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Distribution and clustering of two highly repeated sequences in the A and B chromosomes of maize *

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Summary. Clones from a family of highly repeated sequences present in a heterochromatin rich maize line have been characterized by sequencing and chromosome location. The repeats differ from each other in length and degree of sequence homology, and show areas rich in purine and pyrimidine. In "situ" hybridization experiments indicate that the repeats are mainly located in the knob heterochromatin of the A chromosomes and the centromeric heterochromatin of the B chromosome. However, in addition to previously published data, some copies are also distributed in euchromatic regions of the A chromosomes and in the distal heterochromatic block of the B chromosome. The results are discussed in relation to the centromeric activity of maize heterochromatin.

Key words: Maize – Repetitive DNA – A and B chromosomes – Centromere activity

Introduction

The genome of *Zea mays* is largely constituted by repetitive DNA (60–80%) mainly dispersed with unique or different repetitive sequences (Flavell et al. 1974; Hake and Walbot 1980). Studies on the genome organization of maize by DNA reassociation kinetics (Cot analysis) showed that short repetitive sequences of 500–1,000 bp are interspersed with unique sequences 2,100 bp in average length; middle repetitive sequences are also interspersed with highly repetitive sequences (Hake and Walbot 1980).

In most plant and animal genomes highly repetitive DNA is clustered at specific chromosomal sites, the heterochromatic regions (Jones 1970; Pardue and Gall 1970; Gall 1973; Flavell 1980). In maize, four types of heterochromatin can be recognized by their appearance, chromosomal location, replication pattern and genetic effects: (i) knob heterochromatin, (ii) NOR heterochromatin, (iii) centromeric heterochromatin and (iv) block heterochromatin of the B chromosome. Heterochromatic knobs are sharply delimited and are located at an appreciable distance from the centromere; moreover both number and size of knobs are not constant from variety to variety. The heterochromatin at the nucleolus organizer region (NOR) is morphologically similar to knob heterochromatin but it is always located at the secondary constriction on chromosome 6; hybridization studies localized most of the rDNA cistrons within the NOR heterochromatin (Givens and Phillips 1973). Most of the B heterochromatin is located in few large cushion-like blocks in the distal half of the long arm. Finally, centromeric heterochromatin is distinguishable from the adjacent chromatin only by the degree of heteropycnosis and not by its peculiar shape. The centromeric heterochromatin of the B chromosome, although similar in its appearance to knob heterochromatin, differs from it for its pericentric location.

Recently we have cloned several repetitive sequences from DNA fragments obtained by digestion with restriction enzymes that cut frequently. The distribution on A and B chromosomes and the reiteration frequency in maize lines with different heterochromatin and B chromosome content for three of these clones are reported in the present work.

Materials and methods

Maize and teosinte stocks

Stocks of maize with varying number of knobs and B chromosomes were used in the experiments. Three stocks of Black

