Multiple polyadenylation sites are active in the α1-tubulin gene from *Zea mays*

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Received 17 May 1990; revised version received 17 September 1990

Two cDNA clones (MR2 and MR29) encoding the same α-tubulin isotype (α1) have been identified and characterized from maize roots. The sequence of these two cDNA clones is identical to a previously described cDNA clone (MR19) except in the location of their polyadenylation site. The sequence of the cDNA and its homologous genomic clone (MG19/14) shows two putative polyadenylation signals which could direct the variable 3′ processing of the observed transcripts. Endonuclease S1 protection analysis in this 3′ flanking region confirms the presence in the α1-tubulin gene from *Zea mays* of these two main functional polyadenylation sites and possibly other related ones. The relative accumulation of RNAs bearing the two main polyadenylation sites has been tested by using a RNA-slot analysis of several tissues of the plant. It appears that a higher proportion of shorter mRNA species is found in actively dividing tissues.

α-Tubulin; Maize; Polyadenylation; cDNA cloning; 3′-Cleavage; Endonuclease S1

1. INTRODUCTION

Tubulins are major compounds of cytoskeleton playing important structural roles in different phases of the cell cycle. They are found in all eukaryotic cells. The protein is a dimer formed by two subunits, namely α and β, whose sequence has been highly conserved through evolution. In vertebrates α-tubulins are coded by complex multigenic families encoding different isotypes [1]. To date, scarce information is available from plants but the number of α-tubulin genes seems to be lower (around 5 genes/subunit in *Arabidopsis thaliana* or *Zea mays*) [2,3]. Many of the organisms where the question has been addressed have α-tubulin isotypes showing a specific distribution that may arise from a differential pattern of expression of their genes, in particular organs or through developmental stages [2–4]. Moreover α- (and β-) tubulin have a number of post-translational modifications (tyrosination, acetylation, phosphorylation, etc....) which seem to take part in the fine regulation of the specific function of the protein [1,4]. Here we describe the characterization of two cDNAs corresponding to the α1-tubulin gene of maize [2] which present their polyadenylation sites at a different position than another previously reported homologous cDNA clone, MR19 [2]. This observation adds a new source of variability in the transcripts coming from the same α-tubulin gene that could play a role in the global regulation of these genes in maize.

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The sequences cited in the present paper appear in the EMBL/GenBank/DDBJ nucleotide sequence Database under the accession number X15704

2. EXPERIMENTAL

A cDNA library of 2-month-old root of maize was constructed in λgt-10 as described [2]. Upon differential screening with single strand cDNA probes from roots and leaves, a clone coding for α-tubulin was detected (MR19) [2]. Other clones coding for the same α-tubulin were identified by hybridizing the library with the MR19 insert. Restriction analysis and sequencing were performed as reported before [2]. The pUC Sequencing Kit (Boehringer) was used. Endonuclease S1 protection analysis was carried out essentially as in [5]. The genomic fragment *Sau*3A-*Rsa*I (298 nt) was 3′-end labelled with Klenow and [^{32}P]dATP being purified by isotachophoresis [6]. Hybridization was carried out with 5 μg of polyA$^+$ RNA from 3-day-old roots. The products of digestion with nuclease S1 were phenol extracted, ethanol precipitated and loaded on a 5% acrylamide, 8 M urea sequencing gel. The RNA-slot analysis was performed following previously described methodology [7].

