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Genetics

Asynchronous replication of heterochromatin in maize

(B chromosomes/nondisjunction/late replication)

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ABSTRACT **Replication patterns of four classes of heterochromatin in the chromosomes of maize were analyzed. Centromeric heterochromatin, distal heterochromatin of the B chromosomes, and knob heterochromatin all have asynchronous late replication, knob heterochromatin being the last chromosomal DNA to complete replication. Nucleolus organizer heterochromatin replicates during the period of euchromatin DNA replication. The proportion of the S period involved in asynchronous late replication is directly dependent on the amount of heterochromatin in the nucleus. Both additional knob and B-chromosome heterochromatin participate in this effect. Each additional B chromosome produces an increment in the proportion of nuclei with asynchronous replication; in nuclei with 12 B chromosomes, the proportion of the S phase showing asynchronous late replication is more than 3 times greater than that in nuclei without B chromosomes. The data are consistent with the hypothesis that delayed DNA replication of knob heterochromatin is responsible, in some genotypes, for the loss of chromosome segments and for B-chromosome nondisjunction in the second pollen grain mitosis.**

Delayed replication of heterochromatin in the knobs of maize chromosomes and in the supernumerary B chromosomes has been implicated as the cause of both the loss of chromosome segments and the nondisjunction of B chromosomes at the second pollen grain mitosis (1, 2). Genetic studies using translocations between the normal chromosomes of the complement and B chromosomes have shown that two regions of the B chromosome are involved in nondisjunction (3–5). The proximal region near the centromere, which contains a heterochromatic knob, and the distal tip must both be present in a microspore for nondisjunction to occur. When a B chromosome is dissected into two pieces by a translocation, only the translocated chromosome with the B centromere undergoes nondisjunction. Rhoades and Dempsey (1, 2) suggested that delayed replication of the heterochromatic knob adjacent to the centromere of the B chromosome prevents the separation of the two chromatids at anaphase and results in nondisjunction. The other phenomenon, the loss of chromosome segments, depends upon an interaction of B chromosomes and heterochromatic knobs on the A chromosomes. In some genetic backgrounds, the presence of two or more B chromosomes results in chromosome breakage and loss of genetic markers. Breakage is restricted to chromosome arms that bear a knob and occurs in the region between the knob and the centromere, resulting in the loss of the knob and the chromosome segments distal to the breakpoint. Rhoades and Dempsey (2) proposed that failure of knob replication in the second microspore mitosis causes the formation of a chromosome bridge at anaphase and that subsequent breakage of the bridge results in loss of chromosome segments containing the knob. The rationale for this hypothesis is that heterochromatin in many organisms is known to be late replicating (6). In maize, Abraham and Smith (7) reported that B chromosomes replicate during the second half of the S period, as do some regions of the A chromosomes.

We have examined the time of replication of both the euchromatic and heterochromatic segments of the maize genome and find that the different classes of heterochromatin have their own periods of DNA replication during the S phase of the mitotic cycle.

MATERIALS AND METHODS

The experimental material was the primary root meristem of germinating seedlings of maize lines differing in numbers of knobs on the normal A chromosomes and in numbers of supernumerary B chromosomes. The knob and B-chromosome constitutions of each line were determined in both meiotic and mitotic cells (Table 1 and Fig. 1).

Seeds were surface sterilized and germinated for 3 days in the dark at 30°C. Root tips were then immersed in an aerated aqueous solution of [^{3}H]thymidine (5 Ci/mol, 10 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) at 30°C for 30 min. After they were washed in distilled water three times, they were returned to the incubator. Tissue samples were fixed (ethanol/acetic acid, 3:1, vol/vol) at various times. Slides were prepared by a "cell cloud" technique: the terminal 1–2 mm of the primary root was excised and immersed in 45% acetic acid for approximately 2 min. A needle was used to tease the cut end of the root tip segment, producing a cloud of separated meristematic cells. The remaining cellular debris was removed, an equal volume of 45% acetic orcein was added to the drop containing the dispersed meristematic cells, and a squash preparation was made. Cover slips were removed after freezing in liquid N_2; the slide was dehydrated in 95% ethanol and air dried. Slides were dipped in a 1:1 aqueous dilution of emulsion (Ilford K2), exposed for approximately 3 weeks, and developed in a 1:1 dilution of developer (Dektol).