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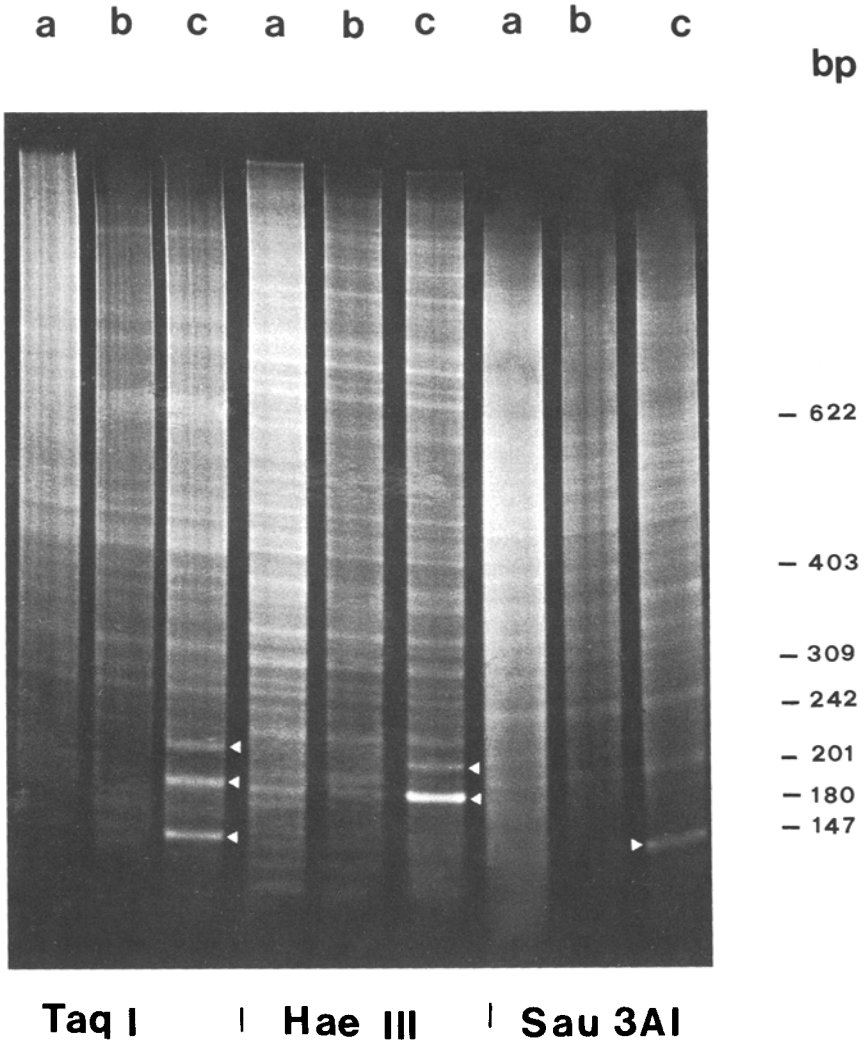

Fig. 1. Agarose-acrylamide gel electrophoresis of DNA from maize lines. The DNA was digested with restriction enzymes as indicated: (*a*) Black Mexican with 6 B chromosomes; (*b*) Black Mexican without B chromosomes; (*c*) W64A. Fragment size is reported on the side. Prominent bands are indicated by *arrow heads*

Mexican with 0, 2 and 6 B chromosomes, respectively were used; all were knobless except for two small knobs on chromosome 6. The inbred lines KYS and W64A showed five and six knobs, respectively. Teosintes, *Zea mays* spp. *mexicana*, var. 'Chalco' and *Zea mays* spp. *parviglumis*, var. 'Batan', have 11 very large knobs.

Purification of DNA and cloning

DNA extracted from unfertilized ears of maize and seedlings of teosinte was purified as previously described (Viotti et al. 1982). For cloning, the DNA was digested with restriction enzymes, electrophoresed on 5% acrylamide gel and stained with ethidium bromide. The DNA of the more heavily stained bands in the heterochromatin rich line (W64A) was eluted from the gel slices and cloned in the pUC8 plasmid vector linearized by Hinc II, Acc I or BamHI restriction enzymes.

Southern hybridization and DNA sequencing

Three μg of DNA from each maize line were digested with ten units of restriction enzymes for 3 h at 37 °C. The digested DNA was electrophoresed on 0.7% agarose, 2.5% acrylamide composit gel, 2 h at 10 V/cm in Tris-borate buffer (Tris 90 mM, EDTA 2 mM, boric acid, pH 8.2). After staining, the gel (2 mm tick) was treated for 30 min with NaOH 0.4 M, NaCl 0.5 M and then blotted with 20×SSC (NaCl 3 M, sodium citrate 0.3 M, pH 7.2) on Biodyne A membrane (Pall) for 5 h at room temperature. The gel was then stained to control the transfer. Filters were treated as usual for Southern blot and hybridized as previously described (Viotti et al. 1982). Washing of the filters was carried out under stringent conditions, 0.2×SSC at 68 °C for 2 h. The DNA sequences on both strands were determined on fragments labelled at the 5′ end by polynucleotide-kinase and purified by strand separation, by the chemical degradation reactions G, G+A, T+C, C and A>C (Maxam and Gilbert 1980).

