Offprints From
MAIZE BREEDING AND GENETICS
Edited By Dr. David B. Walden
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*Chapter 43*

# MOLECULAR CYTOGENETICS OF THE NUCLEOLUS ORGANIZER REGION

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The nucleolus organizer region (NOR) offers many intriguing opportunities to the molecular, classical, and applied cytogeneticist. From the molecular viewpoint, the NOR contains the DNA coding for 18S and 28S rRNA, thereby permitting the study of transcription and genetic regulation of these important cellular macromolecules. The interaction of these ribosomal components (18S and 28S rRNA) with others, such as 5S RNA and ribosomal proteins, must be elucidated to understand the formation and role of the nucleolus in plant development. Classically, the region is interesting because the locus of the 18S and 28S rRNA genes, that is, the NOR, can be directly observed cytologically with the light or electron microscope and evaluated for various structural details. In addition, the functional capacity of the region can be revealed

Paper No. *1608*, Miscellaneous Journal Series, Minnesota Agricultural Experiment Station.

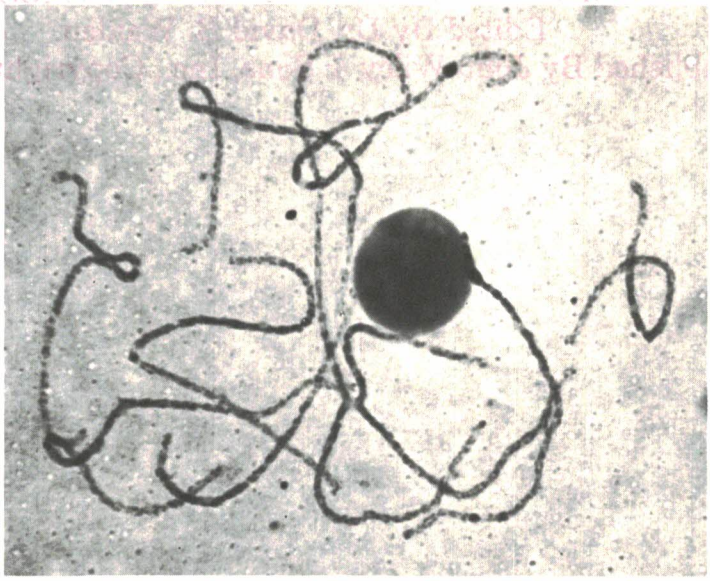

**Figure 1.** Pachynema of microsporogenesis displaying the 10 bivalent chromosomes including chromosome 6 associated with the nucleolus at the NOR. Magnification × 1600. Photograph courtesy of J. T. Stout.

cytologically as manifested by the presence, size, and structure of the nucleolus in various genetic and cytogenetic stocks. And since ribosomes are involved in the synthesis of all proteins in every cell of the plant, the applied cytogeneticist can manipulate the NOR to attempt to correlate the effects with economic traits. Finally, the ribosomal RNA system is controlled by genes reiterated thousands of times. Studies on such genetic systems should provide unique insights to maize genetics and breeding.

Information bearing on the molecular cytogenetics of the NOR in maize has not been extensively reviewed. This paper attempts to assess the current state of knowledge on the maize NOR, giving information on various molecular, classical, and applied cytogenetic aspects of the ribosomal RNA system along with our current interpretations and speculations on the NOR's genetic nature and function. A maize pachytene cell illustrating the chromosome-6 NOR–nucleolus assocation is shown in Figure 1.

## THE MAIZE NUCLEOLUS

### Nucleolar Cycle

The cell cycle represents a complex and highly controlled series of events that includes interactions between nuclear and cytoplasmic processes. The

nucleolus, located within the nucleus, is central to many of these processes and interactions. The nucleolus functions in maize, as in other organisms, as the site of synthesis and maturation of a ribosomal precursor RNA into ribosomal components. The nucleolus undergoes cyclic changes that usually result in its disorganization in prophase and reorganization in telophase, but this varies with cell type. De la Torre and Clowes (1972) have shown that the dissolution and reorganization of the nucleolus is differentially timed relative to the chromosomal cycle in different root zones. In the root-cap initials, the cells that generate the cap cells, the nucleolus starts to disorganize before prophase and has completely reorganized by midtelophase. In contrast, nucleoli in cells of the quiescent center of the root meristem start to disorganize during prophase but do not reorganize until after telophase. Cells of the stelar region are more typical in that the nucleolus commences disorganization in prophase and undergoes reorganization in telophase.

In meiosis the nucleolus reaches its maximum size at midpachynema. Das (1965) and Das and Alfert (1966) found that nucleolar RNA synthesis had essentially ceased by this point of maize microsporogenesis. Autoradiography following a 2-h label with $^{3}$H-cytidine or $^{3}$H-uridine showed rapid RNA synthesis during premeiotic interphase, and as prophase progressed there was a decrease in RNA synthesis in the nucleolus. The nucleolus remained inactive in RNA synthesis from pachynema to the end of prophase (through diakinesis). Although inactive in RNA synthesis, the nucleolus remained prominent and intensely stainable with Azure B. The remainder of the genome continued to be involved in RNA synthesis at pachynema, but the rate decreased significantly at diplonema and diakinesis. Lin (1955) measured RNA amounts in the nucleolus by UV microspectrophotometry. The amount of nucleolar RNA doubled between leptonema and midpachynema. Nucleolar RNA synthetic rate, however, decreased during these stages and ultimately ceased. This elevation of the amount of RNA in the nucleolus while the RNA synthetic rate was decreasing led Das (1965) to suggest that there is a reduced rate of transport of nucleolar RNA to the cytoplasm from leptonema to midpachynema in microsporogenesis.

### Nucleolar Composition

Pollister and Ris (1947), using microspectrophotometry, concluded that the pachytene nucleolus of maize contains $5.0 \times 10^{-11}$ gram of protein. Comparing UV absorption before and after hot trichloroacetic acid (TCA) or ribonuclease treatment of maize pachytene nucleoli, Pollister and Leuchtenberger (1949) observed a reduction of 53% in the extinction value after treatment. Swift and Stevens (1966) reported that maize microspore nucleoli of the normal as well as diffuse (multiple, small, and irregularly sized nucleoli) types stain purple with Azure B, characteristic of the presence of RNA. These observations suggested that the nucleolus contains nucleic acid almost exclusively of the RNA type. Lin

(1955) estimated that maize early-pachytene nucleoli contain from 7.35–14.55 × $10^{-12}$ gm RNA, depending on the strain. This value for RNA coupled with the Pollister and Ris (1947) value for protein suggests that the maize pachytene nucleolus is approximately 80% protein. Lin's (1955) UV absorption spectrum of pachytene nucleoli also indicates a large amount of protein. McLeish (1964) reported the existence of DNA in isolated nucleoli of maize root tips even though they are Feulgen negative.

Maize ribosomal RNA is rich in guanine and cytosine. Three maize genotypes gave similar results (Pollard 1964) with the following average values (mols %):

$$\text{28S rRNA} - \text{A} = 21.2,\ \text{U} = 16.9,\ \text{C} = 28.0,\ \text{G} = 33.9\ \frac{\text{A} + \text{U}}{\text{C} + \text{G}} = 0.62$$

$$\text{18S rRNA} - \text{A} = 22.0,\ \text{U} = 20.2,\ \text{C} = 25.0,\ \text{G} = 32.8\ \frac{\text{A} + \text{U}}{\text{C} + \text{G}} = 0.73$$

Since the rRNA is G–C rich (61.9% for 28S and 57.8% for 18S) the complementary DNA would be G–C rich and more dense in a CsCl density gradient than the bulk DNA. Ingle, Timmis, et al. (1975) and Doerschug (1976) have shown this to be true. Ingle, Timmis, et al. list the buoyant density as 1.710–1.711 for maize rDNA and 1.701 for bulk DNA.

### Ultrastructure of the Nucleolus and NOR

Swift and Stevens (1966) indicated that most of the nucleolar mass of the normal microspore nucleolus consists of a finely filamentous or nonparticulate region. The nucleolus is surrounded by a narrow ring of particles; the particulate component is especially prominent in a nucleolar cap that possesses thread-like nucleonemal structures. Nucleolar vacuoles also are observed. In early stages of microspore development, quartet or early interphase, the particulate component is not present. In diffuse nucleoli, generated in interchange T6-9a heterozygotes, particulate regions usually are not seen. The diffuse nucleolar structure resembles the inner component of normal nucleoli, the finely filamentous portion. Diffuse nucleoli are morphologically distinctive from normal nucleoli.

