

## Highly repeated DNA sequence limited to knob heterochromatin in maize

(satellite DNA/cloning/*in situ* hybridization)

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**ABSTRACT** A highly repeated DNA sequence has been isolated from the maize genome as a satellite in actinomycin D/CsCl gradients. By using maize stocks differing in their heterochromatin content we have established that the sequence is a major constituent of one class of heterochromatin, knob heterochromatin, which can occur at 23 locations in the chromosome complement. The repeating unit, of 185 base pairs, has been cloned in plasmid pBR322 and its nucleotide sequence has been determined. The presence of this DNA sequence in knob heterochromatin and its absence from centromeric, nucleolar, and B chromosome heterochromatin parallels the cytogenetic differentiation previously described for these classes of heterochromatin in maize. Because knob heterochromatin has a distinctive cytological appearance and is unique in showing neocentric activity at meiosis, its association with a particular repeated DNA sequence may reflect a functional role for the sequence in the cell cycle.

Maize has blocks of heterochromatin in a number of different chromosomal regions. Each centromere is flanked by heterochromatin (centromeric heterochromatin) and there is a large block of heterochromatin at the nucleolus organizer region (NOR heterochromatin). The chromosomes may also have heterochromatin at 23 other sites (knob heterochromatin). The number of knobs varies in different races of maize and can be manipulated in genetic crosses. Heterochromatin is also a major constituent of the supernumerary B chromosomes found in some races of maize.

Each class of heterochromatin is distinguishable by its cytological appearance, knob heterochromatin being smooth and sharply delimited in prophase and prometaphase of both mitosis and meiosis. Knobs occur at a number of locations in the normal A chromosomes and there is a small knob in the proximal region of the long arm of the B chromosome. This knob has the same cytological appearance as the A chromosome knobs and it differs from the large diffuse blocks of heterochromatin that occur in the distal half of the long arm of the B chromosome. All knobs have a particular Giemsa staining behavior different from other heterochromatin (1). NOR heterochromatin resembles knobs in its cytological appearance but is sometimes diffuse and vesiculated.

The four classes of heterochromatin are also differentiated by their times of replication in the mitotic cycle (2) and by their specific genetic effects. For example, B chromosome heterochromatin enhances recombination frequencies and induces loss of chromosomal segments from knobbed A chromosomes in the second microspore division (3, 4). Some, and possibly all, knobs affect the levels of recombination in particular regions of the chromosome complement. The heterochromatin of the large knob (K10) on abnormal chromosome 10 has the remark-

able property of causing preferential recovery of knobbed chromosomes from all knobbed/knobless heterozygotes (5). This departure from normal genetic behavior appears to be a consequence of another known property of K10, the induction of neocentromeres at knobs in both the first and second meiotic divisions. The K10 knob also enhances recombination, especially in structural heterozygotes in which crossing-over frequencies are depressed. Another type of heterochromatin is involved in the differences in levels of crossing-over in male and female meocytes observed in several chromosomes; these differences tend to occur in proximal regions, which are composed primarily of centromeric heterochromatin (5).

Although the different kinds of maize heterochromatin are well characterized in their unique genetic and cytogenetic effects, nothing is known of the sequence or composition of their DNA. In this paper we show that the DNA isolated from heterochromatic knobs is composed largely of one repeating sequence that is restricted to knobs and does not occur in centromeric heterochromatin, in the distal heterochromatin of the B chromosome, or in the NOR heterochromatin.

### MATERIALS AND METHODS

**Maize Stocks.** Stocks of maize differing in the numbers of knobs on the normal A chromosomes and in the numbers of supernumerary B chromosomes were used in the experiments. Two Black Mexican stocks were used; both were knobless except for two small knobs on chromosomes 6, one on the short and one on the long arm. One Black Mexican stock had no B chromosomes and the other contained an average of six B chromosomes. The stock containing the highest number of knobs was P100, with large knobs on 3L, 6L, 7L, 8L, and 10L and medium-sized knobs on 1L, 1S, 5L, 6L, and 8L. Small knobs were present on 6S, 6L, and 9S chromosome arms. Among the other stocks used was KYS, with large knobs on 5L and 7L and small knobs on 6S, 6L, and 9S. Two versions of stock 34704 were used. They differed in that one had and the other lacked the large K10 knob on 10L. Both lines were segregating for large knobs on 2L, 3L, 8L, and a medium-sized knob on 4L. They had various numbers of B chromosomes. Because large numbers of seedlings were used, the K10 and no-K10 versions of stock 34704 possessed on the average the same frequency of these knobs and differed only in the K10 knob.

