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Short Communication

A Cell Cycle-Regulated Histone H3 Gene of Alfalfa with an Atypical Promoter Structure

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The control of cell cycle expression of histone genes in plants is incompletely understood. A new histone H3 gene was cloned from alfalfa (*Medicago sativa*) that codes for the replication-dependent histone H3.1 variant protein. Despite lacking all promoter sequence motifs that have been associated with cell cycle-dependent histone gene expression in plants, northern analysis of synchronized cells clearly linked gene expression to DNA replication. TTAATNA was recognized as a new sequence element in the 3′ untranslated regions of this and all other cell cycle-dependent histone H3 genes of dicotyledonous plants. It is not found in the replication-independent histone H3 genes.

Keywords: Cell cycle, DNA replication, histone H3, *Medicago sativa*, plant, S phase

INTRODUCTION

Histone genes are the typical example of cell cycle-dependent genes. They are expressed in tight coordination with DNA replication during the eukaryotic cell cycle to package newly replicated DNA into nucleosomal chromatin. In animal cells, this tight control operates at multiple levels, transcription, pre-mRNA processing and regulation of mRNA stability (Stein *et al.*, 1994; Williams *et al.*, 1994). A stem-loop structure with an associated downstream sequence in the 3′ untranslated region (3′UTR) of the non-polyadenylated histone gene transcripts are prerequisites for proper post-transcriptional regulation (Marzluff, 1992). The mechanisms that link histone synthesis with DNA replication in animals are absent in plants. In higher plants, some histone-specific promoter sequences can produce cell cycle-regulated gene expression (Brignon and Chaubet, 1993; Kapros *et al.*, 1993; Mikami and Iwabuchi, 1993; Terada *et al.*, 1995) but the dynamic range of expression was always lower than seen *in vivo* for these promoters in their natural combination with histone coding and 3′UTR sequences (Kapros *et al.*, 1992;

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Tanimoto *et al.*, 1993). Recently, we have described the destabilization of histone H3 mRNA in non-S phase alfalfa cells (Kapros *et al.*, 1995). These experiments suggest that sequence elements outside of the promoter play a role in cell cycle-regulated expression. The 3'UTRs of higher plant histone mRNAs often but not always contain predicted secondary structures. All contain loosely conserved, histone-specific polyadenylation signal sequences (Wu *et al.*, 1989; Mikami and Iwabuchi, 1993).

Histone H3 genomic clone msH3g423 (Genbank accession No. U09459) was obtained as the second cell cycle-regulated histone H3 gene of alfalfa. It lacks the cell cycle-control promoter sequences that are present in the first alfalfa H3 gene (Wu *et al.*, 1988), but it shares a new 3'UTR sequence element with all replication-dependent histone H3 genes of dicots.

RESULTS AND DISCUSSION

Isolation of msH3g423, A New Alfalfa H3 Genomic Clone

To isolate a new H3 gene, we used a differential screening method. Replica colony lifts of an EMBL3 SP6/T7 library of *Sau*3A partially digested DNA from *Medicago sativa* cultivar Chief, purchased from Clontech (Palo Alto, CA), were screened with the coding region of cDNA plasmid pH3c11 as a general probe for any histone H3 (Wu *et al.*, 1989). Among 500,000 plaques screened, hundreds were positive for histone H3 sequences. However, all plaques, except 20, were also recognized by either of two hybridization probes that are specific for the two known families of histone H3 genes of alfalfa. The 3'UTR region of pH3c11 is specific for the 3 known gene copies of the cell cycle-independent histone H3.2 genes that exist in the alfalfa per haploid genome (Wu *et al.*, 1989; Robertson *et al.*, 1996). The 3'UTR region of cDNA clone pH3c1 is specific for the 56 cell cycle-dependent histone H3.1 variant genes of alfalfa (Kapros *et al.*, 1992, 1995), one of which is genomic histone H3.1 clone ALH3-1.1 (Wu *et al.*, 1988, 1989; Robertson *et al.*, 1996). The 20 plaques that might represent a new histone H3 gene family, were re-screened at high stringency will all probes and 12 were selected for analysis by sequencing using the histone H3 coding region sequencing primer shown in Figure 1. Four clones failed to yield sequence information. The remaining 8 clones all predicted amino acids at codons 87 and 90 that are characteristic for the replication-dependent variant H3.1 protein form (Waterborg, 1993). Additional sequencing showed that three gene loci were represented, based on C *versus* T differences at leucine codons 60 and 92, positions which are also variable among the known H3.1 loci (Fig. 1). The most sequence information was obtained for clone msH3g423. This clone contains a single large open reading frame, highly homologous to ALH3-1.1 (Wu *et al.*, 1989). However, the homology abruptly stops at the borders of the protein coding region (Fig. 1). Thus, clone msH3g423 contains a new alfalfa histone H3.1 gene, most likely present in several copies in the alfalfa genome.