Stout (1973) noted that in maize anthers of normal inbred lines prepared for electron microscopy the nucleolus is associated with the nuclear membrane from leptonema through diakinesis of prophase I. During leptonema he observed small blebs of ribosome-sized particles extending from the inner membrane into the intramembrane space of the nuclear envelope. Cytoplasm with densely packed ribosome-sized particles appears to bud off and may be found near the plasma membrane. This phenomenon was not observed after

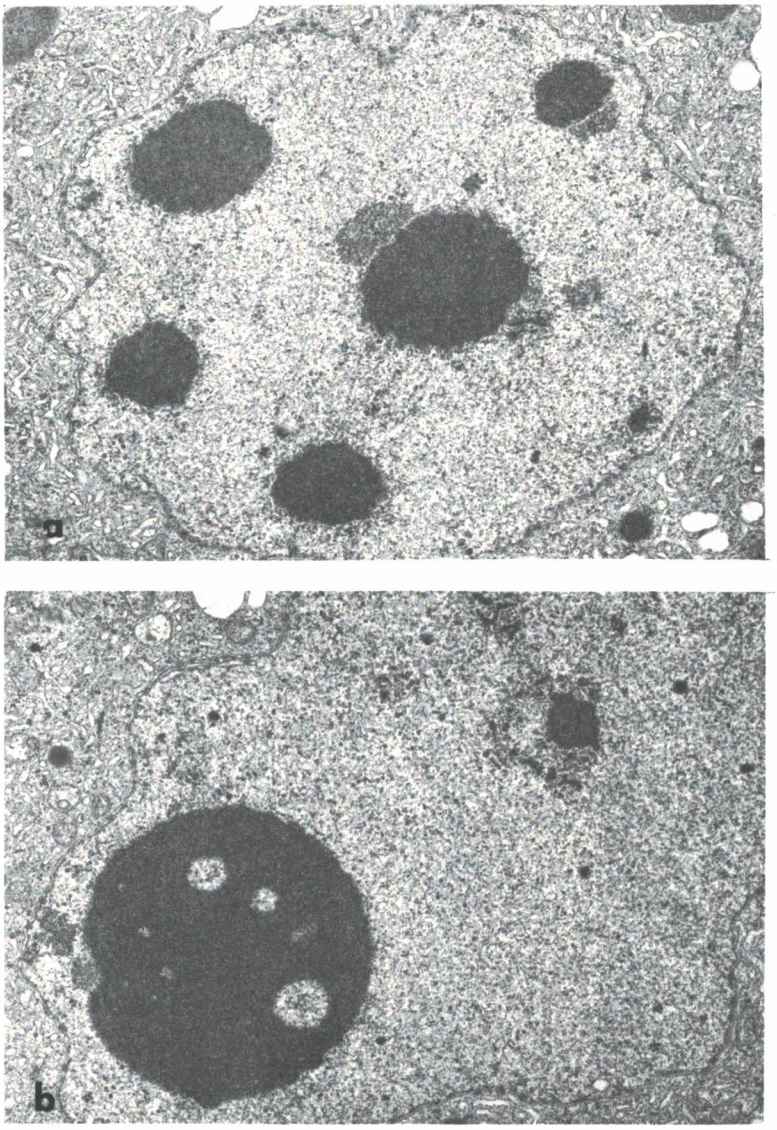


**Figure 2.** "Diffuse" (a) and normal (b) nucleoli in a 2-diffuse quartet of T5-6c heterozygote showing absence of lacunae (vacuoles) in diffuse nucleoli. Magnification × 11,400. Photograph courtesy of J. T. Stout.

zygonema. This would agree with Das's (1965) interpretation that reduced transport rate of nucleolar RNA to the cytoplasm could account for the increase in nucleolar RNA in prophase I found by Lin (1955) while RNA synthetic rate decreases. Stout also observed a lower cytoplasmic ribosome density in zygotene as compared with leptotene cells. The cytoplasmic ribosome density from diplonema to telophase II appeared to remain the same.

Stout (1973) also studied normal versus diffuse nucleoli in quartets of T5-6c heterozygotes. The diffuse nucleoli consistently lacked vacuoles or lacunae. Normal nucleoli in other cells of the same quartet contained vacuoles (Figure 2). He suggested that a functional NOR produces a vacuolated nucleolus. The diffuse nucleoli, which contained no vacuoles, may be aggregations of ribonucleoprotein normally produced by chromosome regions other than the NOR. Nonvacuolated diffuse nucleoli may indicate the absence of a functional NOR. Similar observations were made on diffuse nucleoli from T6-9a heterozygotes by Swift and Stevens (1966). The T6-9a diffuse nucleoli usually, but not always, lacked vacuoles. The T6-9a has a break in the NOR-heterochromatin, and microspores with diffuse nucleoli have a portion of the NOR present in duplicate. Perhaps a complete or partial NOR associated with the observed small vacuolated nucleoli obscured Stout's correlation of nonvacuolated nucleoli and absence of the NOR.

Stout also observed that the meiotic prophase nucleolus consists of a core of finely filamentous material and a cortex of more particulate nature. The core was not centrally located in the nucleolus but displaced to one side, such that a portion of the core became the outside surface of the nucleolus. Always at this point the NOR was associated with the nucleolus (Figure 3).

### Nucleolus Organizer Region

The NOR, as observed ultrastructurally, protrudes into the core portion of the nucleolus (Figure 3). The association of the NOR and the nucleolus is complex and gives the impression of a large amount of chromatin entering the nucleolus from the NOR, with the satellite emerging nearby. The satellite in Figure 3 terminates at the nuclear envelope. Underbrink, Ting, et al. (1967) also noted a complex junction between the NOR and the nucleolus.

Gillies (1973) reconstructed the maize pachytene nucleus from electron micrographs of serial sections and also noted a complex NOR–nucleolus association. His techniques allowed the differentiation of the NOR into a dark-staining region corresponding to the NOR-heterochromatin and a lighter-staining region interpreted as corresponding to the NOR-secondary constriction. The lighter-staining region was associated with the nucleolus in a complex manner. The serial sectioning technique allowed the reconstruction of the synaptonemal complex path through the NOR and the satellite region between

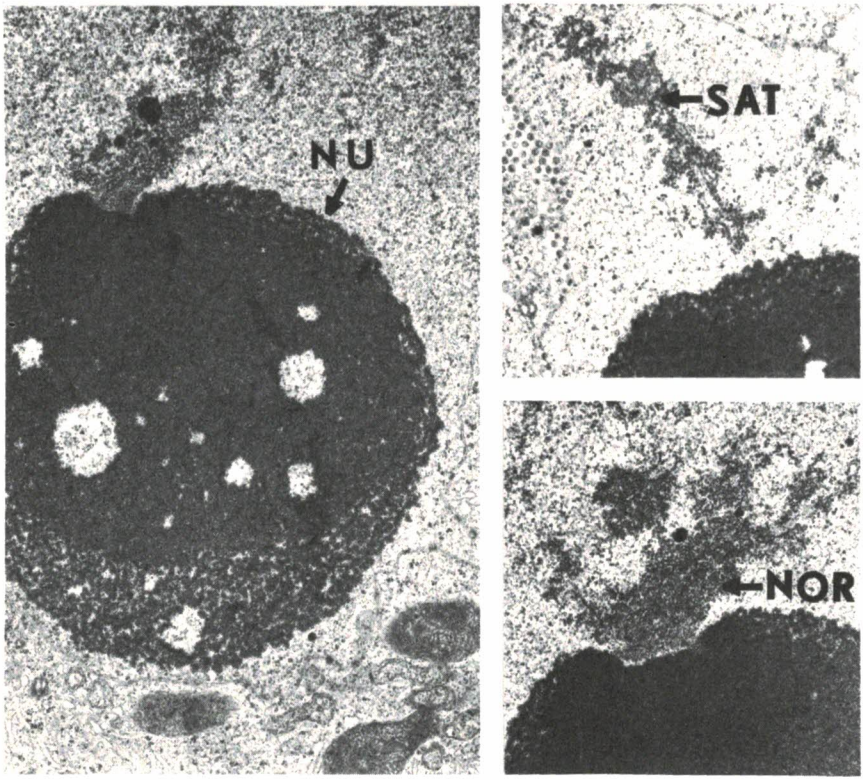


**Figure 3.** Three closely related sections through a pocket in the nucleus of a pollen mother cell at late zygonema containing the nucleolus (NU), NOR, and satellite (SAT). Magnification × 9200. Photographs courtesy of J. T. Stout.

the chromosome 6 homologs. The synaptonemal complex was present through the NOR-heterochromatin as well as the lighter-staining region and the satellite. An interesting point is that the synaptonemal complex traversed the lighter-staining region (secondary constriction), which has the complex junction with the nucleolus, without entering the nucleolus or undergoing significant morphological alterations. In light-microscopic observations of the NOR-secondary constriction, a fine thread of chromatin is often seen forming a direct and apparently uninterrupted connection between the NOR-heterochromatin and the satellite. This chromatin-thread connection seemed difficult to interpret if one assumes the argument that the NOR-secondary constriction represents a region where the chromatin is dispersed to some extent into the nucleolus. However, such a direct thread-like connection might be expected between the NOR-heterochromatin and the satellite by light microscopy if the synaptonemal complex does not enter the nucleolus but maintains a certain

amount of attached chromatin while the remainder is dispersed into the nucleolus. Gillies also reported that the fibrillar chromatin of the lighter-staining region was finer in diameter suggesting a more active state. Although perhaps reasonable to assume, there is no direct evidence that the maize nucleolus contains DNA, with the exception of preliminary biochemical evidence on isolated nucleoli referred to by McLeish (1964).

