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Occurrence of 9 homologous repeat units in the external spacer region of a nuclear maize rRNA gene unit

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ABSTRACT

A 14 kb maize DNA fragment carrying nuclear rRNA genes and spacer regions was isolated and characterised by restriction enzyme mapping. A complete 3020 bp long external spacer region was sequenced and revealed 9 tandemly arranged 200 bp long repeat units with high homology. The repeat units lie upstream from two prominent S1 mapping signals. The sequence of a typical repeat unit is compared to a corresponding 130 bp long wheat repeat unit. The possible functional relevance of the repeat units is discussed.

INTRODUCTION

In higher eucaryotes ribosomal RNA genes are organised in tandemly repeated units of up to several thousand copies. They are localised in the nucleolus, where transcription and processing of the primary transcript takes place (1). Transcription of the ribosomal RNA genes is mediated by RNA polymerase I together with additional, species specific factors (2-4).

A ribosomal RNA gene unit typically consists of the 18S, 5.8S and 25S rRNA coding regions and internal and external spacer regions. The genes are cotranscribed as a single large precursor molecule containing additional transcribed parts of external spacer regions 5` and 3` to the rRNA coding genes and small internal spacers lying between them (see Fig. 1a) (1). The transcription units themselves are separated from each other by nontranscribed external spacer regions, the size of which varies from approximately 1 to 30 kb depending on the organism (5-8). The external spacer regions very often contain repeats of 60-700 nucleotides in length (7-13). The number, arrangement and DNA sequence of the repeats have been found to be highly variable between closely related species and even between dif-

ferent organisms of the same species (8,10,12-14).
The location and sequence of the initiation site of transcription of the precursor RNA is different from species to species, although some homology around the transcription start has been observed in the case of rat, mouse and man (15). In some organisms, such as Xenopus (16) and Drosophila (7) sequences homologous to the transcription initiation site are also found further upstream within the repeat units. Attempts to identify the triphosphate groups at the 5` end of the primary transcripts have been successful (17-20).
Little is known about structure and function of the external spacer region, in particular with respect to plants. The most advanced knowledge is that obtained from wheat (Triticum aestivum L.), where two 130 bp repeat units from the spacer region have been identified and sequenced (13). These repeat units from wheat do not show cross hybridisation with the spacer region of rye (Secale cereale L.), which is related to wheat (21).
In maize (Zea mays L.) the ribosomal RNA gene units occur in 3300-23000 copies per diploid genome and map all to the nucleolus region of chromosome 6 (22). Hybridisation analysis of several maize varieties with a rRNA gene specific probe revealed great similarity of the rRNA gene units (23). As yet only the coding region of the 18S rRNA of maize has been sequenced (24). Since, in other organisms, the external spacer region has been found to contain the RNA synthesis initiation site and also seems to be important for the regulated expression of the rRNA genes we have concentrated our efforts on a detailed analysis of the external spacer region lying upstream from the 18S rRNA gene. To this end a clone containing a complete transcription unit together with complete spacer regions has been used to analyse the external spacer region by sequencing and S1 mapping.

MATERIALS AND METHODS

Reagents

Restriction endonucleases were from Boehringer, Biolabs or PL/Pharmacia. T4 polynucleotide kinase was from Amersham, T4

DNA ligase from Boehringer. Guanidiniumthiocyanate was purchased from Fluka (Switzerland).

RNA and DNA preparation

Total RNA from 10 days post pollination maize kernels of the variety A619 (from Mike Brayton Seeds, Ames, USA) was prepared by the guanidiniumthiocyanate method as described by Maniatis et al. (25) omitting the proteinase K digestion. Ribosomal RNA was isolated from total RNA by fractionation on 1.5% agarose gels containing 7M urea followed by electroelution. Phage λ DNA and plasmid DNA were isolated according to established procedures (25).

5` end labelling of the rRNA species with polynucleotide kinase following partial hydrolysis

Partial hydrolysis was carried out by incubating 3 µg rRNA for 5 min. in 15 µl 50 mM Tris/HCl, pH 9.5, at 90°C. After cooling down on ice 50 µCi γ [^{32}P] ATP (5000 Ci/mMol) and 2 units polynucleotide kinase were added together with buffer to make up 30 µl. The reaction was performed for 1 hr at 37°C and stopped by addition of 30 µl 4M ammonium acetate. The labelled RNAs were separated from the unincorporated radioactivity by running over a Sephadex G-50 column. Incorporation was approximately $5x10^7$ dpm/µg RNA.

Isolation of a clone containing a complete genomic rRNA unit and subcloning

A genomic library of partially digested BamHl fragments of the maize variety A619 cloned into the phage λ EMBL3 vector (26) was screened with 5` end labelled maize rRNA and yielded several clones containing rRNA sequences. Regions of one particular clone, λ Mrl, were subcloned into the plasmid pBR329 (27).

