1988

DNA Methylation, Chromatin Structure and Expression of Maize Ribosomal RNA Genes.

Eldon Ralph Jupe
Louisiana State University and Agricultural & Mechanical College

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DNA methylation, chromatin structure and expression of maize ribosomal RNA genes

Jupe, Eldon Ralph, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1988
DNA METHYLATION, CHROMATIN STRUCTURE
and
EXPRESSION OF MAIZE RIBOSOMAL RNA GENES

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Biochemistry

by
Eldon Ralph Jupe
B.S., Texas A&M University, 1981
M.S., Texas A&M University, 1985
December 1988
ACKNOWLEDGMENTS

I would like to thank my major advisor Dr. Elizabeth A. Zimmer for providing academic and financial support during the development and performance of this research project. I am especially grateful to Dr. Zimmer for providing me with numerous opportunities to travel and present portions of this research at various meetings during my graduate career. Her enthusiasm and encouragement throughout this project were greatly appreciated. I also thank Drs. Russell L. Chapman, Walter A. Deutsch, Sue G. Bartlett and Simon H. Chang for serving on my advisory committee.

My graduate career at Louisiana State University was enriched by the friendship and assistance of many colleagues. I would like to start by thanking Dr. A.L. Rayburn for sparking my interest in working on maize. I thank Vishal Sachdev for working on various maize projects with me during three of my four years. His research project on maize ribosomal RNA genes was a productive effort which was complementary to my own research. I would like to thank Keith Hamby, Mike Arnold, Lynne Sims and Laurie Issel for their assistance with the rRNA sequencing experiments, and Dr. Kathleen Morden and Marty Beasley for their assistance with Maxam-Gilbert sequencing of oligonucleotides. I am especially grateful to Dr. Morden for her valuable suggestions concerning my postdoctoral proposals. I also thank Joey Spatafora, Sami Guzder, Tim Fawcett, Mark Miller and Michael LoMonaco for helpful suggestions and discussions as well as their friendship. I am also grateful to Kelly Mullen, Dianne Dennis, Gretchen Stein, Bryan Peavy, and Sebrena Kolodziej for their friendship.
I thank Dianne Dennis and Stacy Spradley for typing the major portion of this dissertation and Elaine Riley for her help with a portion of the typing. I thank Jeralyn Miller for assisting with a portion of the photography and Vishal Sachdev for helping with figure drafting.

Finally, I would like to express my deepest appreciation to my wife, Johna, for her continuous confidence, patience, understanding and love throughout this learning experience. I also thank my parents, Mr. and Mrs. Ralph Jupe, and my grandparents, Mr. and Mrs. D. W. Ellis for their support and encouragement throughout my undergraduate and graduate education.
FOREWORD

In an attempt to make this dissertation a cohesive and focused text, some of the productive research that the author was involved with was omitted. This other research included a project, performed under the guidance of Dr. Russell L. Chapman, which led to a publication (Jupe, E. R., Chapman, R. L. and Zimmer, E. A. (1988) Nuclear ribosomal RNA genes and algal phylogeny - the Chlamydomonas example. Biosystems 21:223-230). Some experiments involving mapping and inheritance of ribosomal RNA genes in maize and its ancestors which are not directly applicable to my dissertation were not included (Zimmer, E. A., Jupe, E. R. and Walbot, V. (1988) Ribosomal gene structure, variation and inheritance in maize and its ancestors. Genetics 120:in press). Finally, a manuscript describing experiments performed with the author's supervision by an undergraduate research student is currently being prepared (Sachdev, V., Jupe, E.R. and Zimmer, E.A. (1989) Quantitative variation in ribosomal RNA gene copy number and methylation in maize and teosinte, in preparation).
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ABSTRACT

The restriction endonuclease *HpaII* was utilized to examine ribosomal RNA gene (rDNA) methylation in maize and teosinte leaf DNA. Much of the rDNA was inaccessible to *HpaII* cleavage indicating that these repeat units were completely methylated. In all of the DNAs examined, a significant fraction (10-25%) of the rDNA was cleaved at least once by *HpaII* into repeat unit length (9.1 kbp) fragments. The undermethylated *HpaII* sites mapped to the intergenic spacer (IGS) region of the rDNA. The major site of undermethylation was located in a region near the transcriptional start site.

An *EcoRI* polymorphism, present in the 26S gene of certain maize inbred lines, was utilized to correlate rDNA undermethylation, DNaseI sensitivity and expression. In double digest experiments with *EcoRI* and *HpaII*, the fragments originating from repeat units with two *EcoRI* sites (8.0 kbp) are sensitive to *HpaII* digestion, but the fragments originating from repeat units with a single *EcoRI* site (9.1 kbp) in polymorphic lines are resistant to *HpaII* digestion. To examine rDNA chromatin structure, intact nuclei were purified and digested briefly with increasing amounts of DNaseI. Analysis of this DNA with *EcoRI* showed that the 8.0 kbp fragments were extremely sensitive to DNaseI digestion, but the 9.1 kbp fragments were relatively resistant even at high levels of DNaseI. Specific sites hypersensitive to DNaseI cleavage were located in the IGS of the rDNA in a region near the major undermethylated site.

Experiments utilizing the polymerase chain reaction and direct rRNA sequencing indicated that the *EcoRI* polymorphism is due to sequence change in the rDNA. Oligonucleotide probes specific for the region surrounding and including the *EcoRI* polymorphic site were used to examine
rRNA transcripts in inbred lines and hybrids created by crossing EcoRI polymorphic and nonpolymorphic inbred lines. Results from these experiments indicate that the majority of the rRNA transcripts in a hybrid originate from the EcoRI polymorphic arrays when the maternal parent is EcoRI polymorphic. In the reciprocal cross, the EcoRI nonpolymorphic rRNA transcripts seem to be predominate. This suggests that a transcription factor in the maternal genome may determine which rDNA arrays are transcribed.
INTRODUCTION

Ribosomal RNA genes and nucleolar dominance

In eukaryotic organisms the ribosomal RNA genes (rDNA) exist in the genome as tandemly repeated units with each copy containing coding DNA interspersed with noncoding DNA. One repeat unit consists of a noncoding intergenic spacer (IGS) and a region coding for the 18S, 5.8S and 26S (in plants) cytoplasmic ribosomal RNAs (rRNA). The 5.8S gene is flanked on either side by short internal transcribed spacer (ITS) sequences (Appels and Honeycutt, 1986). Transcription by RNA polymerase I is initiated in the IGS region, and a transcript containing the 18S, 5.8S and 26S rRNAs is synthesized. This primary transcript is cleaved in the ITS regions flanking the 5.8S RNA and processed further to yield the mature 18S, 5.8S and 26S cytoplasmic rRNAs. In eukaryotes rRNA genes are highly reiterated and are present at 1 to 5 chromosomal locations as tandem arrays containing tens to thousands of essentially identical repeat units (Appels and Honeycutt, 1986).

Chromosomal location of the rDNA repeat units is detectable cytologically as a distinct structure known as the nucleolus or nucleolar organizer (NOR). The NOR is characterized by a prominent secondary constriction which separates a terminal heterochromatic satellite from the rest of the chromosome arm (Figure 1). The cytological morphology of the NOR was first described independently in 1934 by Navashin (1934) and McClintock (1934). Their investigations on the formation of secondary constrictions in different genetic backgrounds represent the first studies on differential gene regulation in eukaryotes. Navashin, while working on chromosome morphology in the plant genus Crepis, observed that the metaphase chromosome sets of each species of Crepis had a pair of large
Figure 1. Standard maize karyotype showing secondary constrictions. The secondary constrictions are present on the short arm of chromosome 6. Arrows point to the secondary constriction on the two homologous chromosomes. Photograph courtesy of Dr. A.L. Rayburn, University of Illinois, Dept. of Agronomy.
distinct chromosomes with a prominent secondary constriction. When certain interspecific hybrids were examined, the chromosomes of only one parent species were found to form a secondary constriction.

McClintock (1934) was working to define the NOR in maize by using plants carrying chromosomal interchanges. Normally in maize a single secondary constriction is present on the short arm of chromosome 6 (Figure 1). For these experiments McClintock (1934) used maize stocks in which a break in chromosome 6 had occurred at the base of the secondary constriction and undergone a reciprocal translocation with chromosome 9. The new translocated chromosomes 6⁹ and 9⁶ were examined in separate haploid microspores and both were found to form normally sized nucleoli. When both chromosomes were present in a diploid nucleus, there was a competition between the two with the 9⁶ chromosome forming a larger nucleolus and secondary constriction. Thus, these studies showed that regulation of the NOR in maize was dependent upon chromosomal position and genetic background.

In the same report, McClintock (1934) reviewed Navashin’s (1934) data and suggested that the nucleolus organizers of Crepis species could be ranked in a dominance hierarchy based on their ability to suppress nucleolar formation or to be suppressed themselves in interspecific hybrids. The testing of this dominance hypothesis did not occur until 1971 when Wallace and Langridge (1971) repeated Navashin's experiments and analyzed additional Crepis hybrids and reciprocal crosses for the pattern of nucleolus formation. These studies indicated a dominance hierarchy could indeed be applied to predict the active nucleolus in Crepis hybrids. Two additional important conclusions were reached from these experiments (Wallace and Langridge, 1971): (1) The same species is
dominant regardless of whether it supplies the sperm or the egg, and (2) The suppressed nucleolus organizer can be reactivated in the appropriate backcrosses. It was also observed that in hybrids the suppressed nucleolar organizers activate and form a nucleolus as soon as they are separated from the dominant organizers by a nuclear membrane during meiosis (when haploid spore nuclei form). Numerous other plant genera including *Salix* (Wilkinson, 1944), *Ribes* (Keep, 1962), *Solanum* (Yeh and Peloquin, 1965), *Hordeum* (Kasha and Sadasivaiah, 1971) and *Triticum* (Crosby, 1951; Longwell and Svhla, 1960; Flavell and O'Dell, 1979) exhibit nucleolar dominance in interspecific hybrids. This phenomenon is not confined to plants, and has also been described in *Xenopus* (Blackler and Gecking, 1972; Cassidy and Blackler, 1974), *Drosophila* (Durica and Krider, 1977; Durica and Krider, 1978), and mammalian somatic cell hybrids (Elicieri and Green, 1969; Croce et al., 1977; Perry et al., 1979).

The molecular basis for nucleolar dominance is best understood for the *Xenopus* system. In F₁ hybrids from crosses between *X. laevis* and *X. borealis*, the *X. borealis* ribosomal genes are repressed (Honjo and Reeder, 1973; Macleod and Bird, 1982). The difference in base composition and length of the external transcribed spacers in these species make it possible to distinguish the rRNA precursors of *X. laevis* from *X. borealis*. Earlier experiments which relied on base composition differences indicated that these hybrids synthesized predominantly *X. laevis* rRNA precursor (Honjo and Reeder, 1973). Later refinements based on length differences provided the quantitative result that 97-98% of the rRNA precursor in hybrid tadpoles was *X. laevis* precursor (Macleod and Bird, 1982). Together, these experiments showed that nucleolar dominance occurs at the level of transcription. In *Xenopus* the transcriptional dominance is
also not caused by a maternal effect because *X. laevis* rDNA was preferentially transcribed regardless of which species supplied the egg or the sperm (Reeder, 1985).

The ability to analyze transcription by the injection of cloned genes into either oocytes or developing embryos has provided a molecular explanation of the mechanism of nucleolar dominance in *Xenopus* (Moss, 1983; Reeder *et al.*, 1983). Microinjection of *Xenopus* oocytes or developing embryos with constructs containing the subrepeat portion of the IGS indicated that this sequence could influence transcription from the ribosomal gene promoter. Other experiments showed that a group of 60/81-bp repetitive IGS elements which can vary in number (within the genes of an individual or within a species) function as enhancer elements to promote RNA polymerase I transcription (Moss, 1983; Busby and Reeder, 1983). In an analogous situation to that found with RNA polymerase II enhancers, these 60/81-bp elements function in either orientation, both at a distance of several kilobases from the gene promoter or start site, or even when they are inserted within the transcribed region of the gene (Labhart and Reeder, 1984; Reeder, 1985). Sequence comparisons of these IGS repeats identified a 42-bp "core element" in each 60/81-bp repeat which is 90% homologous to a 42-kp region in the ribosomal gene promoter (Boseley *et al.*, 1979; Sollner-Webb and Reeder, 1979; Moss, 1983).

The importance of these 60/81-bp elements in controlling nucleolar dominance was demonstrated by duplicating the phenomenon *in vitro* (Reeder and Roan, 1984). They coinjected cloned ribosomal gene plasmids with different numbers of enhancer elements into oocytes and embryos and found that the plasmids with the greater number of 60/81-bp repeats
(without regard to species origin) were preferentially transcribed. These results are in line with the natural situation where the average _X. laevis_ spacer contains 17 of the enhancer repeats while the average _X. borealis_ spacer has only four copies of the enhancer repeats. This can be accounted for by an enhancer imbalance mechanism which predicts that in interspecific hybrids the spacer with the greater number of enhancer elements will always be preferentially transcribed. This model clearly accounts for the experimental observations in crosses between _X. laevis_ and _X. borealis_ as well as the reciprocal cross (Reeder, 1985).

However, a substantial amount of evidence from the _Xenopus_ system and others suggests that a species specific factor mechanism may also be involved in mediating nucleolar dominance (Reeder, 1985). The major support for this hypothesis is the lack of cross reactivity in _in vitro_ rDNA transcription systems between mouse and human (Grummt _et al._, 1982; Learned and Tijian, 1982, Mishima _et al._, 1982; Miesfeld and Arnheim, 1984) or between _Drosophila virilis_ and _D. melanogaster_ (Kohom and Rae, 1982). Several studies have also identified a species specific factor which, when partially purified, can be added to extracts from other species to stimulate transcription (Mishima _et al._, 1982; Miesfeld and Arnheim, 1984; Learned _et al._, 1985).

In plants the molecular mechanism of nucleolar dominance has not been well studied. The presence of multiple copies of a repetitive element in the IGS is known to occur widely in plants (Rogers and Bendich, 1987). In general, the sequence of these elements is species specific, they are 100-200 bp in length and vary in number from 5-10 copies. They are located in plants in the IGS near the 3' end of the 26S gene similar to the location in _Xenopus_. The IGS sequence from maize,
rye, wheat, radish and squash have been determined and sequences in the presumptive promoter region are also present in the subrepea

et elements (McMullen et al., 1986; Toloczyki and Feix, 1986; Rogers and Bendich, 1987). In addition to the sequence data and the fact that the number of subrepea

et elements vary widely, the best evidence that these elements may play a role in nucleolar dominance in plants comes from studies on Aegilops umbellulata X Chinese spring wheat hybrids. When the A. umbellulata chromosome is present in wheat, the wheat nucleolus organizers are suppressed while the A. umbellulata chromosome forms an active nucleolus and secondary constriction (Martini et al., 1982). Recently, restriction enzyme mapping experiments have shown that the A. umbellulata IGS is longer than the wheat IGS and that this increased length was due to the presence of more copies of the subrepea

et units (Flavell, 1986). Undermethylation of IGS DNA (to be discussed later) was also found to be correlated with nucleolus activity in these hybrids (Flavell et al., 1983; Flavell, 1986).