## RIBOSOMES AND POLYMERASES

The maize monoribosome has a sedimentation coefficient of 80S and can be dissociated into two subunits, 60S and 40S (Hsiao, 1964). The purified monoribosome is 43% RNA and the remainder consists of ribosomal proteins about which little is known. The RNA is characterized by high guanine and relatively high cytosine contents. The large subunit contains the 28S rRNA (Tang, 1971). The small subunit presumably contains the 18S rRNA; however, Tang (1971) obtained apparent RNA degradation products instead of the intact 18S rRNA. By using *E. coli* rRNAs as markers, Jacobson and Williams (1968) reported that maize cytoplasmic rRNAs have sedimentation coefficients of 16S and 26S. Since it is common to refer to eukaryotic ribosomal RNA subunits as 18S and 28S (Tang 1971), the more conventional nomenclature is used in this chapter. Loening (1968) gives the molecular weights for maize 18S and 28S rRNA as $0.7 \times 10^6$ and $1.3 \times 10^6$ daltons, respectively. Pring and Thornbury (1975) recently reported molecular weights of $0.67 \times 10^6$ and only $1.19 \times 10^6$ for maize 18S and 28S rRNA, respectively. The values of $0.7 \times 10^6$ and $1.3 \times 10^6$ have been used in this chapter for the rRNA gene-number determinations. Ruppel (1969) showed that maize ribosomes also contain 5S RNA. Whether the 5S RNA is located in the large ribosomal subunit is unknown.

Two DNA-dependent RNA polymerases have been shown to be present in maize nuclei (Stout and Mans, 1967; Strain, Mullinix, et al., 1971). Ribonucleic acid polymerase I is resistant to $\alpha$-aminitin inhibition, suggesting that it is the nucleolar RNA polymerase and involved in 18S and 28S rRNA synthesis (Roeder and Rutter, 1969). Ribonucleic acid polymerase II is strongly inhibited by $\alpha$-aminitin and can be resolved into two activities. Ribonucleic acid polymerase IIa is active with denatured nuclear DNA of maize, whereas IIb is more active with native DNA (Mullinix, Strain, et al., 1973). Ribonucleic acid polymerase IIa is composed of several polypeptide chains of different molecular weights.

Although the size and maturation processing of the ribosomal precursor RNA that gives rise to 18S and 28S rRNA has not been described for maize, the scheme in plants appears similar to other eukaryotes in that ribosomal RNAs of molecular weight $1.3 \times 10^6$ and $0.7 \times 10^6$ daltons both arise from the

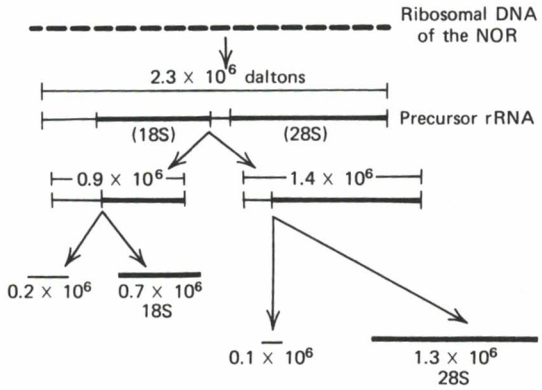


**Figure 4.** Processing of ribosomal precursor RNA to 18S and 28S rRNA. The 18S rRNA is complexed with proteins and rapidly transported to the cytoplasm where additional ribosomal proteins attach making the 40S subunit. The 28S rRNA is complexed with proteins and 5S RNA from genes in chromosome 2 (and perhaps 7S RNA also from the original $2.3 \times 10^6$ precursor rRNA) and transported to the cytoplasm where additional ribosomal proteins attach making the 60S subunit. Drawing is schematic.

selective cleavage of a larger precursor molecule. In plants, the rRNA genes transcribe a rRNA precursor molecule of about $2.3 \times 10^6$ daltons (Rogers, Loening, et al., 1970). This molecule is cleaved into two molecules of $1.4 \times 10^6$ and $0.9 \times 10^6$ molecular weights. The $0.9 \times 10^6$ molecule is rapidly cleaved to produce the $0.7 \times 10^6$ molecule (18S). The $1.4 \times 10^6$ molecule is cleaved to produce the $1.3 \times 10^6$ rRNA molecule (28S). During these maturation processes, the RNA becomes associated with characteristic proteins to produce the 60S (which also is expected to contain 5S and possibly 7S RNA) and 40S ribosomal subunits. The fate of the RNA in excess of the 18S and 28S produced by cleavage of the precursor molecule is unknown. The general scheme is illustrated in Figure 4.

In the maize cytoplasm the 60S and 40S subunits unite to form the 80S monoribosome. The 80S monosomes become associated with mRNA to form polyribosomes active in protein synthesis. Sucrose gradient profiles of maize polyribosomes reveal a peak of monosomes and several polymers of increasing size. Rapid changes in the polyribosome profile occur in maize as the result of altered physiological states, induced by such factors as water stress (Hsiao, 1970) and light patterns (Travis, Huffaker, et al., 1970).

## LOCALIZATION OF 18S AND 28S rRNA GENES

In 1955 Lin showed that nucleolar RNA content could be elevated linearly by increasing the dosage of a portion of the NOR. He concluded that "the func-

tion of the nucleolar organizer involves the actual synthesis of nucleolar material; the organizer is doing something more than merely serving as a pump or a reservoir for the collection and organization of the matrix material, or some other material produced by the chromosomes, into a single body." This work pioneered the understanding of the NOR in terms of its synthetic capacity.

Now there are at least six lines of evidence indicating that the NOR of maize is the chromosomal site of the DNA complementary to 18S and 28S rRNA. The first demonstration, shown by our laboratory in 1971 (Phillips, Kleese, et al., 1971), involved the comparison of rRNA gene numbers by DNA/rRNA hybridization techniques of inbreds W23 and A188 versus a genetic marker strain possessing a NOR consisting of two heterochromatic portions separated by a secondary constriction. This NOR condition was termed "2NOR" (Figure 5). The nucleolar volume of the 2NOR strain at midpachynema was 64% larger than the average of the two inbreds. The large 2NOR–nucleolus segregated with the 2NOR–chromosome 6. The percent DNA hybridizable to 18S and 28S rRNA for the 2NOR strain was found to be twice the amount for A188 and more than twice W23. The NOR of maize was concluded to be the chromosomal site of the 18S and 28S rRNA genes. Further support for this conclusion was provided by showing that plants trisomic for chromosome 6

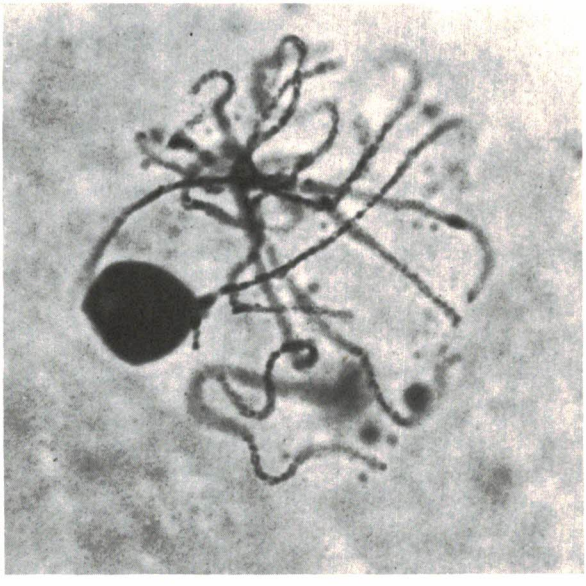

**Figure 5.** Pachytene cell of the 2NOR strain showing the two heterochromatic segments separated by the secondary constriction with attached nucleolus. Magnification × 1600.

possess approximately 50% more rRNA genes than their diploid sibs and that plants monosomic for chromosome 6 carry approximately half as many rRNA genes as the parent that contributed the chromosome to the monosomic progeny (Phillips, Weber, et al., 1974).

Perhaps the best evidence for any eukaryote that the rRNA genes are actually within the NOR and not in neighboring regions is that presented by Givens and Phillips (1976). The procedure involved the production of duplications by the method of Gopinath and Burnham (1956), where selfpollinations of intercrosses between appropriately chosen interchanges involving the same two chromosomes yield duplications of the between-breaks segments of each chromosome. Plants were produced carrying a duplication of either the NOR-heterochromatin or the site giving rise to the secondary constriction. These plants also carried a short duplication in either chromosome 1 or 2, but the previous evidence cited indicates that these chromosomes are not expected to carry rRNA genes. Hybridization of DNA from plants heterozygous for the duplication of the NOR-heterochromatin was approximately 50% higher than the controls. The heterochromatic portion of the NOR, therefore, contains most of the rRNA genes present in maize. As a check, similar experiments were performed using DNA from plants heterozygous for the duplication of the secondary constriction. Given that most of the rRNA genes are in the NOR-heterochromatin, the hybridization level using DNA from plants carrying the duplication of the secondary constriction would not be expected to be higher than the controls; this was observed. Such a result does not necessarily indicate that the secondary constriction carries no rRNA genes, but that it carries either none or a number below the limits of our error in the DNA/rRNA hybridization procedure. As this error is 10% or less, the conclusion is that the NOR-heterochromatin possesses 90% or more and the NOR-secondary constriction 10% or less of the total rRNA genes. Since maize has a high number of rRNA genes, 10% represents a rRNA gene multiplicity that is not dissimilar to that of many animals.