msH3g423 has an Atypical Promoter Structure

The promoter region of msH3g423 was compared with that of all known plant H3 genes for previously recognized sequence elements (Fig. 2). The common promoter structure of cell cycle-dependent histone H3 genes (Fig. 2, sequences 1–13) has been described as an octamer sequence, upstream of a nonamer element. In the 'reverse' orientation, the octamer is typically associated with a hexamer motif. The same promoter structure is seen in histone H4 genes of wheat, maize and Arabidopsis (Brignon and Chaubet, 1993; Mikami and Iwabuchi, 1993). The immediate upstream region of the promoter containing these elements, within 300 bp of the TATA box, conferred cell cycle-dependent gene expression in homologous and heterologous transformants (Wu *et al.*, 1989; Nakayama *et al.*,

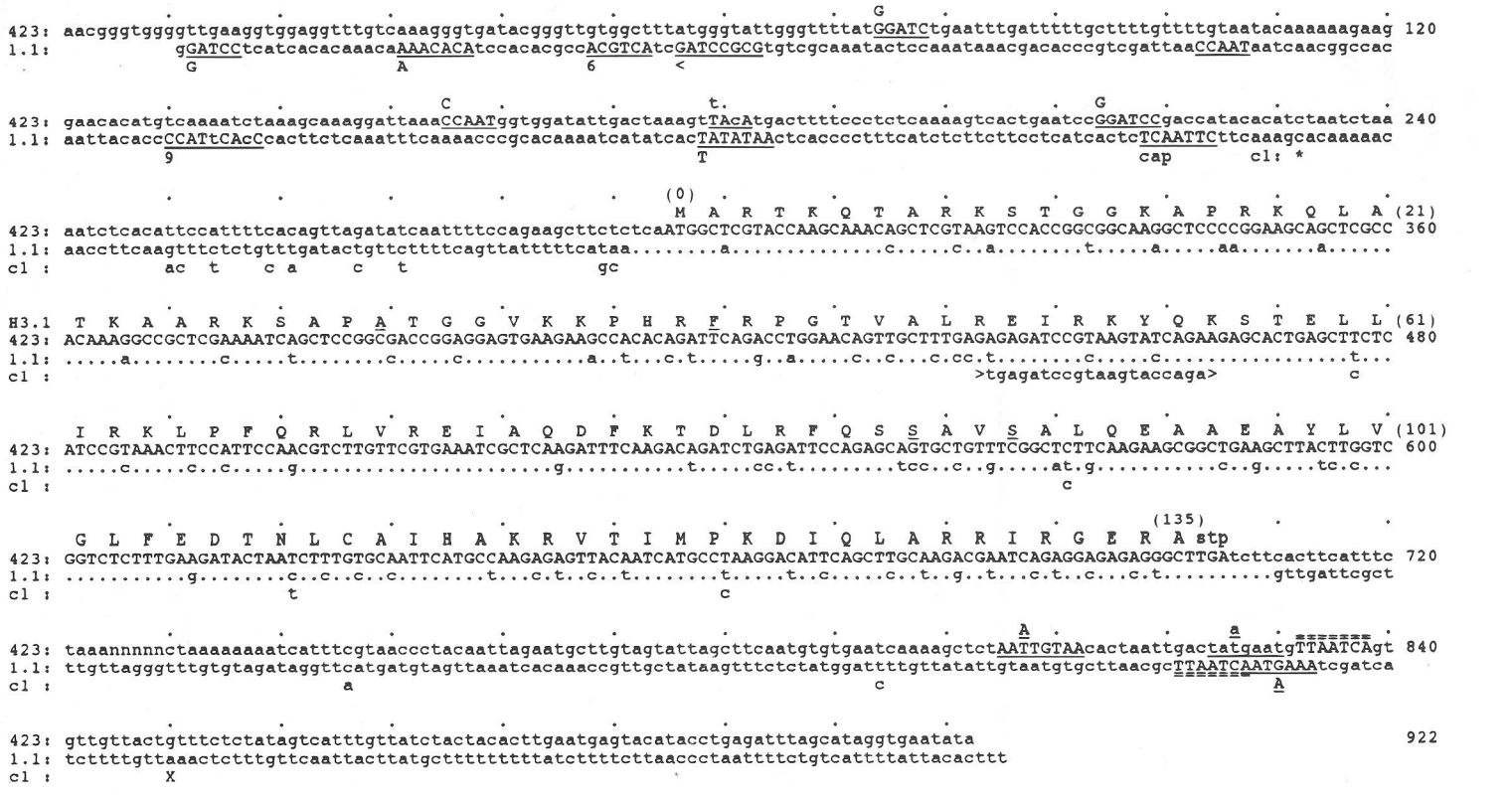


FIGURE 1 Histone H3.1 gene sequences. The sequence of histone H3.1 genomic clone msH3g423 is given as the upper sequence (marked as '423'), and is consecutively numbered. Aligned with its coding region (upper case) are the sequence of genomic clone ALH3-1.1 (Genbank No. X13673) (marked as '1.1') and the sequence of cDNA clone pH3c1 (Genbank No. X13674) (marked as 'c1'). Shown for the cDNA clone are only the start of the cDNA clone (star), the start of the polyA tail (cross) and in lower case nucleotides that are different from the ALH3-1.1 sequence. In the flanking sequences of the genomic clones regulatory sequences are underlined with matches to the consensus sequences in capitals for the AC box (A), CCAAT box (C), GC box (G), TATA box (T) and TATA-like box (t), histone hexamer (6), reverse-orientation octamer (<), nonamer (9), the cap site (cap) and the histone-specific (*A*) and general plant (*a*) polyadenylation consensus sequences (Wu *et al.*, 1989). The TTAATNA 3'UTR motif is marked by the double lines. The protein sequence of histone variant H3.1 is shown in capitals above the coding region in single letter abbreviations for amino acids and stp for stop codon. Amino acid numbering is shown between brackets, numbered as in the mature protein. Amino acids 31, 41, 87 and 90, which are specific for replication variant histone H3.1 protein, are underlined. The sequencing primer used in the screening of isolates was from position 445 to 463 as shown between 5' to 3' directional arrow heads.

