Mouse Satellite DNA, Centromere Structure, and Sister Chromatid Pairing

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Abstract. The experiments described were directed toward understanding relationships between mouse satellite DNA, sister chromatid pairing, and centromere function. Electron microscopy of a large mouse L929 marker chromosome shows that each of its multiple constrictions is coincident with a site of sister chromatid contact and the presence of mouse satellite DNA. However, only one of these sites, the central one, possesses kinetochores. This observation suggests either that satellite DNA alone is not sufficient for kinetochore formation or that when one kinetochore forms, other potential sites are suppressed. In the second set of experiments, we show that highly extended chromosomes from Hoechst 33258–treated cells (Hilwig, I., and A. Gropp, 1973, *Exp. Cell Res.*, 81:474–477) lack kinetochores. Kinetochores are not seen in Miller spreads of these chromosomes, and at least one kinetochore antigen is not associated with these chromosomes when they were subjected to immunofluorescent analysis using anti–kinetochore scleroderma serum. These data suggest that kinetochore formation at centromeric heterochromatin may require a higher order chromatin structure which is altered by Hoechst binding. Finally, when metaphase chromosomes are subjected to digestion by restriction enzymes that degrade the bulk of mouse satellite DNA, contact between sister chromatids appears to be disrupted. Electron microscopy of digested chromosomes shows that there is a significant loss of heterochromatin between the sister chromatids at paired sites. In addition, fluorescence microscopy using anti–kinetochore serum reveals a greater interkinetochore distance than in controls or chromosomes digested with enzymes that spare satellite. We conclude that the presence of mouse satellite DNA in these regions is necessary for maintenance of contact between the sister chromatids of mouse mitotic chromosomes.

SATELLITE DNAs were first discovered over twenty years ago as species of DNA which, due to their unusual base composition, band at densities distinct from bulk DNA upon equilibrium sedimentation (Kit, 1961). Subsequently, it was shown that these DNAs are highly repetitious, that they are arranged in long tandem arrays (Waring and Britten, 1966), and that they are localized typically in pericentric or telocentric heterochromatin (for a recent review, see Singer, 1982). Many of these DNAs, including mouse satellite DNA (Horz and Altenburger, 1981), have been sequenced. Despite detailed knowledge of the structure and location of satellite DNAs, their potential function(s) have only been hypothesized. These range from none (i.e., selfish DNA [Crick and Orgel, 1980; Doolittle and Sapienza, 1980]) to roles in many events including enhanced or reduced recombination (John and Miklos, 1979), spindle attachment (Avila et al., 1983), gene amplification (Bostock and Clark, 1980), chromosome pairing and/or segregation (for a discussion of possible functions, see John and Miklos, 1979, and Brutlag, 1980). Unfortunately, most of these hypotheses do not readily lend themselves to experimental investigation.

Mouse chromosomes offer several advantages for investigating the possible role of specific DNA sequences in centromeric heterochromatin structure and function. Centromere regions are readily visible as highly condensed chromatin both by light and electron microscopy. In addition, there is one major species of satellite DNA with a density of 1.691 g/cc which is present almost exclusively within the highly condensed centromeric heterochromatin of all mouse chromosomes with the possible exception of the Y chromosome (Pardue and Gall, 1970). Finally, mouse satellite DNA, with a repeat length of 234 base pairs, is almost totally resistant to certain restriction enzymes but highly susceptible to others. Thus centromeric heterochromatin can be enriched for by selective digestion (Lica and Hamkalo, 1983).

When L929 cells are grown in the (A + T)-specific dye Hoechst 33258 (Hilwig and Gropp, 1973), there is a profound alteration in the condensation of centromere regions. Fortuitously, this cell line contains a large marker chromosome with multiple C-banded regions (Hutchison, 1982; Vig, 1984). We have used these cells to investigate the effects of Hoechst and the solubilization of the major satellite spe-

cies on centromere structure by combining these treatments with fluorescence and electron microscopy of mitotic chromosomes. In this communication we show that, although there is usually a spatial correlation between the presence of mouse satellite DNA and the presence of kinetochores in metaphase chromosomes, there are situations in which long tandem arrays of satellite DNA are present without the manifestation of kinetochores. In addition, we observed that sister chromatids are held in juxtaposition at sites containing satellite DNA. Finally, we show that treatment of chromosomes with specific restriction endonucleases which solubilize the major satellite DNA species results in the disruption of this chromatid contact.