That the NOR of maize is the site of the 18S and 28S rRNA genes also is supported by DNA/rRNA hybridization experiments (Doerschug, 1976; Ramirez and Sinclair, 1972) with a translocation between the NOR of chromosome 6 and a B chromosome (TB-6a). Due to the property of nondisjunction of the B chromosome centromere at the second postmeiotic division in microsporogenesis (Roman, 1947), maize plants can be obtained with varying numbers of the interchanged $B^6$ chromosome. Since the break of TB-6a is near the center of the NOR-heterochromatin (Phillips, unpublished), plants may be obtained with multiple doses of the segment distal to the break. This segment includes the distal half of the NOR-heterochromatin and the secondary constriction, as well as the satellite. Based on DNA/rRNA hybridization results using DNA from hyperploid plants with from one to seven $B^6$ chromosomes, Doerschug (1976)

estimates that the break occurred such that approximately half of the rRNA genes are in the $6^B$ chromosome and half in the $B^6$ chromosome.

The last line of evidence to be mentioned that the maize NOR is the site of rDNA comes from *in situ* hybridization experiments by Wimber, Duffey, et al. (1974). The placement of the ribosomal RNA genes to the distal end of the short arm of chromosome 6 was confirmed by annealing $^{125}$I-labelled 18S and 28S rRNA to pachytene chromosomes. They state that "the only obvious labelled region was the nucleolus."

The placement of most if not all of the rRNA genes to the NOR appears to be more precise for maize than for any other higher organism. The well-known rDNA localization studies using DNA extracted from such stocks as the NOR-duplications and deficiencies in *Drosophila* (Ritossa and Spiegelman, 1965), the anucleolate mutant of *Xenopus* (Birnstiel, Wallace, et al., 1966) and the *bobbed* mutants of *Drosophila* (Ritossa, Atwood, et al., 1966) place the rRNA genes only in the vicinity of the NOR and not unequivocally within the structure. The *in situ* DNA/rRNA hybridization technique (Pardue, Gerbi, et al., 1970) also does not provide the resolution required for as precise a placement as has been accomplished for maize.

## RIBOSOMAL RNA GENE MULTIPLICITIES

Variation in rRNA gene multiplicity is great among higher plant species. Cullis and Davies (1974) indicated that the range represents a 17-fold difference, from a DNA hybridization percentage of 0.022 or 1580 rRNA gene/2C nucleus for the artichoke, *Helianthus tuberosus* (Ingle and Sinclair, 1972), to a 3.1% hybridization value or 27,000 rRNA genes for cucurbit, *Cucurbita maxima* (Goldberg, Bemis, et al., 1972). We have found the range in rRNA gene multiplicities for maize to be nearly as great as the range reported for all higher plant species. Table 1 includes the rRNA gene multiplicities for various inbred lines utilized in the corn-breeding industry, and thus they may be considered as agronomically desirable lines. The lines were chosen from a list of the 25 most widely used publicly developed lines of corn (Horsfall, 1972). Seventeen of the 25 were tested along with four additional lines (Wf9, A188, A153, and W23). The range in rRNA gene multiplicities among the inbreds is 5,000–12,000 per 2C nucleus. A somewhat smaller range based on data from 10 of these inbreds was reported previously (Phillips, Wang, et al., 1973).

Feulgen microspectrophotometric DNA measurements of these 20 lines indicated that although there were differences in DNA values among the various lines, no correlation existed between DNA amount and rRNA gene multiplicity.

TABLE 1. Variation in rRNA gene multiplicity among inbred lines of maize[a]

| *Inbred* | *Ribosomal RNA gene multiplicity* ± *S.E.*[b] | *Inbred* | *Ribosomal RNA gene multiplicity* ± *S.E.* |
|---|---|---|---|
| W117 | 12,000 ± 450 | A634 | 7,400 ± 370 |
| Mo17 | 10,600 ± 240 | W64A | 7,300 ± 900 |
| B37 | 10,100 ± 250 | A619 | 7,200 ± 390 |
| C103 | 9,900 ± 270 | A188 | 7,000 ± 370 |
| Oh43 | 9,400 ± 270 | C123 | 6,500 ± 300 |
| B14 | 8,500 ± 270 | A153 | 6,400 ± 435 |
| Wf9 | 8,400 ± 250 | A635 | 6,200 ± 150 |
| A632 | 8,300 ± 200 | A554 | 6,100 ± 240 |
| N28 | 8,000 ± 120 | H84 | 5,500 ± 60 |
| B57 | 7,600 ± 600 | W23 | 5,000 ± 100 |
| A239 | 7,400 ± 250 | | |

[a] Deoxyribonucleic acid/rRNA hybridization followed previously published procedures (Phillips, Kleese, et al., 1971; Phillips, Weber, et al., 1974) using 6-day-old seedlings. Ribosomal RNA gene multiplicities based on minimum of six determinations per inbred. Hybridization done as one experiment using A188 $^{3}$H-uridine-labeled rRNA under saturating conditions at 10 $\mu$g rRNA/ml. All gene multiplicities are per 2C nucleus.

[b] S.E. = standard error.

Certain inbreds in Table 1 with a high level of relatedness have retained comparable levels of rRNA gene multiplicities, while others have not. For example, A632 and B14 are closely related and have similar rRNA gene multiplicities, whereas A634 and B14 are related to the same degree but have some divergence in their rRNA gene multiplicities. An explanation cannot be offered without further study. This range in rRNA gene multiplicities among inbred lines of a single species should in the future provide the basis for relating the number of rRNA genes to various biochemical or agronomic characteristics.

The lowest rRNA gene multiplicity observed in maize is that of the *sticky chromosome* (*st*) mutant conditioned by a single locus in the short arm of chromosome 4 discovered by Beadle (1932). The mutant is generally characterized by the stickiness of the chromosomes at the meiotic anaphases giving chromatin bridges, but chromosomes of other stages also are abnormal in various ways. High ovule and pollen sterility, scarring of the endosperm in mature *st* kernels, and small plants with leaf striations were common characteristics. The *st* lines currently available give a variable expression of the *st* phenotype in terms of cytology, sterility, and endosperm and plant phenotypes (Stout, 1973).

Stout (1973) made several observations that bear on a possible relationship between the *st* locus in chromosome 4 and the NOR of chromosome 6. The

NOR of *st* plants was decidedly different from normal (Figure 6). The NOR-heterochromatin was abnormally small and often not particularly apparent. Instead, the locus of the normally heterochromatic portion of the NOR frequently possessed chromatin that appeared diffuse. The secondary constriction was quite large as was the nucleolus. Stout also observed a chromatin vesicle in the *st* homozygotes from the original seed and in certain $F_3$ families. The vesicle had a dark-staining reticulate core and a lighter-staining surrounding matrix. The vesicle was associated with the NOR or nucleolus more often than with any other part of the nucleus, either attached or unattached to other

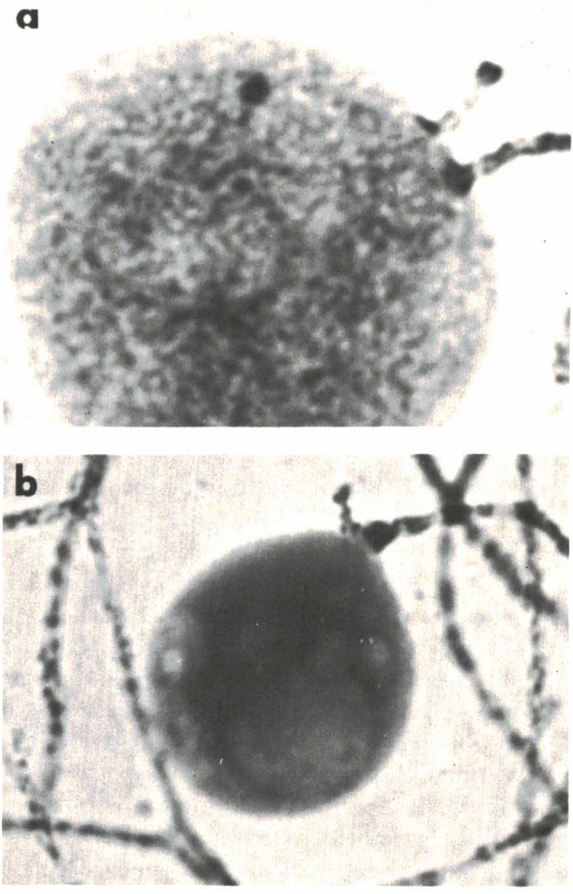


**Figure 6.** Comparison of the *st* NOR (*a*) and the NOR of the next lowest rRNA gene multiplicity line (W23) (*b*) at pachynema. Note small NOR-heterochromatin and large secondary constriction in *st* plant. Magnification × 4200. Photographs courtesy of J. T. Stout.