In the B-chromosome experiment, metaphase figures were accumulated by cold treatment. Seedlings were transferred 3 hr after the thymidine pulse from 30°C to 4°C for 20 hr and then immediately fixed.

RESULTS

Knob Heterochromatin Replication. Two classes of labeled interphase nuclei were observed, one having general labeling with grains distributed over the whole nucleus (Fig. 2*a*), the other a discrete label pattern with localized areas of labeling (Fig. 2*b*). The latter pattern shows that there is marked asynchrony of replication among chromosomal segments in maize. The frequencies of these two classes of label pattern were scored in each of the three genotypes containing low, medium, and

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Table 1. Cytological constitution of maize lines

Line	Knob content	Knob constitution: Size	Knob constitution: Chromosome arm
P98-WFK	Low	Small	6S
KYS	Medium	Large	5L, 7L
		Small	6S, 6L, 9S
P100	High	Large	3L, 6L, 7L, 8L, 10L (=K10)
		Medium	1L, 1S, 5L, 6L, 8L
		Small	6S, 6L, 9S
Black Mexican* (0 B)		Small	6S, 6L
Black Mexican* (+Bs)†		Small	6S, 6L

* Lines differing in no. of B chromosomes.
† B chromosome numbers were determined for each seedling.

Table 2. Patterns of label incorporation in interphase and dividing nuclei in seedlings with different numbers of heterochromatic knobs

Fixation time	Knob no. Low	Medium	High
	Interphase nuclei		
1	6.8 (1166)	7.4 (1421)	18.2 (1079)
2	4.9 (1121)	10.3 (1448)	13.0 (1131)
3	4.3 (707)	11.5 (1280)	16.2 (1040)
4	4.4 (1054)	8.9 (1208)	16.8 (1107)
5	3.4 (834)	9.4 (1084)	17.8 (1055)
	Dividing nuclei		
1	46.1 (37)	58.4 (301)	78.2 (58)
2	21.0 (107)	48.9 (271)	68.3 (266)
3	21.0 (89)	39.9 (227)	43.7 (214)
4	16.1 (230)	20.3 (322)	20.3 (439)
5	10.7 (127)	19.4 (297)	27.2 (466)

Values are given as % discrete label. Total no. of nuclei are in parentheses. See Fig. 3 for specific time intervals.

high levels of knob heterochromatin (Table 2). The distinction between the general and discrete label patterns was clear even though in some discretely labeled nuclei there was some spread of label.

Because the cell cloud dissected from each root tip did not consist solely of meristematic cells, it was not meaningful to