"In situ" hybridization

Recombinant plasmid labelled at a specific activity of 1–3×10^7 dpm/μg were obtained by nick translation using ^{3}H-TTP (90 Ci/mmole, NEN). "In situ" hybridization experiments were made essentially as described in a previous paper (Viotti et al. 1982). Denaturation was carried out in 90% deionized formamide, 0.1×SSC for 150 min at 65 °C, according to Singh et al. (1977). The slides were hybridized for 24–36 h at 68 °C and washed in cold 0.1×SSC three times for 10 min each, then in 1×SSC at 65 °C for 30 min, and finally in 0.2×SSC at 65 °C for 20 min. The slides were dipped in a Kodak NTB-2

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H2a             GGCCACACAACCCCCATTTTTGTCGAAAATAGCCATGAACGACCATTTTCAATAACATCGAAGGCTAACACCTACGGATT   80
H2b                      G           TAT               G               T CTAGA     G
S1       GATCAAT                     * C                          T    T C              G
T2  TCGAT   T        G G             * C                               T C              G
T3                                                                     T C              A   AG
P                    G                                                 T C

                TTTGACCAAGAAATGGTC*TCCACCAGAAATCCAAGAATGTGATCTACGACAAGGAAACATATGTGGGGTGAGGTGTATG  159
                   A              C
                                  *                          end
                         T        *                            T G                       A
                A                 *                            T G                      T
                                  *                            T G

                AGTCTTCGGTGGACGATCAATGGCC
                  CG CT   C

                  C  CT   C  end
                  CG CT   C  end
                  CG CT   C  T
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Fig. 2. Nucleotide sequences of recombinant clones. Each sequence was determined on both strands. *Asterisks* represent gaps introduced to maximize homology. Restriction sites for TaqI and Sau 3AI are underlined. Base changes with respect to H2a are reported at their occurrence. For comparison, satellite sequence of Peacock et al. (1981) is reported (P)

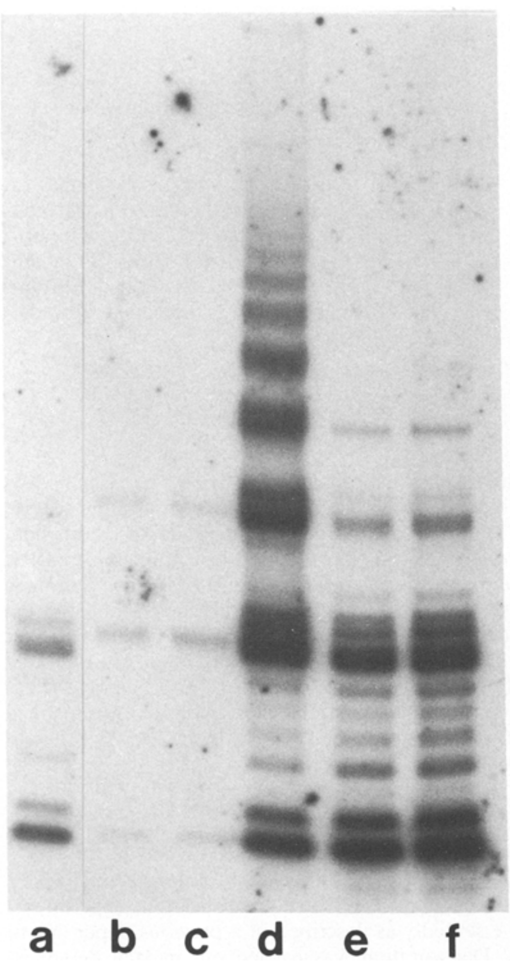

Fig. 3. Hybridization to Southern blots of HaeIII-cut DNA from different maize and teosinte lines. The H2a sequence was hybridized to (*a*) W64A; (*b*) Black Mexican without B; (*c*) Black Mexican with 6 B's; (*d*) partial digest of W64A-DNA; (*e*) teosinte Batan; (*f*) teosinte Chalco

emulsion diluted three times and exposed for 7 days at 4 °C. Development and staining were as reported by Viotti et al. (1980). At least 100 microsporocytes for each line were considered for grain counting.