The studies discussed thus far have addressed differential gene activity in separate nucleoli which have been brought together in interspecific hybrids. A related, intriguing phenomenon involves the differential regulation of gene activity within a single nucleolus. In most eukaryotic cells, each nucleus houses a large number of rRNA gene copies. Animal cells may have from 100 to 1000 copies per diploid cell (Appels and Honeycutt, 1986), but plant cell rDNA copy numbers are even higher, with 500 to 40,000 copies per diploid cell being reported (Appels and Honeycutt, 1986; Rogers and Bendich, 1987). Ribosomal RNA gene copy number has been shown to vary substantially between individuals from the same population and between genotypes of a single species.
(Macgregor et al., 1977; Cullis, 1979; Rogers and Bendich, 1987). These observations suggest that the number of ribosomal RNA genes far exceeds that normally required to supply ample cytoplasmic rRNA to differentiated cells. Thus, within each nucleolus of a cell only a portion of the rRNA genes would be actively transcribed while the rest would remain inactive.

A variety of data suggests that this is the case in maize. As mentioned previously, maize has a single nucleolus organizer region located on the short arm of chromosome 6. Extensive surveys of inbred lines of Zea mays have shown that the rRNA gene copy number may vary from 2,500-24,000 repeat units per diploid genome (Phillips, 1978; Rivin et al., 1986). Cytological and genetic studies of maize have described the heterogeneous nature of the NOR. Figure 2 shows a schematic summarizing the features of an interphase NOR. When observed at the pachytene stage of microsporogenesis the nucleolus is clearly subdivided into two distinct cytological structures, a large block of heterochromatin and a euchromatic extended region (Givens and Phillips, 1976; Phillips, 1978). Givens and Phillips (1976) used rRNA/DNA saturation hybridization experiments on maize plants containing duplications of either the NOR-heterochromatin or NOR-euchromatin to determine that at least 90% of the rRNA genes are present in the NOR-heterochromatin and 10% (or less) of the rRNA genes are in the NOR-euchromatin. Other studies have investigated various NOR-interchange homozygotes by in situ hybridization of labelled rRNA to pachytene chromosomes (Phillips et al., 1983). These experiments also suggested that a majority of the rDNA (70%) was present in the NOR-heterochromatin with the remainder (30%) localized in the NOR-euchromatin. The authors of this study pointed out that due to the diffuse nature of the euchromatin vs. the compact nature of the
Figure 2. Schematic of an interphase nucleolus. This schematic shows the organization of the rDNA in the nucleolus into heterochromatic (inactive) and euchromatic (active) structures (adapted from Flavell, 1986).
heterochromatin, they are probably overestimating the amount in the euchromatic regions and underestimating the amount in the heterochromatic regions. Thus, all of these studies suggest that only a small fraction of the rRNA genes may be active (euchromatic) while the vast majority of the genes are in a tightly compacted (heterochromatic), inactive state. This differential packaging of the NOR is not related to length heterogeneity in the intergenic spacer. The ribosomal gene repeat units found in a single plant are homogeneous in inbred lines of maize (McMullen et al., 1986; Zimmer et al., 1988). In addition, almost all inbred lines of maize thus far characterized have a single repeat unit length of 9.1 kbp (Zimmer et al., 1988). Because of this length homogeneity, other molecular parameters which are associated with differential transcriptional activity have been examined in previous studies in an effort to understand the differential regulation of the rRNA multigene family in maize. These studies are discussed below.

**DNA methylation**

The predominant modified base found in eukaryotes is 5-methylcytosine (5-mC). It occurs as a minor base in the genomic DNA of almost all eukaryotes that have been studied (Doerfler, 1983). In plant genomes, 5-8% of the total base composition consists of 5-mC, but in animals 5-mC comprises 1% (or less) of the total base composition. In several grasses including maize approximately 30% of the cytosines are methylated in nuclear DNA (Bedbrook et al., 1978).

The role of 5-mC in eukaryotic chromosome structure has been examined for many years. Numerous earlier studies in plant and animal systems showed that highly repeated satellite DNA sequences have very high levels of 5-mC (Doerfler, 1983). For example, in the highly repeated
sequence known as the HS-β satellite DNA of the kangaroo rat each 10 bp repeat unit has been found to contain one 5-mC residue (Fry et al., 1973). A fluorescent antibody to 5-mC was used to show that the centromeric heterochromatic regions (which are known to be the location of the HS-β satellite) of the kangaroo rat chromosomes are enriched in 5-mC (Schreck et al., 1977). A similar situation has been described in the bluebell plant (*Scilla siberica*) for a 34 bp satellite DNA which has more 5-mC than cytosine (Deumling, 1981). In this satellite DNA approximately 25% of the total bases are 5-mC. *In situ* hybridization of the satellite DNA to metaphase chromosomes showed that these sequences were localized in heterochromatic regions of the chromosomes. Numerous other studies on base composition of highly repeated sequences in plants and animals have also found high levels of 5-mC in these sequences (Shmookler-Reis et al., 1981; Ehrlich et al., 1982; Gama-Sosa et al., 1983; Grisvard, 1985). These studies indicate that high levels of 5-mC are found in the nontranscribed heterochromatic regions of chromosomes.

The study of 5-mC distribution in eukaryotic DNA was greatly facilitated by the discovery of the isoschizomeric restriction enzyme pair, *HpaII* and *MspI*. Both of these restriction enzymes recognize the sequence 5′-CCGG-3′, but differ in their ability to cleave a methylated sequence when a 5-mC residue occurs at the internal cytosine (i.e., 5-mCpG) *HpaII* cleavage is inhibited by 5mC at this position but *MspI* cleavage is not affected by methylation at this sequence (McClelland and Nelson, 1985). Conversely, *MspI* cleavage is inhibited by methylation of the external cytosine (5mCpC), but *HpaII* cleavage is not. These enzymes have proven to be a powerful tool for studying 5-mC in specific genes because in eukaryotic DNAs a purine (usually guanine) is present on the
3' side of the majority (>95%) of the 5-mC residues (Gruenbaum *et al*., 1978; Doerfler, 1983). Several studies have indicated that the distribution of 5-mC residues in the 5'-CCGG-3' sequence reflect the overall level of methylation in a specific region (Bird, 1980).

Initially, tissue specific methylation levels of highly expressed vertebrate genes were analyzed with Southern blots of *HpaII* or *MspI* digested genomic DNA. Undermethylation of certain regions in the flanking sequence was found to be associated with active transcription of numerous genes, including the rabbit β-globin gene (Waalwijk and Flavell, 1978), the chicken ovalbumin gene (Mandel and Chambon, 1979) and the chicken β-globin gene (McGhee and Ginder, 1979). In contrast, in tissues where these genes were not expressed, these flanking sequences were fully methylated. At this point, studies on a substantial number of single copy genes in vertebrates have shown that, with very few exceptions, specific undermethylation of 5' flanking sequences is correlated with active transcription (Doerfler, 1983). Whether or not a similar situation occurs in plants is not known, as only two low copy number genes, those coding for the storage protein, zein and for alcohol dehydrogenase, have been studied. The zein genes were undermethylated in tissues in which they were active (Spena *et al*., 1983; Bianchi and Viotti, 1988) but no differences in methylation of alcohol dehydrogenase genes was found in inactive vs. active tissues (Nick *et al*., 1986).

The majority of the studies on plant gene methylation have concentrated on the rRNA genes (Flavell *et al*., 1983; Flavell *et al*., 1986). As in vertebrate systems, the restriction endonucleases *HpaII* and *MspI* have proven to be a useful tool for studying plant rDNA methylation. Several studies have provided circumstantial evidence suggesting that
sequence specific rDNA methylation is involved in regulating transcriptional activity (Flavell et al., 1983; Phillips et al., 1985; Flavell et al., 1986; Phillips et al., 1988).

In the maize inbred line A188, DNA purified from seedling leaf tissue has a single HpaII cleavable region in approximately 10% of the rDNA arrays, while 90% of the arrays are fully methylated (Phillips et al., 1985). This unmethylated site maps to a position in the IGS near the previously determined transcriptional start site (McMullen et al., 1986; Toloczyki and Feix, 1986). Developmental changes in A188 rDNA methylation patterns were observed in endosperm tissues. rDNA from endosperm tissue harvested 10 days after pollination was identical to leaf tissue in methylation pattern; however endosperm tissue harvested 17 days after pollination displayed four bands when cleaved with HpaII (Phillips et al., 1985). These results indicated that additional regions of the rDNA were undermethylated in developing endosperm during the time of high synthetic activity (Phillips et al., 1983).

Developmental changes in rDNA methylation levels have also been reported in specific tissues of pea seedlings (Watson et al., 1987). The major HpaII cleavage site, located approximately 800 bp upstream of the 5' end of the mature 18S rRNA, was cleaved only in the short rDNA variant. Methylation levels at this site were low in young seedlings, but this site was heavily methylated in apical buds. When the senescent apical buds were allowed to develop under continuous white light, the rDNA methylation levels decreased.

The relationship between rDNA methylation and rDNA copy number was investigated in wheat (Chinese spring) aneuploids containing different doses of the chromosomes carrying the nucleolus organizer (Flavell et al.,
1983). As the gene number increased there was a nominal increase in the number of undermethylated genes as assayed by HpaII cleavage at a single region in the IGS. These results suggested that the repeat units added over a certain threshold level were not active and, therefore, were completely methylated. This study also examined rDNA methylation in wheat lines carrying the A. umbellulata nucleolar organizer chromosome. In these lines, the A. umbellulata NOR is dominant over wheat NORs. The wheat rDNA was completely methylated while the A. umbellulata rDNA contained a single unmethylated site in a fraction of the rDNA arrays.

Studies in other plants including rice (Olmedilla et al., 1984), Lilium (von Kalm et al., 1986), flax (Ellis et al., 1983; Blundy et al., 1987), spinach (Steele-Scott et al., 1984) and tobacco (Steele-Scott et al., 1984) have also shown that rDNA methylation is heterogeneous. In general, a large fraction (80-90%) of plant rDNA arrays are methylated at all available HpaII sites. A significant fraction of the rDNA (10-20%) is unmethylated at one or a few regions which are in the IGS near the transcriptional start site. All of these studies indirectly suggest that heavily methylated rDNA may be inactive while undermethylation in a specific region is associated with transcriptional activity. A major difficulty in thoroughly examining this situation lies in the nature of the rDNA arrays. They are highly repeated and highly homogeneous in sequence, making it difficult to distinguish the rDNA arrays which are transcribed from those which are not. Since essentially all cell types require ribosomes and rRNA, there are no tissues in which these genes can be found in a totally inactive state. A limited number of studies on animal and plant rDNA have taken advantage of unique model systems to
investigate rDNA undermethylation and transcription activity as assayed by the presence of DNaseI sensitive chromatin structures. These studies will be discussed in detail below.

**Chromatin structure and DNaseI sensitivity**

The structure of eukaryotic chromatin has been well characterized. Electron micrographs of chromatin identified a regular repeating subunit structure of nucleosomes as a beaded chromatin fiber (Felsenfeld, 1978; McGhee and Felsenfeld, 1980). Electron microscopy was also used to visualize the ultrastructure of transcriptionally active regions, particularly ribosomal RNA genes and hormonally induced genes. In general, these transcriptionally active regions have a relaxed or extended conformation in contrast to the nucleosome bound nontranscribed regions (Scheer, 1980; Pruitt and Grainger, 1981). This relaxed structure extends into both 5' and 3' flanking regions. These extended areas seem to represent functional chromatin domains that are essentially nucleosome free and are accessible to the transcriptional machinery.

Further evidence that actively transcribed genes have a chromatin structure different from bulk chromatin came from the finding that active or potentially active genes are more sensitive to digestion by various nucleases (Weisbrod, 1982). Digestion of bulk chromatin with micrococcal nucleases generates a repeating unit that corresponds to the length of DNA wound around the nucleosome. However, analysis of individual genes or specific chromatin fractions showed that micrococcal nuclease distinguishes between transcriptionally active and inactive regions of chromatin with the active regions being more accessible to digestion than bulk chromatin (Weisbrod, 1982).

DNaseI has proven to be the most useful enzyme for studying
chromatin structure. Initially, Weintraub and Groudine (1976) found that the DNA in chromatin of actively transcribed genes is more sensitive to DNaseI digestion than the DNA in inactive chromatin. Shortly thereafter several studies reported that specific transcribed sequences and nearby flanking regions were sensitive or hypersensitive to DNaseI (Wu et al., 1979a,b; Stalder et al., 1980). Specific DNaseI hypersensitive sites were identified within 1000-kbp of the 5'-ends of genes that are active or potentially active (Elgin, 1981; Elgin, 1982). The structure of these hypersensitive sites is not well understood, but local absence of nucleosomes and binding of specific proteins is associated with these structures (Elgin, 1984).

The chromatin structure of rRNA genes has been examined thoroughly in only a few animal and plant systems. The first study to suggest a relationship between DNA methylation and DNaseI sensitivity utilized mouse rRNA genes (Bird et al., 1981a). Both methylated and unmethylated fractions of rRNA genes were identified in liver and several other tissues of several strains of mice. The rRNA genes of the mouse strain Balb/c have three different IGS lengths, designated α, β, γ. The α and β containing repeat units (which are longer than γ) are unmethylated at one or more sites while the γ repeat units are completely methylated. In Balb/c liver nuclei the α and β repeat units are hypersensitive to DNaseI digestion, while the γ repeat units are relatively resistant to DNaseI. Although this study did not offer direct proof that the unmethylated and DNaseI sensitive arrays were transcribed, it did establish a correlation between these parameters.

The chromatin structure and methylation of rRNA genes in hybrids between X. laevis and X. borealis has been studied thoroughly (Macleod
and Bird, 1982). As discussed previously, in these hybrids 97-98% of the rRNA precursor is synthesized from the *X. laevis* genes. In purified hybrid nuclei the preferentially transcribed *X. laevis* rRNA genes were hypersensitive to DNaseI compared to *X. borealis* rRNA genes. However, in this case the DNaseI hypersensitivity and transcription were not related to DNA methylation, as both gene sets had identical methylation patterns.

One study in plant systems has correlated DNaseI sensitivity and undermethylation with rRNA gene activity. The plant system utilized was a Chinese Spring wheat cultivar containing the dominant, NOR-bearing *A. umbellulata* chromosome (Martini *et al.*, 1982). As discussed previously, rRNA genes of *A. umbellulata* have a longer IGS and are undermethylated. In isolated nuclei, rRNA genes of *A. umbellulata* are also more susceptible to DNaseI digestion than the wheat genes (Flavell *et al.*, 1986). Thus, the rRNA genes contained in the active nucleolus are undermethylated and DNaseI sensitive, in contrast to those genes in the suppressed nucleolus which are completely methylated and resistant to DNaseI. The positions of DNaseI hypersensitive sites were mapped to the IGS of the rDNA. Preferential cleavage sites were located in the spacer subrepeats as well as the putative promoter site.