**Isolation of Maize DNA.** Kernels were surface sterilized and germinated, and seedlings were grown in the dark for 5 days. Excised shoots were ground to a fine powder in liquid N<sub>2</sub>. Sufficient buffer (0.15 M NaCl/0.1 M EDTA, pH 8.0) was added to form a slurry and sodium dodecyl sulfate was added to 1%.

Abbreviations: NOR, nucleolus organizer region; bp, base pairs; cRNA, complementary RNA.

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The preparation was heated at 60°C for 10 min and digested with protease (0.4 mg/ml) for 2 hr at 45°C. Debris was pelleted at  $2500 \times g$  for 15 min and the supernatant was extracted with an equal volume of phenol. Two volumes of ethanol was added to precipitate the DNA. The DNA was redissolved in 0.01 M Tris·HCl/0.001 M EDTA, pH 8.4. CsCl was added to a density of 1.71 g/ml and the solution was centrifuged at  $130,000 \times g$  for 40 hr. The yield was approximately 25–50  $\mu\text{g}$  of DNA per g of shoots.

**Separation of Satellite DNA.** Analytical gradients contained 5  $\mu\text{g}$  of maize DNA complexed with 4  $\mu\text{g}$  of actinomycin D. The density was adjusted to 1.64 g/ml with CsCl and the sample was centrifuged at  $130,000 \times g$  for 16 hr. For preparative isolation of the satellite, conditions were established analytically and scaled up.

**Cloning of Maize Satellite DNA.** Purified satellite DNA was digested with restriction endonuclease *Hae* III, ligated to *Hind*III synthetic linkers, cut with *Hind*III, and ligated to *Hind*III-cut plasmid pBR322 (6). *Escherichia coli* K-12 strain RR1 was transformed and colonies containing maize satellite DNA were selected by colony hybridization using nick-translated satellite DNA. Cloning experiments were conducted under containment conditions recommended by the Australian Academy of Science Committee on Recombinant DNA.

**DNA Sequence Analysis.** *Eco*RI-cut pBR322 was end-labeled with [ $\alpha$ - $^{32}\text{P}$ ]dATP by using DNA polymerase I (Klenow fragment). The plasmid was cut with *Hha* I and its sequence was determined (7). The sequence of the second strand was determined by cutting at the *Hind*III linkers, labeling the 3' end, and cutting the segment with *Sau*3A.

**In Situ Hybridization.** Meiotic and mitotic spreads were prepared as described (2). The total satellite DNA or plasmid DNA containing satellite sequence inserts was used as template for the transcription of  $^3\text{H}$ -labeled complementary RNA (cRNA), which was hybridized to chromosome spreads as described (8).

## RESULTS

**Isolation of Knob Satellite DNA.** DNA isolated from a range of maize genotypes, including some with B chromosomes and several knobs, forms a single peak in CsCl gradients at a density of 1.698 g/ml. With the inclusion of actinomycin D, a satellite peak is formed on the heavy side of the main-band DNA (9). The amount of the satellite DNA is proportional to the amount of knob heterochromatin present in the genotype (Fig. 1). The satellite DNA forms a single symmetrical peak in neutral CsCl with a density of 1.698 g/ml and shows strand separation in alkaline CsCl with densities of 1.744 and 1.751 g/ml.

The reassociation experiments showed the satellite sequences to have a  $C_0t_{1/2}$  of 1.0 mol·sec·liter $^{-1}$  in 15 mM NaCl/1.5 mM sodium citrate at 40°C ( $C_0t_{1/2}$  is the product of DNA concentration in mol of nucleotide per liter and incubation time in sec for 50% renaturation). Under the same conditions, the 1.705 g/ml *Drosophila melanogaster* satellite has a  $C_0t_{1/2}$  of 0.1, and the 1.688 g/ml satellite a  $C_0t_{1/2}$  of 2.0. Because the former consists of 5- and 7-base pair (bp) repeats and the latter of a 365-bp repeat, the maize satellite repeat is estimated to have a length between 50 and 200 bp.

