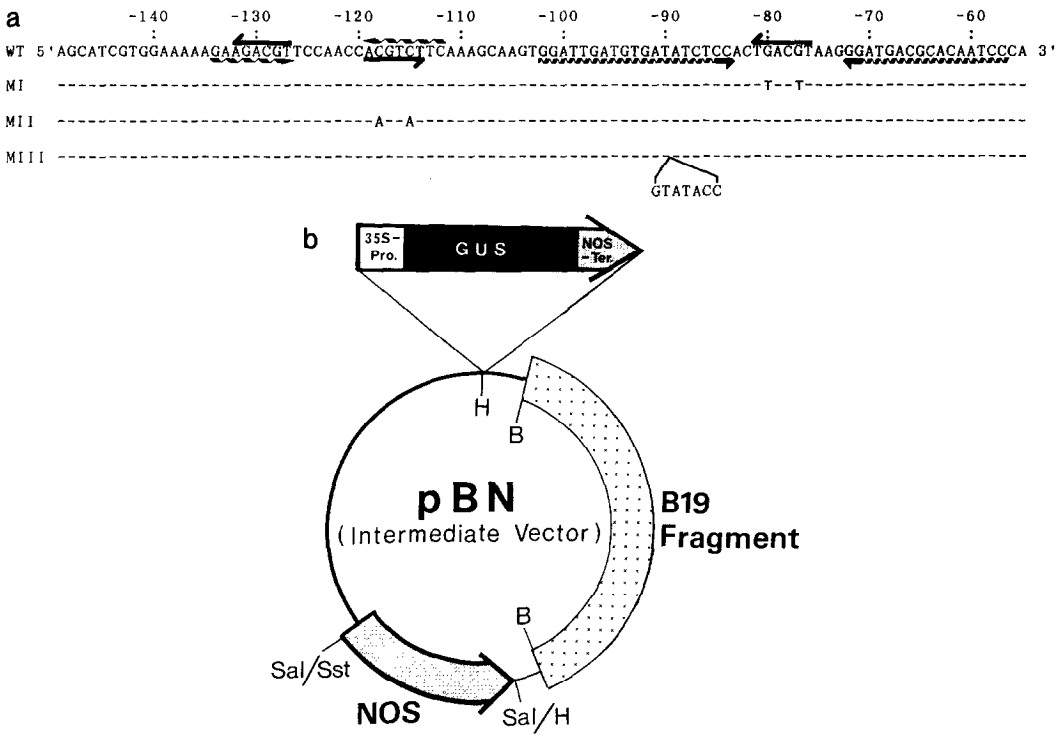


Fig. 1. Nucleotide sequences of a portion of the wild-type and mutant CaMV 35S promoters (a) and the structure of the intermediate vector pBN (b). a: The wild-type sequence is shown at the top by the negative number from the transcriptional start site. Arrows indicate the hexameric and hexamer-like sequences, wavy lines with an arrowhead the inverted repeat structures (see text). In the mutants only the replaced nucleotides are shown. b: The 35S-pro/GUS/NOS-ter chimeric gene or its mutants (in respect to the promoter) have been ligated into the HindIII site of the intermediate vector pBN (7), as described in MATERIALS AND METHODS. The chimeric gene unit (shown at the top of the figure) and the NOS gene (stipled arrow) used as internal reference genes were designed to be transcribed in opposite orientations. B19 is a fragment derived from T-DNA.

point mutations within its hexamer-like sequence located between -119 and -114 (Fig. 1a, short wavy lines) which can disrupt an inverted repeat structure. In MIII, a short sequence has been introduced into a long inverted repeat that is believed to be a cis-element of the 35S promoter (13) (Fig. 1a, long wavy lines). The activities of these three mutated 35S promoters were assayed by northern blot analysis of the transcript from the 35S/GUS/NOS fusion gene (Fig. 1b) of transformed sunflower cells.

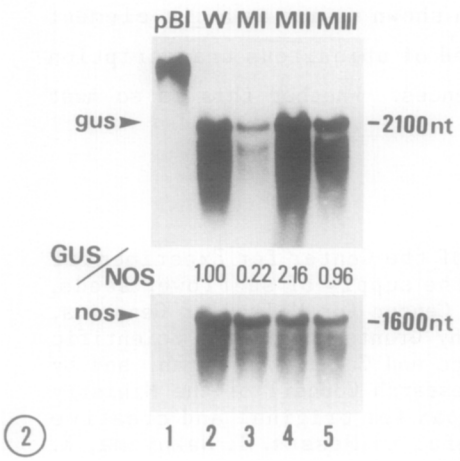
Results of northern blot analysis are shown in Figure 2. The promoter activities have been standarized with the amount of transcript as the internal reference from the NOS gene which had been cotransfected together with the 35S/GUS/NOS gene. In the MI mutant, transcriptional activity was reduced to about 20% that of the control (lane 3); whereas, there was no change in MIII (lane 5). This means that the hexameric sequence is an essential element for transcription from the CaMV 35S promoter. Interestingly, in the MII mutant, transcriptional activity increased about 2-fold (lane 4), but the reason why is not known.

Specific Binding of Nuclear Protein(s) to the Hexameric Sequence of the CaMV 35S Promoter.