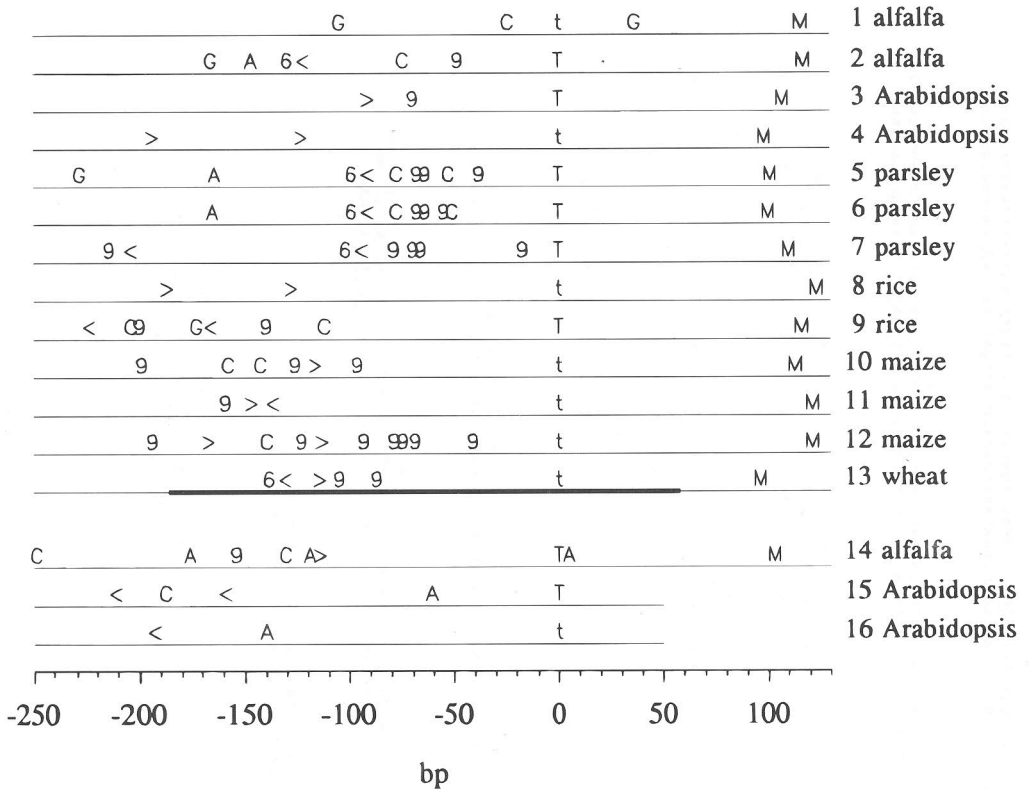


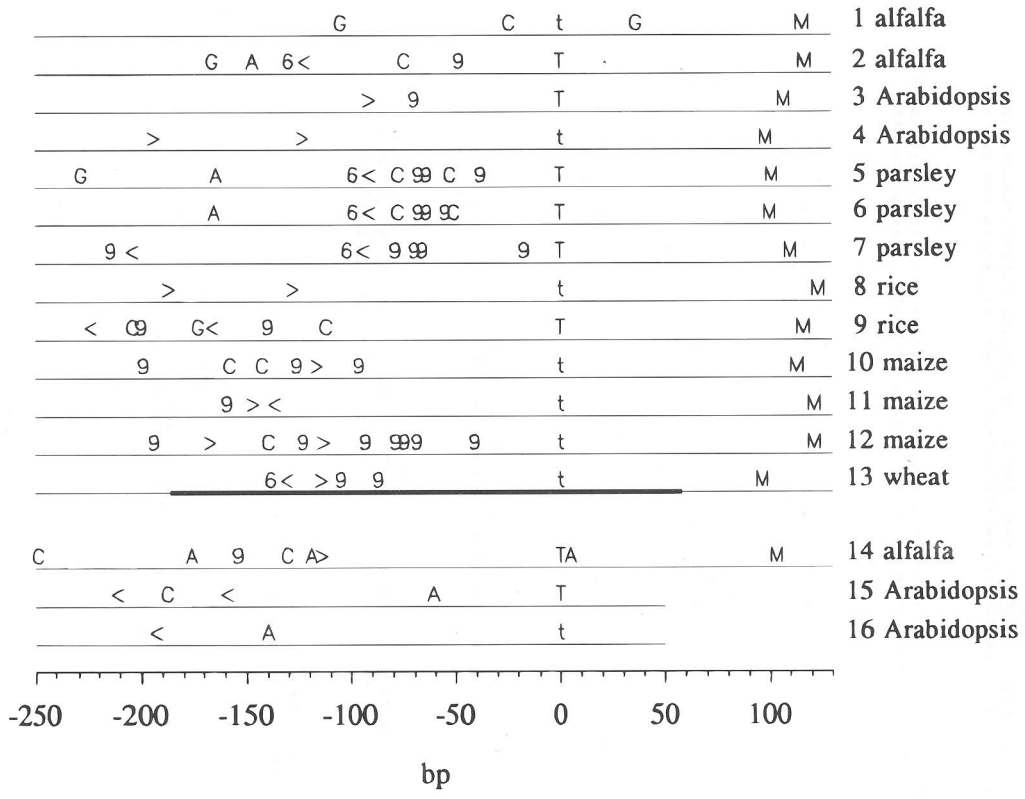
FIGURE 2 Comparison of promoter elements in plant histone H3 genes. All known histone H3 genomic clones of plants (identified by their Genbank accession numbers) were analyzed for the presence of consensus AC box (A = AAACACA), CCAAT box (C), GC box (G = GGATC in both orientations), hexamer (6 = ACGTCA) motifs or for sequences with at least 87% homology with the histone octamer (> = GATCCGCG; < = CGCGGATC) and nonamer (9 = CCATC(N)$_{0-2}$CANC) elements (Mikami and Iwabuchi, 1993). The results were aligned on the TATA box (T) or TATA-like sequences (t = TATTA, TACA) and the position of start codon methionine was marked as M. This information is not given for the two bottom sequences which show genes with 5'UTR introns (Chaubet *et al.*, 1992). Sequences 1 to 13 are replication-dependent genes from alfalfa (1, gene msH3g423, U69459; 2, gene ALH3-1.1, X13673), Arabidopsis (3, M17130; 4, M17131), parsley (5, M77494; 6, M77493; 7, M77495), rice (8, X13678; 9, X13680), maize (10, M13378; 11, M36658; 12, M13379) and wheat (13, X00937). The lower three sequences represent the replication-independent H3 genes of alfalfa (14, U69458) and Arabidopsis (X60429: 15, gene 1; 16, gene 2). The wheat minimal cell cycle-regulated promoter (Ohtsubo *et al.*, 1993) is marked by a thick line in 13.