Results

Sequence cloning and characterization

The DNA from maize lines with different heterochromatin and B chromosome content were digested with the Hae III restriction enzyme and fractionated by electrophoresis on agarose/acrylamide gel. Fragments that lit up in ethidium bromide staining (Fig. 1) were purified and cloned in the pUC8 vector. Two fragments of similar length from the more intense staining band (H2) were further investigated. Figure 2 reports the nucleotide sequence of the fragments H2a and H2b, of 184 and 185 bp, respectively. The comparison shows a high degree of homology along all the sequences but with three regions of 3–6 nucleotides that diverge. At the two edges, stretches of purine or pyrimidine rich regions are present in an alternate fashion that may determine intramolecular pairing. Some restriction sites for Taq I and Sau 3AI enzymes are also present. The reiteration frequency in lines having differing contents of B chromosomes and knob heterochromatin was investigated by Southern blot analysis. Figure 3 shows the autoradiogram of the DNA from the W64A line and from two Black Mexican lines with 0 or 6 B's digested with Hae III and probed with H2a sequence. As indicated by band intensity, the knob rich W64A line has a higher H2a content than the other maize lines. The intensity of hybridization of the DNA from the teosinte lines (Fig. 3 e and f) correlates with their higher knobs heterochromatin content.

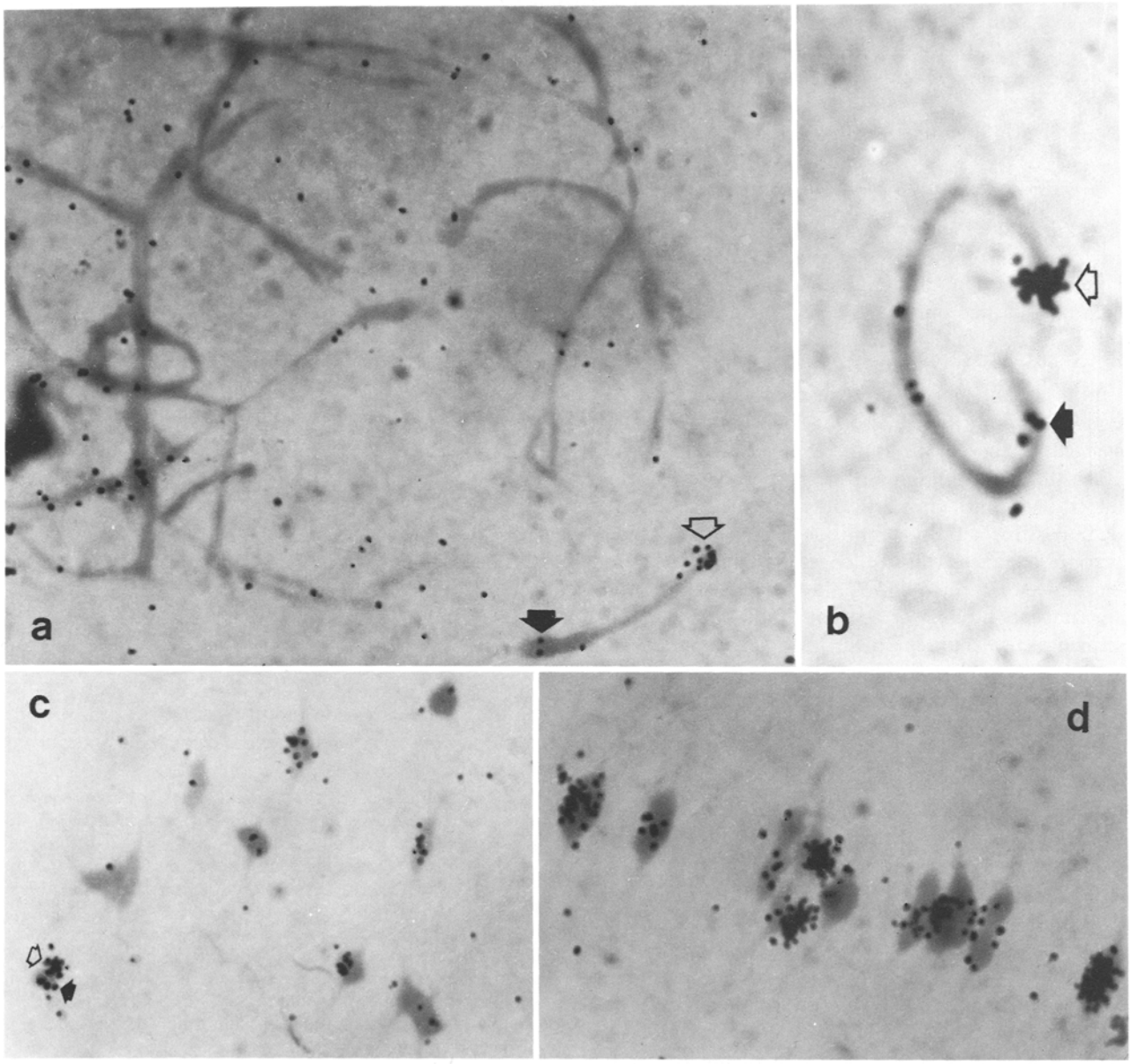

Fig. 4. **a** diplotene chromosomes of Black Mexican line containing a pair of B chromosomes hybridized with H2 sequences. **b** B chromosome pair from a pachytene nucleus hybridized with H2 sequences. **c** Metaphase I microsporocyte of a Black Mexican line containing a pair of B chromosomes hybridized with H2 sequences. Three or more grains are present on five A chromosomes. **d** Meiotic metaphase I chromsomes of the W64A line hybridized with H2 fragment. Silver grains, present on all chromosomes, preferentially accumulate on the 4 knob-bearing A chromosome. *Closed arrow* indicates silver grains on the distal heterochromatic block of B chromosome. Open arrow indicates silver grains around the centromere region of B chromosome