We used the gel mobility shift assay to determine whether a nuclear protein(s) homologous to wheat HBP-1b (a DNA-binding protein for the hexameric sequences of wheat histone H3 and H4 genes and the CaMV 35S promoter [Mikami et al., unpublished]) exists in sunflower. As shown in Figure 3, when the wild type 35S promoter probe was incubated with sunflower nuclear extract, several retarded bands were present (lane 2), some of which behaved like the HBP-1b complex because the addition of an H3 fragment that contained the hexameric sequence prevented formation of those complexes (lane 6). Similar results were obtained when a synthetic oligonucleotide that contained the hexameric sequence was used as the competitor DNA (Mikami et al., unpublished). These HBP-1b homologue complexes were not found with the MI probe (lane 3); but, when the MII (lane 4) and MIII (lane 5) probes were used, the retarded band patterns were identical to those of the wild type probe. This means that a nuclear protein(s) that interacts specifically with the hexameric sequence located in the -80 area of the 35S promoter also exists in sunflower cells.

DISCUSSION

A region that includes the hexameric sequence in the CaMV 35S promoter has been reported to be necessary for efficient transcription from this promoter (11-14), but there have been no studies published on the existence and importance of this sequence. We here have shown that the hexameric sequence of the 35S promoter functions as a cis-acting element. The amount of gene transcript does not always represent the promoter activity per se of that



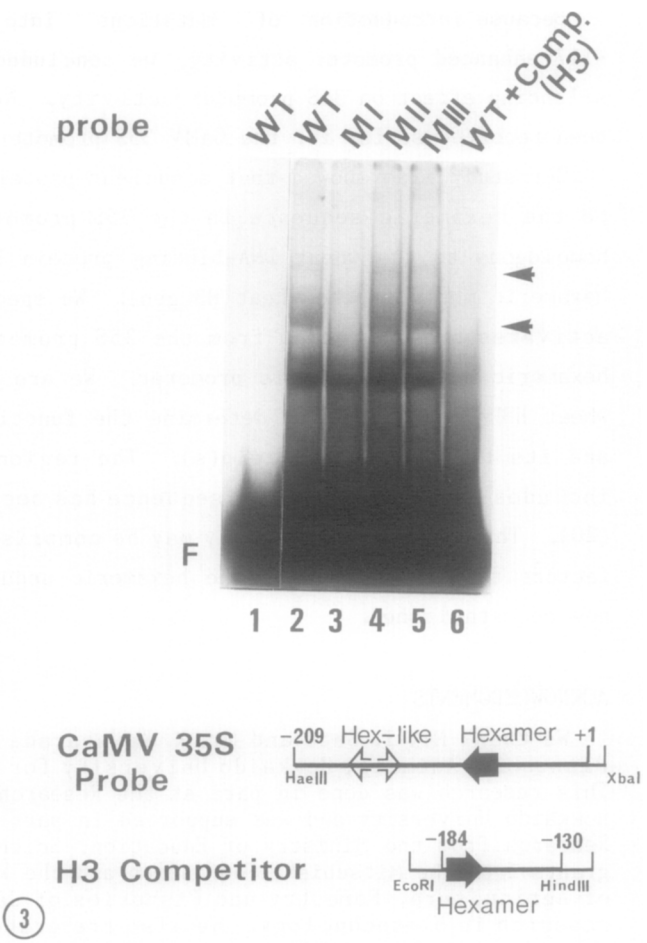


Fig. 2. Northern blot of the GUS transcript of the fusion gene in transformed sunflower cells. Total RNA was isolated from transformed calli. Equal amounts of RNA (50 µg) were electrophoresed on a 1.5% formaldehyde agarose denaturing gel, then hybridized independently with ^{32}P-labeled NOS (lower panel) and GUS (upper panel) probes. Lane 1, 100 ng of pBI121 digested with EcoRI and HindIII; lanes 2-5, RNA samples from sunflower calli transformed by pBN-35S (wild-type) (lane 2), pBN-35S MI (lane 3), pBN-35S MIII (lane 5). The GUS/NOS ratios are shown between the two panels. The ratio for the wild-type is taken as 1.00. The GUS and NOS transcripts are indicated by arrowheads.

Fig. 3. Specific binding of sunflower nuclear protein(s) to the CaMV 35S promoter shown by the mobility shift assay. Lane 1, a free CaMV 35S wild-type promoter probe; lanes 2-5, DNA-protein binding with wild-type (35S-WT) probe (lane 2), 35S-MI probe (lane 3), 35S-MII probe (lane 4) and 35S-MIII probe (lane 5); and lane 6, binding patterns with the 35S-WT probe in the presence of a 100-fold molar excess of competitor DNA containing the hexameric motif of the wheat H3 promoter. The binding reaction mixture contained crude sunflower seedling nuclear extract (16.8 µg protein/20 µl/assay). The fragment used as the probe and the H3 competitor DNA are shown at the bottom. F, free probe; arrowheads, HBP-1b-like protein-DNA complexes.

gene; but, the results of our northern analysis do faithfully reflect the activity of the 35S promoter because the life time of the GUS transcript is thought to be the same in all transformed cells.

Because introduction of mutations into the hexamer-like sequence at -117 enhanced promoter activity, we concluded that this sequence may have a silencing effect on 35S promoter activity. No silencing cis-elements have been reported so far for the CaMV 35S promoter.

Our study also showed that a nuclear protein(s) from sunflower cells binds to the hexameric sequence in the 35S promoter. This protein seems to be homologous to the wheat DNA-binding protein HBP-1b which interacts with the hexameric motif of the wheat H3 gene. We speculate that the HBP-1b homologue activates transcription from the 35S promoter through its binding to the hexameric sequence in this promoter. We are now cloning a cDNA that encodes wheat HBP-1b in order to determine the function(s) of the hexameric sequence and its DNA-binding protein(s). The region within the NOS promoter that includes the same hexameric sequence has been shown to act as a cis-element (20). Thus, the HBP-1b family may be comprised of ubiquitous transcription factors that interact with the hexameric sequences. Whether this is so must now be established.

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