DNA sequencing

DNA sequencing was carried out by both the dideoxy chain termination method according to Sanger et al. (28) and the chemical degradation sequencing method according to Maxam and Gilbert (29). DNA fragments for Maxam/Gilbert sequencing were labelled at the 5`end with polynucleotide kinase, followed by either strand separation or a second restriction endonuclease digestion. For the dideoxy method the sequencing reagents and a 15 base universal primer were purchased from Bethesda Research

Laboratories. DNA fragments to be sequenced were isolated following NcoI, TaqI or SmaI/TaqI restriction endonuclease digestion and subcloned into the NcoI, AccI or SmaI site respectively of M13 mp18 or M13 mp19 phage (BRL). After the sequencing reactions the DNA fragments were separated in thermoregulated (50°C), 1 m long, 0.26 mm thick 4%, 6%, 8% and 16% polyacrylamide gels containing 7M urea according to Ansorge and Barker (30).

Nuclease S1 mapping procedures

100 ug of total RNA was coprecipitated with $1x10^5$ cpm of end labelled DNA fragment, dissolved in 50 µl 80% formamide, 400 mM NaCl, 1mM EDTA, 40 mM MOPS, pH6.7, heated up to 90°C for 5 min and slowly cooled to the hybridisation temperature of 60°C. Hybridisation at 60°C was performed for 16 hrs. To this mixture 1000 units of S1 nuclease, dissolved in 450 µl icecold 30 mM NaAc, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6, was added and incubated at 37°C for 1 hr. The reaction mixture was then extracted with 200 µl phenol/chloroform, 5 µg tRNA was added and the nucleic acids were ethanol precipitated. The pellet was redissolved in 50 µl 0.3 M NaOH, 5 mM EDTA and incubated at 65°C for 30 min. 60 µl Tris/HCl, pH 7.5 were added for neutralisation, and the solution was further extracted with phenol/chloroform. After addition of 5 µg tRNA and ethanol precipitation the pellet was redissolved in 2-4 µl of loading buffer and S1 resistant fragments were separated on 6% sequencing gels.

Primer extension analysis

These experiments were performed according to the S1 mapping procedures (above) with the following modifications. After the hybridisation the nucleic acids were ethanol precipitated, washed with 70% EtOH, dried and dissolved in 50 µl of 100 mM Tris/HCl pH 8.3, 8 mM $MgCl_2$, 145 mM KCl, 20 mM ß-Mercaptoethanol, 1 mM of each deoxynucleotide. 50 units of reverse transcriptase (Anglian Biotechnology Ltd.) were added and the solution was incubated for 3 hrs at 42°C. After incubation the samples were extracted with phenol/chloroform and further handled as described for the S1-mapping procedure.

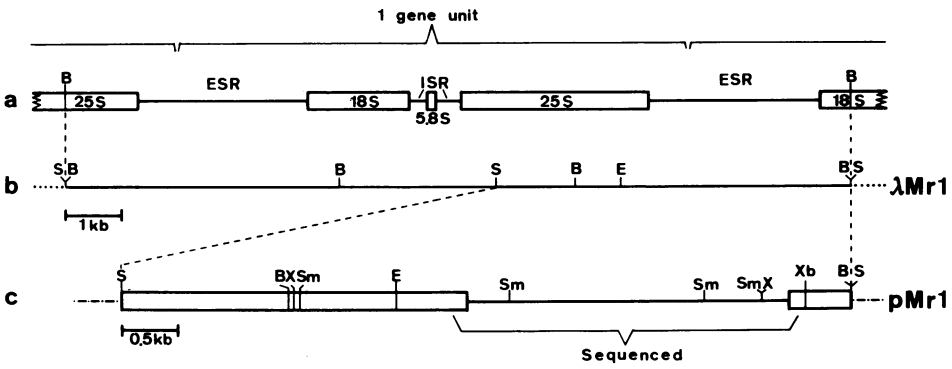


Figure 1. Organisation and localisation of rRNA gene units and cloned fragments. a) Schematic representation of rRNA gene units; b) BamHl fragment cloned into EMBL3; c) the 6.5 kb Sall fragment (using the unique Sall site in the gene and one of the two Sall sites in the linker of EMBL3) cloned into pBR329. Boxed-in areas: rRNA coding regions; ESR: external spacer regions; ISR: internal spacer regions; -·-·-·-: EMBL3 sequences; ·····:pBR329 sequences; B: BamHl; E: EcoRl; S: Sall; Sm: Smal; X: Xhol; Xb: Xbal.