The only other study available on plant rDNA chromatin structure examined pea nuclei for light regulated changes in DNaseI hypersensitive sites (Kaufman *et al.*, 1987). The rDNA was found to have several DNaseI hypersensitive sites in both the coding and noncoding regions. The number and location of the sites were different in light grown vs. etiolated tissues. The DNaseI hypersensitive sites found within the coding regions were observed only in the dark grown seedlings and there were
two hypersensitive sites in the IGS that occurred in a short length variant only in the light. A set of constitutive DNaseI hypersensitive sites were present in the IGS of both light and dark grown seedlings and in both rDNA length variants. These sites were spaced regularly over the subrepeats and in the promoter region analogous to the regions of sensitivity observed in wheat (Flavell et al., 1986).

Research Objectives

The objective of the current research was to investigate rRNA gene methylation, chromatin structure and expression in maize. The research was initiated by characterizing the patterns of variation in rDNA methylation in maize and teosintes. These studies led to the observation that an EcoRI polymorphism, present in the 26S gene of certain maize inbred lines, could be utilized to correlate rDNA undermethylation, DNaseI sensitivity and expression. The specific questions addressed in this study and the rationale for each are listed below.

1. What is the pattern of rDNA array methylation in maize and teosintes? Although one maize line had been previously characterized for rDNA methylation (Phillips et al., 1985), only variation in rDNA restriction enzyme cleavage sites for six base pair-specific restriction endonucleases had been documented previously among inbred lines of maize (Rivin et al., 1983; Zimmer et al., 1988). In the present study, the extent of variation in rDNA methylation was documented for eleven inbred lines, one hybrid, and three species of teosinte.

2. Where do the undermethylated sites map in the rDNA array? The location of the conserved and variable sites of undermethylation were determined by restriction site mapping.

3. How are undermethylated rDNA arrays organized in the genome?
Double digest experiments with \textit{HpaII} and other restriction enzymes that produce polymorphic rDNA cleavage patterns were used to examine the organization of undermethylated rDNA.

4. What is the chromatin structure of methylated and undermethylated rDNA? During the course of restriction enzyme mapping experiments it was observed that an \textit{EcoRI} polymorphism could be used as an independent marker for undermethylated rDNA. This facilitated the design of experiments to determine the relative DNaseI sensitivity of the undermethylated and completely methylated rDNA in a maize hybrid.

5. Where do DNaseI hypersensitivity sites map in the rDNA? Two previous studies suggested that plant rDNA chromatin has DNaseI hypersensitive sites (Flavell \textit{et al.}, 1986; Kaufman \textit{et al.}, 1987). The location and relative sensitivity of DNaseI hypersensitive sites across the rDNA were determined using indirect end-label experiments.

6. Do rDNA transcripts originate from the undermethylated and DNaseI sensitive arrays? Direct sequencing of rRNA transcripts with reverse transcriptase was used to determine the sequence of this region. The nature of the sequence in this region allowed the design of oligonucleotide probes specific for transcripts from different inbred lines. These probes were used to examine the level of transcripts in maize parents and in hybrids.
MATERIALS AND METHODS

Plant Materials

Inbred lines of *Z. mays* were obtained from various sources. B37N, B73, Mo17 and the hybrid SX19 (B73xMo17) were from Pioneer Hi-Bred International. Additional hybrids of B73 x Mo17 and the reciprocal Mo17 x B73 were produced at Louisiana State University. An independent sample of B37N originally from K. Newton (University of Missouri) and maintained at Louisiana State University was also used in this study. Tx601, Ny302 and K10 were from J.D. Smith (Texas A&M University) and Tx303 was from A.J. Bockholt (Texas A&M University). Ohio Yellow Popcorn (OYP), Black Mexican Sweet (BMS), Wilbur’s Knobless Flint (WKF) and Gaspe Flint (GF) were from the Maize Genetics Cooperation Stock Center (Champaign-Urbana, Illinois). The teosintes *Z. luxurians* (G-42) and *Z. diploperennis* (1190) were provided by J. Doebley (University of Minnesota). *Z. perennis* was from D.H. Timothy (North Carolina State University).

DNA Purification

High molecular weight DNA was purified from light-grown seedling or mature plant leaves by ultracentrifugation through cesium chloride as previously described in detail (Rivin *et al.*, 1982). DNA was isolated from individual 10-day old seedlings as previously described by Zimmer and Newton (1982).

RNA Isolation

Total RNA was purified from pooled seedling leaves (5 grams) using minor modifications of previously published procedures (Hall *et al.*, 1978) as detailed by Hamby *et al.* (1988). This procedure was further modified
to purify RNA from etiolated individual 10-day old seedlings (average weight, 0.5 grams). Seedling tissue was harvested, frozen in liquid N₂, and ground to a fine powder in a pre-chilled mortar and pestle. The powdered tissue was added to 10 volumes (5 ml) of extraction buffer (0.2M Na borate, pH=9.0, 30 mM ethylene diamine tetraacetate: 2H₂O (EDTA), 5 mM dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS)) that had been heated to 100°C. The mixture, in a 15ml plastic tube, was ground with a Polytron (Brinkman) homogenizer twice, in 30 second pulses, and then filtered through one layer of miracloth. Proteinase K (100ul of 10mg/ml solution) was added, and the extract was incubated at 37°C for 1 hour. Following the incubation, 300ul of 2M KCl was added. To precipitate the SDS and proteins, the extract was chilled on ice for 10 minutes at 4°C and centrifuged at 10,000 rpm in an HB-4 rotor (Sorvall). The supernatant, containing the RNA, was decanted into a fresh tube, and 10M LiCl (300ul) was added. This mixture was stored overnight at -80°C and thawed slowly at 4°C. The RNA was pelleted by centrifugation (9,000 rpm, 15 min. in the HB-4 rotor), resuspended in 0.6 ml of 2M LiCl, and centrifuged again at 9,000 rpm as described above. This pellet was resuspended in 0.6 ml of 2M potassium acetate, pH=5.5. The RNA was precipitated by adding 2.5 volumes of cold 95% ethanol. After storage overnight at -20°C, the RNA was pelleted by centrifugation (8,500 rpm, 10 min. in HB-4), redissolved in 0.6 ml of STE (10mM Tris-HCl, pH=7.5, 10mM NaCl and 1mM EDTA), and reprecipitated overnight with ethanol as described above. The RNA was pelleted again by centrifugation (8,500 rpm, 10 min in HB-4) and this final pellet was resuspended in 500ul of TE (1mM Tris, pH=7.5, 0.1mM EDTA). The concentration of the RNA was determined by diluting an aliquot of the sample 1:50 and measuring the
absorbance at 260, 280, and 330nm in a spectrophotometer. The yield was calculated by the formula: \[ \text{Yield} = (A_{260} - A_{330}) \times \text{dilution factor} \times 40 \text{ ug/ml}. \] The calculated concentrations were confirmed by analyzing dilutions of the RNA on 0.8% agarose minigels along with standards of known concentrations. Routinely, 100-200 ug of total RNA was obtained from a single seedling.

**Purification of nuclei**

Seedlings for nuclei preparation were germinated in large trays, sandwiched between saturated sterile paper towels. Trays were stacked to allow ventilation and covered with a black cloth. After 10-14 days the dark-grown seedling leaves (3-6cm in length) were harvested for immediate purification of nuclei. The technique used was a modification of previously published procedures (Wurtzel *et al.*, 1987). All manipulations were carried out on ice using prechilled glassware and sterile solutions. All centrifugation steps were at 4°C. Freshly harvested seedling tissue (100-300g) was flash frozen in liquid nitrogen and ground to a powder with two 30-second bursts in a blender. The powdered tissue was added to 10 volumes of homogenization buffer (10mM PIPES, pH=7, 10mM NaCl, 10mM MgCl₂, 1M hexylene glycol, 20% glycerol, 250 mM sucrose, 5mM 2-mercaptoethanol). After the slurry was stirred for 5 minutes with a plastic fork, it was homogenized using a Polytron (Brinkman) for 1 minute at a setting of 5. To remove fibrous material, the homogenate was gravity-filtered through 2 layers of Miracloth followed by a second gravity filtration through a 50uM nylon mesh. The filtrate was then centrifuged at 3000 rpm in a Sorvall GSA rotor (250 ml bottles) for 10 minutes at 4°C with the brake off. This centrifugation collects a crude nuclear pellet. These pellets were not firm, so it was
necessary to remove the supernatant by aspiration.

The pellets were resuspended in a wash buffer (10mM PIPES, pH=7, 10mM NaCl, 3mM MgCl$_2$, 0.5 hexylene glycol, 5mM 2-mercaptoethanol), and a 10 ml aliquot of this mixture was layered over 10 ml of a 30% Percoll solution (in wash buffer, volume/volume). Generally, the nuclei from 50g of starting tissue were resuspended in 20 ml of wash buffer and layered onto two 10 ml Percoll cushions. The nuclei were pelleted through the Percoll solution by centrifuging in a Sorvall HB-4 swinging bucket rotor at 1500 rpm for 30 minutes. The brake was also left off for this spin. The supernatant was carefully removed from the tube by aspiration. The pelleted nuclei were diluted in 10 ml of wash buffer and washed twice by centrifuging them through the wash buffer in a Sorvall SS-34 fixed angle rotor at 1500 rpm for 15 minutes. The supernatant was decanted off, and the final nuclear pellet was resuspended in 9ml of DNaseI digestion buffer (10 mM PIPES, pH=7.0, 10mM NaCl, 3mM MgCl$_2$, 5mM 2-mercaptoethanol, 250 mM sucrose).

**DNaseI digestions in intact nuclei**

To standardize the DNaseI digestions the approximate DNA concentration was estimated spectrophotometrically from an aliquot of sample that was lysed with 1% SDS and 5mM EDTA (Murray and Kennard, 1984). Digestions routinely contained 3-5 $A_{260}$ units/ml of total nucleic acid (corrected for scattering at 320nm). The actual DNA concentration, determined after DNA purification, was from 100-250 ug/ml. Generally, the nuclei from 300g of starting material were diluted into 9 ml of DNaseI digestion buffer and split into nine 1 ml aliquots. One of these aliquots served as an unincubated control and was lysed immediately. Another control aliquot was incubated in the absence of DNaseI. The
remaining seven aliquots were treated with pancreatic DNaseI (Worthington) at a range of enzyme concentrations (0.001 - 5 U/ml). All incubations were for 15 minutes at 15°C. Control experiments were performed to show that the DNaseI hypersensitive sites detected were chromatin specific. Purified DNA was digested with a series of DNaseI concentrations and analyzed with the same restriction enzymes and probes. Site specific digestion of rDNA was not observed in purified DNA. The DNaseI reactions were stopped by the addition of two volumes of a lysis buffer (7M urea, 0.35 M NaCl, 10mM Tris, pH=7.6, 1mM EDTA, 2% (w/v) sarkosyl). An equal volume of phenol: chloroform: isoamyl alcohol (3:1:0.5) was added and gently mixed by inversion. The upper aqueous layer, containing deproteinized DNA, was removed, and one volume of 95% ethanol was added. The DNA was precipitated overnight at -20°C, collected by centrifugation, and resuspended in TE (1mM Tris, pH=7.5, 0.1 mM EDTA).

Ribosomal Gene Probes

The cloned ribosomal gene fragments used in this study are shown in Figure 3. Initially, rDNA methylation patterns were characterized with pGmrl, a plasmid containing a single rDNA repeat unit cloned from soybean, Glycine max. A subclone of pGmrl, pXbrl, was utilized in mapping experiments to generate indirect end label maps from the conserved XbaI site located in the 18S coding region. Details of the construction of these clones and subclones are presented elsewhere (Zimmer et al., 1988). The soybean rDNA clones are highly similar to maize in their coding regions and have been used previously to detect Z. mays rDNA fragments (Rivin et al., 1983; Zimmer et al., 1988). In order to detect the IGS specific fragments, the maize rDNA clone pZmS1 was
Figure 3. Location of the probes and primers used in this study. The positions of the cloned rDNA probes (above the repeat unit) and oligonucleotides (below the repeat unit) used in this study are shown. The conserved and polymorphic restriction sites of importance in this study are also shown (X=\textit{XbaI}; Ev=\textit{EcoRV}; Er=\textit{EcoRI}; B=\textit{BamHI}). The asterick by the \textit{EcoRI} site indicates that cleavage does not occur in all repeat units (Zimmer \textit{et al.}, 1988).
utilized. It was obtained from M. McMullen (University of Minnesota) and its construction has been described in detail previously (McMullen et al., 1986). pZmS1 is a subclone which contains the complete IGS specific region from the exotic maize line Black Mexican Sweet (BMS). Identical results were obtained with the maize and soybean probes except in the case of fragments that contained primarily IGS specific sequences. These fragments were not detected or were detected only faintly with pGmr1 (see Zimmer et al., 1988).

Nick Translations

The plasmid DNAs containing cloned rDNA sequences were labeled with $\alpha^{32}$P-dCTP by standard nick translation procedures (Rigby et al., 1979) as follows: 1 ul of a 1 mg/ml DNaseI (Worthington) solution was placed in 19 ul of DNaseI activation buffer (10mM Tris, pH 7.5, 5mM MgCl₂, 1 mg/ml bovine serum albumin) and incubated on ice for 1 h, then 1 ul of the dilution was added to 1 ml of DNaseI activation buffer. The final nick translation reaction mixture contained 0.5 ug of plasmid DNA in a volume of 4 ul, 10 ul nick-translation buffer (20mM each of dATP, dGTP, dTTP, 40mM Tris, pH=7.5, 20mM MgCl₂, 0.1 mg/ml BSA), 0.5 ul of E. coli. DNA polymeraseI (New England Biolabs, 20 U/ul), 0.5 ul diluted, activated DNaseI and 6 ul of $\alpha^{32}$P-dCTP (ICN 650 mCi/mM). Reactions were incubated for 1 h at 15°C and stopped by adding 5 ul of 0.5M EDTA and 25 ul of a dye solution (1% lauryl sulfate, 40 mg/ml blue dextran, and 0.1 mg/ml bromophenol blue). The solution was chromatographed on a Sephadex G-50 column (in a pasteur pipette) to separate the labeled plasmid DNA from unincorporated nucleotides. The labeled DNA migrated with the blue dextran dye which was the first dye to elute from the column. The specific activity of the labeled DNA ranged from 0.5-
$2 \times 10^8$ cpm/ug of input DNA as determined by quantitation of radioactivity in a 2 ul aliquot of the column eluate.

**Oligonucleotides used as Probes and Primers**

Several oligonucleotides were utilized as probes and primers during various portions of this project. These oligonucleotides were synthesized by the College of Basic Sciences DNA facility with an Applied Biosystems Automated DNA synthesizer (Model 380A) using phosphoramidite chemistry. (Beaucage and Caruthers, 1981; Mattiucci and Caruthers, 1981). The oligonucleotides were desalted and purified by electrophoresis on 20% acrylamide/8M urea gels as previously detailed (Hamby et al., 1988). The oligonucleotide band was detected by UV shadowing, sliced from the gel and purified using a Sep-Pac (Waters Associates) cartridge (Hamby et al., 1988).