Materials and Methods

Cell Culture and Mitotic Selection

Semiconfluent monolayers of L929 cells grown in Joklik's modified minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (KC Biological Inc., Lenexa, KS) were used in all experiments. Cells were incubated for 11 h in the presence of Colcemid (Gibco) at a concentration of 100 ng/ml in order to obtain metaphase arrest. Metaphase cells were harvested by selective detachment, pelleted at 2,500 g for 5 min, and resuspended in medium. In some experiments, cells were grown in the presence of Hoechst 33258 for 36 h following the procedure described by Hilwig and Gropp (1973).

Electron Microscopy (EM)

Metaphase chromosomes from untreated or Hoechst-treated cells were deposited on EM grids essentially as described by Rattner and Hamkalo (1978)

except that preparations used for enzyme digestions or in situ hybridization were deposited on gold grids. EM in situ hybridization was carried out with minor modifications of the technique described by Hutchison et al. (1982). Briefly, mouse satellite DNA was nick translated in the presence of C-16-biotin-dUTP (the generous gift of Dr. David Ward), hybridized to fixed, denatured chromosomes on EM grids, and the hybrids were identified by a two-step antibody procedure using 20-nm colloidal gold particles for EM detection. Specimens to be subjected to restriction digestion were centrifuged onto grids followed by air drying out of 0.4% Photoflo (Eastman Kodak Co., Rochester, NY). Each grid was floated on a 50-μl drop of Joklik's minimum essential medium containing 0.8 U of restriction endonuclease AvaII or AluI. Digestions proceeded at room temperature for 1 to 2 h after which grids were rinsed thoroughly in Photoflo, dried, and stained with 1% alcoholic phosphotungstic acid. Grids were examined in a JEOL 100C electron microscope operated at 80kV.

Restriction Digestions for Fluorescence Microscopy

Suspensions of metaphase chromosomes were digested in petri dishes. A coverslip was placed in each dish before the addition of chromosomes. Chromosomes were digested with 5 U of enzyme per μg DNA for 1-2 h at room temperature. As the digestion proceeded, chromosomes settled onto the coverslips.