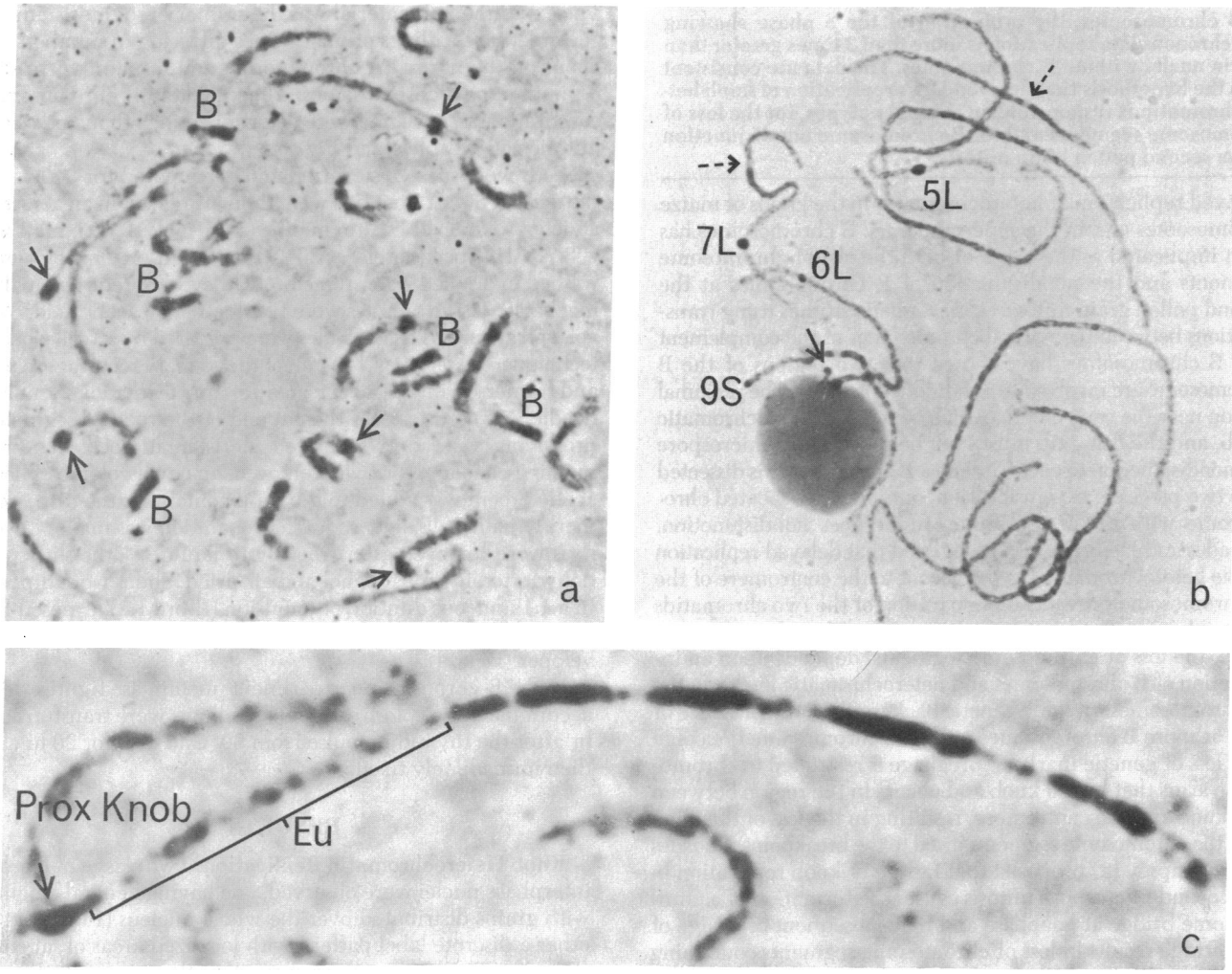

FIG. 1. Mitotic and meiotic nuclei of maize lines. (*a*) Prometaphase of root tip cell of P100, a line with a high content of knob heterochromatin and supernumerary B chromosomes. Some prominent knobs are indicated by the arrows, and the five B chromosomes are labeled. (×1600.) (*b*) Pachytene of KYS, a line with a medium content of knob heterochromatin. Large knobs are present on the chromosome arms 5L and 7L, small knobs on 9S, 6L, and 6S. The small terminal knob on 6S distal to the nucleolus organizer region is indicated by a solid arrow. Broken arrows indicate the centromeres of chromosomes 7 and 10, which are flanked by centric heterochromatin. (×725.) (We are indebted to Janice Lovett of Indiana University for this figure.) (*c*) B chromosome pair from a pachytene nucleus showing the proximal knob (arrow) near the centromere which is separated by euchromatin from the distal blocks of heterochromatin. (×3500.)

