Volume 223, number 2, 273–278 FEB 05231 November 1987

Nuclear protein(s) binding to the conserved DNA hexameric sequence postulated to regulate transcription of wheat histone genes

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Received 4 August 1987

Nuclear protein(s) that specifically bind(s) to the upstream hexamer motif, ACGTCA, of wheat histone H3 and H4 genes has (have) been identified. Sequences homologous to this hexamer are found to be conserved in the upstream region of not only wheat histone genes but also other plant and animal histone genes. This suggests a possible role(s) for the hexamer and the nuclear protein(s) in the transcriptional regulation of the wheat histone genes. This hexamer is homologous to the upstream core sequence, TGACGTCA, which is highly conserved in some animal genes whose expression is regulated by cAMP.

Wheat histone gene; Hexamer motif; Nuclear protein; Mobility shift assay; Methylation interference analysis

1. INTRODUCTION

The regulatory mechanisms controlling histone gene transcription, which is limited to the early S phase and coupled with nuclear DNA synthesis during the cell cycle [1,2], have not been fully understood as yet. Recently, many experiments to define the interactions between specific DNA sequences and regulatory factors have been carried out in order to help toward an understanding of the molecular mechanisms for transcription of eukaryotic genes. Recent experiments of the human histone H4 gene have suggested that a gene-specific transcription factor was also regulated during the cell cycle [3,4].

In order to elucidate regulatory mechanisms for plant histone genes, we have been studying the regulation for transcription of cloned wheat histone genes [5–8]. In a comparison of the upstream regions of four cloned wheat histone genes (two H3 and two H4), two conserved motifs have been detected: one was a hexamer, ACGTCA (in the present paper), and the other was an octamer, CGCGGCAT [9]. Since these motifs are seen in the corresponding regions of all plant histone genes analysed to date, the possibility can be considered that they are *cis*-acting control elements to interact with *trans*-acting factors. Here, we present the identification of the nuclear protein(s) that specifically bind(s) to the upstream sequence of wheat histone H3 and H4 genes containing the hexamer motif by using a mobility shift assay [10,11] and methylation interference analysis [12,13]. Our findings suggest that the hexamer motif and the nuclear protein(s) may play a role in the transcriptional regulation of wheat histone genes.

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2. MATERIALS AND METHODS

2.1. *Preparation of nuclear extracts*

Wheat germ or seedlings were homogenized in buffer A (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF),

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

1.6 mM salicylhydroxamic acid, 1 μg/ml *t*-butylated hydroxytoluene, 5 mM dithiothreitol (DTT)) by using an Ultradisperser (Janke & Kunkel) at high speed for 1–2 min. The homogenate was filtered through a double layer of cheesecloth and a double layer of miracloth. The filtrate was centrifuged in a Hitachi 20-2 rotor at 7000 rpm for 10 min. The crude nuclear phase was washed once with buffer A, and resuspended at an equal weight to volume (for germ nuclei) or at 1/4 weight to volume (for seedling nuclei) in buffer B (10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 5 mM EDTA, 25% glycerol, 1 mM PMSF, 2 mM DTT). Then, 4 M NaCl was added slowly to give a final NaCl concentration of 0.4 M. Nuclei were extracted for 30 min with gentle stirring. The resultant highly viscous solution was centrifuged in a Hitachi 20-3 rotor at 16000 rpm for 20 min to sediment DNA. The clear supernatant was dialyzed for 5 h against 100 vol. buffer C (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, 2 mM DTT) with three buffer changes. The dialysate was centrifuged in a Hitachi 20-3 rotor at 16000 rpm for 20 min. The supernatant, designated crude nuclear extract, was quick-frozen in small aliquots in liquid N_2, and stored at −85°C. For column fractionation, the nuclear extract was dialyzed against buffer D (20 mM Hepes-KOH, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, 2 mM DTT) containing 0.1 M KCl, and applied to a phosphocellulose column (Whatman P-11) equilibrated with the same buffer. After washing with three column volumes of buffer D containing 0.1 M KCl, three successive step elutions were performed with two column volumes each of 0.35, 0.6 and 1.0 M KCl in buffer D. Four peak fractions were dialyzed against buffer C, and stored as described above. All procedures were carried out at 0–4°C.

