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Highly conserved hexamer, octamer and nonamer motifs are positive *cis*-regulatory elements of the wheat histone H3 gene

Takuya Nakayama[a], Ayako Sakamoto[a], Ping Yang[a], Maki Minami[a,b], Yoshinobu Fujimoto[a,c], Takuya Ito[a] and Masaki Iwabuchi[a,b]

[a]*Department of Botany, Faculty of Science, Kyoto University, Kyoto 606-01, Japan,* [b]*Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444, Japan* and [c]*Nippon Oil Company Ltd., Kudamatsu 744, Japan*

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Base substitution mutations were introduced into the promoter region of the wheat histone H3 gene, and promoter activity was assayed in stably transformed sunflower calli or in wheat protoplasts transfected transiently. At least four positive regulatory elements, a hexamer motif (ACGTCA), two octamer(-like) motifs of a direct (CcCGGATC) and a reverse (aATCCGCG) form, and a nonamer motif (CATCCAACG) were identified within the −185 region of the H3 promoter. Analyses of the type I element (CCACGTCACCaATCCGCG) consisting of the hexamer and reverse-oriented octamer motifs, and which is conserved in other plant histone genes as well, predicted the presence of an octamer-binding protein(s).

Transcription; Wheat histone H3 gene; Hexamer; Octamer; Nonamer; HBP-1 family

1. INTRODUCTION

The wheat (*Triticum aestivum*) histone H3 gene (TH012) [1] is expressed specifically in the S phase of the cell cycle (Ohtsubo, N. et al. unpublished). Several researchers have found nucleotide motifs of candidates for *cis*-acting control elements in the promoter region of plant histone genes; the hexamer (ACGTCA) motif that binds HBP-1a and HBP-1b [2], the nonamer (CATCCAACG) motif that is the binding site of HBP-2 [3], and the octamer (CGCGGATC) motif that is conserved in all the plant histone genes known to date [4]. Our long-range goal is to clarify the molecular mechanism that functions in the cell cycle-dependent expression of plant histone genes. As a first step to this end, we must determine whether the hexamer, nonamer, octamer motifs, or all of them, are *cis*-acting elements. Here we show evidence that these motifs are positive *cis*-regulatory elements of the wheat histone H3 gene and predict the presence of an octamer-binding protein(s).

2. MATERIALS AND METHODS

2.1. *Construction of Ti-plasmid vectors containing H3 gene mutants*

A 1.6-kilobase (kb) *Hin*dIII fragment of the wheat histone H3 gene (TH012) [1] was recloned into the *Hin*dIII site of M13mp18 in order to produce the mutants. Introduction of the base substitution to the H3 gene was done according to Kunkel's method [5]. After confirmation of the DNA sequences of the H3 mutants, the *Hin*dIII fragment containing the substitution was recloned to the *Hin*dIII site of pBN19 as described elsewhere [6]. Sequences of the mutants are shown in Fig. 1.

Abbreviations: HBP, histone gene-binding protein; GUS, β-glucuronidase; NOS, nopaline synthase.

Correspondence address: M. Iwabuchi, Department of Botany, Faculty of Science, Kyoto University, Kyoto 606-01, Japan. Fax: (81) (75) 753-4122.

2.2. *Transformation of plant cells, RNA preparation and the S1 assay*

Recombinant Ti-plasmid vectors were transferred to *Agrobacterium tumefaciens* cells as described elsewhere [6]. Transformation of sunflower cells, preparation of RNA and the S1 assay were done as described previously [6], except that mpNOS5′ was used to generate the probe to detect the 5′ end of the NOS [7] mRNA that was the internal standard. mpNOS5′ was made by recloning NOS3′Δ+276 (refers to +1 for A of the protein initiation codon ATG) into M13mp18. The S1-protected band obtained was ca. 310 b.

2.3. *Construction of chimeric genes*

The H3 mutant (3′Δ+57) was constructed as described elsewhere [6]. It was cleaved with *Eco*RI, blunted with the Klenow fragment and dNTPs, then digested with *Hin*dIII. The resulting fragment (−185 to +57) was recloned into the *Hin*dIII and *Sma*I sites of pBI221 (purchased from Clontech Laboratories Inc.). A *Hin*dIII–*Eco*RI fragment containing H3 promoter that had been fused to the *Escherichia coli* GUS coding region was prepared then recloned into the corresponding pUC118 site giving p185H3/GUS$_{118}$.