3. RESULTS AND DISCUSSION

The MR19 cDNA clone coding for an α-tubulin of maize showing a preferential expression in the radicular tissues of the plant and its corresponding genomic clone have already been described [2]. It is possible to observe a tandem of two genes (α1 and α2), both coding for slightly different α-tubulin isotypes, and one of them being homologous to the MR19 clone [2]. After a screening of the library with the MR19 insert, two new cDNA clones (MR2 and MR29) were identified. Upon sequencing the insert by the dideoxy method in pUC vectors they showed a sequence identical to the MR19 clone except on their ends. At 5′ both clone MR2 and

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00145793/90/$3.50

MR29 were incomplete extending up to nucleotide positions 805 and 523 of the MR19 clone. More interesting, they showed heterogeneity at their 3′ ends. MR19 3′-non-translated end was 278 nt long (up to the polyA tail insertion). In contrast, MR2 and MR29 clones share the same 3′ end, but this is only 205 nt long. In the sequence of the longest clone (MR19) it was possible to find two signals departing in a single nucleotide from the established consensus AATAAA for polyadenylation [8], located at +176 and +253 from the TAG stop codon. About 20 nt downstream the first polyadenylation signal there is a G/T rich region which seems to take part in the cleavage of the transcript [9]. All these data are summarized in Fig. 1. The experimental finding of two cDNAs (MR2 and MR29) having their polyA tail at +24 nt from the first polyadenylation signal, and the other cDNA (MR19) at +20 nt from the second polyadenylation signal indicates that both signals could be functional in the α1-tubulin gene. The first one would direct the 3′ processing in some cases (MR2, MR29) whereas the second would be functional in other ones (such as MR19). In order to verify the existence of this heterogeneity observed from cDNA cloning and sequencing, an experiment of endonuclease S1 protection at the 3′ end of the α1-tubulin gene was carried out. The result is shown in Fig. 2 (and summarized in Fig. 1). The indicated genomic *Sau*3A-*Rsa*I fragment (298 nt long), from the MG19/14 clone [2] (α1-tubulin gene) was 3′-end labelled (in the *Sau*3A end) and used to hybridize with 5 μg of polyA$^+$ RNA from 3-day-old roots, the point of maximum mRNA accumulation of this gene [2]. In parallel, 10 μg of yeast tRNA were used as a control. The products of the S1 digestion were fractionated in a sequencing gel. In Fig. 2, indicated by filled triangles, it is possible to distinguish the specific protection of the probe at the expected length of 152 ± 1 nt (MR2 and MR29) and 225 ± 1 nt (MR19). These two groups of protected fragments have been marked in Fig. 1 with asterisks. Some additional protections (not found in the tRNA lane) appear at −10 ± 1 and −35 ± 1 from the first polyadenylation signal and −8 ± 1 and −25 ± 1 from the second one. The same results were obtained by increasing both up to 0.25 × SSC and decreasing down to 5 × SSC the stringency of the standard (2 × SSC) conditions of hybridization (data not shown). It can therefore be concluded that there exist minor polyadenylation sites, other than those observed by cDNA cloning, at distances approximately multiple of 10 nucleotides from the main attachment sites (indicated by open triangles in Fig. 2).

To test the occurrence of this 3′-end variability in the α1-tubulin mRNA population of different organs a RNA-slot analysis was performed. The results are presented in Fig. 3. Two consecutive probes were prepared by *Sfa*NI digestion of the 3′-end of the gene. Probe 1, 190 nt long, may be used to detect the transcript population directed by the polyadenylation signals downstream from the first one (±176 from TAG). Probe 2, 110 nt long, recognizes only the transcripts governed by the second polyadenylation signal (+253 from TAG) (see Fig. 3). 10 μg of total RNAs from several tissues of the plant were blotted onto two different filters and hybridized with the above-described probes. The overall intensity was higher when using the first probe. Upon densitometrical scanning of the RNA-slot autoradiographies, an arbitrary signal of 100% was assigned, in both cases, to the hybridization with the root tip RNA, the rest of the signals being referred to it. The histogram is also showed in Fig. 3.