Table 1 describes the oligonucleotides used in this study and Figure 3 shows their location on the rDNA. The oligonucleotide designated 18B was used as a probe in indirect end label experiments on DNA purified from chromatin preparations. This oligonucleotide is complementary to a region of the 18S gene (Hamby et al., 1988) immediately adjacent to the XbaI site (see Figure 3). The oligonucleotide ER3 was utilized in the polymerase chain reactions as a primer for synthesis of a 247 bp fragment. This oligonucleotide was designed from the rice 26S sequence (Takaiwa et al., 1988) and spans positions 1681-1699 in rice. A primer designated 26E (Hamby et al., 1988) was used for synthesis of the other strand in the polymerase chain reaction. 26E spans positions 1911-1928 in the 26S gene of rice. This primer was also used to directly sequence rRNA from the same region. Two oligonucleotides ERI$^+$ and ERI$^-$, used as probes to detect specific RNAs were designed from RNA sequences.
Table 1. Oligonucleotides used as probes and primers

<table>
<thead>
<tr>
<th>Primer Designations</th>
<th>Sequencea</th>
<th>Region of Homologyb</th>
<th>Experimental Use</th>
<th>Td(°C)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>18B</td>
<td>TCCATGGCTTAATCTTT GAGACAAGCATATG</td>
<td>25-55</td>
<td>Indirect end-label</td>
<td>NA</td>
</tr>
<tr>
<td>ER3</td>
<td>CTGCTTAACGG CCCGCCAAC</td>
<td>1681-1699</td>
<td>Primer for PCR</td>
<td>71</td>
</tr>
<tr>
<td>26E</td>
<td>CTTATCCCAG AAGTTACG</td>
<td>1911-1928</td>
<td>Primer for PCR; Direct Sequencing</td>
<td>49</td>
</tr>
<tr>
<td>26F</td>
<td>CAGAGCACTGGG CAGAAATCAC</td>
<td>2172-2193</td>
<td>Control Probe for RNA blots</td>
<td>63</td>
</tr>
<tr>
<td>ERI+</td>
<td>TGGACGGAATT CGGTCCCTC</td>
<td>1798-1816</td>
<td>Specific Probe for RNA blots</td>
<td>55</td>
</tr>
<tr>
<td>ERI-</td>
<td>TGGACGGTATTC GGTCCTC</td>
<td>1798-1816</td>
<td>Specific Probe for RNA blots</td>
<td>55</td>
</tr>
</tbody>
</table>

aThe sequences are written (5’-3’) from left to right and continued on the line below.

bRefers to approximate region of homology on soybean or rice sequence (see Hamby et al., 1988).

cCalculated as described in Materials and Methods.
collected in this study using 26E. The oligonucleotide 26F was used as a control in these experiments because it is a 100% match with all of the samples examined in this study. The rationale for designing ERI+ and ERI− are described in detail in the Results.

**End Labeling of Oligonucleotides**

The oligonucleotides used as hybridization probes were end labeled with T4 polynucleotide kinase and γ-32P dATP by minor modifications of a previously described procedure (Berent et al., 1985). A solution of 100 ug of oligonucleotide in 30 ul of distilled H2O was heated for 5 minutes at 65°C and chilled on ice. Then 10 ul of 5X kinase buffer (0.25M Tris, pH=7.5, 0.05M MgCl2, 25mM DTT, 0.05mM spermidine, and 0.05mM EDTA), 8 ul of [γ-32P] ATP (ICN, 7000 mCi/mmole, 22.5mM) and 2 ul of T4 polynucleotide kinase (New England Biolabs, 20 U/ul) were added. This reaction was incubated for 1 h at 37°C and was stopped by adding 10 ul of 0.5M EDTA. The labeled oligonucleotide was separated from unincorporated nucleotide by Sephadex G-50 chromatography using the blue dextran dye solution markers as described above for nick translations. The incorporation was determined as described above for nick translations. Assuming 100% recovery of input oligonucleotide the specific activity of the end labeled preparations were routinely 1x10⁹ cpm/ug.

**Restriction Endonuclease Digestions**

Restriction endonuclease digestions were performed using the salt and temperature conditions recommended by the supplier. Restriction enzymes were obtained from the following suppliers: HpaII, EcoRI, XbaI (Bethesda Research Laboratories and Boehringer Mannheim); MspI, EcoRV
(New England Biolabs). For single digest experiments, one ug of each DNA sample was digested with 5-10 units of restriction endonuclease for approximately 6-15 h. Double digestion experiments were performed with two ug of DNA for 6-15 h with each enzyme. The order of double digests was determined by considering optimal salt conditions for the restriction enzymes used. HpaII digestion patterns remained unchanged in the presence of large excesses of enzyme. In addition, HpaII andMspI digests of plant genomic DNA were routinely spiked with two ug of lambda phage DNA (Bethesda Research Laboratories) to monitor completeness of digestion.

Southern blot hybridization

Restriction enzyme fragments were separated on 0.8% horizontal agarose gels. Southern transfers, hybridizations and washes were done as previously described (Jupe et al., 1988; Zimmer et al., 1988). Hybridized filters were exposed for 18-36 h to either XAR or XRP film (Kodak) at 80°C with Lightning Plus Intensifying screens (Dupont). Autoradiograms used for quantitation were produced using XRP film preflashed as previously described by Laskey and Mills (1978).

RNA Slot Blots and Hybridizations

RNAs were diluted from stock solutions with sterile DEPC-treated water and 3 volumes of 6.15M formaldehyde in 10X SSC (1X SSC is 0.15M NaCl plus 0.015M sodium citrate) were added to give a final concentration of 10-150 ug/ml (Berent et al., 1985). These RNA dilutions were heated to 65°C for 15 min and quick chilled on ice. This denatured stock was further diluted with 4.16M formaldehyde in 7.5X SSC such that the desired concentration of RNA could be applied to each slot in a total
volume of 0.2-0.4 ml.

The Zetabind (AMF) nylon membrane was prewet in water and then soaked in 10X SSC for 20 minutes. Slot blots were done using a BRL-Hybri-Slot Manifold. The samples were applied under a water vacuum. After the samples were blotted through, each well was washed with 0.4 ml of 10X SSC. The membrane was removed from the apparatus, air dried for 15 minutes and then baked in a vacuum oven at 80°C for 2 hours.

The filters were prehybridized a minimum of two hours in a solution containing 25mM potassium phosphate, pH=6.5, 1% sarkosyl, 5X SSC, 1X Denhardt's solution, and 200 ug/ml salmon sperm DNA. The initial prehybridization fluid was discarded and a fresh aliquot containing 1x10^6 cpm/ml of end-labeled oligonucleotide was added. Hybridizations were typically incubated overnight at 55°C in a shaking incubator. Filters were washed twice in 6X SSC, 0.1% SDS for 20 min. at room temperature. This was followed by two high stringency washes in the same solution at 60°C. The temperatures at which hybridization reactions and washes were performed were estimated by using a formula to calculate the approximate dissociation temperature for the oligonucleotides (Suggs et al., 1981). This formula is $T_D$ (in °C) = $[2 \times (A+T)] + [4 \times (G+C)]$. The temperature of hybridization is 5°C below the $T_D$. This formula was accurate for all of the oligonucleotides used in this study. Following the washes, the filters were exposed to preflashed X-ray film as previously described (Laskey and Mills, 1978).

Polymerase Chain Reaction

Specific regions of maize genomic DNA were amplified with TaqI polymerase (Perkin Elmer-Cetus) by performing the polymerase chain reaction (Saiki et al., 1985) in an automated DNA thermal cycler (Perkin
Elmer-Cetus). Reactions were performed as described by the enzyme supplier, in 0.5 ml microfuge tubes in a final volume of 100 ul. The reaction components added included 10 ul of a 10X reaction buffer (500mM KCl, 10mM Tris, pH=8.3, 15mM MgCl$_2$, 0.1% (w/v) gelatin), 16ul of dNTP mix (1.25mM in each dNTP), 10ul of each of two oligonucleotide primers (10mM), 1-2ul of template DNA (200-500 ug/ml) and 0.5 ul of Taq polymerase (50 U/ul). The Taq polymerase was vortexed and spun down briefly in a 4°C tabletop centrifuge before pipetting. The samples were overlayed with 100 ul of sterile mineral oil to prevent evaporation.

The thermal block was programmed for an initial template denaturation step of 94°C for 1 minute and 30 seconds. Immediately following this, the temperature was lowered to facilitate annealing of the primers. The temperature of annealing was calculated by using $T_H = [4 \times (G + C)] + [2 \times (A + T)] -5°C$ (Suggs et al., 1981). Primers were annealed for 2 min. at the lowest $T_H$ for the two primers. The next step in the cycle was the extension at 72°C for 1 minute/150bp (of DNA to be synthesized). The next denaturation step, at 94°C for 1 minute, starts the cycle over again. This series of denaturation, annealing, and extension steps were repeated for a total of 25 cycles. At the end of the 25 cycles the heat denaturation step was omitted, and the extension step extended for an additional 7 minutes. The completed reactions were extracted once with an equal volume of chloroform. The DNA samples were diluted to a volume of 2 ml with TE (10mM Tris, pH=8.0, 1 mM EDTA). A 10 ul aliquot of the reaction mixture was checked by agarose gel electrophoresis to determine the success of the reaction. Successfully amplified DNA samples were further purified on Centricon 30 cartridges (Amicon). The Centricon 30 cartridge was washed by applying 2 ml of TE
and centrifuging it in an SS34 rotor at 4800xg (6400 rpm) for 10 minutes at 4°C. The 2 ml of DNA solution was then applied to the cartridge and centrifuged as above for 15 minutes. The solution collected in the reservoir was discarded. The purified DNA sample was collected by inverting the cartridge and centrifuging at 200xg (2500 rpm) for 2 minutes. The final volume of the DNA sample was approximately 100 ul. The yield of DNA from a single amplification was in the range of 7-10 ug of the specific fragment synthesized.

Direct Sequencing of rRNA

The rRNA was sequenced directly by primer extension with reverse transcriptase using dideoxynucleotide chain termination. The detailed procedure used for these reactions has been published recently (Hamby et al., 1988) and will be described here briefly. RNA (6 ug in 6 ul) was denatured at 90°C for 5 minutes and chilled briefly on ice. Two pmoles of the primer were added to the RNA along with 1ul of 20X reverse transcriptase buffer (400mM Tris, pH=8.3, 150mM MgCl2, 150mM KCl, 40mM DTT) and incubated at 42°C for 30 minutes to allow the primer to anneal to the RNA. The RNA/primer solution was distributed to four separate tubes in 2 ul aliquots. Then 1 ul of a different dideoxynucleotide stock (individual stocks were 0.8mM ddGTP, 1.5mM ddATP, 2.0mM ddTTP and 0.024mM ddCTP) was added to each tube. The extension reactions were started by adding 2 ul of a solution containing dATP (1.5mM), dGTP (1.5mM), TTP (1.5mM), dCTP (0.12mM), α32P labeled dCTP (650 mCi/mmole specific activity, 5uM) and 2 U/ul of reverse transcriptase (Life Sciences). The reactions were incubated at 42°C for 10 minutes, and at 50°C for 10 minutes. Then, 0.8 ul of a chase mix containing 2.5mM dGTP, 5mM dATP, 5mM TTP, 10mM dCTP and 3.5 U/ul
of reverse transcriptase was added. The reactions were incubated again for 10 minutes at 50°C followed by a 10 minute incubation at 60°C. These steps completed the primer extension reaction with reverse transcriptase. When sequencing reactions terminated at this point are analyzed on acrylamide gels, greater than 95% of the sequence can be read. However, the sequence in the particular region of interest was obscured by bands present across all lanes making it unreadable. In order to collect sequencing data in this region, it was necessary to resolve the sequencing ambiguities by adding a terminal deoxynucleotidyl transferase (TdT) reaction following the completion of the extension reactions (DeBorde et al., 1986). This reaction was carried out by immediately adding 1 ul of a mixture of dATP, dCTP, dGTP, and TTP (each at 1mM) and 10 U of TdT (BRL) to each reaction tube. The reactions were incubated an additional 30 minutes at 37°C. The reactions were terminated by adding 4 ul of a formamide dye solution (80% v/v deionized formamide, 5% v/v 10X TBE, 1mg/ml xylene cyanol blue, 1mg/ml bromophenol blue) to each tube. The sequencing gels were 9% w/v acrylamide, 8M urea, in 1X TBE. Gels were run at 1800 volts for 3-5 hours. Following electrophoresis the gels were transferred to Whatman 3MM paper, dried in a vacuum gel dryer (3 hours at 80°C) and autoradiographed at room temperature with Kodak XRP film.

Data Analysis

DNA fragment sizes were determined using a nonlinear regression analysis computer program. Standard markers used were phage lambda DNA that had been digested with HindIII and EcoRI. Maize genomic DNA digested with BamHI was also used. This maize digest produces fragments band of 9.1, 5.2, and 3.9 kb on autoradiograms probed with pGmr1.
Preflashed autoradiograms were scanned using a Biorad Model 620 Video densitometer equipped with integration programs. The areas of the peaks were used for quantitative calculations. Densitometry was used to collect quantitative data in three types of experiments in this study as follows: (1) The percentage of the rDNA not susceptible to HpaII digestion was determined by comparing the signal in single digests with EcoRI to that remaining in the HpaII/EcoRI double digests. (2) The relative sensitivity to DNaseI across the rDNA repeat unit was quantitated by comparing the peak areas and peak heights of specific fragments produced by DNaseI digestion. (3) The percentage of rRNA hybridizing to a specific oligonucleotide probe was determined. This was accomplished by determining the areas under the peaks for the specific probes and a control probe which gives a 100% signal (see Results).
RESULTS

Patterns of rDNA methylation in maize and teosinte

Maize and teosinte DNAs were screened with *HpaII* and *MspI* to characterize methylation of genomic DNA and rDNA. Figure 4A shows representative results of a survey of DNA samples (spiked with lambda DNA as a control) digested with either *HpaII* or *MspI*, separated on a 0.8% agarose gel, and stained with ethidium bromide to visualize genomic DNA fragments. Identical lambda DNA patterns (fragments within bracket) were obtained with *HpaII* and *MspI*, indicating that there was no inhibition of *HpaII* activity in the digests. Incubation with *HpaII* (Figure 4A, lanes 1, 3) does not cleave the genomic DNA to any extent as evidenced by the lack of fragments below 20 kbp. In contrast, *MspI* digestion (Figure 4A, lanes 2, 4) produces an even distribution of DNA fragments with significantly fewer high molecular weight fragments relative to the *HpaII* digests. These results show that there are numerous CCGG sites in the genome and that the majority of these sites in genomic DNA are methylated at the sequence CpG (not accessible to cleavage by *HpaII*) while the CpC sequence is undermethylated (accessible to cleavage by *MspI*). Variation in the extent of *MspI* digestion of genomic DNA (Figure 4A, lane 4) was found in some of the maize and teosinte samples surveyed. This indicates that there is variation in the levels of CpC methylation in *Zea* genomic DNA.