RESULTS

Isolation and restriction site analysis of a 14 kb DNA fragment containing an rRNA gene unit

A genomic library of partially digested BamHl fragments of the maize variety A619 was prepared in phage λEMBL3 and screened by hybridisation with [^{32}P] labelled ribosomal RNA for clones containing rRNA coding sequences. Several clones containing 13-14 kb long inserts were isolated and shown to display the same restriction sites for EcoRl, BamHl, Sall and Smal. One clone, λMrl, was submitted to a more detailed restriction site analysis and characterised by hybridisation against [^{32}P] labelled 18S and 25S rRNA (Fig. 1a and b). λMrl consists of a complete transcription unit, two complete external spacer regions and the 3` and 5` regions of a 25S and an 18S rRNA gene respectively. The complete rRNA gene unit for maize as measured from λMrl is 9 kb in length. The insert of λMrl contains a unique Sall site, which was used for the subcloning of a 6.5 kb fragment containing one complete spacer region (Fig. 1b and c). The plasmid thus obtained, termed pMrSl, contained 2.5 kb of 25S rRNA 3` coding region, 550 bp of 18S rRNA 5` coding region, and

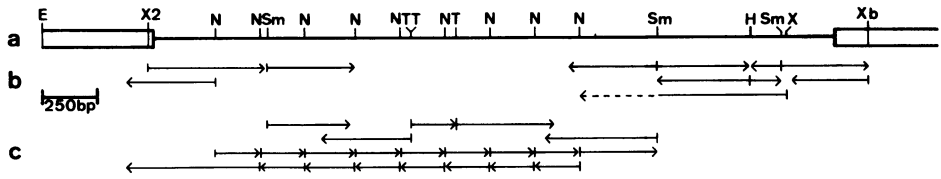


Figure 2. Sequencing strategy of the spacer region and of flanking rRNA coding regions. a)Restriction map of enzymes used for sequencing, b) fragments sequenced by the Maxam/Gilbert technique, and c) fragments sequenced by the dideoxy method. E: EcoRl; H: Hinfl; N: Ncol; T: Taql; Sm: Smal; Xb: Xbal; X: Xhol; X2: Xho2.

3.0 kb representing one complete spacer region (Fig. 1c).

Sequencing strategy

For an assessment of the sequencing strategy the insert of pMrSl was digested with a number of different restriction enzymes. It was found that restriction enzyme Ncol generated from the spacer region several fragments with an approximately equal size of 200 nucleotides. When a limited Ncol digestion was performed, a ladder of fragments separated from each other by about 200 nucleotides could be detected. Since there is only one further Ncol site in the insert of pMrSl, lying in the 18S rRNA gene, the Ncol fragments in the spacer could be used for subcloning in phage M13 mpl8. Clones were obtained containing all fragments in both orientations and consequently sequenced with the dideoxy chain termination method. To determine the order of the Ncol fragments, the 1.8 kb Smal fragment of the spacer was isolated and digested with Taql. The two resulting Smal/Taql fragments were cloned into the Smal/Accl sites of M13 mpl8 and 19 and sequenced. For an unequivocal determination of the Ncol fragment arrangement in the middle of the 1.8 kb Smal fragment, the 200 bp Taql fragment was also subcloned in M13 mpl8 and sequenced.

The region 5` to the 1.8 kb Smal fragment was sequenced by both the Maxam/Gilbert technique and the dideoxy chain termination method. The region 3` to the 1.8 kb fragment was sequenced with the Maxam/Gilbert procedure. The various fragments used for the sequencing are shown in Figure 2b and c by arrows. It should be noted, that due to severe compression some parts of the se-