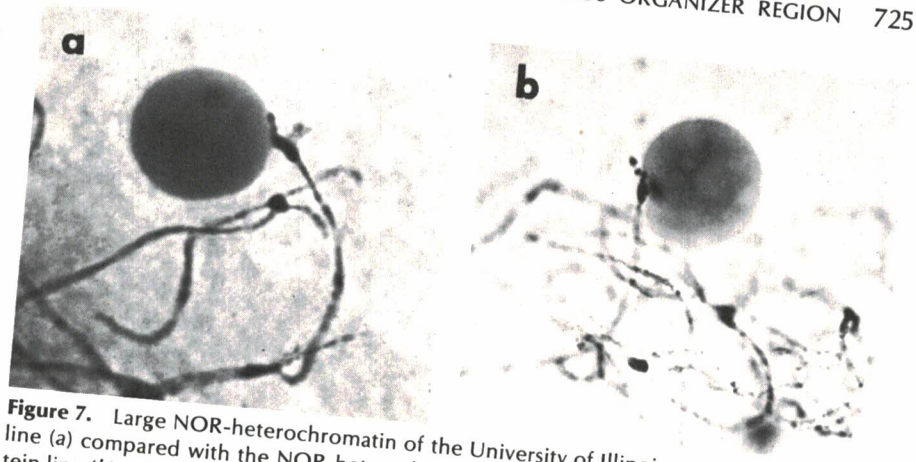


**Figure 7.** Large NOR-heterochromatin of the University of Illinois reverse high-protein line (*a*) compared with the NOR-heterochromatin of the University of Illinois high protein line (*b*). Magnification × 1500.

chromosomes. The abnormal appearance of the *st* NOR-heterochromatin and the occurrence of a vesicle led us to perform DNA/rRNA hybridization experiments using DNA from the *st* homozygote (Stout, 1973). A value of about 3300 rRNA genes was obtained—the lowest rRNA gene multiplicity observed to date for any maize line. The cytologically anomalous NOR is probably a manifestation of this low rRNA gene multiplicity. Whether the observed vesicle contains rDNA and whether the NOR phenotype is somehow controlled by the *st* locus in chromosome 4 await further experimentation. Two other single gene variants of maize have been studied for rDNA content. Both mutants, *elongate* (*el*) and *ragged* (*rgd*) located in chromosome 6 near the NOR, have normal rRNA gene multiplicities (Stout 1973; Phillips, Weber, et al., 1974).

The highest rRNA gene multiplicity thus far observed for maize was discovered while investigating the protein lines selected by University of Illinois investigators since 1896 (see pp. 736–738). The reverse high protein (RHP) line was found to possess 23,100 rRNA genes. Since this value was unusually high, the strain was studied cytologically with special attention focused on the NOR. A strikingly large NOR was observed at pachynema; the NOR-heterochromatin was several times larger than comparable segments in other strains (Figure 7).

Another line with a high rRNA gene multiplicity is the 2NOR strain. In 1971 we compared the 2NOR strain with inbreds W23 and A188 and found that the 2NOR strain possesses twice the A188 number of rRNA genes (Phillips, Kleese, et al., 1971). Our current estimate of the rRNA gene multiplicity for A188 is lower than reported in 1971 (the W23 rRNA gene multiplicity was not reported) as the result of modifying our estimate of the amount of DNA/2C nucleus used for calculating rRNA gene numbers (Phillips, Weber, et al., 1974)

TABLE 2. Ribosomal RNA gene multiplicities of Wf9 lines with normal and T, C, S, and J male-sterile cytoplasms[a]

| *Wf9* | *Wf9 Tcms* | *Wf9 Ccms* | *Wf9 Scms* | *Wf9 Jcms* |
|---|---|---|---|---|
| 8100[b] | 7600 | 6600 | 6600 | 6500 |

[a] Seed kindly provided by C. A. Laible, Funk Seed International, Bloomington, Illinois. The number of backcrosses to Wf9 for the T, C, S, and J *cms* lines is 12, 10, 10, and 7, respectively.

[b] Data average of two experiments each with eight determinations using saturating levels of A188 $^3$H-rRNA. Deoxyribonucleic acid was extracted from 6-day-old seedlings. Duncan's multiple range test was used to determine significance. The cms lines are not significantly different; however, the Wf9 C, S, and J cms lines are significantly different from Wf9.

and a calibration problem with the spectrophotometer. Thus our estimate for the 2NOR rRNA gene multiplicity is 14,000. Genetic alterations in rRNA gene multiplicities have been demonstrated in animal species as the result of aneuploidy or gene mutation. Such changes in rRNA gene multiplicities, termed rDNA compensation (Tartof, 1973) for somatic alterations and rDNA magnification (Ritossa, 1968) for germ-line changes, were sought in maize by generating plants monosomic for chromosome 6 and determining their rRNA gene multiplicity as well as that of their progeny (Phillips, Weber, et al., 1974). The results were as expected assuming additivity and no disproportionate replication of the rDNA. Additiveness seems to be a general principle for rRNA gene multiplicities in maize. Hybrids between lines of known rRNA gene multiplicities (Phillips, unpublished) and various aneuploids (Doerschug, 1976, Phillips, Weber, et al., 1974) have been shown to behave in an additive fashion. To my knowledge, no well-documented exceptions to this general rule exist at the present time.

Another question of interest regarding rRNA gene multiplicity is whether it is influenced by the cytoplasm. Through extensive backcrossing, lines are available with the nuclear genetic constitution of inbred Wf9 and different cytoplasms. Beckett (1971) grouped the various sources of cytoplasms that confer male sterility into the S, C, and T groups. The J cytoplasmic male sterile (CMS) was included in the S group, according to the methods employed. Deoxyribonucleic acid from Wf9 T, C, S, and J CMS lines and normal cytoplasm Wf9 were hybridized to A188 rRNA. The results (Table 2) indicate that the sterile cytoplasms do not cause an increase in rRNA gene multiplicity. The various Wf9 CMS lines were not significantly different. However, the Wf9 C, S, and J CMS lines were significantly different from Wf9 with normal cytoplasm. Since these converted lines are the result of seven to twelve backcrosses to Wf9, it would be unlikely that the differences are simply a matter of not recovering the Wf9 rRNA gene constitution. Crossing over at the NOR during the conversion process could give rise to these differences.

The foregoing discussion focused on the multiplicity of 18S and 28S rRNA genes. Since 5S RNA is also part of the ribosome and is present in the nucleolus prior to its transport to the cytoplasm as part of the large subunit, knowledge on the 5S RNA gene multiplicity is important to an understanding of the NOR function. Based on *in situ* DNA/5S RNA hybridization, the genes for 5S RNA appear to be clustered near the end of the long arm of chromosome 2 (Wimber, Duffey, et al., 1974). Multiple copies of the 5S RNA genes are indicated by the autoradiographic observation of a heavily labeled chromosomal region. A high multiplicity would be expected, extrapolating from animal systems where the number of 5S RNA genes is usually greater than the number of 18S and 28S rRNA genes (Ford, 1973). Our preliminary DNA/5S RNA hybridization experiments suggest that 5S RNA genes of maize also are present in many thousands of copies; the data are not sufficient to give a gene multiplicity value at this time.

## FUNCTIONAL MAP OF THE NOR

In 1931 Heitz reported on the physical relationship between the nucleolus and a particular chromosomal site. The nucleolus was shown to form in a similar manner in association with the same daughter chromosomes in daughter telophase nuclei. The nucleolus appeared to develop in association with the secondary constriction. The theory was advanced that the secondary constriction actually formed the nucleolus. McClintock (1934) challenged this idea in 1934 as a result of her study of nucleolus–chromosome associations in maize. She studied a chromosomal interchange which divided the NOR-heterochromatin in two parts (Figure 8). The break in this interchange (T6-9a) occurred such that the proximal two-thirds of the NOR-heterochromatin was separated from the distal one-third of the heterochromatin and the contiguous secondary constriction. In the homozygote, the proximal NOR-heterochromatic

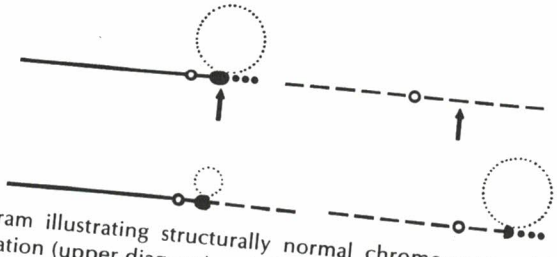

**Figure 8.** Diagram illustrating structurally normal chromosomes with typical NOR–nucleolus association (upper diagram) and chromosomes of an NOR-interchange with a break in the NOR-heterochromatin showing differential functional capacities of the NOR-segments (lower diagram).

segment formed a small nucleolus, whereas the distal part formed a larger nucleolus. Thus, the heterochromatic body located adjacent to the secondary constriction had the capacity to form a nucleolus. The fact that in T6-9a homozygotes a larger nucleolus is formed by the distal one-third of the NOR-heterochromatin while possessing 50% or less of the total rRNA genes (Doerschug, 1976), suggests that all of the rRNA genes may not be concomittantly functional in nucleolar formation. The question then is whether the secondary constriction has a nucleolus-forming capacity as believed by Heitz. Through elegant analyses of T6-9a and several other stocks, McClintock concluded that whereas the nucleolus formed as a result of activity of the NOR-heterochromatin, the secondary constriction formed as a passive result of nucleolar growth. The heterochromatin was referred to as the "nucleolar organizing body or element." In addition to the interchange strain, McClintock studied two other strains in which the nucleolus appeared to form at places other than the distal end of the NOR-heterochromatin. The nucleolus of one strain formed near the center of the heterochromatin. In another strain the nucleolus formed at the proximal end; in this case the NOR-heterochromatin was on the satellite side of the nucleolus creating a large satellite. These observations suggested that the functional site of the NOR in terms of nucleolar formation may be at any one of three locations— distal, central, or proximal.