To construct H3 promoter mutants, a *Hin*dIII–*Sac*II fragment (−184 to −159) of p185H3/GUS$_{118}$ was replaced by the following synthetic double-stranded oligomers:

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HexM: 5'- AGCTTTCGGCCAAGTAACCAATCCGC    3'
      3'-     AAGCCGGTTCATTGGTTAGG      5'

OctM: 5'- AGCTTTCGGCCACGTCACCCCGAAGC    3'
      3'-     AAGCCGGTGCAGTGGGGCTT      5'

[illegible]: 5'- [illegible]    3'
             3'- [illegible]    5'
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Nucleotide sequences of the mutants were confirmed by the chain-

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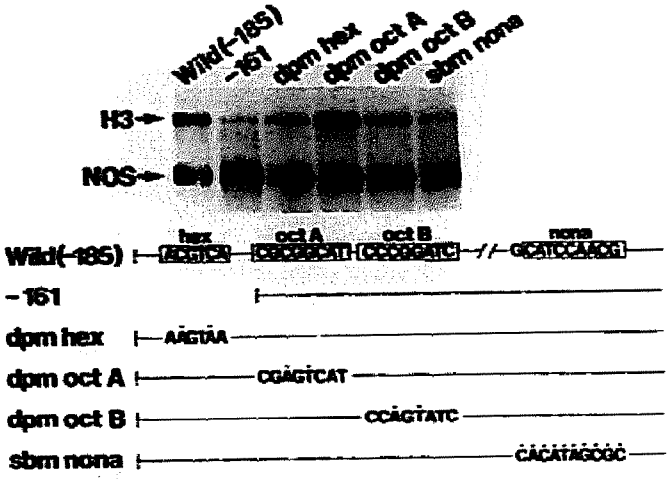


Fig. 1. S1 analyses of the promoter activity of base-substitution mutants of the wheat histone H3 gene. The total RNA from sunflower calli transformed with wild-type or mutated H3 genes (depicted schematically) was analyzed by the S1 assay as described elsewhere [6]. S1-protection patterns are shown in the upper panel: H3 indicates transcripts from the wild-type or mutated H3 genes (shown above the lanes); NOS, the transcript from the NOS gene used as the internal standard. Wild(–185), gene having the upstream sequence up to –185 [6]; hex, hexamer motif; octA, upstream octamer(-like) motif; octB, downstream octamer(-like) motif; nona, nonamer motif; –161, the 5′ deletion mutant [6]; dpm, double-point mutation; sbm, substitution mutation. The mutated bases are marked by dots. See text for details.

termination DNA sequencing method with the Sequenase Version 2.0 (USB).

2.4. Protoplast preparation and electroporation

The wheat suspension cell line, HY-1, was subcultured weekly on a gyratory shaker (100 rpm) at 26°C in the dark after 5 ml of packed cells were diluted with 100 ml of fresh medium (pH 5.6) consisting of MS salts [8], 2,4-D (2 mg/l), *myo*-inositol (100 mg/l), thiamine-HCl (0.4 mg/l) and 3% (w/v) sucrose.

For protoplast isolation, 3-day-cultured cells were harvested by centrifugation (100 × *g*, 3 min), then incubated with 100 ml of an enzyme solution (pH 5.6) consisting of 4 % (w/v) Cellulase 'onozuka' R-10 (Yakult Honsha Co.), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co.), 0.1% (w/v) $CaCl_2$, and 9% (w/v) mannitol with rotatory shaking (60 rpm) for 3–4 h at 26°C in the dark. Protoplasts were filtered twice through nylon meshes (70 and 30 μm), then washed by repeated centrifugation (100 × *g*, 3 min) with electroporation buffer (pH 5.8) consisting of 5 mM MES, 70 mM KCl, 4 mM $CaCl_2$ and 0.4 M mannitol to remove enzymes. Lastly, protoplasts were resuspended at a density of 2 × 10^6/ml in electroporation buffer. 800 μl of this suspension was mixed with the test (20 μg) and carrier (sonicated salmon sperm, 200 μg/ml) DNAs in a cuvette (Gene Pulser Cuvette with a 0.4 cm electrode, Bio-Rad), kept on ice for more than 10 min, then electroporated with a Gene Pulser apparatus (Bio-Rad) at 960 μF and 200 V.

After electroporation, protoplasts were cooled on ice for 10 min, then collected by centrifugation (100 × *g*, 3 min) and resuspended in 8 ml of a protoplast culture medium (pH 5.6) consisting of MS salts, 2,4-D (4 mg/l), 2% (w/v) sucrose and 7 % (w/v) glucose, after which they were incubated at 26°C in the dark for about 40 h.

2.5. Fluorometric assay of GUS activity

This assay was done according to the method of Jefferson et al. [9].

2.6. Bacterial expression of HBP-1a and the DNA mobility shift assay

The preparation of bacterially expressed HBP-1a(17) and the DNA mobility shift assays were performed according to the method of Tabata et al. [10].