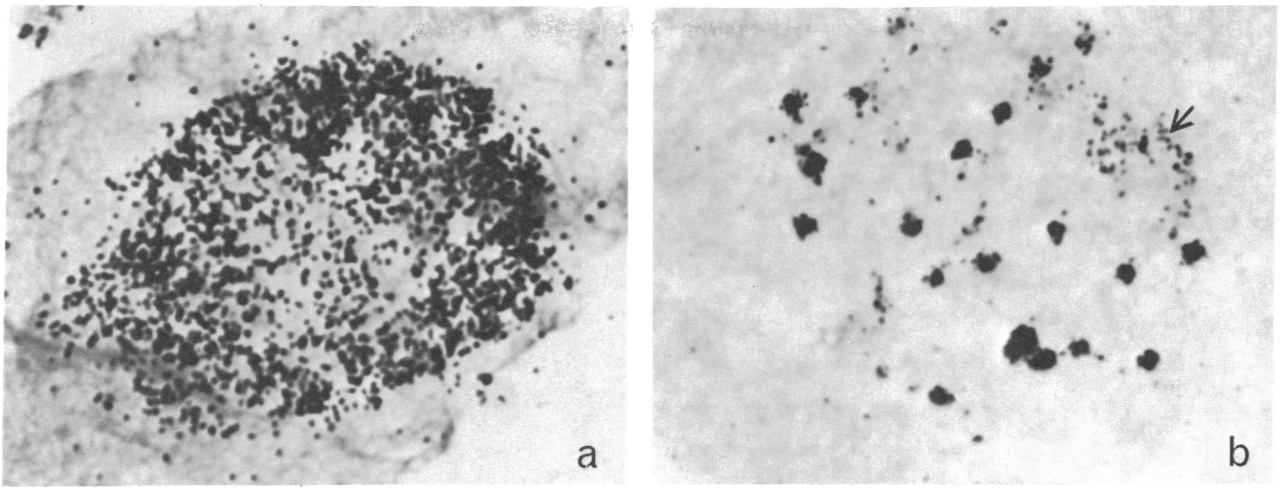

FIG. 2. Label incorporation patterns in interphase nuclei. (*a*) General label pattern in a line with a high knob content (P100). The less intensely labeled region in the center of the nucleus reflects the presence of a large nucleolus. (×1475.) (*b*) Discrete label pattern in line P100. The arrow indicates a small amount of "label spread," which may represent DNA replication in the centromeric heterochromatic regions, mostly grouped at one "pole" of the nucleus. (×1320.)

score the frequency of unlabeled nuclei to obtain an estimate of the length of the S period in the mitotic cycle. Our data (Table 2) provide a measure of the proportion of the S period in which there is asynchronous replication. In Fig. 3 the mean percentage of discrete labeled interphases for each level of knob content at five fixation times is plotted. These data show that, apart from sampling error, the proportions of discrete and general label patterns are not affected by the time of fixation after the label pulse. This is expected in an asynchronous population of cells such as occurs in the meristematic region of the root tip. Another feature evident in these data is that the proportion of discrete labeled nuclei is directly related to the proportion of knob heterochromatin in the genome. In the lines with low, medium, and high knob content, the asynchronous label pattern occupies (mean ± SEM) 4.8 ± 1.3%, 9.5 ± 1.5%, and 16.4 ± 2.1% of the S period, respectively.