Figure 4B shows the results obtained when the fragments from the gel in Figure 4A were transferred to a filter and probed with ³²P labeled pGmr1, an rDNA clone homologous to the maize coding region (Figure 3). The majority of the rDNA is resistant to *HpaII* cleavage (Figure 4B, lanes 1, 3) and remains in a high molecular weight fraction
Figure 4. Survey of *Z. mays* nuclear and rDNA with *HpaII* and *MspI*. Nuclear DNA purified from leaf tissue (spiked with λ DNA as a control) was digested with either *HpaII* (lanes 1, 3) or *MspI* (lanes 2, 4). Restriction fragments were separated by electrophoresis through 0.8% agarose gels. DNA samples shown are from the maize inbred lines B37 (lanes 1, 2) and B73 (lanes 3, 4). Panel A shows the ethidium bromide stained agarose gel photographed under UV (330 nm) light. The fragments enclosed in the brackets are characteristic for *HpaII* or *MspI* complete digests of the λ DNA included as an internal control. Fragment sizes (kbp) of a λ DNA marker double digest with *HindIII*/EcoRI are indicated at the left. In panel B, the fragments were transferred to a nylon membrane by Southern blotting and hybridized with 32P-labeled pGmr1, a cloned probe specific for the rDNA coding region. Fragment patterns were detected autoradiographically after overnight exposure. The lengths of rDNA standard marker fragments (kbp) are shown at the left of panel B.
that is greater than 20 kbp in size. However, it is evident from the discrete fragments produced that a significant fraction of the rDNA is sensitive to cleavage by \textit{HpaII} at one or more sites in the repeat unit. \textit{MspI} cleavage (Figure 4B, lanes 2, 4) produces a smear of low molecular weight rDNA fragments indicative of multiple CCGG cleavage sites within the rDNA. This pattern of digestion by \textit{MspI} was characteristic of all of the rDNA samples examined in this study.

The \textit{HpaII} fragment patterns obtained for the rDNA varied among the \textit{Zea} species examined in this study. Representative digestion patterns for maize and teosinte rDNAs screened with \textit{HpaII} and pGmr1 are shown in Figure 4B and Figure 5. Single digestion of rDNAs from inbred lines of maize with \textit{HpaII} produced either one, two, or three discrete fragments (Figure 4B; Figure 5A). The three species of teosinte (\textit{Z. diploperennis}, \textit{Z. luxurians} and \textit{Z. perennis}) surveyed had a single fragment present (Figure 5B). When a single fragment was produced in maize or teosintes, it was always of repeat unit length (Figure 4B, lane 3; Figure 5A, lanes 3, 4; Figure 5B, lanes 1-3). A summary of the \textit{HpaII} fragment patterns observed in our survey are presented in Table 2. \textit{HpaII} digestion produced a single fragment of repeat unit length from the fraction of accessible rDNA arrays in half of the inbred lines (Table 2). In the other inbred lines, two or three distinct fragments were present. In these cases, one fragment was of repeat unit length and additional fragments were lower in molecular weight. A fragment of 6.9 kbp is clearly evident in the rDNA samples with two fragments (Figure 5, lanes 1, 2). A three fragment pattern was unique to the inbred line B37. In addition to the 9.1 and 6.9 kbp fragments observed in other inbred lines, an 8.0 kbp fragment is present in B37N (Figure 4B, lane 1). Thus, inbred
Figure 5. Patterns of maize and teosinte rDNA methylation surveyed with HpaII. Nuclear DNA purified from leaves was digested extensively with HpaII, electrophoresed on 0.8% agarose gels, transferred to nylon membranes and probed with $^{32}$P-labeled pGmr1. Samples loaded in lanes are as follows: Panel A, inbred lines of maize (1) Tx303, (2) OHYP, (3) GFC, (4) Mo17; Panel B, species of teosintes (1) Z. diploperennis, (2) Z. luxurians, (3) Z. perennis. The lengths of rDNA fragments (kbp) are indicated on the left and right of the figure.
Table 2. Summary of maize and teosinte rDNA methylation patterns and undermethylated sites

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of fragments present</th>
<th>Size of fragments (kbp)</th>
<th>Location of unmethylated sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>B37N</td>
<td>3</td>
<td>9.1, 8.0, 6.9</td>
<td>A, A+B, A+C</td>
</tr>
<tr>
<td>BMS</td>
<td>2</td>
<td>9.1, 6.9</td>
<td>A, A+B</td>
</tr>
<tr>
<td>K10</td>
<td>2</td>
<td>9.1, 6.9</td>
<td>A, A+B</td>
</tr>
<tr>
<td>OHYP</td>
<td>2</td>
<td>9.1, 6.9</td>
<td>A, A+B</td>
</tr>
<tr>
<td>Tx303</td>
<td>2</td>
<td>9.1, 6.9</td>
<td>A, A+B</td>
</tr>
<tr>
<td>WKF</td>
<td>2</td>
<td>9.1, 6.9</td>
<td>A, A+B</td>
</tr>
<tr>
<td>B73</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>GF</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>Mo17</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>Ny302</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>Tx601</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>Sx19 (B73 x Mo17)</td>
<td>1</td>
<td>9.1</td>
<td>A</td>
</tr>
<tr>
<td>Z. luxurians</td>
<td>1</td>
<td>9.3</td>
<td>A</td>
</tr>
<tr>
<td>Z. diploperennis</td>
<td>1</td>
<td>9.6</td>
<td>A</td>
</tr>
<tr>
<td>Z. perennis</td>
<td>1</td>
<td>9.5</td>
<td>A</td>
</tr>
</tbody>
</table>

*a*Fragments observed in *Hpa*I single digests probed with pGmr1.

*b*See Figure 7 for location of *Hpa*I-sensitive sites in the IGS region.
lines of maize are heterogeneous with respect to the number and position of undermethylated sites. This heterogeneity was further examined by mapping the position of the undermethylated sites in these maize and teosinte.

**Location of HpaII-sensitive regions in maize and teosinte rDNA repeat units**

Initially, the positions of the \textit{HpaII}-sensitive sites in the rDNA repeat unit were determined by mapping them relative to the conserved \textit{XbaI} site located at the 5' end of the 18S gene (Figure 3). Southern blots of \textit{HpaII}/\textit{XbaI} double digests were probed with the subclone \textit{pXbr1} (Figure 3) to generate indirectly end-labeled (Wu, 1980) fragments, the length of which reflect the distance of the \textit{HpaII}-sensitive sites from the \textit{XbaI} site. Figure 6A shows examples of these mapping experiments with three samples having different \textit{HpaII} single digestion patterns. In all cases, digestion with \textit{XbaI} alone cleaves the rDNA into a single fragment of repeat unit length (Figure 6A, lanes 1, 3, 5). In \textit{HpaII}/\textit{XbaI} double digests, fragments produced from the undermethylated fraction of repeat units are clearly evident. \textit{HpaII}/\textit{XbaI} double digestion of \textit{Z. diploperennis} produced a single fragment of 8.5 kb in length (Figure 6A, lane 2). In the inbred line \textit{Tx303} (Figure 6A, lane 4) fragments of approximately 8.0 and 6.0 kbp are evident. \textit{B37N} (Figure 6A, lane 6) shares the 8.0 and 6.0 kbp fragments with \textit{Tx303}, but an additional 7.2 kbp fragment is also present. The lengths of these fragments indicate the distance of the respective \textit{HpaII}-sensitive sites from the \textit{XbaI} site. It should be noted that since the \textit{Z. diploperennis} repeat unit is 0.5 kb larger than the repeat unit of inbred lines (Figure 6A), the 8.5 kb fragment maps the \textit{HpaII}-sensitive site to the same approximate location as the 8.0 kb
fragment observed in inbred lines of maize.

The fragment lengths observed in the \textit{HpaII/XbaI} digests indicated that all of the \textit{HpaII}-sensitive sites detected occur in the IGS region of the rDNA repeat unit. This conclusion was further substantiated when these blots were subsequently analyzed with pGmr1 which is also only able to detect maize coding region rDNA. These probes detected the same fragments as pXbr1, thus indicating that there were no detectable \textit{HpaII}-sensitive sites in the coding region and that the \textit{HpaII}-sensitive sites were confined to the IGS.

The experiments described above detected fragments extending 3' to the \textit{XbaI} site. In order to confirm the location and number of \textit{HpaII}-sensitive sites in the IGS, \textit{HpaII/EcoRV} double digests probed with pZmS1 (Figure 3) were used. As with \textit{XbaI}, \textit{EcoRV} cuts at a single conserved site in the rDNA repeat unit, producing a single fragment of repeat unit length. In \textit{Z. diploperennis} fragments of 7.0 (not clear due to overexposure) and 2.5 kb are found (Figure 6B, lane 1). The 7.0 kb fragment extends from the \textit{EcoRV} site through the 26S gene and the IGS to the A (Figure 7) site while the 2.5 kb fragment extends from the \textit{EcoRV} site through the 18S gene to the A site in the IGS. These two fragments are observed in all in teosintes and in inbred lines of maize in which only the A site is undermethylated. However, the large fragment varies in size from 6.2 - 6.7 kb depending on repeat unit length (Figure 6B, lane 4). In Tx303 (Figure 6B, lane 2) and B37N (Figure 6B, lanes 3, 4), as expected from the \textit{XbaI} digests, more than two fragments were obtained. In Tx 303, a 1.9 kbp fragment that is IGS specific is found. This fragment spans the IGS A and B sites. Additional fragments that would indicate cleavage between these sites are not seen. In the B37N
Figure 6. Mapping of undermethylated HpaII sites in maize and teosinte rDNA. In panel A, samples with different HpaII patterns were digested with either XbaI (lanes 1, 3, 5) or HpaII/XbaI (lanes 2, 4, 6). In panel B, HpaII/EcoRV (lanes 1, 2, 3, 4) double digests are shown. XbaI single digests (lanes 1, 3, 5) as well as EcoRV single digests (not shown) produce a single repeat unit length band. HpaII/XbaI digests (lanes 2, 4, 6) were probed with pXBrl to indirectly end label the fragments. The HpaII/EcoRV double digests (lanes 1-3) were probed with pZmS1. The B37N double digest in lane 4 was probed with pGmr1. The autoradiogram of the EcoRV experiment is overexposed to clearly show the smaller IGS specific fragments. The sizes of fragments (in kbp) are designated at the right of the figures. DNA samples shown in panel A are Z. diploperennis (lanes 1, 2), Tx303 (lanes 3, 4), and B37N (lanes 5, 6). In panel B, the samples loaded are Z. diploperennis (lane 1), Tx303 (lane 2), B37N (lanes 3, 4).
Figure 7. Location of undermethylated regions in the IGS of maize and teosinte rDNA. The map depicts the major structural features of the maize rDNA repeat unit. The positions of the conserved restriction enzyme cleavage sites used in this study to map undermethylated regions are shown (X=|Xba|1; Ev=|Eco|V; Er=|Eco|R). The |Eco|R site marked with a star is not susceptible to cleavage in all arrays. The expansion at the bottom of this figure details the features of the undermethylated regions in the IGS. The Hp|II sites are marked as Hp A, B, or C based on their frequency of occurrence. The solid circles show the location of potential Hp|II cleavage sites found in the sequence of BMS (McMullen et al., 1986). The asterisks mark the location of the transcriptional start sites (McMullen et al., 1986; Toloczyki and Feix, 1986). The open boxes show the position of the subrepeated region. The location of the rDNA specific cloned probes used in this study are shown at the top.
arrays which are cleaved at the A, B and C sites, a population of IGS specific fragments in the 2.0 - 1.7 kb range are produced. In B37 additional fragments are also present. The 5.4 kb fragment (Figure 6, lane 4) results from cleavage at the C site in a subset of rDNA repeats which are also cleaved at the A site to produce a 1.1 kb fragment. There is also a 1.0 kb fragment present which may result from cleavage at the A, B, and C sites in a fraction of the arrays, but it may also be due to an IGS length variant. The exact nature of the additional fragments observed in B37 is difficult to determine. They may result from length variants in the IGS region. The IGS specific fragments which are produced by HpaII cleavage are from the region known to vary in length in Zea and Tripsacum (McMullen et al., 1986; Zimmer et al., 1988). B37N is know to contain two IGS length variants which could account for this variation (Zimmer et al., 1988).

Overall, it is clear from these mapping experiments that the A region is HpaII-sensitive in those maize and teosinte rDNAs which produce only a 9.1 kbp fragment in HpaII single digests (Table 2). Those inbred lines with two fragments present in HpaII single digests contain a population of repeat units with the A region undermethylated and a separate population of repeat units with both the A and B regions undermethylated. About one-half of the inbred lines surveyed (five of eleven) have a portion of their repeat units with the additional HpaII-sensitive site in the B region while most other inbred lines and all of the teosintes have only the A region undermethylated (Table 2). Results with the inbred line B37N indicate that there were three distinct populations of repeat units present. One had only the A site, one had both the A and B sites, and the other had both the A and C sites.
We can compare our mapping results with the sequence for the IGS of BMS published by McMullen et al. (1986), in which seventeen CCGG sites are present. The positions of these sites are shown by the solid circles in Figure 7. The most frequently undermethylated region identified, region A, maps to at a position with a cluster of six HpaII (5'-CCGG-3') sites. This region is also within the boundaries of the transcriptional start sites represented by the asterisks in Figure 7 (McMullen et al., 1986; Toloczyki and Feix, 1986). Undermethylation in this region also has been observed in the leaf rDNA of the inbred A188 (Phillips et al., 1985). Undermethylation in the B region corresponds to a cluster of CCGG sites near the beginning of the IGS. The C region is located near the middle of the IGS. In the BMS sequence only a single CCGG site is present in this area. However, it is possible that there are a number of CCGG sites clustered in this region in B37N. The significance of the additional undermethylated regions (B and C) in a population of repeat units in some inbred lines of maize is unclear. If sites of undermethylation function in regulating rDNA transcription as is suggested by the position of the A site (Phillips et al., 1988), it is possible that a subset of repeat units in these inbred lines may initiate transcription at more than one start site.

**Organization of polymorphic rDNA repeat units**

Based on cytogenetic and genetic translocation (Phillips et al., 1988) studies, it has been suggested that the active rDNA arrays are clustered in the secondary constriction of the NOR. The HpaII single digest patterns described above suggest that undermethylated rDNA arrays are arranged in adjacent clusters rather than being interspersed with methylated arrays (Phillips et al., 1988). We have investigated the
molecular organization of undermethylated rDNA arrays further by utilizing an independent restriction enzyme polymorphism. In most inbred lines of maize, rDNA digestion with EcoRI produces a single fragment of repeat unit length as the result of cleavage at a conserved site in the 26S gene (Figure 7). However, several inbred lines have been shown to contain an additional EcoRI site (Figure 7) located about 1.1 kbp to the left of the conserved site (Rivin et al., 1984; Zimmer et al., 1988). The EcoRI rDNA polymorphism does not segregate in F1 or F2 individuals which indicates that individual rDNA arrays on a chromosome are heterogenous for cleavage by this enzyme (Zimmer et al., 1988). In individuals with the additional EcoRI site, cleavage at the conserved site and the polymorphic site occurs in a fraction of the rDNA arrays of an individual to generate 8.0 and 1.1 kbp fragments in addition to the 9.1 kbp fragment (Figure 8; lanes 1, 2). This EcoRI polymorphism is present in the inbred line B73 (Figure 8, lane 1) as well as the hybrid SX19 (Figure 8, lane 2) which is produced by crossing B73 (female) x Mo17 (male), but is absent in Mo17 (Figure 8, lane 3). Figure 9A shows the result of HpaII/EcoRI double digestion experiments in the hybrid, Sx19. Two separate probes (pXbrl and pZms1) were utilized to examine separate regions of the rDNA. The EcoRI single digests show both the 9.1 and 8.0 kbp fragments (Figure 9A, lanes 1, 3) with both probes (the 1.1 kbp fragment is not spanned by either probe). When the HpaII/EcoRI double digests are probed with either pXbrl or pZms1, the additional fragments produced by HpaII digestion originate from the 8.0 kbp fragments. The 9.1 kbp fragments appear to be completely resistant to HpaII digestion and therefore completely methylated.