2.2. *Probes and competitors for mobility shift assay*

A 54 bp *Hin*dIII-*Fok*I fragment (−184 to −130) for an H3 probe and a 79 bp *Hin*dIII-*Taq*I fragment (−169 to −94 and segment of a M13 polylinker) for an H4 probe were excised from the recombinant plasmid pTH012 [5] and pH45′ −169 (unpublished), respectively, labeled at the 3′-end with [α-^{32}P]dCTP and Klenow enzyme, and isolated by polyacrylamide gel electrophoresis. To prepare the specific competitor DNA, the H3 probe fragment was cloned into the *Sma*I site of pUC118, and a 91 bp fragment containing the probe fragment and a segment of pUC118 polylinker was prepared by digestion with *Hin*dIII and *Sal*I. For non-specific competitor DNA, pUC118 was digested with *Hin*dIII and *Eco*RI, and a polylinker region was isolated.

Fig.1. Binding of a nuclear factor(s) to the upstream region of the wheat histone H3 gene (pTH012) and competition analysis. (A) 5′-region of the histone H3 gene segment. The 'hexa', 'octa' and 'TATA' motifs start at positions −173, −161 and −34 (relative to the cap site), respectively. (B) Mobility shift assay and competition analysis of the 54 bp *Hin*dIII-*Fok*I fragment of the H3 gene with nuclear extracts from wheat germ (lanes 2–5) and seedlings (lanes 6–9). Lanes: 1, free probe; 2 and 6, control reactions in the absence of additional competitor DNA; 3–5 and 7–9, binding reactions carried out in the presence of 10-, 30- and 50-fold molar excess, respectively, of specific competitor DNA.

2.3. *Mobility shift assay and methylation interference analysis*

Binding reactions were carried out in a total volume of 10 μl containing 17 mM Hepes-KOH, pH 7.9, 60 mM KCl, 7.5 mM $MgCl_2$, 0.12 mM EDTA, 17% glycerol, 0.6 mM PMSF, 1.2 mM DTT, 1 μg poly(dI-dC)·poly(dI-dC), 6–12 μg nuclear protein, labeled probe (~1 ng, 3000–5000 cpm), and competitor DNA. The assay mixtures were incubated for 30 min at 25°C. Electrophoresis and methylation interference analysis were performed as described in [11,13], respectively, with slight modifications.

3. RESULTS AND DISCUSSION

For mobility shift assay, an H3 probe (fig.1A) was incubated with nuclear extracts plus increasing amounts of a sequence-specific competitor. In the absence of the specific competitor, several bands with slower electrophoretic mobilities than the free probe were observed (fig.1B). Because the mobility-shifted bands disappeared after treatment of nuclear extracts with protease or heat (not shown), it is certain that they were DNA-protein complexes. When the specific competitor was included in the binding mixture, a band indicated by an arrowhead (fig.1B) was specifically attenuated in the assay containing both the germ and seedling extracts. This band, however, was not affected by the addition of a non-specific competitor (not shown). These data demonstrate that both the germ and seedling nuclear extracts contained the DNA binding protein(s) which specifically bound to the upstream sequence of the H3 gene.