The partial digest of the W64A DNA with the Hae III enzyme probed with H2a (Fig. 3 d) suggests a tandem array of the sequence. The cluster should contain at least 8–9 units distributed in a continuous fashion. An alternative interpretation may be the interspersion of the H2's with one or more repetitive sequences with the periodic occurrence of Hae III site every 200 bp. Obviously, from this type of experiment it can not be excluded that H2 may be also randomly interspersed as single copy.

As for the H2 fragments, in Sau 3AI and Taq I digests of the W64A DNA, some bands lit up in ethidium bromide staining (Fig. 1). Clones from these bands were obtained and some of them further investigated. Their sequences compared to those of H2a and H2b (Fig. 2) showed homologies of between 91%

and 95% with base changes in some of the Taq I and Sau 3AI sites of the H2 sequences. Sequence and size comparison (and Southern blot analysis, data not shown) indicates that the unit length is about 200 bp with small differences in sequence and length.

Chromosomal location by "in situ" hybridization

The "in situ" hybridizations with H2a and H2b on pachytene chromosomes of a Black Mexican line containing two B chromosomes showed silver grains preferentially located on the pericentric heterochromatin of the B chromosome (Fig. 4a and b). A lower number (4–5) of silver grains was observed in the two small knobs present in chromosome 6 of this line; one to three silver grains were also found in the distal heterochromatic block of the B and in several other position along the A chromosomes. In metaphase I cells, where the chromosomes are physically contracted, at least five (and possibly all) A chromosomes appear to contain the H2 sequences (Fig. 4c). Grain counts indicate that the pericentric heterochromatin of the B chromosome contains about half of the total number of the H2 sequences present in the Black Mexican genome.