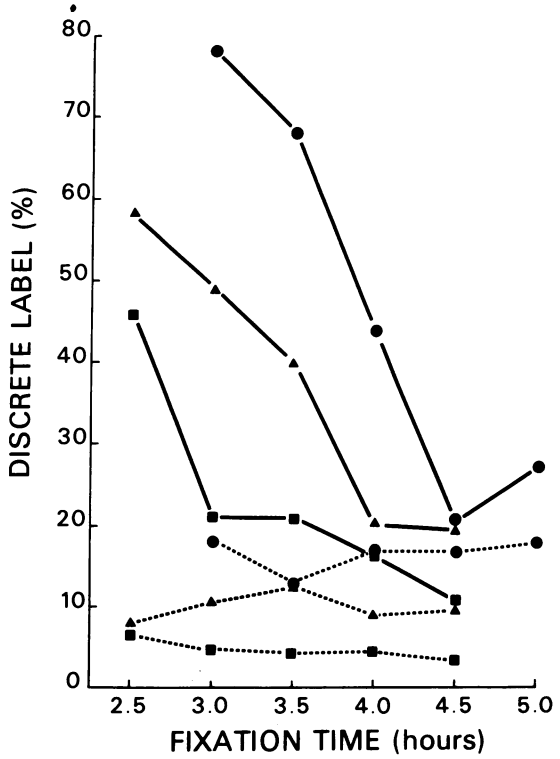

FIG. 3. Percentage of labeled nuclei with discrete label patterns in three lines having low (■), medium (▲), and high (●) numbers of knobs. ---, Interphase nuclei; —, mitotic division figures.

When does this asynchronous labeling occur during the S period, and how are the labeled segments involved distributed in the chromosome complement? Answers to both these questions were obtained by examination of the distribution of label in prometaphase and metaphase figures at the sequential times of fixation after the labeling pulse (Table 2 and Fig. 3). Because the proportion of discrete division figures is highest at the first fixation time and decreases in subsequent fixations, we conclude that the asynchronous labeling occurred at the end of the S period. The distribution of these late replicating segments was confined to the known knob positions in each line (Fig. 4 *a* and *b*). An occasional exception was the late replication of centric heterochromatin, which was easily scored in anaphase because grains were restricted to the polar regions of the two chromosome groups. Figures having late replication of centric heterochromatin and not of knobs were rare and were found mostly in fixations 3–5. Knob replication, although overlapping with replication of centromeric heterochromatin, must be completed later in the S period. The NOR heterochromatin replicates during that period of S when euchromatin replicates.

B Chromosome Replication. In asking whether the heterochromatin of B chromosomes was also late replicating we used a stock with two small knobs (on the long and short arms of chromosome 6) which provided an internal marker for the time of replication of the segments of the B chromosome. The euchromatin of the B chromosome replicated at the same time as the euchromatin of the A chromosomes. The large blocks of heterochromatin on the long arm of the B chromosome (Figs. 1*c* and 4*d*) were late replicating and finished their replication subsequent to the completion of the replication in the centric heterochromatin of the A chromosomes. The proximal heterochromatic knob was the last B-chromosome segment to undergo replication. It replicated at the same time as the marker knobs on chromosome 6 (Fig. 4 *c* and *d*). In those seedlings in which there were two or more B chromosomes, no evidence was