The signal present in single and double digest bands was quantitated
Figure 8. *EcoRI* digestion patterns of maize rDNA. *EcoRI* single digests of B73 (lane 1), Sx19 (lane 2) and Mo17 (lane 3) rDNA blotted and probed with pZmS1 are shown. The 1.1 kbp fragment is not detected by the probe used in this experiment. The fragment sizes (in kbp) are indicated at the left of the Figure.
Figure 9. Clustering of undermethylated rDNA arrays in EcoRI polymorphic arrays. In panel A, DNA from Sx19 was digested with EcoRI (lanes 1, 3) or HpaII/EcoRI (lanes 2, 4). The hybridization probes used were pXBrl (lanes 1, 2) and pZmS1 (lanes 3, 4). In panel B, double digests with EcoRI and BstEII (lane 1), EcoRII (lane 2) or HindIII (lane 3) probed with pGmr1 are shown. The sizes of fragments (in kbp) are indicated by the arrows.
by densitometric scanning of prefilled autoradiograms. For the 9.1 kbp fragment band approximately 95% of the EcoRI single digest signal remains in the 9.1 kbp double digest fragment band. In contrast, for the 8.1 kbp band only 17% of the single digest signal remained in the double digest lane. Thus, the 9.1 kbp band contains the completely methylated repeat units while the 8.0 kbp band contains the undermethylated (HpaII-sensitive) repeat units.

This conclusion is also substantiated by the size of the fragments detected by pXbr1 in the HpaII/EcoRI double digests (Figure 9A, lane 2). The fragment detected is 5.6 kbp in length as expected from cleavage at a single HpaII site in the A region in the IGS of rDNA arrays with the extra EcoRI cleavage site (Figure 7). If a substantial number of undermethylated rDNA arrays were in the EcoRI non-polymorphic arrays, fragments of 6.6 kbp would also be observed in this experiment (Figure 9A, lane 2). The 2.5 kbp fragment detected by pZms1 hybridization (Figure 9A, lane 4) to the double digest confirms the location of the single HpaII-sensitive site at position A (Figure 7) in Sx19. The EcoRI polymorphic inbred lines B73 and B37N also have been examined with HpaII/EcoRI double digests. In both of these inbred lines, the 9.1 kbp bands were also about 95% resistant to HpaII digestion while the 8.0 kbp arrays were HpaII-sensitive. The double digest fragment patterns were also similar to those observed for Sx19 except for B37N in which the additional undermethylated sites produced additional fragments in the EcoRI/HpaII double digest.

The organization of rDNA in the hybrid Sx19 was further examined by using double digests with EcoRI and other restriction enzymes. The clustering of undermethylated arrays within EcoRI polymorphic arrays was
also observed with the restriction enzyme EcoRII which is sensitive to cytosine methylation in the C(A/T)G sequence (McLelland and Nelson, 1985). The isoschizomer BsrNI is not sensitive to methylation at this sequence and cleaves maize rDNA into fragments of less than 500 bp (data not shown). Digestion of rDNA with EcoRII produces a limited number of rDNA fragments (Figure 9B, lane 2). Published sequences indicate that there are 6 (BMS) to 8 (A619) EcoRII recognition sites in the IGS (McMullen et al., 1986; Toloczyki and Feix, 1986). Thus, as with HpaII, the majority of these sites are completely methylated. Similarly, those that are undermethylated repeat units are confined to those arrays with the additional EcoRI site (Figure 9B, lane 2).

Maize rDNA has also been shown to be polymorphic for cleavage by BstEII and HindIII, both of which cleave in the IGS region (Zimmer et al., 1988). These two restriction enzymes are sensitive to cytosine methylation (McLelland and Nelson, 1985), but the lack of appropriate isoschizomers hinders further definition of this situation. As shown in Figure 9B (lanes 1, 3), in double digests of Sxl9 rDNA with EcoRI and either HindIII or BstEII the 8.0 kbp arrays are digested while the 9.1 kbp arrays are resistant.

Chromatin Structure of Undermethylated rDNA

The observation that undermethylated rDNA repeat units occur primarily in EcoRI polymorphic repeat units proved valuable for designing experiments to examine the relationship between rDNA methylation and chromatin structure using the maize hybrid Sx19 as a model system. Utilizing the EcoRI polymorphism as an independent marker of undermethylated rDNA, an experiment was designed to examine the chromatin structure of EcoRI polymorphic vs. nonpolymorphic rDNA. The
enzyme DNasel, which is known to digest active genes preferentially (Weintraub and Groudine, 1976), was used for the experiments. Intact nuclei were purified, and aliquots were digested briefly (15 minutes, 15°C) with increasing concentrations of DNasel. The DNA was purified from the individual aliquots of nuclei and digested with EcoRI. The fragments were separated on 0.8% agarose gels, transferred to nylon membranes and analyzed by blot hybridization with specific rDNA probes. Figure 10 shows the result from a DNasel digestion series from these experiments probed with pGmr1.

Several important points are illustrated by this experiment. On the left, the first two lanes (U and I) are the controls. Lane U (unincubated) contains the DNA from nuclei that were not incubated with DNasel. Lane I contains DNA from nuclei incubated along with the experimental samples but without DNasel. Both of these lanes show the EcoRI digestion pattern typical for Sxl9 DNA. The remaining lanes contain the DNA from DNasel digested nuclei. Two important points are evident from examining these fragments. First, the 8.0 kbp rDNA arrays are extremely sensitive to DNasel compared to the 9.1 kbp arrays. Even at the lowest concentration of DNasel the 8.0 kbp arrays have been digested extensively by DNasel. This preferential sensitivity of the 8.0 kb arrays shows that the arrays that are undermethylated are more susceptible to DNasel than those that are completely methylated (9.1 kbp). Another important observation is that some specific fragments appear to be released by DNasel digestion of the rDNA although the patterns are obscured by the large number of fragments detected. This shows that there are numerous specific locations being cleaved by DNasel in the rDNA.
Figure 10. DNaseI sensitivity of EcoRI polymorphic and nonpolymorphic Sx19 rDNA chromatin. Maize leaf nuclei from Sx19 were incubated, as described in Materials and Methods, with increasing concentrations of DNaseI. DNA was extracted, digested with EcoRI, and analyzed by Southern blot hybridizations with pGmr1. U, unincubated control; I, incubated control. The numbers above the DNaseI treated lanes show the units of DNaseI per ml. The numbers at the left and right indicate the fragment sizes in kbp.
DNaseI sensitive sites in the rDNA

Experiments were then performed to determine the position of DNaseI hypersensitive sites across the rDNA array. When there are numerous cleavage sites across a region it is extremely difficult to accurately determine the location of these fragments with long probes because both ends of a fragment will be detected (Figure 10). Thus, the indirect end-label technique was utilized to identify the specific sites of DNaseI cleavage across the rDNA repeat unit. DNA purified from DNaseI treated nuclei was digested with XbaI and blot hybridization experiments utilizing short probes adjacent to the XbaI site were performed to determine the location of specific DNaseI cleavage sites. To analyze IGS fragments from XbaI digests, the probe 18B was utilized (Figure 11). The probe pXBrl which extends in the opposite direction to 18B was used to detect the coding region patterns. Figure 11 shows the results of these experiments when the probe 18B was utilized. This probe detects fragments which extend 5' from the XbaI cleavage site into the IGS region. The map at the right of Figure 11 shows the approximate location of the sites detected in this autoradiogram. There are numerous specific DNaseI cleavage sites across the IGS region. Two particularly intense fragment bands located upstream of the XbaI site and just down from the subrepeat region marked by the boxed region are located near the A site of undermethylation. A series of regularly spaced distinct DNaseI cleavage sites are also present in the subrepeat region. In the 26S coding region, two smears of fragments representative of multiple cleavage sites are produced. In Figure 12 the DNaseI cleavage profile using the probe pXBrl to indirectly end label coding region fragments is shown. There are several distinct cleavage sites located across the 18S
Figure 11. DNaseI hypersensitive sites in the IGS of Sx19 rDNA chromatin. DNaseI hypersensitive sites in the IGS of maize rDNA chromatin were visualized by using the method of indirect end-labeling. DNA from DNaseI treated nuclei was digested with XbaI and analyzed by Southern blot hybridization using 18B as a probe. Lanes U and I are the unincubated and incubated controls, respectively. The numbers above the DNaseI treated lanes show the units of DNaseI per ml. Numbers at the left of the lanes indicate the size (in kbp) of standard markers. The map at the right of the autoradiogram is included as a reference point to show the approximate location of the DNaseI sensitive sites on the rDNA repeat unit. The location of 18B and the conserved XbaI site are also shown on the map. The open box shows the location of the subrepeat region.
Figure 12. DNaseI hypersensitive sites in the coding region of Sx19 rDNA chromatin. DNaseI sensitivity was assayed as described in Figure 11, except that the hybridization probe pXB1r1 was used to visualize fragments in the coding region. Lanes are identical to those shown in Figure 11. The map at the right is included as a reference point to show the approximate location of the DNaseI sensitive sites on the rDNA repeat unit. The size (in kbp) of standard markers are indicated at the left.
and 26S genes along with smears of fragments across the genes with some distinct points of cleavage.

**Location and sensitivity of DNaseI cleavage sites**

To provide a more detailed analysis, the autoradiograms in Figures 11 and 12 (and those from two additional experimental trials) were scanned with a densitometer equipped with an integrator. The relative intensities and locations of the fragment bands produced by DNaseI cleavage are summarized in Figure 13. Examination of the data compiled on Figure 13 supports several conclusions concerning the chromatin structure of Sxl9 rDNA.

The IGS region has a greater general sensitivity to DNaseI compared to that of the coding region. The IGS contains two sites just downstream of the subrepeatt region that are twice as sensitive to DNaseI as are any of the other sites detected in the noncoding and coding regions. These two sites are near the transcriptional start site and undermethylated region in the IGS. Two other strong DNaseI sensitive sites are found between these sites and the 18S coding region. The subrepeatt region contained nine cleavage sites which were also strongly sensitive to DNaseI. The first five of these sites (5' to the 26S gene) were evenly spaced with approximately 200 bp separating them. This regular spacing was interrupted by two sites spaced 100 bp apart, but the 200 bp spacing resumed for the next three sites. These spacing intervals are characteristic of nucleosomal (200 bp) and half-nucleosomal (100 bp) units in chromatin (Gross and Garrard, 1988). Across the coding regions, many of the fragment bands are much less intense and not as distinct. This is depicted in Figure 13 by joining the lines (to form hatched boxes) over regions where peaks were not distinguished in the densitometer
Figure 13. Map of DNaseI hypersensitive sites in Sx19 rDNA chromatin. The lines below the repeat unit show the approximate location of the DNaseI hypersensitive sites in relation to other major structural features of the rDNA. The length of the lines reflect the relative sensitivity of sites based on the peak area of fragment bands as determined by densitometry. Hatched regions designate areas that are sensitive to DNaseI but do not show a distinguishable fragment pattern. The position of the probes used for indirect end-labeling are indicated by the arrows extending from the XbaI site. Abbreviations for restriction enzymes are as follows: H, HpaII; X, XbaI; B, BamHI; E, EcoRI; E* refers to the polymorphic EcoRI site.
scans but were clearly in a region of increased general sensitivity. There is one region of this type in the 18S gene and one in the 26S gene.

**Analysis of the EcoRI polymorphism with the polymerase chain reaction**

The DNaseI studies of chromatin structure support the hypothesis that undermethylation of maize rDNA is correlated with transcriptional activity. This correlation was established by using the EcoRI polymorphism as an independent marker of rDNA undermethylation. Because this polymorphism is located in the coding region of the 26S gene, it provides the potential for directly examining the rRNA. However, it was first necessary to determine the nature of the EcoRI polymorphism. Two types of rDNA patterns have been observed with EcoRI digestion. As previously shown (Figure 8, lane 3), Mo17 has a single 9.1 kbp fragment while B73 and Sx19 have the 9.1/8.0 kbp fragments (Figure 8, lanes 1, 2). The basis for the lack of EcoRI cleavage in the 9.1 kbp repeat units is unknown, but there are two possibilities. One is that the lack of cleavage is due to methylation in the EcoRI cleavage site. Methylation of the 3' cytosine in the recognition site inhibits EcoRI digestion (McLelland and Nelson, 1985). If this were the case, all of the rDNA arrays would contain the additional EcoRI cleavage site, but cleavage would not occur in the fraction of the arrays that were methylated. The other possibility is that the fraction of the rDNA arrays not cleaved at the additional EcoRI site do not have the EcoRI recognition site in their sequence. The only previously published 26S gene sequence for plants (rice) suggests that base sequence changes may be responsible for lack of cleavage in this region (Takaiwa *et al*., 1985). In the location where the EcoRI site would be in rice, the sequence is 5'-GAGTAC-3'. Thus, two bases differ from the 5'-GAATTCC-
3′ EcoRI recognition sequence.

An experiment utilizing the polymerase chain reaction was designed to determine whether methylation or base sequence change is responsible for the EcoRI polymorphism in maize. Genomic rDNA was amplified with the TaqI polymerase to produce a fragment containing the EcoRI site (Figure 14). The in vitro amplified fragments will not be methylated. These fragments can then be cleaved with EcoRI and analyzed by gel electrophoresis and blot hybridization to determine whether a methylation or sequence polymorphism is present in the genome.

Figure 14 illustrates the two primers used to synthesize a 247 bp fragment. The EcoRI site is located in the center of this fragment and cleavage at this site will produce fragments of 124 and 123 bp. Figure 15 shows an analysis of the EcoRI digestion products from this fragment hybridized to the end-labeled 5′ primer. The in vitro synthesized DNA from B73 and Sx19 (lanes 2 and 6, respectively) is polymorphic for EcoRI cleavage just as it is in the genome. Conversely, in Mo17 (lane 4) there is no cleavage of the 247 bp fragment with EcoRI. The results of these experiments indicate that the lack of EcoRI cleavage in genomic DNA at the polymorphic site in the 9.1 kbp arrays is due to actual sequence variation at the recognition site.

rRNA direct sequencing

The analysis of polymerase chain reaction products suggested that the EcoRI polymorphism which was utilized to correlate rDNA undermethylation and DNaseI sensitivity is the result of base substitution in the cleavage site. To further examine this situation the RNA from B73, Mo17 and Sx19 was directly sequenced using primer extension with reverse transcriptase and chain termination with dideoxynucleotides. The
Figure 14. Schematic illustrating the primers used for the polymerase chain reaction. The primer ER3 is boxed at the upper left, and 26E is boxed at the lower right. The EcoRI cleavage site falls in the center of the synthetic sequence.
Figure 15. Analysis of in vitro amplified DNA with EcoRI. The amplified DNA from B73, Sx19, and Mo17 was digested extensively with EcoRI and fragments separated on a 4% Nu Sieve agarose gel. The gel was blotted and probed with the primer ER3 (Figure 14). Lanes 1, 3, 5 contain the full length fragment not treated with EcoRI, but incubated at 37°C along with the digests. The fragments in lanes 2, 4, 6 were digested with EcoRI. Samples are B73 (lanes 1, 2), Mo17 (lanes 3, 4) and Sx19 (lanes 5, 6). The arrows at the left show the sizes (in kbp) of φX174 DNA digested with HaeIII. The arrows at the right show the estimated sizes of the undigested fragment and the fragments produced by EcoRI digestion.
position of the polymorphic EcoRI site in the parental lines and the hybrid was known from restriction mapping experiments (Figure 8). Based on the position of the site, the conserved primer designated 26E (Hamby et al., 1988) was utilized to determine the sequence of the rRNA in this region. The only plant for which the 26S rRNA sequence has been published is rice. This sequence was determined from a cloned rDNA repeat unit. As previously noted, rice does not have an EcoRI recognition site in this region due to two base substitutions. Thus, as a control, rice rRNA was sequenced in parallel with the maize samples.