Fluorescence Microscopy

Coverslips containing untreated, Hoechst-treated, or restriction enzyme-digested chromosomes were fixed in 3.7-4% paraformaldehyde and then stained with anti-kinetochore antibody and rhodamine-conjugated secondary antibody as described by Lica and Hamkalo (1983). After mounting with Gelvatol (Monsanto Co., Dayton, OH), coverslips were viewed with a Zeiss filter set 48 77 12 for rhodamine detection or 48 77 02 for Hoechst 22358 detection.
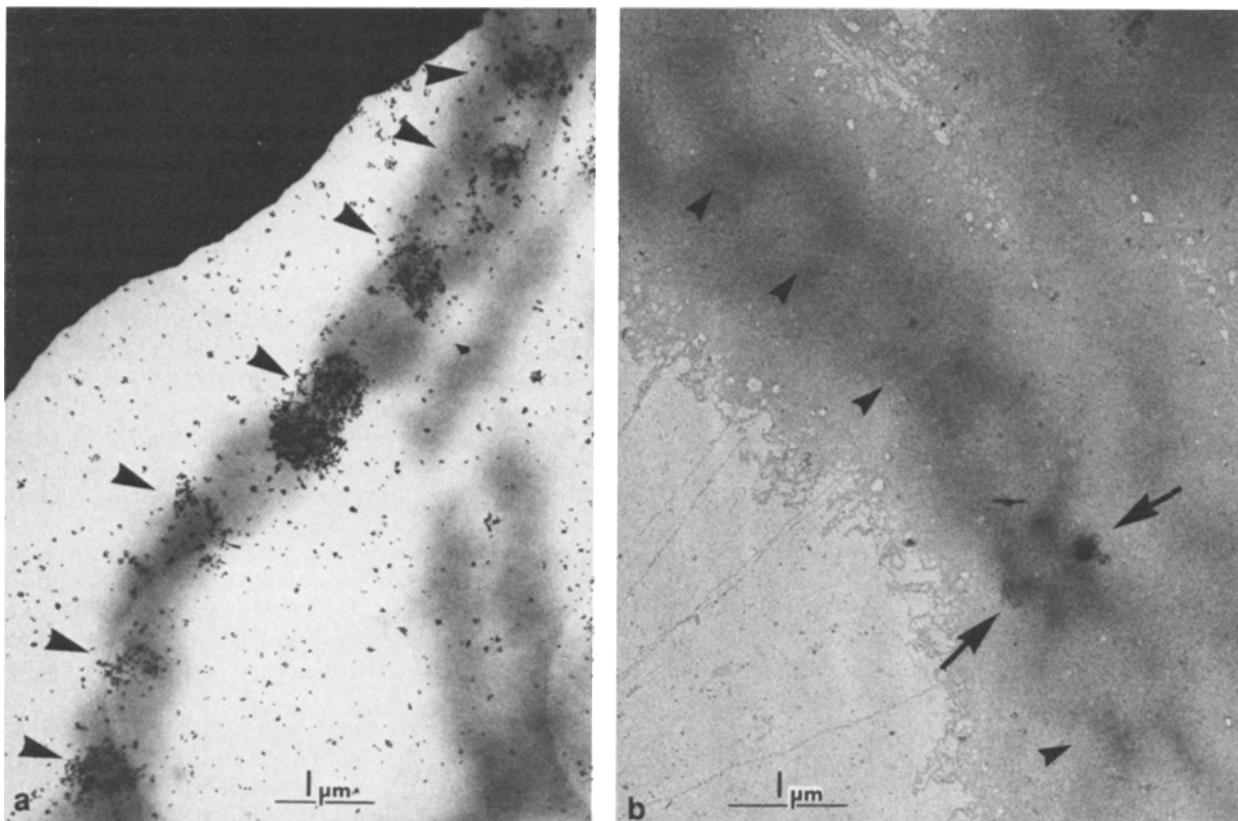

Figure 1. Electron micrographs of the large marker chromosome in mouse L929 cells (a) shows hybridization in situ of biotinylated mouse satellite DNA to multiple sites (*arrowheads*) after immunogold staining. (b) The marker before hybridization illustrating multiple secondary constrictions (*arrowheads*), but a single pair of kinetochores (*arrows*).

Results and Discussion

The Structure of the Large Marker Chromosome in L929 Cells

Large stable marker chromosomes with several secondary constrictions in both arms have been seen in a number of *Mus musculus* cultured cell lines including 500 γβ (White et al., 1975), LA (Hilwig and Gropp, 1973), and L929 (Hutchison, 1982; Vig, 1984; Rattner and Lin, 1985). When biotinylated mouse satellite DNA is hybridized to L929 chromosomes and hybrid sites are detected by colloidal gold, satellite sequences are obvious at all sites of sister chromatid contact in the large marker (Fig. 1 *a*). The central site shows an intense bipartite pattern of hybridization. The secondary sites of labeling are clearly less intense suggesting the presence of fewer copies of the satellite DNA repeat. Analysis of hybridization patterns on a large number of these chromosomes suggests that the major site of labeling is a center of symmetry with approximately equivalent levels of labeling at comparable secondary constrictions. We do not think that the lower labeling at secondary sites is due to a lower degree of accessibility of satellite sequences to probe since ~100 marker chromosomes have been identified with the same labeling pattern. In addition, labeling at these sites was not increased when chromosomes were partially decondensed before centrifugation onto EM grids (Lundgren, K., and B. A. Hamkalo, unpublished data). This observation is consistent with the hypothesis that this chromosome arose as a result of a series of fusions followed by duplication to form an isochromosome (Vig et al., 1984). However, the fact that the secondary constrictions exhibit a lower hybridization signal than the primary constriction of this and other chromosomes in these cells (Narayanswami, S., and B. A. Hamkalo, unpublished data) suggests that some DNA was deleted at these sites during the generation of this marker chromosome.