3. RESULTS AND DISCUSSION

Several conserved nucleotide sequences (hexamer, octamer, nonamer) have been reported in the promoter regions of the H3 and H4 genes of wheat, corn, *Arabidopsis*, rice and alfalfa [3,4,11–15]. On the basis of previous results of 5′ deletion experiments we posited that the hexamer and nonamer motifs are *cis*-acting elements of the wheat histone H3 gene [6]. To confirm whether this is so, we introduced mutations to the hexamer or nonamer motif of the H3 promoter and assayed its promoter activity in transformed sunflower calli. The steady-state levels of the H3 transcripts from the mutant genes were assayed by quantitative S1 nuclease mapping. The band intensities of S1-protected bands were measured densitometrically by scanning the autoradiogram, then normalized to the level of the NOS transcript used as the internal reference [6]. We first examined the effect of a hexamer motif with double-point mutations that inhibits the DNA-binding of HBP-1a and -1b [2]. The promoter activity of this mutant (dpm hex) was reduced to about 30% that of the wild-type (Fig. 1). Similarly, the nonamer motif mutant (sbm nona), in which the 10 bp sequence that includes the nonamer motif had been shuffled randomly without changing the relative nucleotide ratio, showed only 20% of the activity of the normal promoter (Fig. 1). These results indicate that the hexamer and nonamer motifs are both positive *cis*-acting elements of the H3 gene.

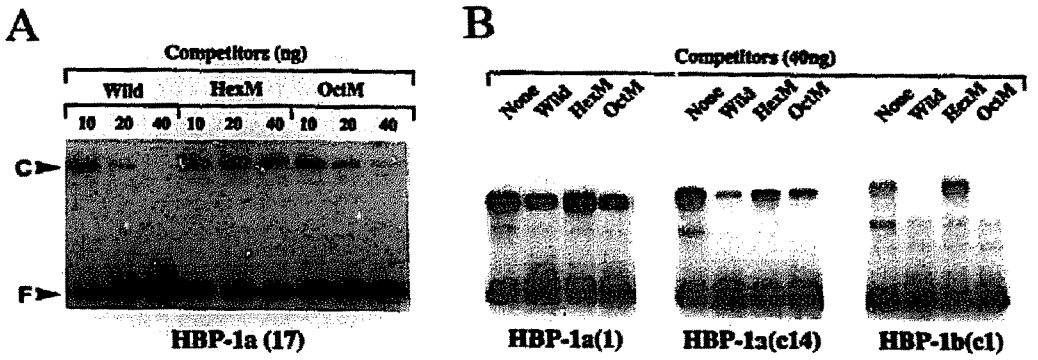
The octamer motif CGCGGATC, first reported by

Table 1

Comparison of sequences around the type I element in wheat histone promoters

Gene	Sequence	Reference
H2B (TH152)	5′-TCCGCC ACGTCA GC GATCCGTG CCAA-3′	unpublished
H3 (TH012)	5′-TCGGCC ACGTCA CC AATCCGCG GCAT-3′	[1]
H4 (TH011)	5′-TCGGCC ACGTCA CC GATCCGCG GCAT-3′	[19]
H4 (M042)	5′-TGAGCC ACGTCA CC TATCCGCG CCCT-3′	unpublished
Type I element	5′-CC ACGTCA CC GATCCGCG -3′	

Hexamer and reverse-oriented octamer motifs are boxed



C

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Wild: 5'-TTTCGGCCACGTCACCAATCCGCGGCATTC-3'
HexM: 5'-TTTCGGCCAAGTAACCAATCCGCGGCATTC-3'
OctM: 5'-TTTCGGCCACGTCACCCCGAAGCGGCATTC-3'
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Fig. 2. Competition DNA mobility shift assay of HBP-1 family. The members of the HBP-1 family were expressed from each of the cDNA clones under the control of the T7 promoter in *E. coli* and subjected to DNA mobility shift assays as described elsewhere [10]. The probe was ^{32}P-labeled DNA fragment that contains the H3 gene fragment (–184 to –130) and flanking polylinker sites [10], and the assay was conducted with ~1 ng of the probe in the presence or absence of 30 bp oligonucleotide competitors. F, free probe; C, DNA-protein complex. (A) HBP-1a(17). (B) HBP-1a(1), HBP-1a(c14) and HBP-1b(c1). (C) Sequences of the upper strand of the competitors. The mutated bases are bold-faced. Hexamer and reverse-oriented octamer motifs are underlined.

Chaubet et al. [4], is the most highly conserved in the promoter region of all the plant histone genes known. There are two octamer-like sequences in the promoter region of the wheat H3 gene (TH012), but they are not entirely consistent with the consensus sequence of the octamer motif. Previously, we could not confirm that the octamer-like sequences of the H3 promoter were positive regulators [6]. We therefore constructed H3 promoter mutants that carry double-base substitutions in each of the two octamer-like sequences and assayed their promoter activities. When the upstream octamer (octA) underwent mutation, promoter activity was scarcely affected, whereas mutation of the downstream octamer (octB) reduced the promoter activity to about 30% that of the wild-type (Fig. 1), indicative of octB being a positive regulatory element.