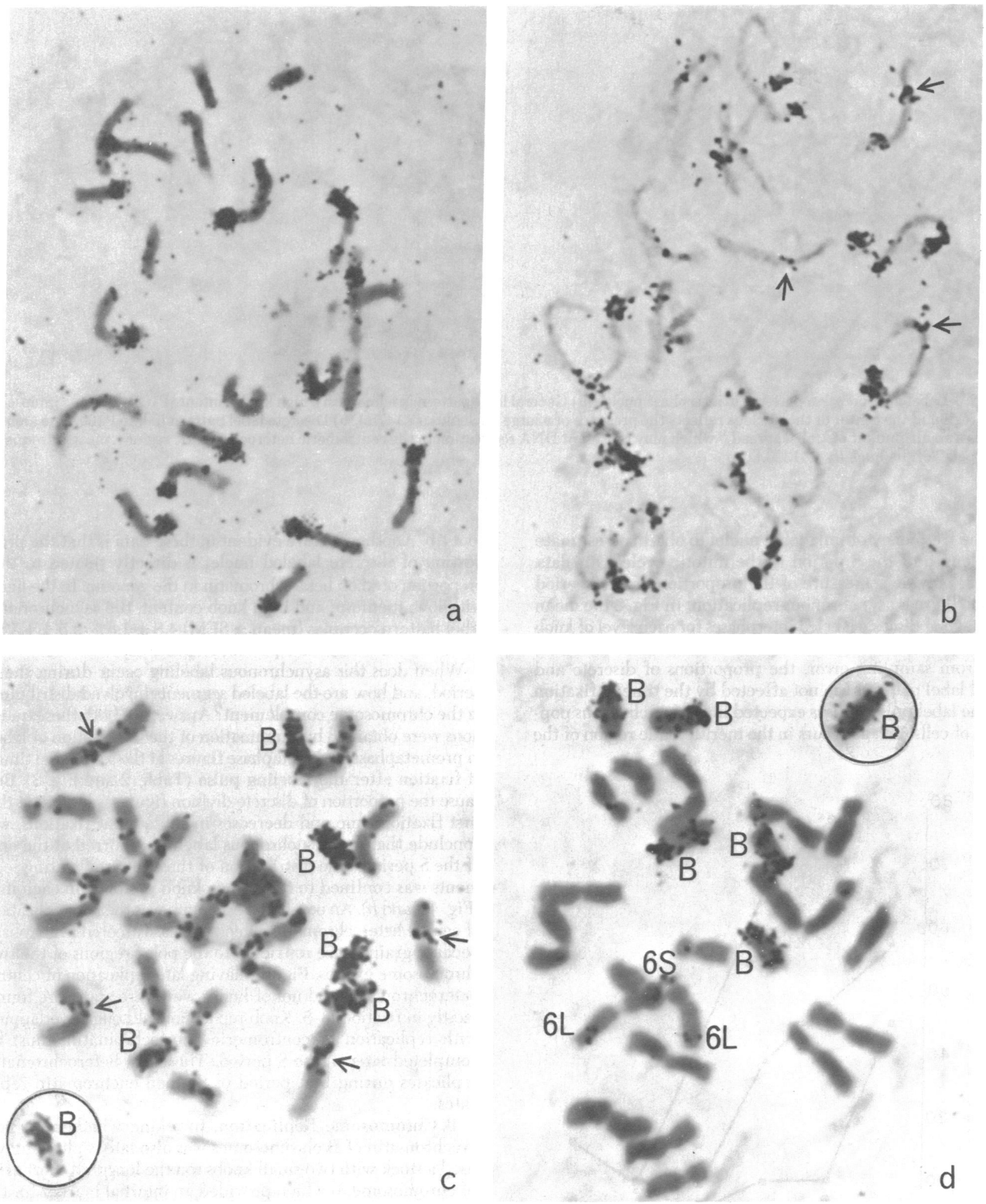

FIG. 4. Late labeling patterns. (*a*) Mitotic metaphase of the line with a high knob content (P100) showing [^{3}H]thymidine incorporation only in knobs. (×2200.) (*b*) Prometaphase of P100 showing late replication of knob regions but with some chromosomes also showing late replication of centromeric heterochromatin (arrows). (×2200.) (*c*) Late labeling of centromeric heterochromatin and supernumerary B chromosomes in line with a low knob content (Black Mexican). Some centromere regions are indicated with arrows, and B chromosomes are marked by B. (×2400.) (*d*) A metaphase showing even later labeling than in *c*. The only knob heterochromatin in this line (Black Mexican) occurs in the small 6S and 6L knobs. They replicate at the same time as the proximal knob in the supernumerary B chromosomes. In some B chromosomes (encircled), late replication of the distal heterochromatin is evident. (×2400.)

found for differential replication among the B chromosomes (see ref. 7).

Because there was a relationship between the extent of asynchronous late replication and the knob heterochromatin

Table 3. Patterns of label incorporation in interphase nuclei in seedlings with different numbers of B chromosomes

No. of B chromosomes	Seedlings scored	Nuclei scored	Discrete label, %
0	3	1056	3.1
2	3	1021	4.5
3	3	1117	4.6
4	4	1988	7.9
5	4	2130	8.6
6	1	526	8.7
7	3	1525	9.7
8	3	1562	9.8
12	1	521	13.7

content of the nucleus, we asked if there was a similar relationship for B-chromosome heterochromatin. Frequencies of general and discrete labeled nuclei were determined in seedlings with known numbers of B chromosomes (Table 3). The regression analysis (Fig. 5) established that, just as for knob heterochromatin, an increasing B-chromosome content was associated with an increase in the proportion of the S period involved in localized late replication.