Sequence heterogeneity between individual repeat units was indicated by increased buoyant density ( $\Delta\rho = 0.006$  g/ml), decreased melting temperature ( $t_m$ ) ( $\Delta t_m = -6^\circ\text{C}$ ), and reduced hypochromicity of the renatured satellite relative to native DNA.

The length of the repeating unit was determined to be 185 bp by digestion of the satellite DNA with the restriction enzyme *Hae* III. Electrophoresis after the *Hae* III digestion showed that a major fraction of the satellite DNA was cut into 185-bp lengths

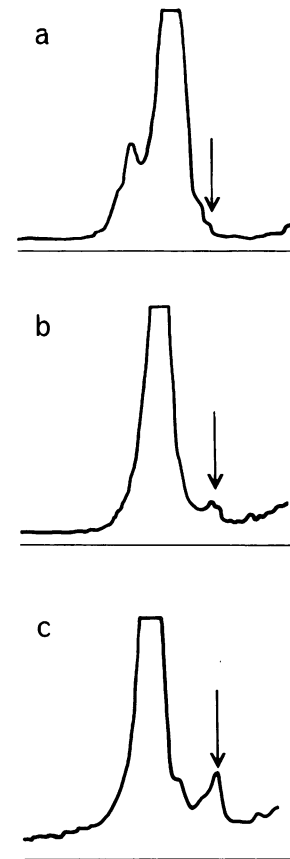


FIG. 1. Buoyant density separation of a satellite DNA in maize lines containing different quantities of knob heterochromatin. (a) Black Mexican without B chromosomes (very low knob content), (b) 34704 (medium knob content), (c) P100 (high knob content). Actinomycin D was added and the density was adjusted with CsCl to 1.64 g/ml. The satellite is indicated with an arrow. The peak on the left of the main band in a contains the ribosomal DNA cistrons; other repeated DNA fractions are visible as shoulders on the main band of DNA in b and c.

and in addition there was a minor band of approximately 215 bp and a small proportion of dimer and higher-order fragments (Fig. 2a). The satellite DNA was not digested by *Bam*HI, *Eco*RI, *Hind*III, *Pst* I, *Sal* I, *Sma* I, *Hpa* II, *Mbo* II, and *Hha* I. When the *Hae* III monomer segments are denatured and reassociated they reform the 185-bp segment and do not pair out of register to form aggregate molecules, and when long molecules of satellite DNA are denatured and reassociated, the renatured molecules are almost all cleaved by *Hae* III into 185-bp monomers. These results show that the monomer does not contain subrepeats and that the pairing frame for reassociation must be precise.

**Cloning of the 185-bp Repeat.** The *Hae* III monomer was recovered from an acrylamide gel after electrophoresis and cloned. The cloned inserts contained recognition sites for *Taq* I and *Sau* 3A, restriction enzymes that digested the native satellite DNA (Fig. 2). DNA sequence analysis of cloned repeats has directly confirmed the *Taq* I and *Sau*3A sites and has shown that there is no subrepeat within the monomer (Fig. 3). Hybridization and sequencing analyses have established sequence homology between the monomer, dimer, and 215-bp segments.

**Relationship Between Knob Size and Number of 185-bp Repeat Units.** Concordance of the amount of the satellite sequence seen in buoyant density analyses (Fig. 1) and the content of knob heterochromatin in the genome has been confirmed by isolation of rapidly renaturing DNA from a number of maize