Paired densely stained structures are present only at the primary constriction of this chromosome (Fig. 1 *b*), in agreement with the observations of Rattner and Lin (1985). When microtubules are stabilized in mitotically arrested preparations, they can be seen attached to these structures supporting their identification as kinetochores (Hamkalo, B. A., unpublished data). When L929 chromosomes are reacted with an anti-kinetochore serum (Lica and Hamkalo, 1983) followed by immunofluorescent staining, no chromosomes were observed with more than one pair of fluorescent dots (data not shown). These results are in agreement with data from Earnshaw and Migeon (1985) but in contradiction with that of Merry et al. (1985). Since many autoimmune sera are

not well characterized it is not possible to define the basis of this apparent discrepancy.

The existence of sites in marker metaphase chromosomes that lack kinetochores but contain satellite sequences has implications for the regulation of kinetochore development. This observation is consistent with either of two hypotheses. Vig (1984) suggested that all C-banded regions of this chromosome possess the information necessary to form a kinetochore but that there is "centromere-dominance" so that formation of one pair of kinetochores results in suppression of secondary sites, perhaps by a substance which travels along the chromosome. This hypothetical phenomenon was previously proposed to explain the existence of stable isodicentric human X chromosomes (Therman et al., 1974). However, the existence of dicentric chromosomes which break during anaphase because they possess two functional centromeres (McClintock, 1939; Mann and Davis, 1983) argues against the idea of centromere dominance for these chromosomes.

An alternative hypothesis to explain the absence of kinetochores at secondary constrictions invokes the existence of DNA sequences other than, or in addition to, mouse satellite which provide information required for kinetochore establishment. If these sequences were deleted along with satellite DNA from the secondary constrictions during the formation of the marker chromosome the secondary sites would not be expected to possess kinetochores. A direct test of this idea awaits the identification of mouse DNA which can function as a centromere.

Absence of Kinetochores in Chromosomes from Hoechst-treated Cells

When mouse cells are grown for an extended period of time in Hoechst 33258, several changes are notable. Cell number increases more slowly than the control and, after one doubling, cells no longer divide although they grow in size. This result agrees with those of Hirschberg et al. (1980) with hamster E-36 cells. As noted above, growth of mouse cells in Hoechst 33258 results in inhibition of normal chromosome condensation, particularly at sites containing mouse satellite DNA. As first noted by Hilwig and Gropp (1973), chromosomal regions which are C-band positive appear as thin threads in the fluorescence microscope after Hoechst 33258 treatment (Fig. 2 *a*). With the exception of reduced condensation, these chromosomes superficially appear normal. However, a significant difference is seen when these chromosomes are stained with anti-kinetochore serum from a CREST scleroderma patient.

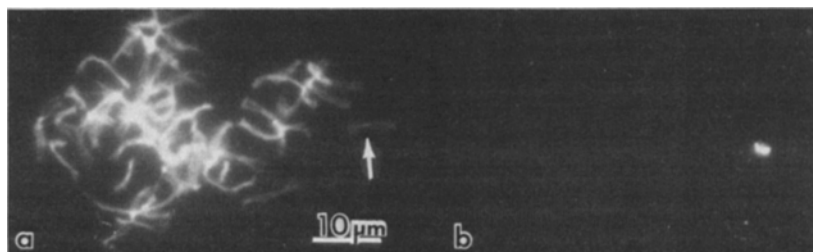Control metaphase chromosomes exhibit a paired-dot

Figure 2. Fluorescence micrographs of chromosomes from L929 cells grown in the presence of Hoechst 33258 according to Hilwig and Gropp (1973). Mitotically arrested cells were lysed on coverslips, stained with anti-kinetochore antibodies and rhodamine-conjugated anti-IgGs as described in Lica and Hamkalo (1983). The same field is shown; using appropriate filters (*a*) fluorescence due to Hoechst permits visualization of chromosomes but (*b*) there is no typical paired dot staining with rhodamine. The arrow in *a* indicates an extended centromere region.

