Specific Binding of Nuclear Proteins to the Promoter Region of a Maize Nuclear rRNA Gene Unit*

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The external spacer region of a nuclear rRNA gene unit of maize was analyzed for binding of nuclear proteins and revealed binding to the promoter region and to the 200-base pair repeat elements upstream of the promoter. The promoter binding site was further characterized by DNase I and exonuclease III footprinting as well as by gel shift and South-Western experiments, revealing the binding of several proteins. Competition experiments indicate that the protein which interacts with the repeat units also binds to the promoter region. A model for the combined action of both binding regions in the regulation of transcription is presented.

Nuclear rRNA genes from a wide range of eucaryotes are organized in gene units containing the 18, 25, and 5.8 S rRNA genes together with internal and external spacer regions. The external spacer region (ESR) is of particular interest as it contains the DNA signal structures responsible for an accurate and regulated transcription. ESRs have been reported to contain signal sequences required for transcription initiation (Grummt, 1982; Sollner-Webb et al., 1983; Skinner et al., 1984; Sollner-Webb and Tower, 1986; Halitzen Jones et al., 1988), enhancer-like sequences (Moss, 1983; Labhart and Reeder, 1984; Elion and Warner, 1986), transcription termination signals (Grummt et al., 1986; Labhart and Reeder, 1987), and hot spots of recombination (Voelkel-Meiman et al., 1987).

Nuclear rRNA gene units have also been isolated and partially characterized from a number of plants (McMullen et al., 1986; Ganal and Hemleben, 1986; Barker et al., 1988). rRNA gene units of maize were recently isolated from the variety A619 and from a Black Mexican Sweet suspension culture (Toloczyki and Feix, 1986; McMullen et al., 1986).

Sequencing of the 3-kilobase-long external spacer region from variety A619 revealed 9 highly homologous 200-bp-long repeat units arranged tandem lying upstream from two S1 signals, which correspond to the transcription start site and a putative processing site (Toloczyki and Feix, 1986).

For a number of organisms it has been shown that transcription initiation by RNA polymerase I (pol I) requires the previous formation of stable preinitiation complexes between initiation factors and the respective DNA promoter sequences (Tower et al., 1986; Kowlin et al., 1987). Likewise, stable complexes with trans-acting factors were also found at enhancer-like structures in rats (Cassidy et al., 1987) and proposed for Xenopus rRNA genes (Reeder et al., 1983). Furthermore, specific interactions with nuclear proteins have been observed at sequences responsible for transcription termination (Kermechev and Grummt, 1987).

Although no interaction of nuclear rRNA gene units with specific binding proteins has been reported in plants, the structural similarity of plant ESRs with the more extensively studied spacer regions from other organisms makes it likely that the basic mechanisms underlying the pol I transcription apparatus are not fundamentally different. We therefore decided to search for specific DNA-protein interactions in the external spacer region of a cloned ESR from maize variety A619, and to characterize further the promoter region which was previously identified by nuclease S1 mapping. Using filter binding assays, footprinting experiments with DNase I and exonuclease III, South-Western experiments, and gel retardation assays it was possible to identify and characterize a protein-binding site immediately upstream of the transcription start site. Furthermore, the repeat region was found to contain additional binding sites.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases were purchased from Bethesda Research Laboratories (EcoRII, SacII), New England Biolabs (SmaI, Hhal), and Boehringer Mannheim (BamHI, BglII, EcoRI, HaeIII, HindIII, HinI, SmaI, and XhoI). Calf thymus DNA, alkaline phosphatase, Klenow polymerase, DNase I, and exonuclease III were from Boehringer Mannheim. DEAE membranes and nitrocellulose membranes were from Schleicher and Schuell. The protein molecular mass markers were purchased from Sigma. Radioactive nucleotides and T4 polynucleotide kinase were from Amersham Corp.

Methods

Preparation of Nuclear Extracts—Nuclear extracts were prepared as described in Maier et al. (1988).