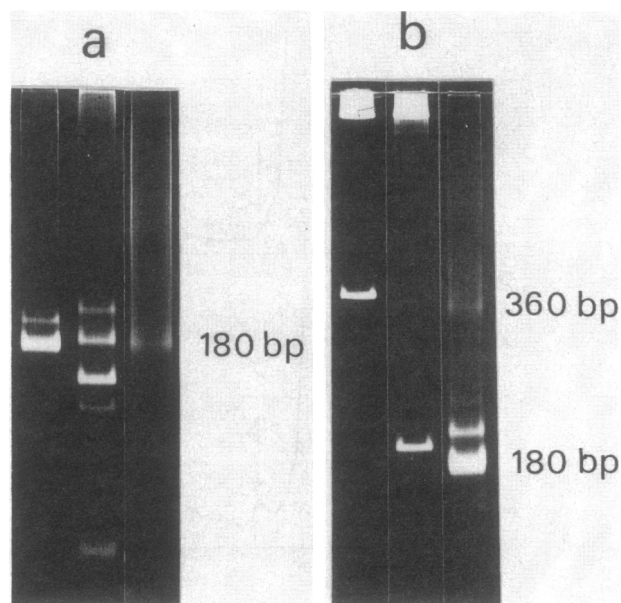


FIG. 2 Restriction enzyme digests of the satellite DNA, electrophoresed in 12% acrylamide gels. (a) From the left, lanes contain *Hae* III digest of the buoyant density satellite DNA, *Taq* I digest of the buoyant density satellite DNA, and *Hae* III digest of DNA from P100 annealed to  $C_{0t_{1/2}}$  of 0.01 mol·sec·liter<sup>-1</sup>. (b) *Hind*III digests of cloned monomer and dimer units together with a *Hae* III digest of the buoyant density satellite DNA. The cloned inserts are slightly larger than the satellite bands because of the ligated *Hind*III linkers.

lines differing in their knob content. *Hae* III digestion of  $C_{0t_{1/2}} = 0.1$  mol·sec·liter<sup>-1</sup> DNA shows that the 185-bp repeat is a major constituent of the rapidly renaturing DNA in high-knob lines (Fig. 2a). The relative concentration of the satellite sequence in the different genotypes was estimated by renaturation kinetic analysis (Table 1). The number of repeat units in the large K10 knob was estimated from the difference in the rate constants of full sib lines that differed only in the presence or absence of the K10 knob. In the line containing the K10 knob (34704, K10) 36% of the seedlings were homozygous K10 and 64% were heterozygous for the knob. This means that on the

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G G C C A C A C A A C C C C C A T T T T
T G T C G A A A A T A G C C A T G A A C
G A C C A T T T T C A A T A A T A C C G
A A G G C T A A C A C C T A C G G A T T
T T T G A C C A A G A A A T G G T C T C
C A C C A G A A A T C C A A G A A T G T
Sau3A
G A T C T A T G G C A A G G A A A C A T
A T G T G G G G T G A G G T G T A T G A
Taq I
G C G T C T G G T C G A T G A T C A A T
G G C C

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FIG. 3. A single-strand nucleotide sequence (5' → 3') of the cloned satellite monomer in the plasmid pZm4.25. Recognition sites for *Taq* I and *Sau*3A are indicated.

Table 1. Renaturation kinetic analysis of satellite sequence content of maize stocks

DNA source	$C_{0t_{1/2}}$ , mol·sec·liter <sup>-1</sup>	$k$ , sec <sup>-1</sup>	Relative amount of satellite sequence
Purified satellite DNA	0.0017	588	1
34704, K10	0.025	40	0.068
34704, no K10	0.055	18	0.031
Black Mexican ± B chromosomes	0.5	2	0.003

Satellite DNA and the DNA preparations from each maize stock were sonicated to approximately 250 bp. The satellite DNA was nick-translated and used as a tracer of renaturation of the satellite sequences present in the different stocks. The extent of hybrid formation was assayed on hydroxylapatite. DNA concentrations, experimental conditions, and  $k$  were as described by Singer (10). The tracer DNA without added driver DNA renatured with a  $C_{0t_{1/2}}$  of  $3.0 \times 10^9$  mol·sec·liter<sup>-1</sup>.  $C_{0t_{1/2}}$  values were used with a DNA content of  $3.6 \times 10^9$  bp (11) to calculate reiteration frequencies of the 185-bp unit.

average there were 0.68 K10 knobs per haploid genome. There are approximately  $3.6 \times 10^9$  bp per haploid genome (11) and in 34704, K10 the satellite is 6% of the genome. This means that there are  $1.2 \times 10^6$  copies of the satellite repeat unit in the K10 and other knobs. In the sib line (34704, no K10) there are only  $5.3 \times 10^5$  repeats, so  $(6.7 \times 10^5)/0.68$  repeats must reside in the K10 knob. As indicated earlier, all other knobs were equally distributed among the sibs. Thus, each K10 knob contains approximately  $10^6$  185-bp repeat units of the satellite.