The pattern obtained with the probe 1 is very similar to those obtained with cDNA probes containing either most of the coding sequence [2] or 3′-end specific sequence [7]. The mRNA population that includes the shorter species (those detected by probe 1) appears to be

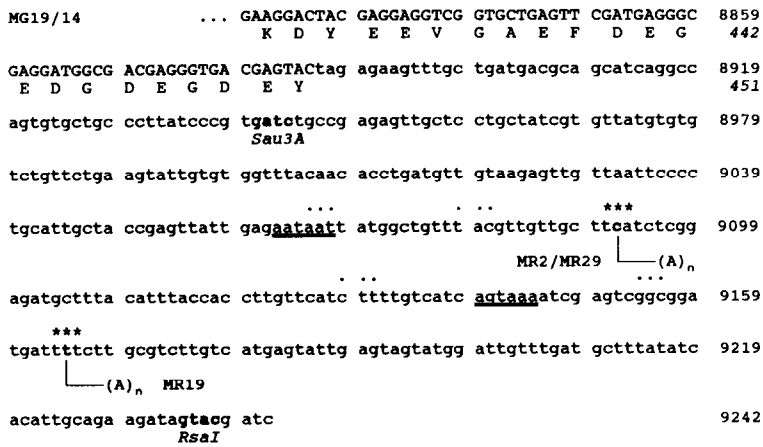

Fig. 1. Partial sequence of the genomic clone MG19/14 containing the 3′-end of the α1-tubulin gene. Putative polyadenylation signals are doubly underlined. The *Sau*3A and *Rsa*I sites (bold characters) used to obtain the genomic probe for S1 nuclease analysis are indicated. The MR2 and MR29 cDNA clones show their polyA tail at position 9092 whereas MR19 does at 9165 (shown in bold characters). The 5′-end of DNA-protected fragments in the S1 analysis are indicated with asterisks, when corresponding to the observed cDNA clones, or dots above the corresponding nucleotide positions in the rest of cases.

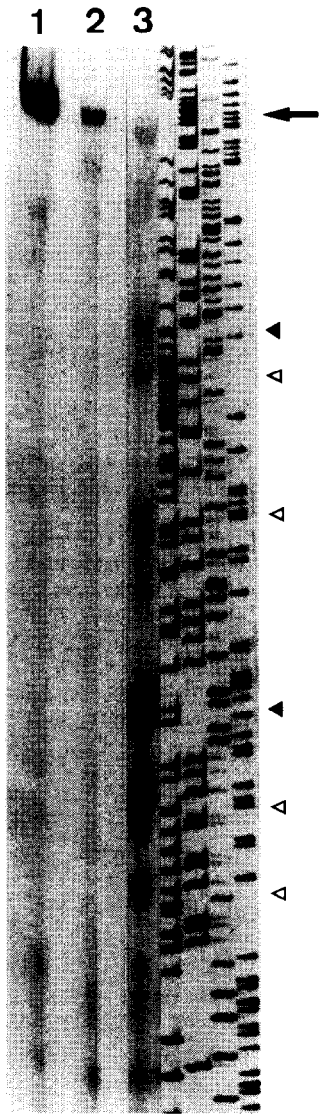

Fig. 2. Endonuclease S1 analysis. Lane 1 contains the undigested probe, indicated by the arrow, lane 2 the control with 10 μg of yeast t-RNA and lane 3 the products of S1 digestion of the 3'-end labelled DNA segment in the presence of 5 μg polyA$^+$ RNA from 3-day-old roots. The four contiguous lanes are nucleotide position markers. Filled triangles show the specific protected fragments corresponding to the observed cDNA clones whereas open triangles correspond, possibly, to other polyadenylation sites.