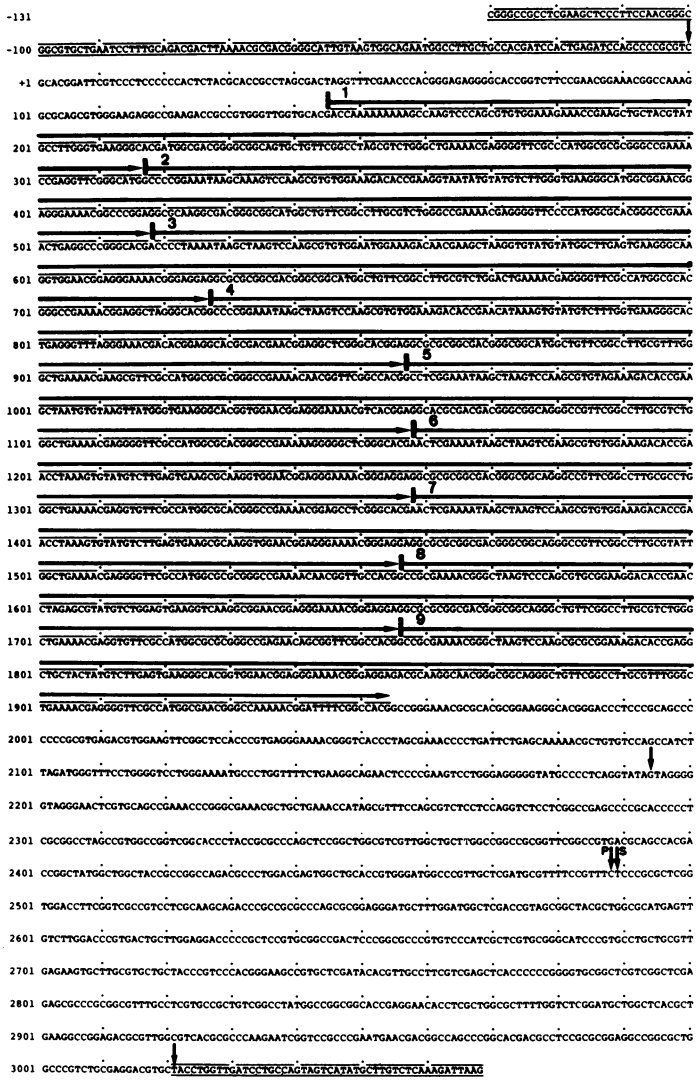


Figure 3. Sequence of the external spacer region. The spacer region extends from position +1 to +3020. Positions -1 to -131 represent the 3` end of the 25S gene, and positions +3021 to +3068 represent the 5` end of the 18S gene. The nine individual repeat units are shown by the thick black lines above the sequence. S1 signals at positions -1, +2194, +2489, and +3021 are indicated by arrows. The S1 signals at positions -1 and +3021 represent the 3` end of the 25S rRNA and the 5` end of the 18S rRNA respectively. The arrow at position +2488 is due to a signal obtained by a primer extension experiment.

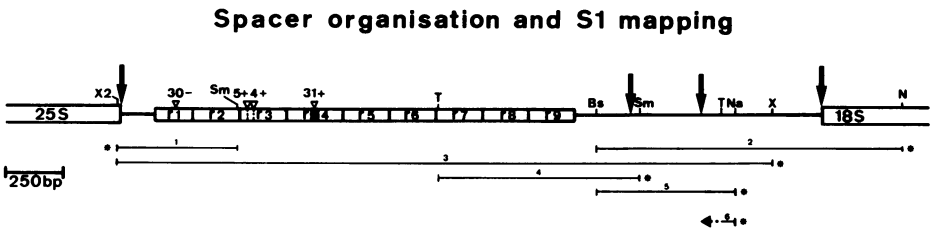


Figure 4. Organisation of the external spacer region. Repeat units are indicated by r1-r9. The position of a 30 bp deletion in r1, a 5 bp and 4 bp duplication in r3 and a 31 bp insertion in r4 are indicated by the open arrowheads above the repeats. The vertical arrows indicate the positions of the S1 signals and the lines below illustrate the fragments used for the S1 (1-5) and primer extension (6) experiments. Asterisks show the labelled ends. Bs: BstE2; N: NcoI; Na: NarI Sm: SmaI; T: TaqI; X: XhoI; X2: Xho2.