A sequencing gel using the 26E primer is shown in Figure 16. The region containing the EcoRI recognition site is pointed out by the arrows at the left. Reading the RNA sequences as the anticoding DNA strand, the rRNA from B73 and Sx19 both contain the EcoRI recognition site, 5'-GAATTC-3'. The sequence of Mo17 rRNA in this region has a single base change to 5'-GAATAC-3'. In the rice rRNA sequence, two base changes, which agree with those in the published DNA sequence are observed. Also shown in Figure 16 is a nineteen base region surrounding and including the EcoRI site which is indicated by the arrows to the left of the first set of lanes. The sequence of this region, excluding the EcoRI site, is conserved in all of the samples examined. The sequences detected in this region are summarized in Table 3.

Some important conclusions can be drawn from these direct rRNA sequencing experiments. First, they identify the base substitution in Mo17 in the EcoRI recognition site which renders it uncleavable by the enzyme. This variation is equivalent to one of the differences observed in the rice sequence (Table 3). In B73, as well as the hybrid Sx19, the EcoRI recognition site is present in the rRNA sequence. In order to
Figure 16. Direct sequencing of rRNA in the EcoRI polymorphic region. RNAs from B73, Mo17, Sx19 and Rice were directly sequenced using the primer 26E and reverse transcriptase. The upper and lower arrows point to the boundaries of the 19 base conserved sequence excluding the EcoRI sequence. The location of the EcoRI sequence and the changes detected in Mo17 and Rice are also shown. The sequence is being read as the anticoding strand of the DNA that produced the RNA.
Table 3. *Sequence of rRNA in the EcoRI polymorphic region and oligonucleotide probes designed from them*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>B73</td>
<td>5'-GAGGACCG[AATT]CCGTCCA-3'</td>
</tr>
<tr>
<td>MO17</td>
<td>5'-GAGGACCG[AATA]CCGTCCA-3'</td>
</tr>
<tr>
<td>SX19</td>
<td>5'-GAGGACCG[AATT]CCGTCCA-3'</td>
</tr>
<tr>
<td>RICE</td>
<td>5'-GAGGACCG[AGTA]CCGTCCA-3'</td>
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<tbody>
<tr>
<td>ERI+</td>
<td>3'-CTCCTGGC[TTAA]GGCAGGT-5'</td>
</tr>
<tr>
<td>ERI−</td>
<td>3'-CTCCTGGC[TTAT]GGCAGGT-5'</td>
</tr>
</tbody>
</table>
detect the sequence in the rRNA, the majority of the transcripts in these samples would probably have to contain the sequence. Thus, these results suggest that the rDNA arrays containing the EcoRI site are preferentially transcribed in B73 and Sx19.

The nature of the sequence in this region indicated that oligonucleotide probes could be designed to specifically detect transcripts from the EcoRI polymorphic and nonpolymorphic rDNA arrays. The sequence of these probes is shown in the lower portion of Table 3. One probe designated ERI+ is homologous to the transcripts containing the EcoRI site from B73 and Sx19. The other probe designated ERI− is homologous to the transcripts from Mo17 which have a single base substitution in the EcoRI site. If ERI+ is hybridized to Mo17 rRNA, a single base mismatch (A-A) would be present near the center of the oligonucleotide, and the converse situation occurs if ERI− is hybridized to B73 or Sx19 rRNA. Thus, it appeared that these oligonucleotide probes could be used to directly detect specific transcripts in the parent inbred lines and hybrids produced by crossing them. In addition, it is important to note that homology to the ERI + oligonucleotide probe is diagnostic for the production of rRNA from undermethylated and DNaseI sensitive rDNA arrays.

Direct detection of rRNA transcripts

The reactivity of the oligonucleotide probes toward different rRNAs was tested by end-labeling and hybridizing them to slot blots. Figure 17 shows a group of the RNAs that were tested. All hybridizations were done at 55°C, and all washes were at 60°C as described in the Materials and Methods. In panel A, set 1, the ERI+ probe was hybridized to total RNA from B73, Mo17, Sx19 and rice. As expected this probe reacts
Figure 17. Characterization of oligonucleotide probes. The oligonucleotide probes were tested by hybridizing to slot blots of total RNA. In panel A, slots from left to right for each sample contain 0.10, 0.25, 0.50, 0.75 and 1.0 ug of total RNA, respectively. The source of each RNA is shown on the figure. The sets of slot blots were probed with the following probes: (1) ERI⁺; (2) ERI; (3) 26F. In panel B, the cross reactivity of ERI⁺ with Mo17 and rice are shown when washed at 55°C. The samples, amounts loaded and the probe are shown on the figure. The 26C designated the use of 26F as a control probe.
strongly with the EcoRI polymorphic samples, B73 and Sx19. At this stringency, no cross reactivity with Mo17 or rice was observed. When washes are done at 55°C some cross reactivity with Mo17 and rice are observed (Figure 17, Panel B). These signals can be selectively removed by washing a second time at the higher stringency. In panel A, set 2, the ERI+ probe was tested with the same samples except that the reciprocal cross of Sx19 was applied. This probe clearly reacts in an opposite manner with the inbred lines B73 and Mo17. It does not detect the B73 RNA, but does give a strong signal with the Mo17 RNA. The RNA from the reciprocal cross (Mo17xB73) is also detected by this probe. Although there should be a single base mismatch between rice RNA and ERI+, the rice RNA reacted strongly. However, increasing the wash temperature to 62°C removes the majority of ERI+ from the rice sample (data not shown). Following analysis with specific probes, all blots were hybridized with a conserved oligonucleotide (26F) known to be a 100% match with all of the RNAs analyzed (Figure 17, Panel A, set 3; Figure 17, Panel B, 26C). This control experiment shows that rRNA samples are present in all of the slots.

The experiments in Figure 17 show that the oligonucleotide probes ERI+ and ERI− are specific for the different transcripts from which they were designed. This observation was reinforced by a control experiment in which hybridizations were done with labeled ERI+ and a 10-fold excess of unlabeled ERI−. The densitometric signal detected under these conditions is the same as that seen without the excess unlabeled oligonucleotide present. A similar result is obtained when labeled ERI− is hybridized in the presence of excess unlabeled ERI+. This further reinforces the conclusion that these oligonucleotide probes recognize
Figure 18. Analysis of rRNA from parental inbred maizee lines and a hybrid with specific oligonucleotide probes. Six replicate samples of rRNA from an individual were applied at 0.5 ug of total RNA per slot. The reciprocal reactivity of the two probes towards the parents and an F$_1$ hybrid are shown. The probes and samples are indicated on the figure.
unique RNA species.

The two probes ERI+ and ERI- were used to analyze the levels of specific rRNAs in B73, Mo17 and Sx19 (Figure 18). Replicates of 0.5 ug of total RNA were applied to six slots in these experiments. Clearly, the ERI+ probe hybridizes strongly with the Sx19 RNA. However, there is also some cross hybridization of the ERI- probe with the hybrid RNA indicating that transcripts from both B73 and Mo17 are present. In order to quantitate the levels of the different RNAs, autoradiograms were scanned with a densitometer to determine the area of the peaks. The sum of the peak areas from Sx19 probed with ERI+ and ERI- was approximately equivalent to the peak area of the same sample when probed with the conserved oligonucleotide. Thus, these values were utilized as the 100% response of the rRNA to the oligonucleotide. Based on this calculation, approximately 82% of the RNA is homologous to ERI+, and 18% of the RNA is homologous to ERI- (Figure 19).

Figure 19 summarizes the results obtained in similar experiments for pooled leaf samples of SX19 and the reciprocal Mo17 x B73. The data presented in the bar graph for these samples was determined by averaging the results of three pooled samples of each genotype. The third experiment on the bar graph shows results from analysis of individuals from the B73 x Mo17 cross which have been found to have approximately 50% of their rRNA homologous to each probe. These values represent an average of the levels from 5 of the individuals surveyed. An example of an experiment performed with the RNA extracted from the reciprocal cross (Mo17 x B73) is shown in Figure 17 (Panel A, set 2). The ERI- probe reacts strongly with this RNA. In this reciprocal cross, about 85% of the RNA is homologous to ERI- and 15% homologous to ERI+ (Figure
Figure 19. Summary of rRNA levels in the hybrids examined in this study. The three bar graphs summarize the relative levels of rRNA homologous to the specific oligonucleotide probes used in this study. All 100% rRNA levels were obtained using 26F as a control probe. The percentage of rRNA homologous to either ERI+ and ERI- in each type of hybrid are shown in pairs. The hybrid parents are indicated below each pair. The values shown in the two sets on the left (B73 x Mo17; Mo17 x B73) are from the analysis of three separate pooled samples of each genotype. The values shown for the B73 x Mo17 cross on the right hand set are from a group of five individuals that deviated from the values observed in the pooled samples.
19). The RNA samples discussed above were from isolations of pooled material. When RNA extracted from individuals was examined, several deviated from the values observed for pooled samples. In particular, RNA from five individuals from a B73 x Mo17 cross contained more RNA homologous to ERI⁻ (58%) than to ERI⁺ (42%) (Figure 19). Thus individual seedlings may contain proportions of parental RNAs that deviate substantially from the values observed in pooled samples. Although preferential transcription of the maternal rDNA has been noted in both pooled and individual samples, at present no hybrid individuals have been observed in which 100% of their rRNA results from transcription of maternal rDNA.
DISCUSSION

The genomic and rDNA methylation patterns observed in inbred lines of maize and species of teosinte agree with those previously observed in other plants and animals. The genomic DNA was highly resistant to HpaII cleavage, while MspI digestion produced an even distribution of fragments. These results show that there is an abundance of CCGG recognition sites in the genome, but the majority are methylated at the 5' CpG-3' dinucleotide. This general pattern of genomic DNA methylation has been observed in numerous eukaryotic organisms (for review see Flavell, 1986). These results further reinforce previous observations that the 5'-CpG-3' sequence is a primary site of cytosine methylation in plants.

The methylation pattern of the rDNA is similar to that of the genomic DNA. The rDNA was highly resistant to HpaII cleavage but was cleaved extensively by MspI. Examination of the published sequences of the IGS of BMS and A619 (McMullen et al., 1986; Toloczyki and Feix, 1986), the 18S gene of W22 (Messing et al., 1986), and the 26S gene of rice (Takaiwa et al., 1985) indicates that there are approximately 70 CCGG sites per repeat unit in the grasses. Although the 26S gene sequence of maize has not been published, one author has reported that there are 75 CCGG sites distributed throughout the maize rDNA repeat unit (Phillips et al., 1988). HpaII digestion of the rDNA showed that in most inbred lines and all teosintes a single fragment of repeat unit length was produced. In some inbred lines one or two additional fragments were produced. The undermethylated HpaII sites were all located in the IGS of the rDNA repeat unit. Based on the mapping experiments, three categories of rDNA array undermethylation were identified in Zea: (1)
Repeats with a single undermethylated site in the A region; (2) Repeats with undermethylated sites in the A and B regions; (3) Repeats with undermethylated sites in the A and C regions. Thus, the A region is undermethylated in all of the array types observed.

The association of undermethylation with transcriptional activity is suggested by the function of the A region. In both BMS and A619 the A region is near the transcriptional start site, suggesting that cleavage of this region indicates undermethylation of a promoter (Phillips et al., 1988). This conclusion is further supported by the evolutionary conservation of this region of undermethylation. Studies of numerous other plant rDNAs have identified the primary site of undermethylation at approximately 0.7 - 1.0 kb upstream of the start of the 18S coding region (Flavell, 1986). Conservation of this site of undermethylation also has been noted in mouse and Xenopus (reviewed in Bird, 1984). In Xenopus this region has been shown directly to serve as a transcriptional start site. The significance of the additional undermethylated sites that are present in a portion of the arrays of certain inbred lines is not known. However, it should be noted that all inbred lines and teosintes have a single undermethylated site located in the A region and that all arrays with additional undermethylated sites also have the A region undermethylated. The undermethylated sites in the B region and C region may augment the transcriptional capability of these arrays since they occur within the subrepeat region of the IGS that is thought to function as a set of repeated enhancer elements.

The occurrence of HpaII-sensitive (undermethylated) rDNA in the portion of polymorphic rDNA arrays containing an additional EcoRI site supports several hypotheses concerning the organization of rDNA.
Because *HpaII* single digestion releases a single fragment of repeat unit length, it has been suggested that the undermethylated rDNA repeat units are tandemly clustered in the genome (Phillips *et al.*, 1988). If there were an interspersion of methylated and undermethylated rDNA repeats, a fragment ladder of different sized digestion products would be expected in single digests. A similar situation would be observed in the *HpaII/EcoRI* double digest where a ladder of fragments between 6.6 and 5.5 kb would be expected if there were considerable interspersion. Instead, a single fragment indicative of cleavage at the undermethylated *HpaII* site and the upstream *EcoRI* site is observed. These results further support the idea that in the nucleolus undermethylated rDNA arrays are tandemly clustered as are the completely methylated arrays. The functional aspects of this arrangement in transcriptional activity are discussed later in this section.

The study of the chromatin structure of rDNA (in which the *EcoRI* polymorphism as an independent marker of undermethylated arrays) showed that in intact nuclei the undermethylated arrays are hypersensitive to DNaseI digestion compared to the methylated arrays which are relatively resistant to DNaseI digestion. These results suggest that the undermethylated and *EcoRI* polymorphic arrays are in an "open" conformation that is highly accessible to DNaseI. In numerous systems increased susceptibility to DNaseI digestion is associated with transcriptional activity (Gross and Garrard, 1988). Thus, these DNaseI sensitivity experiments suggest a correlation between undermethylation and transcriptional activity in maize rDNA.

The mapping of DNaseI hypersensitive sites across the entire rDNA repeat unit and the finding that the strongest sites of preferential cleavage were in the IGS in approximately the same position as the
transcriptional start site and the undermethylated HpaII site, again suggest that undermethylation of the promoter region occurs in transcriptionally active maize rDNA. In both wheat (Flavell et al., 1986) and pea (Kaufman et al., 1987) rDNA chromatin similar locations of DNaseI hypersensitive sites have been observed in the IGS. This type of chromatin structure also has been detected in X. laevis and X. borealis rDNA (LaVollpe et al., 1983).