1992; Kapros *et al.*, 1993; Ohtsubo *et al.*, 1993; Terada *et al.*, 1995).

The comparison in Figure 2 clearly shows that the location of these elements in histone H3 promoters is highly variable, but all contain at least one element. Alfalfa histone H3.1 gene msH3g423 (sequence 1) is the exception. Conversely, the cell cycle-independent histone H3.2 genes of alfalfa and Arabidopsis (Fig. 2, sequences 14–16) contain the octamer element and the alfalfa msH3g1 gene also has the nonamer (Fig. 2) (Chaubet *et al.*, 1992; Kapros *et al.*, 1992; Robertson *et al.*, 1996). Thus, it appears that cell cycle control of histone gene expression in plants is still incompletely understood, and that as yet unknown factors may control the difference in expression between replication-dependent and -independent histone genes.

Expression of the msH3g423 Gene

In order to determine the expression pattern of the msH3g423 histone H3 gene that lacks all putative cell cycle control motifs, mRNA levels were determined by northern analysis. It was very important to establish that the hybridization probes, sequences immediately surrounding the



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H3.1 coding sequence in msH3g423, would detect only the mRNA from this new histone H3.1 gene. To this end, slot-blot hybridization was performed with digoxigenin-dUTP labeled 3'UTR and 5'UTR fragments of the msH3g423 clone on msH3g423 DNA, representing the newly recognized H3.1 loci, and on pH3c1 DNA, representing the previously identified histone H3.1 genes (Wu *et al.*, 1989). This analysis showed the expected strong signal on the homologous template with less than 1% cross-hybridization (Fig. 3, inset), consistent with sequence differences (Fig. 1).