staining pattern (Fig. 4 a and Lica and Hamkalo, 1983) after incubation with this anti-kinetochore serum and rhodamine-conjugated secondary antibody. On the other hand, when chromosomes from Hoechst-treated cells are stained with the same antibody, the characteristic paired-dot pattern is absent and no specific staining is visible. Fig. 2 a shows Hoechst fluorescence of a typical field and Fig. 2 b shows rhodamine fluorescence of the same field. Further evidence for the lack of kinetochore structures in these chromosomes is provided in the electron micrograph shown in Fig. 3. Paired kinetochores, equivalent to those shown in Fig. 1 b, are absent from under-condensed chromosomes.

One interpretation of these data is that Hoechst directly prevents kinetochore proteins from binding to chromosomes. An alternative interpretation is that there are both compositional and spatial requirements for the development of a kinetochore. That is, specific proteins must be associated with centromeric heterochromatin in order for it to fold into a particular higher order structure which then can be recognized directly or indirectly as a site for kinetochore assembly. Extensive binding of Hoechst to centromeric DNA could alter its ability to bind to putative condensation-related polypeptides or displace these polypeptides, resulting in less-condensed centromeric heterochromatin, and the absence of kinetochores. We cannot distinguish between these interpretations with the data available. Nevertheless, the absence of identifiable kinetochores and at least one kinetochore antigen after Hoechst treatment suggests that these chromosomes cannot attach to the mitotic apparatus and, consequently, cannot segregate.

A somewhat unexpected observation noted above is that Hoechst-treated cells cannot divide. One trivial explanation for this is that Hoechst affects a variety of cellular functions in addition to binding to (A+T)-rich DNA. One interesting

speculation is that there is a link between normal mitotic activity and cytokinesis. Such a link could involve a requirement for chromosome segregation before cell division. If, as we propose, Hoechst-treated chromosomes do not attach to the spindle, then correct segregation cannot occur and one might see secondary effects on cytokinesis. Obviously, this hypothesis is purely speculative but suggests experimental approaches to investigate it.

The Effects of Restriction Nuclease Digestion on the Structure of the Centromere Region

In these experiments, a chromosome suspension in a petri dish is digested with a restriction enzyme. During digestion, chromosomes settle onto a coverslip, and kinetochores are visualized using anti-kinetochore antibody and immunofluorescence. Using this protocol, we previously reported that pericentric heterochromatin of mouse chromosomes is resistant to restriction endonucleases that do not recognize or cleave mouse satellite DNA. Digestion of metaphase chromosomes by EcoRI or AluI results in the generation of relatively intact centromeric regions which bind anti-kinetochore antibody in a paired dot configuration equivalent to the undigested control (see Fig. 4 b in Lica and Hamkalo, 1983). However, digestion of chromosomes with enzymes that cleave satellite into small fragments (e.g., AvaII or BstNI) gives a different result. Although the chromosomes retain their ability to bind anti-kinetochore antibody, the members of a pair of fluorescent dots are typically further apart (Fig. 4, c and d) when compared to equivalently stained chromosomes that are mock digested (Fig. 4 a) or digested with AluI (Fig. 4 b). In some areas of the slide the fluorescent dots are so far apart that it is not possible to define pairs (Fig. 4 d).