Because the Black Mexican lines have in their haploid complement only two small knobs on the long and short arms of chromosomes 6, it follows that there are approximately  $3 \times 10^4$  repeat units in each knob. The fact that the Black Mexican line containing B chromosomes has the same rate constant as the line without B chromosomes argues that the proportion of satellite DNA in a B chromosome must be equivalent to the proportion in the chromosomes of the A genome. On the basis of meiotic cytological length it can be estimated that the amount of DNA in one B chromosome is approximately 1/20th of the total DNA of a knobless A genome, and this means that the number of 185-bp repeat units in one B chromosome is approximately  $1.25 \times 10^4$ .

**Chromosome Location of the Satellite Sequence.** Because [<sup>3</sup>H]cRNA made from a pBR322 template was not homologous to any maize sequence, [<sup>3</sup>H]cRNA transcribed directly from chimeric plasmids containing a satellite insert was suitable for *in situ* hybridization experiments. Equivalent results were obtained with monomer and dimer insert plasmids. Usually a dimer plasmid (pZm2.1) was used because of its greater content of satellite sequence.

The maize line KYS has small knobs on the termini of the short arms of chromosomes 6 and 9 and on the long arm of chromosome 6, and much larger knobs on the long arms of chromosomes 5 and 7 (Fig. 4a). Hybridization with pZm2.1 [<sup>3</sup>H]cRNA to mitotic metaphase and meiotic pachytene chromosomes showed that the satellite sequences were localized to these known knobs (Fig. 4b and c). Grain counts over these knobs (Table 2) showed a correspondence between satellite sequence content and knob size (see Fig. 4a), with the exception of the small knob on 6S, distal to the NOR heterochromatin, which had a disproportionately low content of the sequence.

In Black Mexican, the satellite was restricted to the two small knobs on chromosome 6, the only knobs present in this line. In lines with high numbers of knobs the correlation between knobs and sequence was maintained. Of the known knob positions on the genome, we have examined all other than those on the short arms of chromosomes 1, 2, 3, and 7, the most pro-