Standard DNA Manipulations—All DNA manipulations were done essentially as described by Maniatis et al. (1982). Fragments were isolated from agarose gels, treated with alkaline phosphatase, and 5'-labeled with T4 polynucleotide kinase. Fragments used for footprinting and sequencing were subsequently recut with an appropriate restriction enzyme. All 5'-labeled fragments were purified by electrophoresis on a 5% polyacrylamide gel and eluted on a DEAE membrane. Sequencing was carried out as described by Maxam and Gilbert (1980). Nonradioactive DNA used in the competition assays was quantitated by comparison to standards on ethidium bromide-stained agarose gels; calf thymus DNA, used as nonspecific competitor DNA, was sonicated. Random primer extension labeling was carried out as described by Feinberg and Vogelstein (1983).

Filter Binding Assays—The method outlined in Maier et al. (1987) was used to assay DNA-protein binding.

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Filter Binding Assays—The method outlined in Maier et al. (1987) was used to assay DNA-protein binding.
Footprinting Experiments—DNase I footprinting experiments were carried out with the 289-bp BstEII/HhaI fragment labeled at the BstEII site. The total volume of the reaction was 100 μl. Forty μl of nuclear extract, 10 μl of glycerol, 10 μl of 10× binding buffer (100 mM Tris (pH 7.5), 500 mM NaCl, 10 mM dithioerythritol, 10 mM EDTA, 50% (v/v) glycerol), and 10 pg of calf thymus DNA were preincubated for 15 min at room temperature. After this time, 12,000 cpm of the labeled fragment was added and incubated for an additional 15 min at room temperature. Subsequently, divalent cations and DNase I were added in concentrations as specified in the legend to Fig. 3. The DNase I digestion took place at 37 °C for 5 min and was terminated by the addition of 20 mM EDTA and heating for 10 min at 60 °C. The reaction was extracted twice with phenol-chloroform (1:1), once with chloroform:isoamy alcohol (24:1), ethanol-precipitated, loaded on a 6% sequencing gel, and autoradiographed.

ExoIII/ExoIII footprints were performed with the buffers and incubation times identical to those used in DNase I footprinting experiments, except that only 5 μg of nonspecific DNA was used. Fifteen min after the addition of 7000 cpm of the 260-bp EcoRI/BamHI fragment, which was labeled at the EcoRI site, 5 mM MgCl2 was added, immediately followed by the addition of 200 units of exonuclease III. The solution was incubated at 30 °C for various times as indicated in the legend to Fig. 4 and stopped by the addition of phenol-chloroform (1:1). The phenol extraction was repeated once, followed by chloroform:isoamy alcohol (24:1) extraction, ethanol precipitation, and gel electrophoresis on a 6% urea-acrylamide gel.

South-Western Experiments—Nuclear extracts from endosperm were mixed 1:4 with a 5× loading buffer 22.5% sodium dodecyl sulfate, 5% mercaptoethanol, 20% glycerol, 87.5 mM Tris/HCl (pH 6.8), and proteins were solubilized by heating the extract at 60 °C for 2 min. The proteins were separated on a discontinuous 14% sodium dodecyl sulfate gradient gel (Laemmli, 1970) and then electrophoretically transferred to nitrocellulose (Schleicher and Schuell, BA 83) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% sodium dodecyl sulfate) at 100 mA for 1 h at 4 °C. The filters were soaked at room temperature in 5 ml of binding buffer 1 (10 mM Tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 3 mM MgCl2) for 40 min. The blots were incubated with DNA at room temperature in 5 ml of binding buffer 2 (10 mM Tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 3 mM MgCl2, 0.02% bovine serum albumin, 0.02% Ficol, 0.02% polyvinylpyrrolidone, and 1 mM dithiothreitol). First, competitor DNA was added and allowed to react with the proteins for 20 min, followed by the addition of 100 cpm of the promoter fragment (EcoRI/HindIII from pSP-P, see Fig. 1), which was radioactively labeled by random priming. After an additional incubation at 30 °C for various times as indicated in the legend to Fig. 4, the filters were washed three times each for 10 min in binding buffer 1, air-dried on Whatman 3MM paper, and autoradiographed. Protein blots were stained with India ink (Hancock and Tsang, 1983). Molecular mass markers (fructose-6-P kinase (84 kDa), fumarase (48.5 kDa), lactic acid dehydrogenase (36.5 kDa), and triosephosphate isomerase (26.6 kDa)) were used to determine the molecular mass of the proteins.