Methylation interference analysis was used to

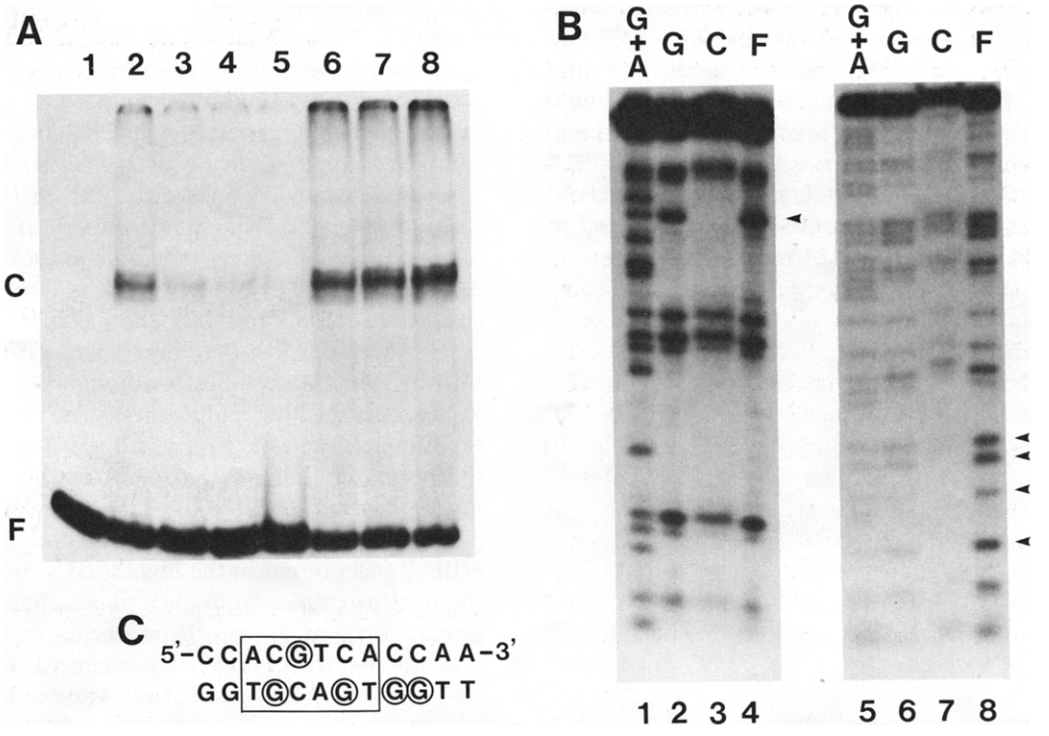

Fig.2. (A) Binding competition analysis in a phosphocellulose 0.6 M KCl fraction of germ nuclear extract. Lanes: 1, free probe; 2, a control reaction in the absence of additional competitor DNA; 3–5, binding reactions carried out in the presence of 10-, 30- and 50-fold molar excess, respectively, of the specific competitor DNA; 6–8, the same reactions using non-specific competitor DNA. (B) Methylation interference analysis of binding to the upstream region of the H3 gene. Chemical cleavage ladders of G + A (lanes 1 and 5) and G (lanes 2 and 6) of the H3 probe were co-electrophoresed to map the binding domain. Lanes: 1–4, assays with probe 3′-labeled on the non-coding strand; 5–8, the same assays with a probe 3′-labeled on the coding strand; 3 and 7, DNA recovered from complexed band; 4 and 8, DNA recovered from free band. (C) A schematic representation of the sequence containing the hexamer (boxed) and the G residues (circled) involved in protein binding, assumed from the methylation interference data.

define the critical contact points for the specific binding protein(s). To facilitate this experiment, the specific binding protein(s) was (were) partially purified from the germ extract by chromatography on a phosphocellulose column to remove almost all of the non-specific binding activity. The mobility-shifted band corresponding to the position of the specific DNA-protein complex appeared only when the labeled probe was incubated with a fraction eluted with 0.6 M KCl. Again, the band formation was attenuated by including the specific competitor and no effect was detected with the non-specific competitor (fig.2A). Methylation interference analysis using the H3 probe and the 0.6 M KCl fraction showed one band corresponding to the G residue at position −171 (relative to the cap site) was decreased on the non-coding strand of the probe in the specific complex as compared with the free probe, whereas four G residues at positions −172, −169, −167 and −166 were decreased on the coding strand (fig.2B). This indicates that the nuclear protein(s) recognized at least the sequence between −172 and −166, which contained the hexamer, ACGTCA (fig.2C). On the other hand, (a) nuclear protein(s) specific for the octamer could not be detected in the 0.4 M NaCl nuclear extracts. Therefore, the nuclear protein(s) identified here may be (a) hexamer-specific binding factor(s), and we have designated the protein(s) HBP-1 (histone DNA binding protein(s)-1).

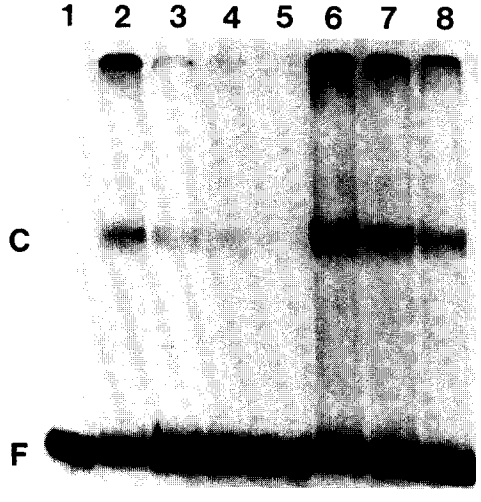

Fig.3. Binding of HBP-1 to the upstream region of the H4 gene and competition analysis. Lanes: 1, free probe; 2, control reaction carried out in the absence of the competitor DNA; 3–5, binding reactions carried out in the presence of 10-, 30- and 50-fold molar excess, respectively, of the H3-specific competitor; 6–8, the same reactions using the non-specific competitor.