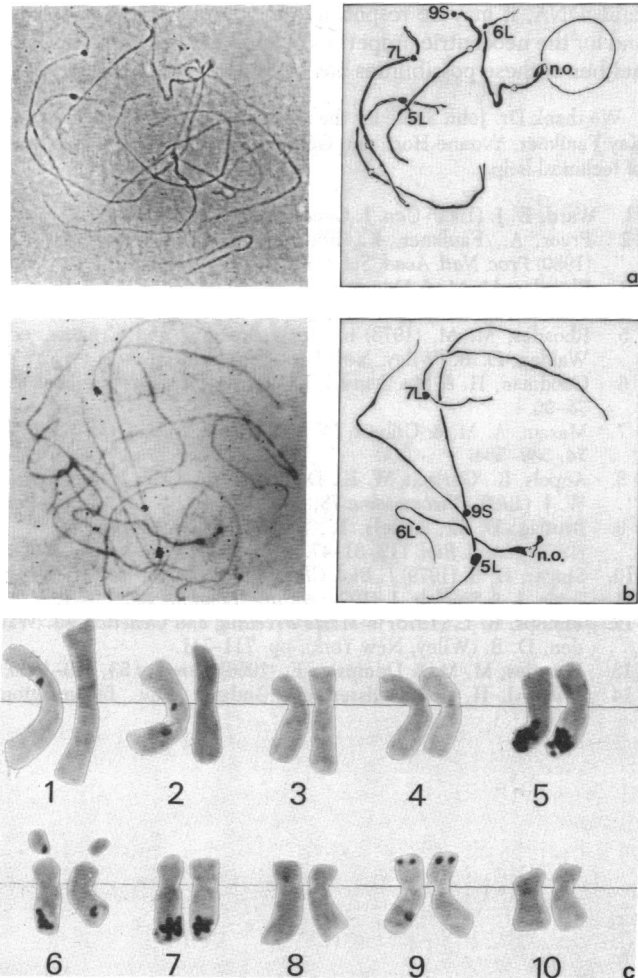


FIG. 4. (a) Pachytene cell of KYS showing knobs on 5L, 6L, 7L, and 9S. (Left) Micrograph, (Right) labeled drawing; n.o., nucleolus organizer. (b) (Left) Autoradiogram of *in situ* hybridization of a cloned satellite dimer plasmid (pZm2.1) to a pachytene cell of KYS. (Right) Labeled drawing of the same cell. (c) Karyotype showing *in situ* hybridization of pZm2.1 to a mitotic metaphase of KYS.

imal knob of 6L, and the 9L knob. Each of the A chromosome knobs we examined contained the satellite and all but K6S showed a correlation between knob size and satellite sequence content. The large K10 knob on abnormal chromosome 10 contained a large amount of satellite, and in pachytene cells where the knob was physically extended the sequence was distributed along its full length.

No sequences were detected in the large block of NOR heterochromatin or in any of the centromeric heterochromatin segments. The only portion of the B chromosome heterochromatin containing the sequence was the small proximal knob on the long arm.

Table 2. Grain counts over knob heterochromatin in KYS autoradiograms

Exp.	Chromosome location of knobs					No. cells	No. grains
	5L	6S	6L	7L	9S		
1	714 (0.42)	25 (0.01)	239 (0.14)	536 (0.31)	214 (0.12)	25	1728
2	320 (0.42)	3 (—)	97 (0.13)	245 (0.32)	102 (0.13)	19	767

The figures in the body of the table represent raw grain counts and the figures in parentheses give the proportion of grains over each knob.

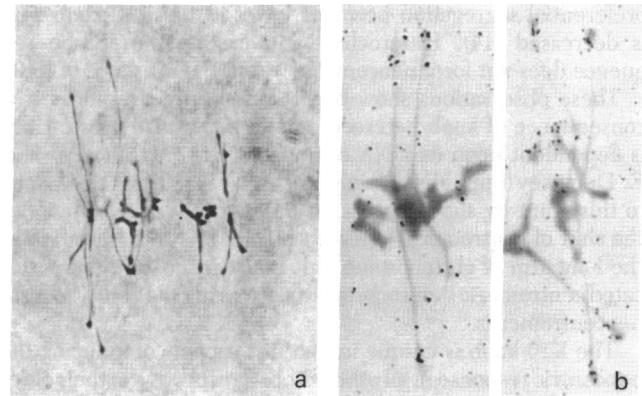


FIG. 5. (a) Meiotic metaphase II cell of a maize stock containing knobbed chromosomes and homozygous for the K10 knob. Strong precocious neocentric activity of knobbed arms is evident. Only chromosome 9, which was knobless in this plant, shows no evidence of neocentric activity. Seven chromosomes were homozygous for a knob in their long arms and two were homozygous for knobs in both arms (5). (b) Autoradiograms of portions of two metaphase II cells of the maize stock 35279-55 after *in situ* hybridization with pZm2.1. Grains are localized on the knobbed extremities of the neocentric chromosome arms.

In meiotic metaphase and anaphase figures showing neocentric activity the satellite sequences were restricted to the extremity of the neocentric arms, the locations of knob heterochromatin (Fig. 5).

## DISCUSSION

*In situ* hybridization has shown that the 185-bp repeated DNA sequence, which can be isolated as a satellite in actinomycin D/CsCl gradients, is located in knob heterochromatin and not in any other class of maize heterochromatin. Quantitation by buoyant density and renaturation analyses has indicated that the sequence is a major component of the knobs on the A chromosomes. There is a correlation between knob size and the frequency of reiteration of the 185-bp unit. The large K10 knob contains approximately  $10^6$  copies of the segment, with small knobs such as those occurring on the long arm of chromosome 6 having approximately  $3-5 \times 10^4$  units of the repeating sequence. The proximal knob on the B chromosome has only about a quarter of the number of repeat units relative to the number occurring in a comparable knob in the A chromosome complement, making it unlikely that the 185-bp DNA is the major component of the B chromosome knob. The same conclusion can be drawn for the small knob on 6S.