The general conservation of DNaseI hypersensitive regions across the IGS of plants and animals probably reflects the specific contacts between the promoter units and chromosomal proteins. The DNaseI cleavage profile observed indicated that a highly ordered set of proteins exist in the IGS. Two regularly spaced fragments of 200 bp occur in the IGS over the first two subrepeat elements immediately following the strongest DNaseI hypersensitive sites. This regular spacing is interrupted by two fragments spaced 100 bp apart then the spacing returns to 200 bp over the next six subrepeat elements. These spacing intervals approximate the nucleosomal repeat interval and may represent cleavage in nucleosomal linker regions (Cartwright and Elgin, 1986). Thus, the strong DNaseI hypersensitive sites are probably followed by two nucleosomes. These seem to be interrupted by two half-nucleosomes, which are followed by six full nucleosomes. A highly ordered structure similar to this has been observed across the hsp 82 promoter region in yeast (Szent-Gyorgi et al., 1987). The strong DNaseI hypersensitive sites have been suggested to represent a chromatin topology which determines a nucleosome phasing arrangement. A similar arrangement has been observed in the small heat shock genes of Drosophila melanogaster (Cartwright and Elgin, 1986).

A rather abrupt transition from this highly ordered arrangement was
observed in the transcriptional unit. There were areas which were sensitive to DNaseI cleavage across the genes. Extensive periodicity of cleavage sites was not observed across the coding regions and two of the fragmentation patterns were present as smeared bands. One of these occurred in the middle of the 18S gene, while the other was in the middle of the 26S genes. DNaseI sensitivity of coding regions has been noted in yeast hsp82 (Szent-Gyorgyi et al., 1987) and D. melanogaster small heat shock genes (Cartwright and Elgin, 1986) when they are being actively transcribed. In D. melanogaster, induction of the small heat shock genes caused a considerable loss of detail in the cleavage profiles and significantly enhanced accessibility, which reflected the relative transcription rate of each gene. Thus, the rather nondescript cleavage profile might be expected to occur over the constituitively expressed maize rDNA.

The intensity of the fragment bands detected across the coding region was about half that of the strongest cleavage sites in the IGS. In the IGS there was a general increase in intensity of fragments from 5'-3' up to the strongest cleavage sites. Beyond that, the intensity of cleavage sites was lower. These strongest sites of cleavage were located just upstream of the transcriptional start site. This profile across the IGS is what might be expected since DNaseI is able to cleave chromatin in an "open" conformation (Gross and Garrard, 1988).

Cytologically the rDNA arranged in the NOR is distinguishable as heterochromatin and euchromatin. The tightly compacted, heterochromatic portion may contain 70-90% of the rRNA genes (Givens and Phillips, 1976). Only a fraction (10-30%) of the genes are in the euchromatic (active) region. It has been suggested previously that the
undermethylated rDNA repeat units are active and present in the euchromatic portion of the NOR (Phillips et al., 1988). Observations on rDNA organization, undermethylation, chromatin structure and expression made in the present study substantiate this hypothesis. Restriction fragment patterns from single and double digests suggest that the undermethylated rDNA repeat units are tandemly clustered in the genome in repeat units that are also polymorphic for cleavage by other restriction enzymes. It follows from this that the completely methylated repeat units are also tandemly clustered. Approximately 22% of the total rDNA arrays are undermethylated at a single \( HpaII \) site in the Sx19 rDNA. Thus, 20% of the undermethylated rDNA arrays would be tandemly clustered in the euchromatic portion of the NOR while the remaining 80% (completely methylated) would comprise the NOR heterochromatin. A clustered arrangement for the undermethylated, transcribed genes would be optimal for maximum transcriptional efficiency. The arrangement would serve to sequester the transcriptional machinery so that it could move contiguously from one unit to the next.

The chromatin structural features of the rDNA detected by DNaseI digestion indicated that undermethylated arrays were transcriptionally active. The undermethylated arrays of rDNA chromatin were preferentially sensitive to DNaseI digestion, and the strongest DNaseI hypersensitive sites were near the major undermethylated region in the IGS. Direct characterization of the rRNA transcripts further substantiates these conclusions. The direct sequence of rRNA from the EcoRI polymorphic samples (B73, Sx19) detected the presence of this site in the transcripts. A single base mutation was found in the rRNA of the EcoRI nonpolymorphic line (Mo17). Further direct analysis of the rRNA
transcripts with oligonucleotide probes designed to be specific for RNA from each parent were performed. The results with ERI+ indicated that in B73 all of the transcripts originated from EcoRI polymorphic arrays. This might be expected since in B73 the EcoRI polymorphic arrays are also undermethylated. The polymerase chain reaction experiments showed that the EcoRI sequence itself is changed in a portion of the arrays. Therefore, it appears that in the inbred line B73, undermethylation is strictly correlated with rDNA transcription. The other probe, ERI-, did not detect transcripts from B73, but was specific for rRNA from Mo17. ERI+ did not detect Mo17 RNA transcripts.

When these probes were used to examine rRNA from hybrids produced by crossing these lines, both probes were able to detect the RNAs. The relative amounts of rRNA levels detected seemed to vary. In pooled samples of Sx19 rRNA the majority (ca. 82%) of the transcripts were homologous to ERI+. The remaining (ca. 18%) of the rRNA cross reacted with ERI-. These results showed that in the Sx19 hybrid the undermethylated, DNaseI sensitive arrays are preferentially transcribed. However, in certain individuals from B73xMo17 crosses, the rRNA levels approach 50% of each type of transcript. Thus, among individuals considerable variation in these levels may be present. These results seem to be in conflict with those observed for the undermethylation and chromatin structure results which suggest preferential transcription of ERI+ arrays. However, several factors should be considered in evaluating this situation. The HpaII mapping experiments and chromatin structure experiments give a general view of the structural aspects of rDNA. There may be a small fraction of undermethylated, DNaseI sensitive, and EcoRI nonpolymorphic arrays that are not detectable in the blot
hybridization experiments. Some indication that this is the case comes from the fact that 5-7% of the signal from the 9.1 kb rDNA arrays are always lost in the *HpaII/EcoRI* double digests. This fraction may represent undermethylated, transcribed Mo17 rDNA arrays which are transcriptionally active. This situation could be further examined by simultaneously purifying DNA and RNA from individuals to determine if the differences in rRNA levels is reflected by changes in the fraction of undermethylated 9.1 kbp arrays.

Developmental changes in the degree of dominance that one nucleolus has over the other have been noted in other systems (for review see Reeder, 1985). The level and number of undermethylated sites has been shown to undergo developmental changes in the endosperm of the maize inbred A188 (Phillips *et al.*, 1985; Phillips *et al.*, 1988). Similar changes have been noted in the hybrid Sx19 (Sachdev *et al.*, 1987). Therefore, it is possible that there may be a certain fluidity associated with maize rDNA methylation in other tissues. In the present study, the individual hybrid samples in which variation in rRNA levels were observed were purified from young (ca. 10 day) seedling tissues. Although young seedlings have the same rDNA methylation patterns as mature plant materials, there may be more subtle developmental changes in rRNA levels that are detected by the oligonucleotide probes.

It is not surprising that there is variation in the levels of rRNA produced in the hybrid made by crossing B73 and Mo17. This is an intraspecific cross and all cases of nucleolar dominance in plants and animals have been reported in interspecific hybrids (Reeder, 1985). Since these inbred lines of maize have the same length IGS, there would not be any competition for transcriptional machinery mediated by different
numbers of enhancer elements being present. Rather, the results from the current study suggest that there may be inbred line specific transcription factors which mediate transcriptional competition.

The preliminary results also suggest that the maternal parent's transcription is dominant in the hybrid. This might be expected since the maternal genome is highly transcribed in the endosperm (Phillips et al., 1988) and is activated first after fertilization. It may be difficult for the paternal genome to become strongly activated in the hybrid. The dominance level mediated by a maternal factor is much weaker than that observed in *Xenopus* where the enhancer subrepeats are responsible for the dominance. In *Xenopus*, no maternal effect has been observed and under normal circumstances, the nucleolus with the most subrepeats is transcribed almost exclusively whether it was of maternal or paternal origin (Honjo and Reeder, 1969). However, cases of maternal dominance have been noted in hybrids of *Xenopus* NOR deletion mutants (Reeder, 1985). The situation in inbred lines of maize may be similar to that in *Xenopus* mutants. The approach for assaying ribosomal RNA gene expression used in the present study will be directly applicable to analyzing hybrids for nucleolar dominance at the molecular level. Hybrids made between maize and teosintes should show more pronounced levels of dominance since the teosinte repeats units probably contain more copies of the subrepeat elements.

In conclusion, the present study has established a general correlation between rDNA undermethylation, DNaseI sensitivity, and gene expression. The results suggest that the undermethylated rDNA is packaged into the euchromatic portion of the nucleolus. The presence of two DNaseI hypersensitive sites in the vicinity of the major undermethylated site
further suggests that this region is involved in establishing a transcriptionally competent DNA topology in the promoter. This region may be preferentially recognized by a maternal transcription factor in hybrids, thus, leading to preferential transcription of the undermethylated, DNaseI sensitive arrays.


91. Schreck, R.R., Erlanger, B.F. and Miller, O.J. (1977) Binding of
antinucleoside antibodies reveals different classes of DNA in the
108:403-409.

DNA methylation and interspersion of melon satellite DNA sequences.

of the initiation and termination sites for ribosomal RNA

 genomic zein sequences: structure, organization and tissue specific

95. Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M. and
 chromatin domain introduced by DNAaseI. Cell 20:451-460.

Methylation of rRNA genes in some higher plants. Plant Sci. Let.
35:213-217.

97. Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J.,
oligodeoxyribonucleotides for the isolation of specific cloned DNA
York.

boundaries demarcate the chromatin structure of a yeast heat-shock


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PERSONAL INFORMATION

Social Security number: 462-25-9641
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EDUCATION

Louisiana State University, Baton Rouge - 8/1984 to present
Ph.D. program in Biochemistry Dept.
Cumulative G.P.A. - 3.84/4.00
Thesis Advisor: Dr. Elizabeth A. Zimmer

Texas A&M University, College Station, TX - 1/1982 to 8/1984
M.S. in Genetics - August, 1985
Cumulative G.P.A. - 4.00/4.00
Thesis Advisor: Dr. Clint W. Magill
Thesis Title: "Genomic DNA Methylation at Specific Stages in the Life Cycle of Two Fungi"

Texas A&M University - 9/1979 to 12/1981
B.S. in Biochemistry with honors, Magna Cum Laude
Cumulative G.P.A. - 3.56/4.00

McLennan Community College, Waco, TX - 9/1977 to 5/1979
Chemistry Major
Cumulative G.P.A. - 3.77/4.00

PUBLICATIONS


variation, and inheritance in maize and its ancestors." Genetics 120:in press.


PUBLISHED NOTES AND ABSTRACTS


**PROFESSIONAL PRESENTATIONS**


6. Jupe, E. (1988) "DNA methylation, chromatin structure, and expression of maize ribosomal RNA genes." Presented at University of Cincinnati Medical Center, Department of Biochemistry and Molecular Biology, Cincinnati, OH.

RESEARCH EXPERIENCE

1984-present Louisiana State University, Department of Biochemistry, Baton Rouge, LA. Graduate student in the laboratory of Dr. E.A. Zimmer.
Doctoral research involves studying chromatin structure, DNA methylation and gene expression in maize and teosinte ribosomal RNA genes. Techniques utilized include nuclear DNA purification from specific tissues, purification of maize leaf nuclei on Percoll gradients, DNasel sensitivity assays, restriction endonuclease mapping, Southern blotting, nick translation, primer extension sequencing of rRNA with reverse transcriptase, oligonucleotide purification and plasmid DNA purification. Other techniques used include performing crosses and increasing maize seed stocks in both field and greenhouse.

1982-1984 Texas A&M University, Genetics Section, Dept. of Plant Sciences, College Station, TX. Graduate Student in the laboratory of Dr. C.W. Magill.

Thesis research involved studying DNA methylation in two fungi, *Phymatotrichum omnivorum* and *Neurospora crassa*. Techniques used include culturing conidia and mycelia of *N. crassa* and sclerotia and mycelia of *P. omnivorum*, purification of nuclear DNA from all of the above tissues, HPLC to determine major and modified base composition, and statistical analysis of data. Other techniques learned included purification of mRNA from various corn, rice, and fungal tissues.

1979-1982 Texas A&M University. Undergraduate research in the
laboratory of Dr. J.R. Gold Genetics Section and Dr. J.M. Magill, Dept. of Biochemistry.

Techniques learned with Dr. Gold included preparing of Giemsa-stained standard karyotypes from both meiotic and mitotic tissues of North American minnows and basic microscopy techniques. Techniques learned with Dr. J.M. Magill included study of purine and pyrimidine metabolism using radioactive uptake experiments, HPLC analysis of cellular extracts, and enzyme purification.

HONORS AND AWARDS

1988  Recipient of a Genetics Society of America, Graduate Student Travel Award to present a poster at the International Congress of Genetics.

1987  Recipient of the Robert S. and Louise Allen Research Award for Outstanding Graduate Student in Biochemistry, Department of Biochemistry, Louisiana State University.

1986  Recipient of Grant-in-Aid of Research from Sigma Xi for proposal to investigate "Chromatin Structural Heterogeneity in the Ribosomal Gene Arrays of Maize."

1986-present  Member of Sigma Xi, the Scientific Research Society
1985-present  Member of Phi Kappa Phi Honor Society - Graduate Division

May 1984-     Awarded Departmental Graduate Research Assistantship for
Aug 1984      proposal to investigate "DNA methylation and gene expression
              in pathogenic fungi."

1981         Graduated Magna Cum Laude with B.S. in Biochemistry

1981         Outstanding Undergraduate Student in Biochemistry Award-
              Department of Biochemistry and Biophysics, Texas A&M Univ.

1981         Member of Gamma Sigma Delta - TAMU Chapter of the Honor
              Society of Agriculture

1981         Distinguished Student List, College of Agriculture

1978-1979    Member of Phi Theta Kappa National Honor Society for Junior
              Colleges

1977-1979    Full Scholarship to McLennan Community College

TEACHING EXPERIENCE

Texas A&M University

Sept 1982-   Teaching Assistant for genetics lab: gave short lectures,
May 1984 prepared and graded lab quizzes, lab preparation for restriction enzyme mapping of plasmids

Jan 1982- Teaching Assistant for question and discussion for genetics
Sept 1982 course: gave lectures and help sessions, graded quizzes and major exams

McLennan Community College

Sept 1978 State employed tutor for math and chemistry courses

OTHER PROFESSIONAL EXPERIENCE

1988 Member of Genetics Society of America

1985-1987 Member of International Society for Plant Molecular Biology and Southern Section, American Society of Plant Physiology

1984 Member of American Society for Microbiology and American Association for Advancement of Science
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Title of Dissertation: DNA Methylation, Chromatin Structure and Expression of Maize Ribosomal RNA Genes

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