To determine whether HBP-1 binds to a DNA sequence of other histone genes, we did the mobility shift assay using an H4 probe containing hexamer (fig.3). When the probe was incubated with the 0.6 M KCl fraction, one major band whose intensity was decreased with increasing amounts of the H3-specific competitor was detected. This result indicates that HBP-1 binds to the H4 probe with the same specificity as to the H3 hexamer.

The present study does not afford direct evidence on the biological function(s) of HBP-1. The significance of this factor(s) for wheat histone gene transcription, however, can be inferred from the existence of similar upstream hexamer sequences in many plant and animal histone genes (table 1). We have shown [8] that the two histone genes used here were transcribed faithfully in sunflower cells transformed by a T_i plasmid vector-mediated gene transfer system. Our unpublished data indicate that deletion of an upstream region of position −191 in the wheat histone H4 gene, which still contained the hexamer, resulted in a great reduction in the efficiency of transcription of this gene in transformed sunflower cells. Therefore, the hexamer may not be connected with the maximum efficiency of histone gene transcription. Although HBP-1 exists in both the seedling and dormant germ nuclei, the binding activity was greater in the former than in the latter (cf. lanes 2 and 6 in fig.1B). Furthermore, in wheat seedlings in which the cell cycle was partially synchronized by aphidicolin treatment, HBP-1 was present in the highest concentration in the nuclear extract from an S phase-abundant cell population (not shown). From the above data, we propose that the hexamer sequence and HBP-1 are *cis*- and *trans*-acting elements, respectively, involved in cell cycle-dependent transcription of the wheat histone genes.

In recent studies on the animal genes whose expression is regulated by cAMP, the highly conserved sequence, TGACGTCA, has been noticed as a regulatory core sequence [14–16]. This core motif includes the hexamer motif reported here. Thus, HBP-1 might be one of the factor(s) involved in the cAMP-regulated gene expression.

Table 1

Comparison of the hexamer homologies in plant and animal histone genes

Plant		
Wheat	H3(pTH012) [5]	$^{-237}$CCACGTCACC^{-226}
	H3(pTH081) [*]	$^{-250}$GAACGCAGGA^{-241}
	H4(pTH011) [6]	$^{-205}$CCACGTCACC^{-196}
	H4(pTH091) [7]	$^{-213}$CAACGTCGCA^{-204}
Corn	H3(C2) [9]	$^{-209}$CCACCTCATC^{-200}
	H3(C4) [9]	$^{-214}$CCACCTCATC^{-205}
	H4(C7) [17]	$^{-222}$CCACGTCAGC^{-213}
	H4(C14) [17]	$^{-187}$CAACGGTTCATC^{-176}
Arabidopsis	H3(A713) [18]	$^{-206}$AGACGTGACG^{-197}
	H3(A725) [18]	$^{-218}$GGATCGTGATA^{-208}
	H4(A748) [18]	$^{-259}$CCACTGTCATC^{-249}
		$^{-230}$CAACCGTCGAT^{-220}
	H4(A777) [18]	$^{-231}$TCACCGTCGAT^{-221}
Animal		
Frog	H2B(X1h3-A) [19]	$^{-140}$TGACGTCATG^{-131}
	H3 (XLHW23) [20]	$^{-217}$TTACGTCACA^{-208}
	H3 (X1h1) [19]	$^{-195}$TGACGTCACA^{-186}
	H4 (X1h3-A) [19]	$^{-615}$GAACGTCAGA^{-606}
Chicken	H2A(CH-01) [21]	$^{-114}$AGACGTGAGC^{-105}
	H2B(pKR1a-1.3) [22]	$^{-135}$ACACGTCACG^{-126}
	H3 (pCH8.4E) [23]	$^{-163}$GTACGTTCCAAA^{-152}
	H4 (pCH8.4E) [23]	$^{-203}$GAACGCATT^{-195}
Sea urchin	H2B(h22) [24]	AGACCTCATA
	H4 (H22) [24]	$^{-249}$TCTCGTCACC^{-255}
	H4 (h19) [25]	$^{-264}$ATTCGTCACC^{-255}
Human	H2B(MP11.PE) [26]	$^{-103}$TGACGTTACC^{-94}

*, Unpublished data

Nucleotide sequences are written in 5′–3′-direction, with respect to the non-coding strand. The position of hexamer homologous sequence is negatively numbered from the translation initiation point, and the homologous sequences are underlined. In the case of sea urchin H2B, the nucleotide sequences surrounding the translation initiation point are not included in [24], so the distance is unknown

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and a grant from the Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan for original and creative research in biotechnology.

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