That the NOR has three potential sites of activity also has been indicated from studies in our laboratory of additional maize interchanges with a break in the NOR. At least 20 interchanges (termed *NOR-interchanges* for the purposes of this chapter) are available today with a break in the NOR at various sites (Phillips and Wang, 1972). At pachynema in plants homozygous for NOR-interchanges, two bivalent chromosomes are associated with a nucleolus, making exact the placement of the break in the NOR. A break immediately proximal to the NOR in the short arm of chromosome 6 or immediately distal in the satellite results in only one chromosome pair associated with the nucleolus. The association of both interchanged bivalents with the nucleolus in NOR-interchange homozygotes also allows the precise placement of breakpositions in the other chromosome involved in the interchange. Thus the cytological placement of the breaks is more accurate than is possible for most maize interchanges. When the break is in the NOR, indicated by two pachytene bivalents associated with a single nucleolus or each with a separate nucleolus, there may or may not be an obvious segment of the NOR-heterochromatin translocated. When a portion of the heterochromatin is translocated, the result is apparent and a breakposition can be assigned in the NOR-heterochromatin by measuring the two heterochromatic portions. When there is no obvious heterochromatin translocated, but there are two bivalents with nucleolar associations, the break is assumed to have occurred in the distal site that forms the secondary constriction in these strains. The two resultant interchanged chromosomes may

each form an obvious secondary constriction. The average lengths of the two secondary constrictions were taken to indicate the position of the break in the site giving rise to the secondary constriction. Although the measurement is subject to error, we have assigned the breaks to the proximal part of the secondary constriction near the heterochromatin, about 0.25 of the heterochromatin-satellite distance, or midway (Table 3). There were no indications of any interchange break beyond midway. Seven of the interchanges have a break in the NOR-heterochromatin and 12 in the secondary constriction.

Nucleolar volumes were determined using pachytene cells with two nucleoli. The data are presented (Table 3 and Figure 9) as the volume of the nucleolus associated with the proximal part of the NOR expressed as a percentage of the total nucleolar volume. Apparent is the fact that nucleolar size and, therefore, the functional capacity of the NOR portion, depends on the position of the break in the NOR-heterochromatin or the secondary constriction. Perhaps the most striking observation is for interchange T2-6(8786). The break is close to the distal end of the heterochromatin, resulting in chromosome bivalent $6^2$ possessing nearly all of the NOR-heterochromatin. An extremely small amount

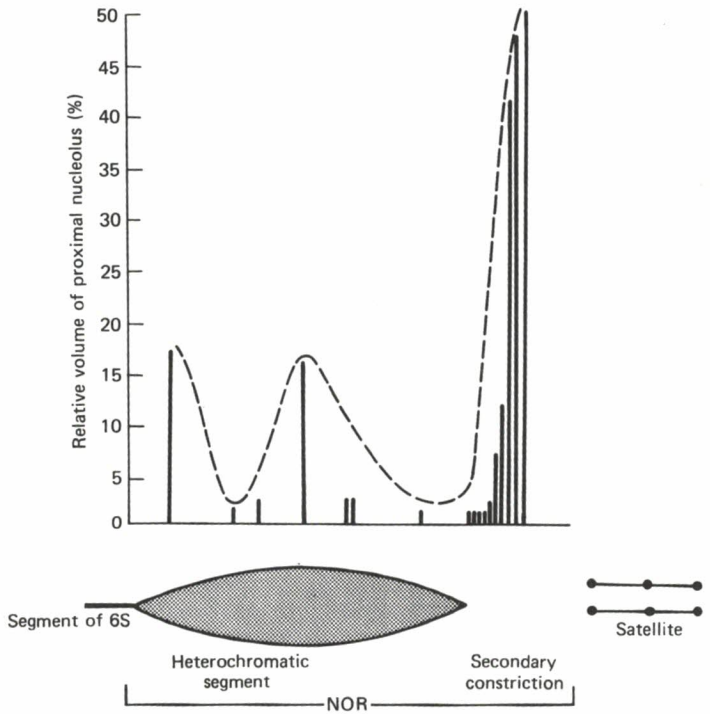


**Figure 9.** Functional map of NOR.

TABLE 3. Functional capacities of portions of the NOR when separated in homozygous NOR-interchange stocks

| | *Breakpoints* | | *Relative proximal nucleolar volume (%)*[a] |
|---|---|---|---|
| *Interchange* | *Chromosome 6* | *Other* | |
| 1-6Li[b] | S.C.[c]—prox. | 1L.81 | 0.2 |
| 1-6(4986) | S.C.—prox. | 1S.11 | 0.8 |
| 1-6(6189) | Het. 0.10 | 1S.50 | 17.8 |
| 1-6(8415) | S.C.—prox. | 1L.31 | 0.01 |
| 2-6(5419) | S.C.—0.25 | 2L.82 | 13.7 |
| 2-6(8441) | S.C.—prox. | 2L.95 | 0.1 |
| 2-6(8786) | Het. 0.88 | 2S.97 | 0.1 |
| 2-6(027-4) | S.C.—prox. | 2L.04 | 0.6 |
| 3-6(030-8) | S.C.—0.25 | 3S.05 | 1.6 |
| 3-6(032-3) | S.C.—midway | 3S.34 | 50.0[d] |
| 4-6(4341) | Het. 0.50 | 4S.36 | 15.5 |
| 5-6f | S.C.—midway | 5S.23 | 47.3[d] |
| 5-6(8696) | S.C.—midway | 5L.79 | 42.0[d] |
| 6-7(4964) | Het. 0.32 | 7L.67 | 1.3 |
| 6-7(5181) | Het. 0.71 | 7L.85 | 2.6 |
| 6-7(035-3) | S.C.—0.25 | 7L.59 | 5.8 |
| 6-9a | Het. 0.67 | 9L.32 | 2.5 |
| 6-9d | Het. 0.46 | 9L.84 | 3.3 |
| 6-10(5519) | S.C.—prox. | 10L.10 | 8.0 |

[a] Nucleolar volumes were determined using diameter measurements from *camera lucida* drawings of three cells from each homozygous interchange and converting to volumes assuming a spherical shape. The relative proximal nucleolar volume is the volume of the nucleolus associated with the proximal portion of the NOR when two nucleoli were formed divided by total nucleolar volume (expressed in percentage).

[b] Originally reported as a T4-6 interchange (Stout and Burnham, 1968).

[c] S.C. = Secondary constriction, Het. = NOR-heterochromatin.

[d] In all three interchanges with breaks that appear to have divided the secondary constriction into two equal parts, two nucleoli were never observed at pachynema, even though over 2000 pachytene cells per interchange were studied. The nucleolar sizes were determined at the quartet stage in these cases.

of nucleolar material, 0.1% of the total nucleolar volume, is associated with nearly the entire NOR-heterochromatin. In these pachytene cells with two nucleoli, the large nucleolus is formed by the small distal piece of the NOR-heterochromatin and the secondary constriction. The nearly complete NOR-heterochromatin has little functional capacity in pachytene cells in which the small distal piece of the NOR-heterochromatin and secondary constriction also

are present. Confirmation of this conclusion comes from an independent study of the previously mentioned stock carrying a duplication of the NOR-heterochromatin generated in the $F_2$ of intercrosses of interchanges involving the same two chromosomes (Givens and Phillips, 1976). The bivalent chromosome carrying only the NOR-heterochromatin (and none of the original secondary constriction) in homozygous condition had no associated nucleolar material in 71% of the pachytene cells ($N = 324$). When that chromosome was associated with a separate nucleolus (13% of the cells), the nucleolus was extremely small representing only 0.6% of the total nucleolar volume. The remaining cells (16%) had both chromosome bivalents associated with one nucleolus.

In contrast to the T2-6(8786) interchange, others with a break in the NOR-heterochromatin revealed a greater functional capacity of the proximal portion of the NOR-heterochromatin. The functional capacity, however, was not a linear function of the NOR breakposition. Maximum activities occurred when the break was either near the midpoint of the NOR-heterochromatin or near the proximal end (Figure 9). Interchanges with breaks between these sites revealed lower activities associated with the proximal NOR segment.

Interchanges with a break in the secondary constriction gave activities that may be a linear function of the breakposition. Little activity was associated with the proximal NOR segment when the break was near the proximal end of the secondary constriction. A larger nucleolus was associated with the proximal portion when the break was at 0.25 in the secondary constriction. Pachytene cells of T3-6 (032-3), T5-6f, and T5-6 (8696) with breaks about midway in the secondary constriction contained only one nucleolus; two nucleoli were not observed in more than 2000 pachytene cells of each interchange. However, two nucleoli of approximately equal volumes were observed at the quartet stage in homozygotes of all three interchanges.

Our interpretation of these results is that the NOR has three potential sites of activity. We hypothesize that these interchanges arose in maize strains in which the distal site was active, at least as expressed in prophase I of microsporogenesis. When the break occurred in this site, activity was divided proportionately between the two segments and formed appropriately sized secondary constrictions. The other two potential sites of activity in these stocks are inactive in meiosis of microsporogenesis unless a break occurs in or near one of them. Such a break, then, allows a small amount of activity and reveals their existence. This conclusion is in basic agreement with McClintock's theory regarding the existence of three potentially functional sites. The difference is that these results suggest that the secondary constriction does have a functional capacity. This idea also is supported by observations on nucleolus–chromosome associations in plants heterozygous for a duplication of the secondary constriction (Givens and Phillips, 1976). In these plants, all three of the secondary constrictions were associated with a nucleolus in 97–100% of the pachytene cells.