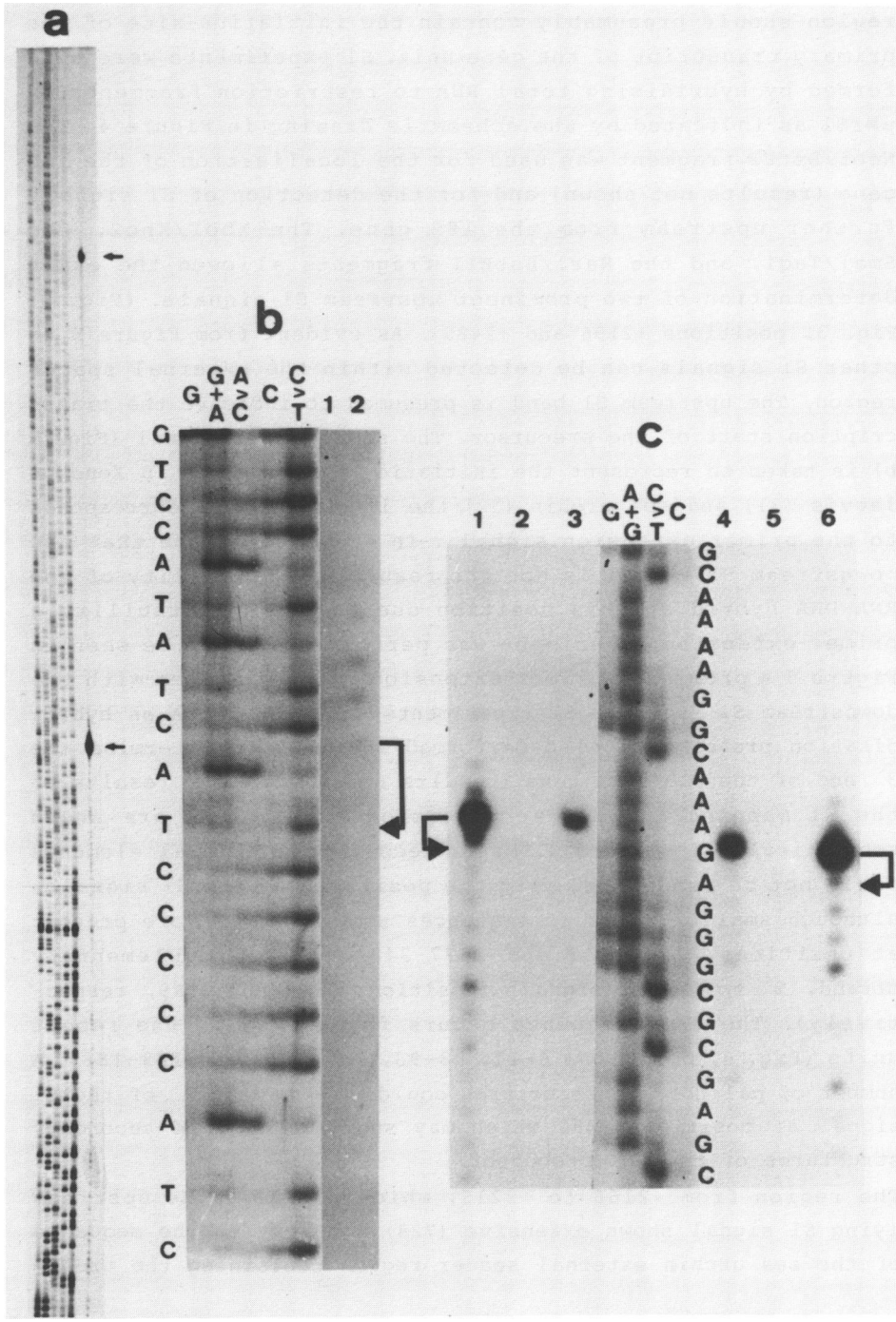
quence could only be resolved by running the sequencing gels at 60°C.

Sequence of the external spacer region

The sequence of the external spacer region is shown in Figure 3. The spacer region is 3020 nucleotides long and is distinguished by its high GC content of 64%. Positions -131 to -1 represent the 3` end of the 25S rRNA gene, as seen by the S1 mapping experiment described below. Positions 1-3020 represent the spacer region. The 5` end of the 18S rRNA gene from positions 3021 to 3068 was identified by S1 mapping (see below) and by sequence comparison with the 18S rRNA coding sequence described by Messing et al. (24). The sequence as analysed by Messing et al. was derived from W22 maize and differs in 15 positions from the sequence described here (positions 2770-3068). The spacer region can be divided into 3 major sections. Firstly, the region immediately flanking the 3` end of the 25S gene (Fig. 3, positions +1 to +144). This section is followed by a block of 9 tandemly arranged repeat units without interruptions (Fig. 3, positions +145 to +1954). From positions +1955 to +1963 eight nucleotides homologous to the beginning of a typical repeat unit can be seen. The remaining part of the spacer (Fig. 3, positions +1964 to +3020) represents the 5` flanking region of the 18S rRNA gene. This part of the spacer

region should presumably contain the initiation site of the primary transcript of the gene unit. S1 experiments were performed by hybridising total RNA to restriction fragments of pMrS1 as indicated by the schematic drawing in Figure 4. The NcoI/BstE2 fragment was used for the localisation of the 18S gene (results not shown) and for the detection of S1 signals further upstream from the 18S gene. The XhoI/Xho2, the SmaI/TaqI, and the NarI/BstEII fragments allowed the exact determination of two prominent upstream S1 signals. (Fig. 5; Fig. 3, positions +2196 and +2489). As evident from Figure 5 no other S1 signals can be detected within the external spacer region. The upstream S1 band is presumed to indicate the transcription start of the precursor. The smallest S1 signal (Fig. 5 b) is taken to represent the initiation point, since in Xenopus laevis (31) and sea urchin (32) the lowest S1 band corresponds to the primer extension signal . In order to ensure that the downstream S1 signal is not the result of instability of the RNA-DNA hybrid at this position due to rU:dA instability a primer extension experiment was performed. As can be seen in Figure 5 a prominent primer extension band coincides with the downstream S1 signal. S1 experiments with total RNA as hybridisation probes were also performed in order to determine the 3` end of the 25S rRNA gene (results not shown). The results of the S1 mapping and primer extension experiments are shown schematically in Figure 4. Major secondary structural elements could not be associated with the positions of the S1 signals, although smaller parts of sequences such as GAAAAC are present at positions 2137-2142 and 2477-2482 in the complementary strand, 5` to the S1 signals (positions 2194 and 2489, respectively). The same sequence occurs frequently in the repeat units (Fig. 6, positions 6-11, 88-93, 150-155, and 183-188). A number of palindromic structures could be detected 3` of the S1 signal at position +2489 which may suggest possible secondary structures of the RNA precursor.