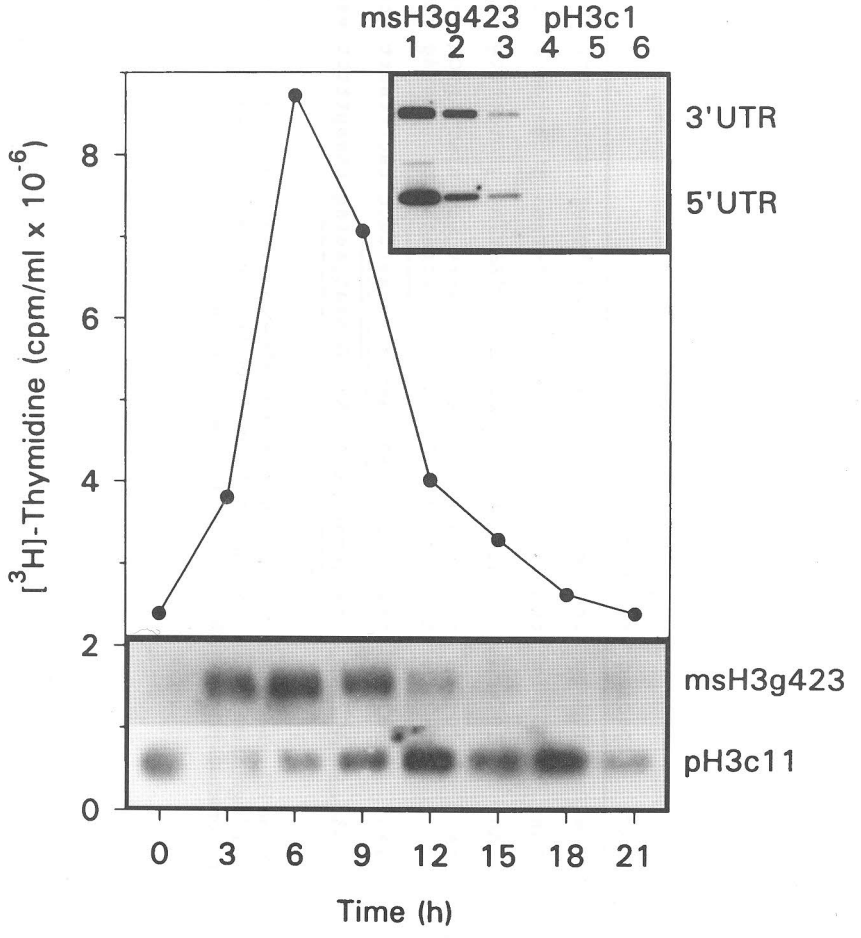

FIGURE 3 Cell cycle expression of histone H3.1 gene msH3g423. Alfalfa (*Medicago varia*) A2 suspension cultures were synchronized by double phosphate starvation (Kapros *et al.*, 1992). Aliquots of 10 ml and 1 ml culture were collected at 3 h intervals, starting 9 h after the second addition of phosphate, for the preparation of total RNA and for incorporation of tritiated thymidine, respectively, exactly as described before (Kapros *et al.*, 1995). DNA synthesis is reported as millions of cpm incorporated per ml of cell culture (see graph). The 3'UTR hybridization probe was produced by random priming with digoxigenin-substituted dUTP, as described for the Genius system (Boehringer, Mannheim), on the 179 bp subcloned fragment of msH3g423 that starts at the *Bsm*I site at position 769 (Fig. 1) and that includes 25 bp of pBluescript (Stratagene, La Jolla, CA) vector sequence. The figure insert shows slot-blot hybridization of this probe, marked as 3'UTR, with 200 pg, 20 pg and 2 pg of purified insert sequences of msH3g423 (922 bp) and pH3c1 (617 bp). This revealed less than 1% cross-hybridization of this probe with ALH3-1.1 histone H3.1 gene sequences. The northern analysis with this probe is marked as msH3g423. The 5'UTR probe template was produced from a 323 bp subcloned fragment that terminates at the internal *Hin*dIII site (position 286 in Fig. 1). This 5'UTR probe was completely specific for the msH3g423 H3.1 gene (see insert). The northern analysis with this probe is not shown. Histone H3.2-specific northern analysis, marked pH3c11, was performed with a probe made from a multimer of the 3'UTR of cDNA clone pH3c11 (Kapros *et al.*, 1995). Electrophoresis, transfer and hybridization of RNA with these probes was performed exactly as described (Kapros *et al.*, 1995) with chemiluminescent CDP-star alkaline phosphatase substrate (Tropix, Bedford MA).