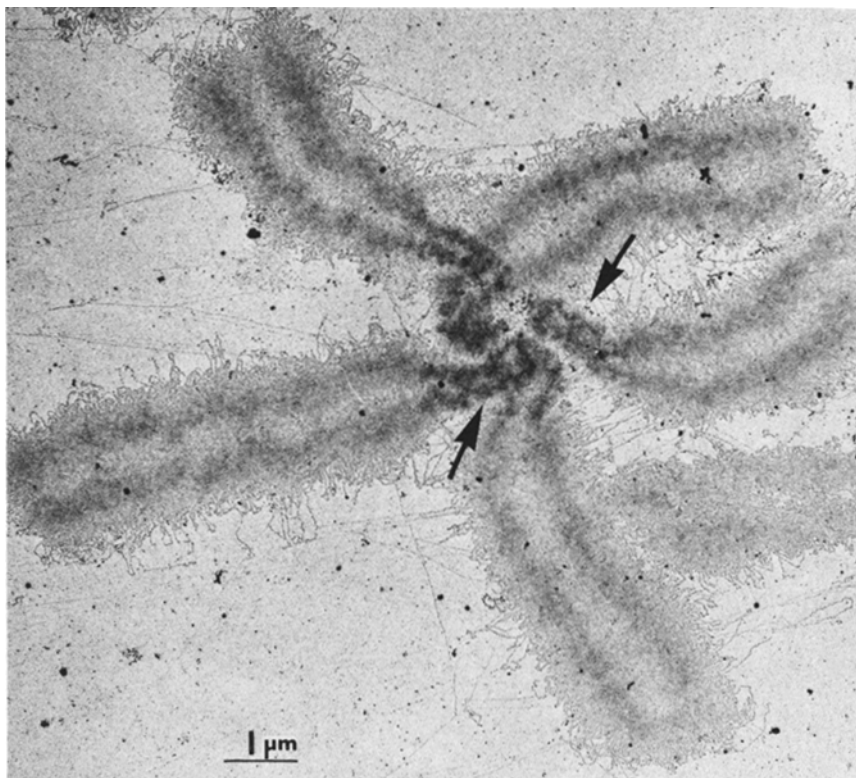Since some chromosomes settle onto the coverslip early in

Figure 3. Electron micrograph of L929 metaphase chromosomes released from cells grown in the presence of Hoechst 33258 (Hilwig and Gropp, 1973). Arrows indicate primary constrictions devoid of kinetochores.
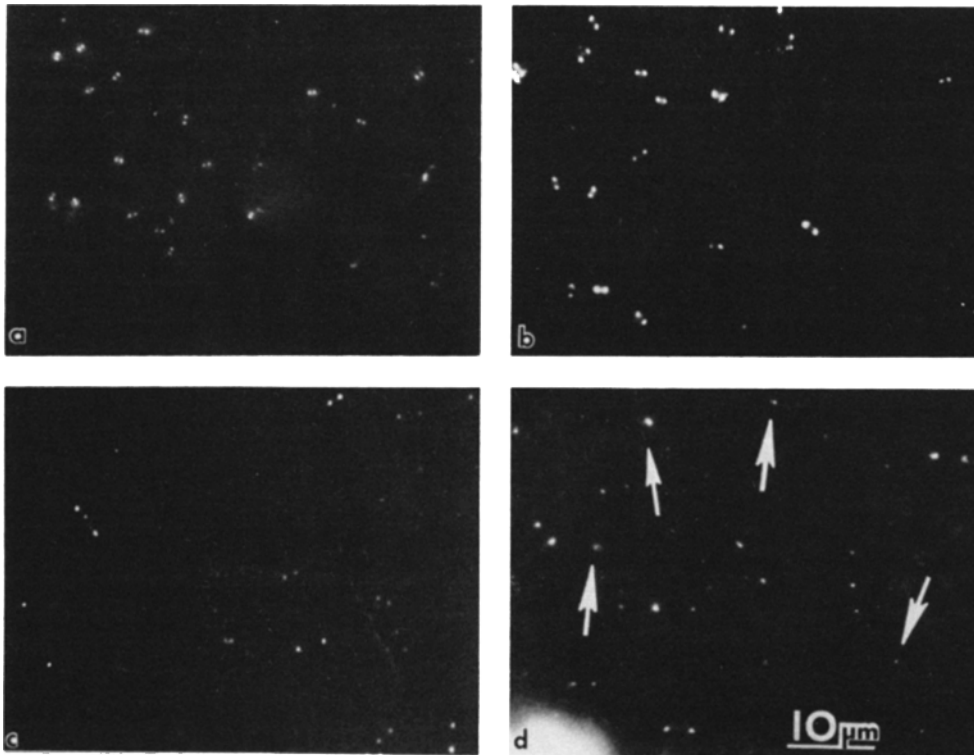
Figure 4. Fluorescence micrographs of restriction endonuclease-digested L929 chromosomes stained with anti-kinetochore antibody and rhodamine-conjugated secondary antibody (Lica and Hamkalo, 1983). Metaphase cells were lysed in the presence of (*a*) no enzyme; (*b*) AluI; (*c*) BstN1, or (*d*) AvaII. Arrows in *d* denote unpaired kinetochores.