The region from +2168 to +2215, which contains the upstream lying S1 signal shows extensive (72%) homology to the sequence of the sea urchin external spacer region containing the trans-


a
b
G
G+A
A>C
C
C>T
1 2
G T C C A T A T C A T C C C C C A T C
c
1 2 3
G
A+G
C>T
C
4 5 6
G C A A A A G G C A A A G A G G G C G C G A G C

cription initiation site (32) (Fig. 7). The region directly surrounding the S1 signal (from +2186 to +2200 or +2190 to +2198, respectively) is very similar also to the regions of the rRNA transcription start in Xenopus species (14,31) and Drosophila melanogaster (33).

A schematic representation of the arrangement of the repeat units in the spacer is indicated in Figure 4. A comparison of the sequences of the repeat units is shown in Figure 6. The complete sequence of repeat 6 is given, while the other repeats are described by indicating the nucleotide positions which differ from repeat 6. The high degree of homology between the repeat units is evident from the relatively few base changes seen. The nucleotides from 78 to 108 are deleted in the first repeat, while the additional sequences TGGAA and TGTA are present at the indicated positions in the third repeat. These two short sequences are direct repetitions of sequences immediately preceding their insertion site which occur in all 9 repeats. Likewise the sequence of the 31 bp insertion in the fourth repeat occurs also in all 9 repeat units from positions 96 to 127 with slight modifications. The repeat units contain regions with a particular high GC content of 82% (positions 93-

Figure 5. Primer extension and S1 nuclease mapping of the 5` ends of precursor molecules of the rRNAs. a) The S1 signals obtained using the 2.8 kb Xhol/Xho2 fragment (Fig. 4, fragment 3) are indicated by arrows. Lanes from left to right: Maxam/Gilbert reactions G, A>C, G+A, C, C>T, S1 control experiment without RNA, S1 experiment with total RNA. b) S1 experiment using the 0.7 kb Smal/Taql fragment (Fig. 4, fragment 4) to show the exact position of the upstream S1 signal obtained in (a). Lane 1 shows the S1 control experiment without RNA, lane 2 shows the S1 experiment with total RNA. The shortest band corresponds to position +2196. c) S1 mapping and primer extension using the 0.6 kb Narl/BstE2 and 75 bp Narl/Taql fragments (Fig. 4, fragments 5 and 6), respectively, to show the exact positioning of the lower S1 signal obtained in (a). Lane 1 and 3 show the primer extension experiment with total RNA, lane 2 the control experiment without RNA. Lane 4 and 6 show the S1 experiment with total RNA, lane 5 shows the S1 control experiment without RNA. In lane 1 and 6 ten times more radioactivity in comparison to lane 3 and 4 was loaded onto the gel. The S1 signal corresponds to position +2488. For the exact alignment with the sequence a correction of 1.5 bases was made for the S1 bands and a correction of 1 base for the primer extension band, as shown by the arrows in the sequence sections. The sequences show the complementary strand of the rRNA coding strand.