Abraham and Smith (7) determined the replication time of B chromosomes of maize by following the incorporation of labeled thymidine in the nuclei of root tip cells. They reported that B chromosomes replicated in the last half of the S period. However, our finding that replication is first completed in the euchromatic region followed by distal heterochromatic segments and finally by the proximal knob adjacent to the centromere indicates heterogeneity within the B chromosome, similar to that found in A chromosomes.

CONCLUSIONS

Our data show two major points: (*i*) Most heterochromatin in maize is late replicating and the different classes of heterochromatin have their own distinctive times of replication. Replication of nucleolus organizer heterochromatin is completed prior to the period of asynchronous late replication. (*ii*) The proportion of asynchronous late replication occurring in the S period is directly dependent on the knob and B-chromosome heterochromatin content of the nucleus.

Differential Replication of Heterochromatin Types. Knob heterochromatin is cytologically distinct from the other classes of heterochromatin in maize (8), and we have found that it is also distinct in that it is the last component of the chromosome complement to complete DNA replication. This is true for the heterochromatic knob in the proximal region of the B chromosome as well as for the knobs on A chromosomes, including the large knob on abnormal chromosome 10. The differentiation of knob heterochromatin from other classes of heterochromatin is also seen in its staining behavior after C-banding treatment (9, 10). Unpublished studies have shown that the major DNA component of knobs is a 185-base-pair sequence, tandemly repeated in many copies in each knob but not detectable in the other classes of heterochromatin.

Proportion of Asynchronous Labeling in S Period. Although our data established that the proportion of discrete labeling relative to general labeling among interphase nuclei increases when heterochromatin content increases, we cannot differentiate between the possibilities of altered proportions within a constant S period as opposed to changes in the length of the S period itself. Change in the length of the G2 period is possible because, in order to detect any labeled divisions in the stocks with a high knob content, we had to delay the time of the first fixation (see Fig. 3). It may be that the heterochromatin content influences several components of the mitotic cycle, but it would be necessary to work with isogenic lines to evaluate this situation. In rye, it has been established that increasing the number of B chromosomes (0 to 4) lengthens the mitotic cycle and changes the relative timing of component phases (11).

We recognize that our analysis has been restricted to the mitotic cells of root tips. Nevertheless, we believe that the distinctly later replication pattern displayed by knobs and by the proximal B chromosome heterochromatin in root tip cells is characteristic of all cell types. We therefore conclude that our demonstration of late replication of the knobs of both A and B chromosomes lends support to the hypothesis advanced by Rhoades and Dempsey (2) to account for B-chromosome nondisjunction at the second pollen grain mitosis and for the loss of A-chromosome segments induced by interaction between knobs and B chromosomes. Further support for this hypothesis comes from Saraiva's findings (12) that the structural changes produced by the interaction of B chromosomes and knobs are precisely those predicted by the delayed replication model.

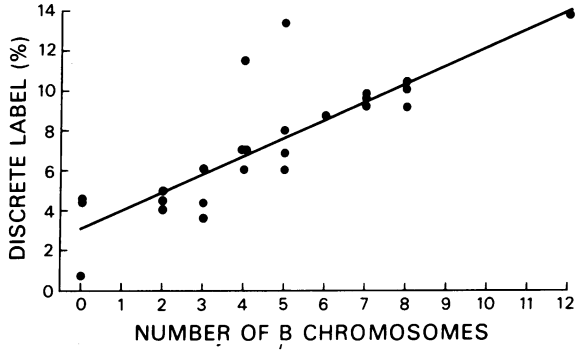

FIG. 5. Percentage of interphase nuclei with discrete label patterns in seedlings with different numbers of B chromosomes (b = 0.0093 ± 0.001).

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