*In situ* hybridization analyses have shown that the sequence is not present in centromeric heterochromatin, in NOR heterochromatin, or in the large distal blocks of heterochromatin of the B chromosome. The 18S and 26S ribosomal DNAs are largely contained within the NOR block of heterochromatin (12), and it is probable that other highly repeated sequences are localized in centromeric heterochromatin.

There is a strict correlation between neocentromere formation, preferential segregation, and the presence of a knob on a chromosome arm. For example, neocentromeres do not arise on the long arm of chromosome 3 unless the 3L knob is present. If the 3L knob is present, neocentromeres form in the presence of K10. This is still the case when the position of the knob is changed relative to the true centromere, as in paracentric inversion 3b (13). The correlation between the presence of the 185-bp sequence and neocentromere formation induced by K10 is emphasized by the correlation between knob size and the amount of the 185-bp DNA, and by the fact that the degree of

preferential segregation becomes lower as the K10 knob size is decreased (14). Heterochromatin lacking the 185-bp sequence does not form neocentromeres in the presence of K10.

These observations show that the neocentric response is a consequence of knob heterochromatin *per se* and presumably is dependent upon its DNA composition. M. M. Rhoades and E. Dempsey (unpublished) have recently established a corollary to this point by showing that, in the paracentric inversion 3c, the shift of centromeric heterochromatin to a distal position in the long arm of chromosome 3 does not result in this translocated centromeric heterochromatin acquiring the ability to form neocentromeres.

The K10 knob is unique in that it is capable of inducing the neocentric response in all other knobs irrespective of their chromosome location. None of the other knobs or combinations of them in the genome can replace K10 in eliciting neocentric activity. The K10 knob has the 185-bp repeat as its major component, but it must contain one or more additional sequences that are responsible for neocentromere induction. This second sequence may be repeated several times in the K10 knob and it may be interspersed with the long tandem arrays of the 185-bp unit.

Knob heterochromatin is different from all other classes of heterochromatin in the genome in its cytological appearance, its time of DNA replication, and its DNA sequence composition. Because the 185-bp sequence is the major component of

knob DNA, it may be responsible for the distinctive cytology and for the neocentric properties of knob heterochromatin, but neither of these possibilities has been established directly.

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1. Ward, E. J. (1980) *Can. J. Genet. Cytol.* **22**, 61–67.
2. Pryor, A., Faulkner, K., Rhoades, M. M. & Peacock, W. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6705–6709.
3. Rhoades, M. M. & Dempsey, E. (1972) *Genetics* **71**, 73–96.
4. Rhoades, M. M. & Dempsey, E. (1973) *J. Hered.* **64**, 12–18.
5. Rhoades, M. M. (1978) in *Maize Breeding and Genetics*, ed. Walden, D. B. (Wiley, New York), pp. 641–671.
6. Goodman, H. & MacDonald, R. J. (1979) *Methods Enzymol.* **68**, 75–90.
7. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
8. Appels, R., Gerlach, W. L., Dennis, E. S., Swift, H. & Peacock, W. J. (1980) *Chromosoma* **78**, 293–311.
9. Brutlag, D. L., Appels, R., Dennis, E. S. & Peacock, W. J. (1977) *J. Mol. Biol.* **112**, 31–47.
10. Singer, D. S. (1979) *J. Biol. Chem.* **254**, 5506–5514.
11. Ingle, J. & Sinclair, J. (1972) *Nature (London)* **235**, 30–32.
12. Phillips, R. L. (1978) in *Maize Breeding and Genetics*, ed. Walden, D. B. (Wiley, New York), pp. 711–741.
13. Rhoades, M. M. & Dempsey, E. (1966) *Genetics* **53**, 989–1020.
14. Miles, J. H. (1970) Dissertation (Indiana Univ., Bloomington, IN).