In KYS and W64A lines, the H2 sequences were mainly located in the knobs, as observed in the pachytene chromosomes. However, several minor hybridization sites identified by few silver grains (1–3) were also found in euchromatic regions, suggesting that the sequences are distributed along the whole chromosome. In meiotic metaphase I figures of KYS and W64A the sequences were present in all chromosomes but at different extent, the Knob-bearing chromosomes showing most of the silver grains (Fig. 4d).

The Black Mexican metaphase I cells with two B chromosomes had about one-fourth of the silver grains observed in KYS and W64A, these latter two having approximately the same number of grains (200–250 grains/cell). The centromeric Knob of the B and the knobs of comparable size in the A chromosomes showed the same number of the silver grains (20–30) suggesting a similar frequency of reiteration for the H2 repeats in these two heterochromatic regions. The chromosomal distribution of the hybridization sites for a clone obtained by Sau 3AI digestion (S1) was identical to that observed for the H2 sequences. However a lower number of silver grains on the centromeric heterochromatin of B chromosome was obtained using the S1 sequence.

Discussion

The "in situ" hybridization results indicate the H2 sequences to be a major component of knob heterochromatin of A chromosomes and pericentric heterochromatin of B chromosome. Except for the distal heterochromatic block of B chromosome these sequences are not present in the other heterochromatic segment of the maize chromosomes. Moreover, several euchromatic regions of A chromosomes and the distal heterochromatic block of the B chromosome have been found to contain few copies of the H2's. These findings are largely in accordance with the results obtained by Peacock et al. (1981) using a highly repeated DNA sequence isolated as a satellite in actinomycin D/CsCl gradients from a range of maize genotypes. By "in situ" hybridization these authors revealed the presence of a satellite sequence in the knob heterochromatin of A chromosomes and centric heterochromatin of B chromosome and its absence from any other class of heterochromatin as well as from euchromatic regions of A and B chromosomes. This satellite sequence of 184 base pairs shows an homology of about 94% to our sequences, with differences in the regions containing Taq I sites, which accounts for the presence of several bands which lit up in the Taq I digestion (Fig. 1). However, the repeats, that are from two different maize lines, are highly conserved, particularly in the pyrimidine and purine rich regions. This constancy is confirmed by Southern blot analysis of DNA from two teosinte subspecies, differently related to maize, indicating a conserved pattern and a constant anatomy of the tandem array (Fig. 3).

Taking into account their strict sequence homology, it is likely that differences in chromosomal location between the H2 repeats and the satellite sequence can be due to differences in the "in situ" hybridization procedures, such as specific activity of the probes, denaturation agents (Sing et al. 1977; Peacok et al. 1977) and hybridization time.

It is well known that knobs are to attach spindle fibers and draw the chromosomes to the poles during both the first and second meiotic division. This centromeric activity is not constitutive but is induced by the presence of a large knob (K10) on a rare kind of chromosome 10 (abnormal 10). Knob heterochromatin is unique in its ability to form neocentromers, the other class of heterochromatin lacking this property. According to Peacock et al. (1981) it seems likely that the neocentric response is a consequence of the DNA "composition" of knob heterochromatin. This heterochromatin must contain one or more centromeric sequences which may be interspersed with the highly repeated H2 sequences. The alternative possibility that the H2 repeats may contain a latent attachment site for spindle structures seems to be limited by the fact that few copies of these sequences are present in several chromosomal sites devoid of neocentric activity.

The pericentric heterochromatin of B chromosome proved to be similar in morphology and DNA composition to knob heterochromatin of A chromosomes. Since knobs are placed at a some distance from the centromere of A chromosomes (Longley 1939), the centromeric heterochromatin of B chromosome is the sole example of knob-like heterochromatin adjacent or surrounding a centromere. A hypothesis can be advanced to explain this uniqueness: the B chromosome

centromere might be derived from neocentromeric sequences present in knob heterochromatin, sequences which became functionally independent of K10 knob control.

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