Gel Retention Assays—Binding reactions (25 μl) contained 2 μg of nuclear proteins, binding buffer, and 2 μg of nonspecific calf thymus DNA. After a 15-min preincubation at room temperature, 10,000 cpm of labeled DNA fragment and the competitor DNA were added at the same time. After 10 min, 4 μl of loading buffer (99.5% glycerol, 0.5% bromophenol blue) was added, and the samples were loaded on 5% acrylamide gels (acrylamide/bisacrylamide (47:1, v/v)) containing 10% (v/v) glycerol which were prerun overnight. The electrophoresis buffer described by Staudt et al. (1986) (50 mM Tris, 380 mM glycine, 2 mM EDTA) was used. Electrophoresis was performed at 4 °C for 3 h with 10 V/cm, and the gel was subsequently dried and autoradiographed.

RESULTS

Protein-binding Sites in the ESR

The nuclear rRNA clone pMRI (Toloczky and Feix, 1986) used in this study contains parts of the 18 and 25 S rRNA coding regions and a complete external spacer region, as shown schematically in Fig. 1.

The ESR of pMRI was screened for protein-binding regions by incubating different restriction fragments with crude nuclear extracts from 8-day-old seedling leaf tissue and analyzing them for protein-binding regions by the nitrocellulose filter binding assay. This assay, DNA restriction fragments are terminally labeled and incubated with a nuclear protein extract under conditions optimized for binding. The mixture is passed through a nitrocellulose filter on which only DNA bound by protein is retained. Fig. 2 shows the results of the filter binding assays for three of the tested fragments, whose positions in the ESR are indicated in Fig. 1. The BstEII/Smal fragment, which shows binding, comprises the region around the transcription start site. Another bound fragment is the EcoRII/Smal fragment, which shows several repeat units. The SacII/XhoI fragment, which is located downstream of the promoter, does not exhibit protein binding.

It is noteworthy that the BstEII/Smal fragment displays a strong binding only after 15 min of incubation, whereas the repeat containing the EcoRII/Smal fragment is bound by nuclear protein after only 2 min of incubation. The EcoRII/BstEII fragment also reveals full binding already after 2 min (data not shown). Binding to both regions has also been observed using extracts from endosperm tissue, indicating the absence of tissue specificity. In order to study the interaction of these two regions with nuclear protein extracts, the restriction fragments containing them were subcloned into pSP 65.

Footprint Analysis of the Promoter Region

DNase I Protection Experiments—For a more accurate localization of the protein-binding site in the promoter region,
footprinting experiments were performed. The BstEI/SacII fragment (see Fig. 1) was 5'-labeled with $^{32}$P and recut with HhaI to generate an asymmetrically labeled fragment. The labeled BstEI/HhaI fragment was then submitted to partial digestion with DNase I with or without prior incubation with nuclear extracts from seedlings. Fig. 3 shows that digestion without prior incubation with protein extract generates a typical ladder pattern (Fig. 3, lanes 1-3 and 6). However, when the fragment is incubated with extract before the DNase I digestion, a gap in the ladder occurs from positions -9 to -40 (relative to the transcription start site), indicating that this region is protected against DNase I digestion on the coding strand (lanes 7 and 8). The strong signal at position -9 can be explained by an enhanced susceptibility to DNase I at this position as a consequence of protein binding to the DNA. In lanes 4 and 5 the higher concentration of DNase I led to an almost complete digestion of the DNA.

Exonuclease III Footprinting—Since the boundaries of DNase I footprints do not always coincide with the ends of the actual recognition sequence of binding proteins (Borgmeyer et al., 1984), additional footprint experiments using exonuclease III were performed. Exonuclease III footprinting experiments have the advantage that only partial protein occupancy of the binding sites is required to obtain a signal, making exonuclease III footprinting a preferred method for an analysis of multifactorial protein-DNA complexes where more than one DNA-binding protein is involved.

The EcoRI/HindIII fragment from pSP-P containing the promoter region was 5'-labeled and digested with BamHI to generate an asymmetrically labeled fragment. Digestion of the EcoRI/BamHI fragment with exonuclease III after prior incubation with nuclear proteins resulted in the pattern shown in lanes 5 and 6 of Fig. 4. In comparison to lane 4, which shows the exonuclease III digestion pattern without prior incubation of the fragment with extract, three additional distinct exonuclease III signals can be observed. The first signal at position -10 maps only 1 base pair away from position -9, which has been determined using DNase I. Two further exonuclease III signals can be seen at positions -17 and -26, reflecting a more complex binding pattern. The footprinting data obtained with DNase I and exonuclease III, respectively, are summarized schematically in Fig. 5, which shows part of the sequence and the transcription start position indicated. The proposed hairpin structure is shown in the lower part of Fig. 5, with the stem-loop being part of the region protected from DNase I digestion by bound proteins. All positions are 5' to the transcription start site. It is interesting to note that the sequence AGGGGGTA, which is flanked by two exonuclease III signals, corresponds exactly to the first eight transcribed nucleotides.