Mikami and Iwabuchi [16] reported a novel conserved sequence, CCACGTCACCGATCCGCG, which they called the 'type I element', in the regulatory region of wheat histone H2B, H3 and H4 genes (Table I). This sequence contains the hexamer motif and a reverse- oriented octamer motif with a 2 bp interval. We previously demonstrated [6] that the H3 promoter which lacks the type I element has only 30% of the wild-type promoter activity, indicating that this element functions as a positive *cis*-acting element.

Because the hexamer motif in this element remains a recognition site for the DNA-binding of HBP-1a and HBP-1b, and because the octamer motif is a highly conserved sequence in plant histone genes, the type I element most probably is composed of two independent *cis*-acting sequences. We therefore constructed H3 promoters with base-substitution mutations in the hexamer, the reverse-oriented octamer motif (or both) of the type I element and assayed their promoter activities. For the assay we used a H3/GUS chimeric gene in which the H3 promoter (from –185 to +57) was fused to the GUS coding region. The promoter activity of this chimeric gene (introduced by electroporation into the wheat protoplasts) was assayed by measuring GUS ac-

Table II

Promoter activities of type I element mutants of the wheat H3 gene

	Sequences	Promoter activity*
Wild:	TTCGGCCACGTCACCAATCCGCGGCAT	100
HexM:	TTCGGCCAAGTAACCAATCCGCGGCAT	40
OctM:	TTCGGCCACGTCACCCCGAAGCGGCAT	32
CaMV:	ATCCCTTACGTCAGTGGAGAGCGGCAT	5
HexM/OctM:	TTCGGCCAAGTAACCCCGAAGCGGCAT	5

Values are averages of duplicate results for three independent preparations of protoplasts (total: six determinations). Similar results were obtained for tobacco protoplasts (not shown). Mutated nucleotides are underlined.

*Promoter activity as measured by GUS activity (%).

tivity. When the hexamer motif underwent mutation promoter activity was reduced to 40% of the wild-type promoter (TablE II), which is consistent with results for sunflower calli obtained for the Ti-plasmid-mediated gene transfer system.

In the nucleotide-shuffling experiment with the octamer motif, promoter activity was reduced to 32% of the wild-type. The same amount of reduction was found when the octamer sequence was disrupted by replacement of the sequence containing the H3 hexamer with the sequence containing the hexamer of the CaMV 35S promoter, evidence that the octamer motif in the type I element also is a positive regulatory element.

Simultaneous mutation of the hexamer and octamer motifs, however, did not have an additive effect (Table II). Mikami et al. [17] showed that in a DNAse I footprinting experiment a mixture (HBP-1) of HBP-1a and HBP-1b bound to the region containing both the hexamer and octamer motifs of the type I element. The octamer motif of the type I element probably also is essential for interaction with HBP-1a, HBP-1b, or both, as is the hexamer motif; therefore, disruption of the octamer motif would abolish the binding activity of HBP-1a or HBP-1b, resulting in the reduction of promoter activity. To substantiate this assumption, we made protein–DNA binding assays between HBP-1a(17) and synthetic oligonucleotides (30 bp) with base-substituted hexamer or octamer sequences. HBP-1a(17), which was bacterially expressed using cDNA, could bind to the oligonucleotide competitor with the octamer mutant, but not to that with the hexamer mutant (Fig. 2A). We assume that the binding of HBP-1a(17) to the type I element is mediated throughout the interaction with the hexamer motif. This assumption led us to speculate that reduction of the promoter activity of the octamer mutant is not due to the inability of the mutant promoter to bind to HBP-1a. Similar results have been obtained with other bacterially expressed members of the HBP-1 family (HBP-1a(1), HBP-1a(c14), HBP-1b(c1): Sakamoto et al. unpublished) (Fig. 2B).

These results suggest that there may be a *trans*-acting factor(s) that interacts with the octamer motif; however, we have not yet found octamer-binding proteins, other than ssDBP-1 and ssDBP-2, which sequence-specifically bind to the single-stranded DNA around the type I element of the wheat histone H3 and H4 genes [18]. It is important to determine whether there is an octamer-binding protein(s) in order to understand the role of the type I element that may function throughout the cooperative interaction of each member of the HBP-1 family and octamer-binding protein(s) during plant histone gene regulation.

We have identified four *cis*-acting elements: a hexamer, two octamers (reverse-oriented and octB) and a nonamer, but their roles in the cell-cycle-regulated expression of plant histone genes is unknown. Further experiments are now being done to establish what these roles are.

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