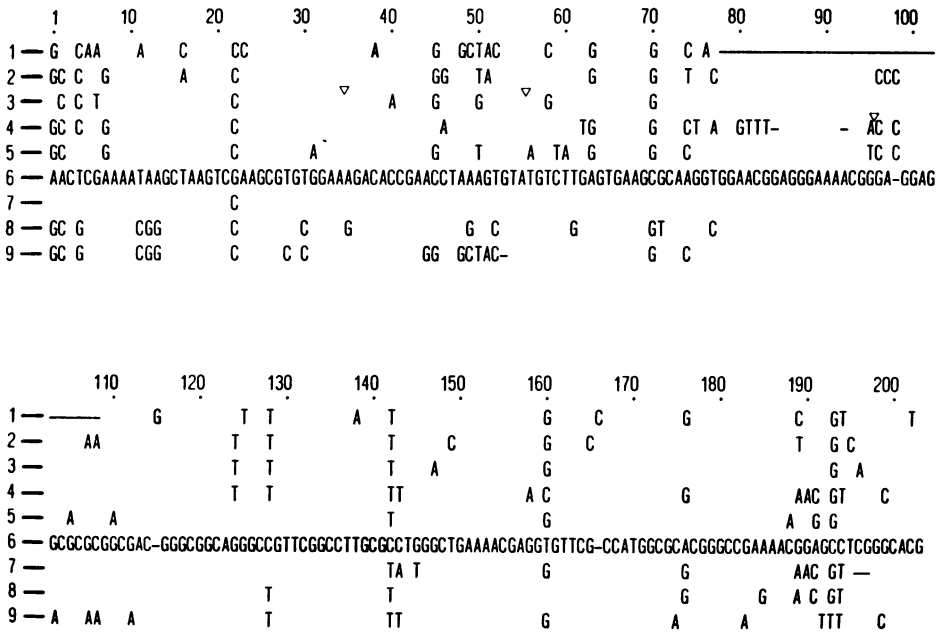


Figure 6. Sequence comparison of the repeat units from the external spacer region in maize. The numbering of the repeats corresponds to the repeat numbers in Figure 3. Numbers on top indicate nucleotide positions of the individual repeats. Repeat 6 is represented in full length while for the other repeats only the nucleotides different from repeat 6 are given. The bars represent positions of deletions. Open arrowheads indicate the positions of sequence insertions: the sequences TGGAA and TGTA are inserted in repeat 3 between positions 34 and 35, and 55 and 56 respectively, and the sequence CACGGAGGCACGCGACGAACGG AGGCTCGGG is inserted in repeat 4 between positions 95 and96.

147). This region is followed in each repeat unit by two consecutive regions of 80% homology (positions 145-159 and 178-192), which are separated by a palindromic sequence of 10 bp (positions 164-174) from each other.

Sequence comparison of the spacer region with a fragment from the wheat ribosomal spacer region

A 130 bp long sequence from the spacer region of a wheat ribosomal RNA unit (cv. Chinese Spring) as described by Appels et al. (13) was compared with the sequence of the maize repeat units. The 130 bp block (termed 130.6) is repeated 11 times in the 3.3 kb long wheat spacer region and differs only in 7 positions to the second sequenced repeat block (termed 130.8).

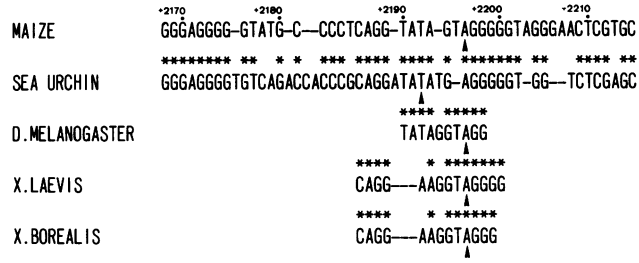


Figure 7. Comparison of the sequence around the upstream S1 signal from maize with sequences around the rRNA transcription initiation sites of sea urchin, Drosophila and Xenopus species. The sequence from +2169 to +2215 from maize is shown on the top line. The S1 signal is shown by the arrowhead at position +2196. Positions homologous to the maize sequence are shown by asterisks above the individual bases. Positions of S1 signals and transcription initiation sites are shown by arrowheads. Spaces introduced to maximise homology are represented by bars.

The maize and wheat repeat units can be aligned as shown in Figure 8. The wheat repeat sequence is compared to the last 69 nucleotides of repeat 6 and the first 125 nucleotides of repeat 7 (Fig. 3, positions 1289-1482). Alignment of the 132 nucleotides wheat sequence with the 194 nucleotides maize sequence reveals several regions of homology separated by regions of non-homology and by four gaps (positions 28 to 32 - 5 bp, 62 to 104 - 43 bp, 116 to 119 - 4 bp, and 127 to 136 - 10 bp) which account for the difference in length between the two repeat units. When the gaps are left out there is an overall homology

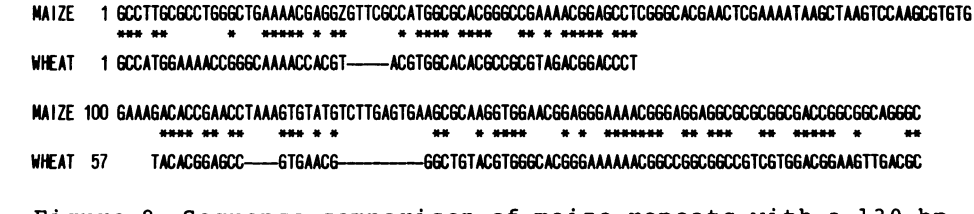


Figure 8. Sequence comparison of maize repeats with a 130 bp repeat from wheat. The wheat repeat is arranged to allow maximal homology with the maize repeat. Homologous positions are shown by asterisks. The maize spacer sequence from +1289 to +1488 (representing the 3` end of repeat 6 and the 5` end of repeat 7) is compared with the 130 bp wheat spacer repeat 130.6 (13).

between the repeats of 60% including one block of 7 homologous nucleotides (positions 158 to 164 - maize) and a number of shorter blocks of complete homology.