This result is unlike that for the duplication of the NOR-heterochromatin where all heterochromatic segments were associated with a nucleolus in only 29% of the pachytene cells. Therefore, the secondary constriction appears to possess a functional capacity, even when duplicated in a cell and physically separated from the NOR-heterochromatin in a chromosome other than chromosome 6.

Coupling the cytological observations on the interchange and duplication stocks with the previously mentioned DNA/rRNA hybridization data on the duplication stocks, the interpretation is advanced that although most of the rRNA genes are in the NOR-heterochromatin the secondary constriction appears to be the site primarily responsible for nucleolus formation (at least in pachynema of microsporogenesis). Also, two secondary sites appear to be located in the NOR-heterochromatin in these strains—one near the midpoint and one at the proximal end.

Electron-microscopic observations of transcribing rRNA genes in primary spermatocyte nucleoli of *Drosophila hydei* show that a maximum of one-half of the rRNA genes are transcribing at any one time and the number varies with developmental stage (Meyer and Hennig, 1974). Regulation of rRNA synthesis appears to occur primarily by the activation or inactivation of groups of adjacent rRNA genes. The NOR primary and secondary sites suggested for maize may represent groups of rRNA genes coordinately controlled and active at various developmental stages.

The question arises as to the purpose of the thousands of rRNA genes present in maize nuclei. The argument can be made that most of them are not active in any one strain since (a) animal species usually possess rRNA genes in the hundreds rather than the thousands, (b) maize strains differ so vastly in rRNA gene multiplicities, and (c) the region that appears primarily responsible for nucleolus formation, the site giving rise to the secondary constriction, contains relatively few rRNA genes. However, one can argue that evolution does not generate an inefficient genetic system and that all of the rDNA must be useful at certain developmental stages or under certain stress conditions. A supporting fact is that all plant genera studied thus far have more than 1500 rRNA genes per 2C nucleus, and, therefore, high rRNA gene multiplicities must be important to plant development and survival. One view is that plants meet peak rRNA demands by transcribing more of the rRNA genes already present in the cell rather than amplifying their rDNA (Phillips, Weber, et al., 1974). The final answer to this question will provide important information on genetic regulation in higher plants.

Considerable information can also be gained on the functional capacity of NOR segments from plants *heterozygous* for NOR-interchanges. Meiotic products of these plants principally are of four chromosome constitutions. For example, T6-9a (breakpoints: NOR-heterochromatin 0.67, 9L.32) produces microspores with chromosome combinations $6 + 9$, $6^{9} + 9^{6}$, $6^{9} + 9$, and $6 + 9^{6}$.

McClintock (1934) showed that chromosome $6^9$ in $6^9 + 9$ microspores formed a normal sized nucleolus at prophase of the first postmeiotic division, whereas it formed a small nucleolus in $6^9 + 9^6$ microspores. Therefore, the proximal 67% of the NOR-heterochromatin has the potential of forming a normal-sized nucleolus and exhibits this potential when the distal portion is not present in the cell. This observation is consistent with the idea of three potential sites of NOR-activity (proximal, central, and distal). The secondary site(s) may become active on deletion of the primary site of that strain. Now that several NOR-interchanges are available, the functional capacity of various proximal portions of the NOR can be assayed in the absence of the distal portion. The results (Table 4) show that the proximal portion of the NOR-heterochromatin can form a normal nucleolus in every case when the distal portion is absent, even in the extreme case of T1-6(6189) in which the break is at 0.10 in the NOR-heterochromatin. The segment proximal to 0.10 in the heterochromatin can function to apparently normal capacity in the absence of the remainder of the NOR. This supports the idea of a site of potential activity at the proximal end of the NOR-heterochromatin.

Microspores from T6-9a heterozygotes of the $6 + 9^6$ chromosome constitution possess a diffuse nucleolus at prophase of the first postmeiotic division as reported by McClintock (1934) and reconfirmed in our laboratory (Table 4). McClintock suggested that the diffuse nucleolus may have been the result of deleting a part of 9L; that is, 9L may carry a gene(s) necessary for normal nucleolus formation. If this interpretation is correct, the gene must be between 9L.32 and 9L.84, since T6-9d does not produce microspores with diffuse nucleoli. Another such gene may reside between 7L.59 and 7L.85, since T6-7(035-3) heterozygotes produce diffuse nucleolated microspores whereas none occur in T6-7(5181) heterozygotes. The T6-10(5519) heterozygotes also produce microspores with diffuse nucleoli at a relatively high frequency (11%), but mostly in the microspore type $6^{10} + 10$, deficient for a portion of the NOR and duplicate for a portion of chromosome 10. Although not understood, it is important to note that either of the two unbalanced chromosome combinations can lead to a diffuse nucleolus.

Another important observation is that the appearance of a diffuse nucleolus at prophase of the first postmeiotic division does not mean that a diffuse nucleolus existed at the quartet stage (Table 4—see T6-9a for example). A nucleolus apparently can form immediately following meiosis in certain cases and then become diffuse. The reverse also is observed as in T1-6 Li in which 16% of the quartet cells possessed a diffuse nucleolus while diffuse nucleoli were not observed at the first postmeiotic prophase. In these cases, a diffuse nucleolus appears able to coalesce into a single nucleolus of normal appearance. The homozygous anucleolate mutant of *Xenopus* also may form nucleoli that appear normal in older embryos (Barr, 1966). The genetic cause of diffuse

TABLE 4. Nucleolar constitution of microspores in the first postmeiotic prophase or quartet stages of NOR-interchange heterozygotes

| Interchange | Breakpoints: Chromosome 6 | Breakpoints: Other | Spore types: 6 + X | Spore types: $6^x + X^6$ | Spore types: $6^x + X$ | Spore types: $6 + X^6$ | Total cells | Prophase microspores with diffuse nucleolus (%) | Quartet microspores with diffuse nucleolus (%) | Total cells (no. quartets × 4) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-6 Li | S.C.[a]—prox. | 1L.81 | 1N(60)[c] | 1N(50)2U(2) | 1N(52) | 1N(60) | 224 | 0 | 16.0 | 1680 |
| 1-6(4986) | S.C.—prox. | 1S.11 | 1N(54) | 1N(41)2U(3) | 1N(42)D(8) | 1N(40) | 192 | 4.2 | 12.5 | 2128 |
| 1-6(5495) | S.C.—prox. | | 1N(51) | 1N(35)2U(1) | 1N(60) | 1N(74) | 221 | 0 | 1.1 | 2488 |
| 1-6(6189) | Het.[b] 0.10 | 1S.50 | 1N(30) | 1N(30) | 1N(34) | 1N(25) | 119 | 0 | 0.8 | 2816 |
| 2-6(027-4) | S.C.—prox. | 2L.04 | 1N(46) | 1N(35)2U(13) | 1N(48) | 1N(38)2U(1)D (5) | 186 | 2.7 | 8.5 | 2152 |
| 2-6(8786) | Het. 0.88 | 2S.97 | 1N(80) | 1N(26)2U(14) | 1N(45) | 1N(55) | 220 | 0 | 17.4 | 2392 |
| 2-6(5419) | S.C.—0.25 | 2L.82 | | | | | | | 1.2 | 2160 |
| 3-6(030-8) | S.C.—0.25 | 3S.05 | 1N(52) | 1N(35)2U(6) | 1N(46) | 1N(48)D(2) | 189 | 1.1 | 2.2 | 2276 |
| 3-6(032-3) | S.C.—midway | 3S.34 | | | | | | | 9.2 | 2328 |
| 4-6(4341) | Het. 0.50 | 4S.36 | 1N(65) | 1N(60) | 1N(54) | 1N(29) | 208 | 0 | 9.7 | 2116 |
| 5-6f | S.C.—midway | 5S.23 | 1N(70) | 1N(69) | 1N(70) | 1N(10) | 219 | 0 | 0.1 | 2292 |
| 5-6(8696) | S.C.—midway | 5L.79 | 1N(45) | 1N(45)2U(9) | 1N(31) | 1N(45) | 175 | 0 | 18.6 | 2768 |
| 6-7(4964) | Het. 0.32 | 7L.67 | 1N(28) | 1N(22)2U(11) | 1N(24) | 1N(23)D(1) | 109 | 0.9 | 9.7 | 2416 |
| 6-7(035-3) | S.C.—0.25 | 7L.59 | 1N(49) | 1N(36)2U(2) | 1N(34) | 1N(16)D(24) | 161 | 14.9 | 0.9 | 2276 |
| 6-7(5181) | Het. 0.71 | 7L.85 | 1N(38) | 1N(34)2U(2) | 1N(24) | 1N(28) | 126 | 0 | 1.0 | 2116 |
| 6-9a | Het. 0.67 | 9L.32 | 1N(54) | 1N(60)2U(4) | 1N(50)D(2) | 1N(8)D(42) | 220 | 20.0 | 0.6 | 2192 |
| 6-9d | Het. 0.46 | 9L.84 | 1N(47) | 1N(32)2U(10) | 1N(45) | 1N(40) | 174 | 0 | 2.8 | 1912 |
| 6-10(5519) | S.C.—prox. | 10L.10 | 1N(60) | 1N(50)2U(12)D(4) | 1N(42)D(24) | 1N(55) | 247 | 11.3 | | |

[a] S.C. = Secondary constriction.

[b] Het. = NOR-heterochromatin.