A number of the pol I promoters sequenced so far could be capable of forming hairpin structures (Financelk et al., 1982; Rogers and Bendich, 1987). The sequence upstream from the S1 signal in maize can also be arranged in such a typical structure. The proposed hairpin structure is shown in the lower part of Fig. 5, with the stem-loop being part of the region protected from DNase I digestion (indicated by the solid line above the sequence). Several Proteins Bind to the Promoter Region

The presence of several exonuclease III signals in the promoter region is an indication of the participation of more than one protein in the binding reaction. In comparison with the faster binding reaction of the repeat fragment, the delayed formation of the DNA-protein complex with the promoter fragment is another hint of the complexity of the binding reaction. To substantiate this assumption, South-Western and gel shift experiments were undertaken.

South-Western Experiments—Nuclear protein extracts from endosperm tissue were separated electrophoretically by sodium dodecyl sulfate-polyacrylamide gel electrophoresis,
transferred to nitrocellulose membranes, and incubated with the $^{32}$P-labeled promoter fragment containing EcoRI/HindIII from pSP-P. The patterns of proteins interacting with the labeled DNA are shown in Fig. 6. The binding reactions were performed in the presence of either an 80 M excess of unlabeled homologous promoter fragment (lane 2) or an excess of nonspecific calf thymus DNA (lane 1). It can be seen that three proteins show binding to the labeled fragment regardless of the competitor DNA used, indicating nonspecific binding. The disappearance, however, of six proteins of the approximate molecular mass 108, 44, 42, 38.5, 37, and 27 kDa in the reaction performed with an excess of the unlabeled homologous fragment indicates a sequence-specific interaction. Whether all indicated protein bands represent separate proteins or if some result form protein alterations cannot be decided at this stage. The protein pattern of a nitrocellulose filter stained with India ink is shown in lane 3.

FIG. 5. Map of the footprinting data. Upper, the footprint positions obtained by DNase I footprinting (Fig. 3) and exonuclease III (EcoHI) footprinting (Fig. 4) are indicated. The TATA box-like element is underlined, and the transcription start site is indicated by an arrow. The two regions of homology are indicated by dots. Lower, a proposed hairpin structure for this region is presented with the solid line showing the nucleotides protected from digestion by DNase I.
from pSP-R, which contains an entire repeat unit, leads to only one retarded band (Fig. 7, right, lane 2). This shift is equally well competed by a 50-fold excess of either the repeat fragment (Fig. 7, right, lane 3) or the promoter fragment (Fig. 7, right, lane 4) but is not competed by an excess of nonspecific call thymus DNA (Fig. 7, right, lane 2). This finding suggests that the protein which binds to the repeat fragment is also able to bind to the promoter region. Although the repeat fragment leads only to one shifted band, this fragment is able to interfere with the formation of all three retarded bands of the promoter fragment when added as a competitor. It is tempting to speculate that the disappearance of all three retarded bands of the promoter fragment by the addition of nonlabeled repeat fragment is the result of interference in an initial DNA-protein interaction at the promoter region. Such a complex could be a prerequisite for the subsequent binding of further proteins.

**DISCUSSION**

This report presents evidence of the specific binding of nuclear proteins to the external spacer region of a maize nuclear rRNA gene unit. Working with crude nuclear extracts of seedling and endosperm tissues, two prominent protein-binding regions were identified. Binding to these regions by nuclear protein extracts of either tissue agrees with the presumed rRNA synthesis in both tissues. One binding site was localized by DNase I footprinting to a region on the coding strand immediately upstream of the transcription start site.