DISCUSSION

The work presented here represents the first report of the complete sequence of the external spacer region from a rRNA gene unit of higher plants. The 3020 nucleotides long spacer region contains 9 tandemly arranged repeat units of about 200 bp in length. The repeat structures follow each other without interruption. With the exception of a 30 bp deletion in the first repeat, two minor insertions in the third repeat and a 31 bp insertion in the fourth repeat, the repeats exhibit the same length and show 80-95% homology to one another. Similar observations have been made for several other organisms (7,9-11,13). This suggests, that the repeat units are submitted to restrictions of mutational change and hints at a functional importance of these structures. In the case of the rRNA genes of Xenopus laevis, which contains a comparable repeat arrangement an enhancing effect of the repeat structures on rRNA synthesis was shown (34,35). These findings ware in support of a previously suggested model, in which the repeats serve as a loading site for RNA polymerase I (36). It is supported by the phenomenon of nucleolar dominance as found in Xenopus, where transcriptional activity correlates positively with repeat copy number (16), and in wheat, where spacer length correlates positively with the size of the nucleolus (37). In the case reported here, a specific binding of regulatory proteins to single repeats or to the complete block of the 9 repeats can easily be envisaged. This could be assisted by specific structural features of the single repeats, such as the region of 82% GC and the two repeat structures following this region.

A homology between promoter structures and repeat units as found in Xenopus (16) and Drosophila (7) could not be detected in maize by computer alignment and homology search programs.

This resembles the situation found in mammals, where only short stretches of sequence could be defined as to be common for repeat units and the regions of transcription initiation sites.

In maize the hexanucleotide GAAAAC present about three times per repeat unit and occuring 25 times in the sequence 5` to the S1 signals in the rRNA coding strand can be detected two times in the complementary strand, from positions -58 to -53 with respect to the S1 signal at position +2196 and from positions -11 to -6 with respect to the S1 signal at position +2489. 3` to the S1 signals it is not present at all.

However, in all cases investigated so far, the repeat units widely diverge in length, nucleotide sequence and copy number between different species, whereas in one and the same species the sequences are highly conserved, a phenomenon termed concerted evolution (38). This sequence conservation may have arisen by gene amplification caused by two basic mechanisms: unequal crossing over and gene conversion (39), which are both known to occur in eucaryotes.

The only other known sequence from an external spacer region in higher plants comes from wheat, where two out of eleven 130 bp units have been sequenced and found to be almost identical (13). The arrangement of the repeats seen in maize resembles the situation as found in wheat by restriction enzyme analysis of the corresponding spacer regions. The sequence comparison with the repeat unit of wheat also reveals certain similarities. As shown in Figure 8 the wheat repeat unit shows regions of high homology to the maize repeat units. Whether these similarities indicate a comparable mechanism of rRNA transcription, remains to be shown. The difference in size between the repeat units of maize and wheat results primarily from four blocks of sequences not present in wheat. In the spacer of other eucaryotes the repeat structures are variant in terms of copy number, arrangement and homology. No repeat unit has been found in the rRNA gene of yeast (6).

In all cases analysed so far the repeat structures are located upstream from the initiation site of the primary transcript. This is also indicated for maize by the occurence of two prominent S1 signals 532 and 827 bp in front of the 18S rRNA gene (see Fig. 3) which makes them candidates for the RNA initiation site. This is in line with the sequence comparison shown in Figure 7, where a striking homology with the sequence surroun-

ding the upstream S1 signal to the rRNA transcription initiation site from other organisms can be seen. Alternatively the S1 signals could be results from RNA processing sites. In general S1 experiments in multigene systems need to be taken with caution because of the presence of many very similar genes. However, in the case presented in this investigation, only two very strong signals were observed. Preliminary experiments to identify the 5` end of the primary transcript by capping were not successful. The functional relevance of the spacer region and in particular of the tandemly occuring repeat units is unknown at present. Preliminary restriction site analyses of further cloned rRNA gene units from the same plant do not exhibit any variation. For an assessment of the importance and functional relevance of the structural elements seen in the spacer region of pMrS1 further investigations are under way such as in vitro transcription experiments with appropriate mutational constructs derived from the spacer region described here.

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