[c] Number of microspores given in parentheses; 1N = normal nucleolus, 2U = two unequal-sized nucleoli, D = diffuse nucleolus.

nucleoli at quartets but not at the first postmeiotic prophase is unknown, but it is not the result of a deficiency for a segment of the nonorganizer chromosome involved in the interchange. Weber (1975 and personal communication) observed that plants monosomic for chromosome 1, 2, 4, 7, 8, 9, or 10 did not produce quartet microspores with diffuse nucleoli. Half of the microspores in these plants would be deficient for the respective chromosome. Therefore, the cause of diffuse nucleoli at the quartet stage in NOR-interchange heterozygotes appears to be due to either the duplication of a portion of the nonorganizer chromosome or a duplication or deletion of NOR segments. Diffuse nucleoli at the quartet versus first postmeiotic prophase stages may have different genetic bases.

## THE NOR-SYNTHESIZER AND ORGANIZER

Our finding that the NOR is the site of DNA complementary to 18S and 28S rRNA (Phillips, Kleese, et al., 1971; Phillips, Weber, et al., 1974) shows that the maize NOR is functioning in a synthetic capacity—synthesizing 18S and 28S rRNA. McClintock (1934) showed that the NOR serves to organize nucleolar material into a nucleolus. When the NOR is absent in maize microspores, nucleolar material is present as small nucleoli or what was termed a "diffuse nucleolar condition." This observation, along with several other considerations, demonstrated the organizing capacity of the NOR.

The hypothesis being tested in our laboratory is that the NOR is a *synthesizer* of 18S and 28S rRNA and an *organizer* of other nucleolar components, such as 5S RNA and ribosomal proteins. The 5S RNA genes of maize are localized in a chromosome other than chromosome 6, most likely chromosome 2 (Wimber, Duffey, et al., 1974). Therefore, 5S RNA is synthesized by a chromosome different from that to which the nucleolus is attached and must move to the nucleolus. No information is available for the genes coding for the various ribosomal proteins, but they also may be in chromosomes other than chromosome 6. The ribosomal proteins are expected to be synthesized on cytoplasmic polyribosomes and transported to the nucleolus within the nucleus. The diffuse nucleolar condition, therefore, may not be the result of all nucleolar material simply remaining dispersed and forming small nucleoli. But based on present knowledge, the nucleolar material observed in diffuse-nucleolated microspores may represent collections of 5S RNA and ribosomal and other nucleolar proteins. These bodies would appear as nucleolar material but be lacking 18S and 28S rRNA. According to the hypothesis, when a cell is deficient for the NOR, 18S and 28S rRNA is not synthesized and the 5S RNA and ribosomal proteins do not become organized into a single nucleolar structure associated with chromosome 6.

Perry (1973) reviewed the evidence that 5S RNA and ribosomal proteins are synthesized independent of 18S and 28S rRNA synthesis in eukaryotes. Persistent synthesis of 5S RNA, for example, occurred in mammalian cells in which low doses of actinomycin D suppressed 18S and 28S rRNA synthesis. Miller (1974) showed that the anucleolate mutant of *Xenopus* synthesizes 5S RNA while unable to synthesize 18S and 28S rRNA; also, the 5S RNA has a shorter half-life than 5S RNA synthesized by normal embryos. Hay and Gurdon (1967) described the existence of multiple nucleolar bodies and pseudonucleoli in embryonic cells of the anucleolate mutant. Thus maize microspores deficient for 18S and 28S rRNA genes (NOR-deficient microspores) may continue to synthesize 5S RNA, ribosomal and other nucleolar proteins that fail to coalesce into a single nucleolus due to the absence of 18S and 28S rRNA synthesis and instead form many small nucleolar bodies.

## RIBOSOMAL RNA GENE MULTIPLICITY AND PROTEIN PRODUCTION

A potential application of the knowledge gained on the NOR is the possible effect of varying numbers of rRNA genes on protein levels. A preliminary

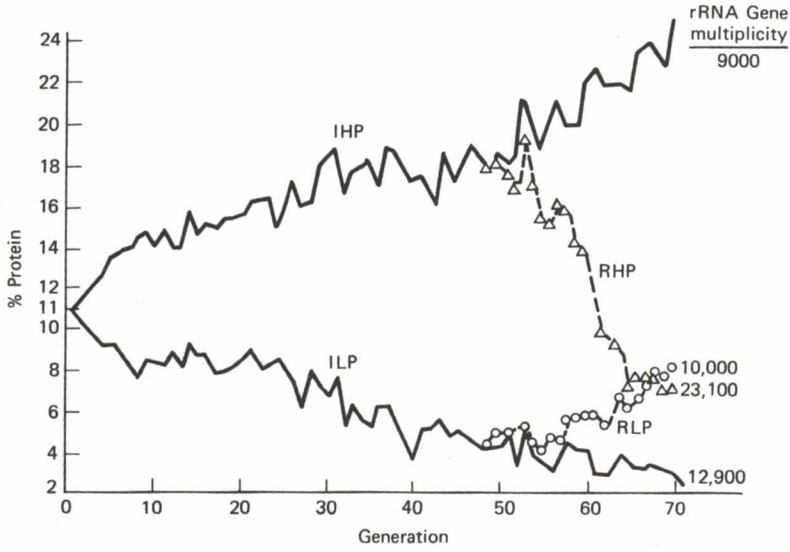


**Figure 10.** Response to forward and reverse selection for modified protein levels and rRNA gene multiplicities of the resultant lines (IHP—Illinois high protein, ILP—Illinois low protein, RHP—reverse high protein, RLP—reverse low protein). Modified from Dudley (1974).

TABLE 5. Ribosomal RNA gene multiplicities of University of Illinois protein and oil lines[a]

| *Protein line* | *Ribosomal RNA gene multiplicity* | *Oil line* | *Ribosomal RNA gene multiplicity* |
|---|---|---|---|
| Illinois high protein | 9,000(a)[b] | Illinois high oil | 10,000(a)[b] |
| Reverse low protein | 10,000(a)[b] | Reverse low oil | 9,000(a)[b] |
| Reverse high protein | 23,100(c)[b] | Reverse high oil | 9,500(a)[b] |
| Illinois low protein | 12,900(b)[b] | Illinois low oil | 5,400(b)[b] |

[a] Hybridization performed using DNA from 6-day-old seedlings. Each value for the protein lines based on average of two experiments each with eight determinations per line using A188 $^3$H-rRNA at saturating levels. Oil lines evaluated in one experiment with eight determinations per line.

[b] a,b,c Significance relationships using Duncan's multiple range test.

report is presented here on research directed toward this goal. Since many factors influence the quantity of protein in the vegetative portion or in the grain of a plant, a system was needed where protein levels had been extensively selected. In the selection process, perhaps many of the genetic factors influencing protein synthesis would have been modified including the rRNA gene multiplicity.

As an initial step, we decided to survey the University of Illinois protein lines selected for high as well as low protein from one initial open-pollinated variety since 1896 (Dudley, 1974). Four protein lines were tested: (a) Illinois high protein (IHP); (b) Illinois low protein (ILP); (c) Illinois reverse high protein (RHP); and (d) Illinois reverse low protein (RLP). The RHP line was developed by selecting for low protein from the IHP line, and the RLP line was the result of selecting for high protein from the ILP line (Figure 10). Seed selected in 1969 and 1971 was used for rRNA gene-number determinations. Since rRNA gene-number estimates are from 6-day-old seedlings, protein values of the lines were determined by the *microkjeldahl* technique using vegetative tissue from 6-day-old seedlings. The protein values for the vegetative tissue reflect the same rankings as for grain protein, although not of the same magnitude. The lines were found to differ considerably in rRNA gene multiplicities from an average of 9000 for IHP to 23,100 for the RHP line (Table 5). As mentioned previously, cytological examination of the NOR of the RHP line revealed a greatly enlarged NOR-heterochromatic segment, supporting the rRNA gene-number estimate. The relationship of these rRNA gene multiplicities with protein level is not obvious. The variation, however, may be related to protein selection, since rRNA gene multiplicities for the University of Illinois oil lines (selected from the same open-pollinated variety) did not display the same degree of variation. Based on one experiment, three of the oil lines were not sig-

nificantly different and possessed 9,000–10,000 rRNA genes, whereas the low oil line possessed fewer (Table 5).

An additional interesting observation is that the RHP line had a dramatically rapid response to selection that was greater than for any of the other protein lines (Figure 10). Perhaps sometime after the reverse selection program was initiated, the RHP line developed a greatly elevated number of rRNA genes as the result of unequal crossing over. Considering the current rRNA gene multiplicity of IHP, an unequal crossover event must have occurred at least twice to achieve the RHP level of 23,100 rRNA genes. If one assumes that the rapid selection response is related to the elevated rRNA gene multiplicity, the unequal crossover event must have occurred soon after the initiation of reverse selection (Figure 10). On the other hand, the selection response has been slow in recent years (Figure 10), and perhaps the sudden slow down in response is related to the high rRNA gene multiplicity. The data presented here show only that variation exists among the protein lines; further tests are underway in an attempt to determine the genetic relationship between protein level and rRNA gene multiplicity.

## ACKNOWLEDGMENTS

The dedicated and careful technical assistance of Dr. S. S. Wang in the studies reported herein is gratefully acknowledged. Graduate research assistants P. J. Buescher, T. C. Murphy, and W. D. Springer also contributed importantly to the maize NOR investigations.

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