Histone H3	stop codon	spacing	sequence
1. alfalfa	TGA	107 bp	cactaattga ctatgaatgT TAATCAgtgt tgttactgtt tctctatagt catttgttat
2. alfalfa	TGA	107 bp	ttgtaatgtg cttaacgcTT AATCAatgaa atcgatcatc ttttgttaaa ctctttgttc
3. Arabidopsis	TAA	107 bp	cgatgaaatg cctcaatgaa tcTTAATTAa gtctgttttg aatttaattc tcatgaatga
4. Arabidopsis	TAG	107 bp	tctatcaaag tattgacttt ttagttgact ttaTTAATCA ctaattgttt ggttaatttg
		192 bp	tcacaaaTTA ATCAgctttg acgctgaaat tgttgtgaag atcattgTTA ATTAgttgtg
5. parsley	TAA	107 bp	taatgcgttT TAATGAatta tgaaattatc tactttaatt gtctcttata actcaatgaa
6. parsley	TGA	107 bp	taatgaaatg tttCTAATGA aaatgaatag ttaagtttct aaataatttt gttataattg

FIGURE 4 Aligned 3'UTR sequences of histone H3 genes in dicotyledonous plants. Histone H3 sequences 1–6 are the same as shown in Fig. 2. The sequence of "7 parsley" (Fig. 2) is identical to "6 parsley". Highlighted are the TTAATNA element (capitals), identified by homology search, histone-specific polyadenylation sequences (underlined) (Mikami and Iwabuchi, 1993) and consensus plant polyadenylation signals (broken underlined) (Wu *et al.*, 1989).

Total RNA was prepared at 3 hour intervals from alfalfa cultures synchronized by the double phosphate starvation and release protocol (Kapros *et al.*, 1992). The msH3g423 3'UTR probe revealed a defined, replication-dependent pattern of expression that paralleled incorporation of tritiated thymidine into replicating DNA (Fig. 3). The 5'UTR probe produced an identical result (not shown). This ruled out the possibility that msH3g423 could be a gene with a 5'UTR intron containing the msH3g423 sequences upstream of the start codon and a promoter in an unsequenced upstream part of the clone. As a control, the blot was also developed with a probe derived from the 3'UTR of plasmid pH3c11, representing the cell cycle-independent histone H3.2 genes (Kapros *et al.*, 1995; Robertson *et al.*, 1996). This revealed the expected replication-independent pattern of expression, somewhat marred by uneven transfer (Fig. 3).

A New Putative 3'UTR Sequence Element of Plant Histone Genes

We have recently reported evidence of differential histone H3 mRNA stability in alfalfa (Kapros *et al.*, 1995). This raises the question which element(s) may be involved in this posttranscriptional regulation. Higher plant histone mRNAs are polyadenylated and lack the 3'UTR hairpin structure and downstream element, the major posttranscriptional control element in animals (Marzluff, 1992). The combination of the histone-specific 3'UTR polyadenylation motif AATG(G)AAATG followed by the $TTT(N)_{13-16}$ GATT sequence, downstream of the poly A addition site (Nakayama *et al.*, 1989; Ohtsubo and Iwabuchi, 1994), cannot be the equivalent of this signal in plants because it is absent in the replication-dependent H3.1 genes of alfalfa (Fig. 1) but present in the cell cycle-independent H3.2 histone genes of alfalfa, barley and Arabidopsis (Wu *et al.*, 1989; Chaubet *et al.*, 1992; Robertson *et al.*, 1996).

Sequence homology analysis (DNASIS program, Hitachi) identified a single consensus element (TTAATNA) in the 3'UTR of all cell cycle-regulated dicot histone H3 genes. It is located in a narrow window, 117 to 141 bp downstream of the stop codon, close to a polyadenylation signal. In most cases, it contains the general plant polyadenylation signal AATGAA (Wu *et al.*, 1989). In the Arabidopsis H3 gene where this association was not seen, the element is repeated close to the recognized polyadenylation signal (Fig. 4). It is absent in all known cell cycle-independent H3 genes (Robertson *et al.*, 1996). Thus, it might play a role in the differential stability of alfalfa histone H3.1 mRNA that we have observed (Kapros *et al.*, 1995).

Acknowledgements

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