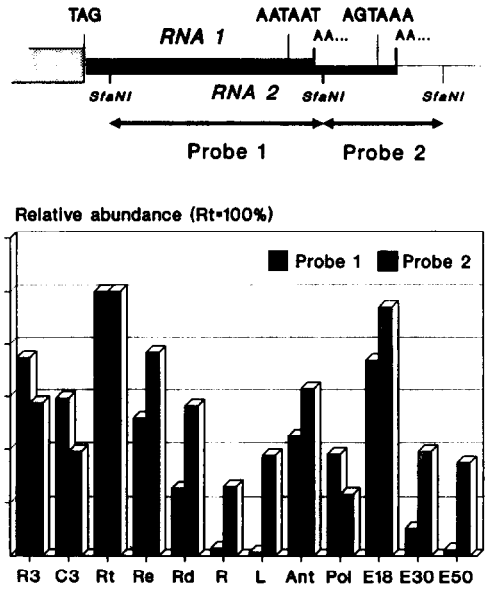

Fig. 3. Relative accumulation of the α1-tubulin mRNAs having different 3'-ends. (Above) Restriction map of the 3'-end of α1-tubulin gene showing the *Sfa*NI sites, used to isolate the DNA probes. The relative length of the transcripts is also marked. (Below) The results of the RNA-slot analysis. The histogram shows the relative abundance of messengers detected by the two probes referring to the accumulation observed in the root tips. The legends of the different tissues analyzed stand for: R3 and C3, young roots and coleoptiles, 3 days old; Rt, Re and Rd, root tips, part of roots rich in elongating cells or differentiating cells, respectively, from 6-day-old roots; R and L, adult (2 months) roots and leaves; Ant and Pol, anthers and pollen from 2-month-old plants; E18, E30 and E50 embryos from 18, 30 and 50 days after pollination.

relatively more abundant in those tissues rich in dividing cells (such as young root tips and embryos 18 days after pollination) whereas the larger ones (probe 2) are more evenly distributed in the different tissues. This fact could be explained as a consequence of the differential stability of both messengers. A higher stability of the shorter species may thus contribute to increasing the steady state level of the α1-tubulin mRNA.

The heterogeneity at the 3'-end of transcripts has been reported in the literature, as well as its relation with the control of gene expression [9]. In plants, the presence of multiple putative polyadenylation signals located at different positions at the 3'-end of mRNA and departing from the consensus sequence seems to be the rule and a small body of evidence has been reported on heterogeneity in the 3' termination point. The barley toxin β-hordothionin gene has been found to have heterogeneity in the polyadenylation sites (from a single polyadenylation signal) as well as different processing due to the presence of two polyadenylation signals [10]. The maize glutathione *S*-transferase mRNAs (I and III) have been proved (from cDNA cloning) to have different polyadenylation sites located from +13 to +51 nt downstream of the nearest polydenylation signal [11]. The maize phosphoenolpyruvate carboxylase mRNA also shows 3'-end heterogeneity due to variations in the attachment sites of the polyA tail [12].,

Regulation of expression of tubulin genes due to the stability of its mRNA has been reported in different organisms. Autoregulation of the β-tubulin production seems to be achieved by the control of the stability of their homologous mRNAs [13]. The length of the polyA tail of α-tubulin mRNAs could vary through the cell cycle of *Physarum polycephalum* and therefore be correlated with the stability of the messengers [14], but no reports on the use of different polyadenylation signals have been reported to date in α-tubulin genes. In

conclusion, the existence of two main polyadenylation sites has been observed in the maize α1-tubulin gene by both cDNA cloning and S1 protection. These are located at similar distances (+20 and +24 nt, respectively) from the putative polyadenylation signals present in the sequence. The other putative polyadenylation sites, located at approximate multiples of 10 nucleotides from the two sites observed in the cDNAs, represent an added variability in the insertion of the polyA tail from a given polyadenylation signal. This is, to our knowledge, the first case that such features at 3'-end have been reported in the α-tubulin genes. This observation introduces a new possibility for the regulation of α-tubulin gene expression and it might contribute to generating transcripts with specific stability depending on the organ or developmental stage where they are functional.

Acknowledgements: The authors are grateful to Plan Nacional de Investigación Científica y Técnica (Grant Bio/88-242) and the European Communities (Grant BAP-374) for their support. We also thank Mr Josep Vilardell for his useful technical experience in S1 assays. L.M. is a recipient of a PFPI fellowship.

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