The footprint extends from positions -9 to -40 relative to the transcription start site which was previously identified by S1 mapping analysis (Toloczyki and Feiz, 1986). This position of the promoter binding site compares well with corresponding footprint positions of animal nuclear rRNA systems which span or immediately precede the respective transcription initiation site (Dunaway and Reeder, 1986; Bateman et al., 1985; Clos et al., 1986; Learned et al., 1986; Kownin et al., 1987; Reeder et al., 1988). As in some other organisms, a part of the promoter region could be folded into a hairpin structure, which might play a role in DNA-protein recognition and/or be involved in the unwinding of the DNA for the transcription process. A "TATA box"-like element at positions -6 to -2 is, however, not protected in the footprint analysis under the conditions used. Since this element is absent from many pol I promoter regions analyzed so far (Grummt, 1982), it apparently does not have the important function as do the TATA boxes in pol II transcribed genes. The 3'-border of the binding site was confirmed with exonuclease III footprinting experiments, revealing an exonuclease III signal at position -10, which maps only one nucleotide upstream of the position determined by using DNase I. It has been shown for a number of pol I promoters that in addition to the "proximal promoter domain" there is one or more "upstream promoter domains" constituting the pol I holopromoter (reviewed in Sollner-Webb and Tower, 1986). It will be important to learn whether such an arrangement applies also to the maize promoter.

As only un fractionated crude nuclear extracts were used in the binding studies, it remains unclear which kind of proteins bind to the promoter region.

In contrast to purified transcription factors (Clos et al., 1986; Learned et al., 1986), purified polymerases I alone fails to show specific binding to promoter templates (reviewed in Roeder, 1978). A preformed complex of transcription factors bound to the promoter region (preinitiation complex) can, however, be bound efficiently by pol I (Iida et al., 1985; Tower et al., 1986; Kato et al., 1986; Cavanaugh and Thompson, 1986; Kownin et al., 1987). Therefore, it seems probable that the footprint on the maize promoter fragment was caused by the binding of transcription factors or a complex of pol I associated with transcription factors rather than by pol I alone. Evidence for the participation of several proteins in the binding reaction comes from the following observations.

1) Kinetic studies using the filter binding assay show that the binding reaction at the promoter region takes 15 min to complete, in contrast to the completion of binding at the repeat units after only 2 min (Fig. 2). 2) The occurrence of two exonuclease III signals within the region protected by bound protein from DNase I digestion suggests a more complex binding reaction (Fig. 4). 3) South-Western experiments suggest the specific binding of several proteins to the promoter-carrying fragment (Fig. 6). 4) Gel retention assays show the specific retention of three bands, reflecting the binding of several proteins or protein-protein interactions (Fig. 7).

These data are in line with the findings that simultaneous binding of several factors to promoter sequences is important for genes transcribed by polymerases I, II, and III (Kownin et al., 1987; Zheng et al., 1987; Jahn et al., 1987).

The repeat units were identified to be the second region of the ESR interacting with nuclear proteins of crude nuclear extracts. Competition experiments using the gel retention assay show that the repeat fragment interferes with the binding of all three retarded bands of the promoter fragment, which led to the proposition of the model shown in Fig. 8. This model reflects the data that the repeat fragment by itself leads only to one retarded band, which can also be competed by an excess of unlabeled promoter fragment (see Fig. 7). The model proposes that the promoter binding of a protein which is also able to bind to the repeat fragment is the prerequisite for the association with further proteins at the promoter region. This model closely resembles that previously proposed by Moss (1983) for the 60/81-bp repeats of *Xenopus laevis*. It is conceivable that the repeat units trap a protein which then subsequently reaches the promoter region where its binding precedes the formation of a preinitiation complex. Repetitive elements in ESRs are generally thought to play an important role in the regulation of transcription by trapping transcription factors (Reeder et al., 1983; Flavell, 1986; Rogers and Bendich, 1987). Experimental evidence was obtained for the 60/81-bp repeat elements in the ESR of *X. laevis* by injecting ribosomal gene plasmids bearing different length spacers into oocyte nuclei in competition with each other. The gene promoter attached to the longer spacer is dominant in transcription, showing an enhancer-like function of the repeat elements (Reeder et al., 1985). Since no in vitro transcription

![Fig. 8. Model for the binding of nuclear proteins to the ESR.](image)
system is yet available for plant nuclear genes, experiments are under way to study pol I expression by transiently transforming maize protoplasts with chimeric gene constructs.

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