the digestion, separation of sister chromatids could be limited. On the other hand, kinetochores associated with chromosomes which settle after significant digestion presumably can dissociate and appear as single fluorescent dots (arrows in Fig. 4 *d*). Table I presents data on center-to-center distances of members of pairs of kinetochores in either undigested controls and after BstN1 or AluI digestion. In those cases where it was possible to measure spacing, sister kinetochores are as much as four times as far apart as undigested, EcoRI-, or AluI-digested controls. In addition, there is wide variation in inter-kinetochore distances in the BstN1- or AvaII-digested chromosomes when compared to the other preparations (Table I; Fig. 5), suggesting that solubilization of satellite DNA relieves a constraint on the centromere region thus permitting kinetochores to move apart.

The large variation in the kinetochore-to-kinetochore distances in the chromosomes subjected to AvaII and BstN1 digestion could be explained by the time chromosomes settle during digestion, as noted above. Thus, extreme values for inter-kinetochore distances of the measurable pairs may be more significant than the average. Based on this type of ex-

periment, we suggest that satellite DNA is involved in maintaining contact between sister chromatids in the centromere region.

This hypothesis is further supported by EM analysis of

Table I. The Effects of Restriction Enzyme Digestion on Interkinetochore Distance

Enzyme treatment*	Mean distance between sister kinetochores	Standard deviation	Number of measurements
	μm		
None	0.82	0.265	23
Alu I	1.21	0.261	44
Bst N1	1.88	0.764	25

* Digestions and immunofluorescence were carried out as described in Materials and Methods.
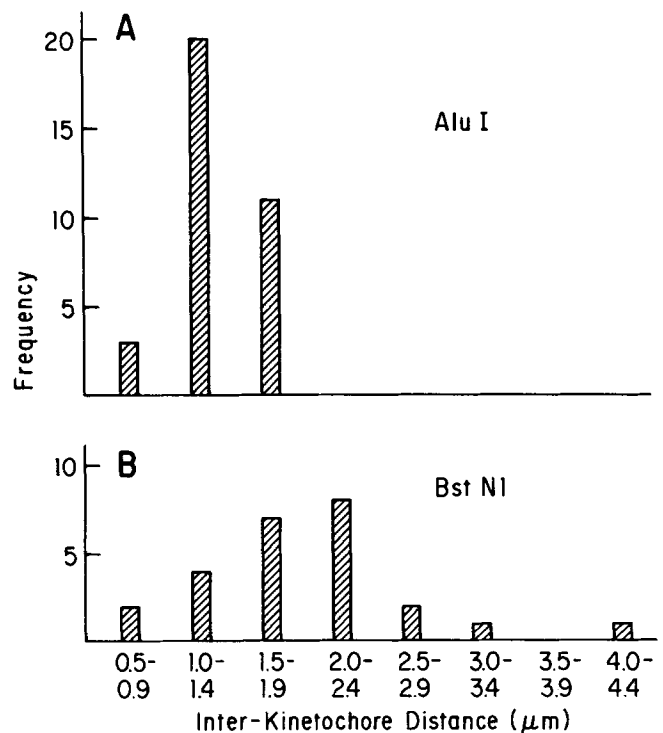
Figure 5. Distribution of inter-kinetochore distances after restriction enzyme digestion. Measurements were taken from negatives of fluorescent micrographs such as those shown in Fig. 4 magnified 1,000×.
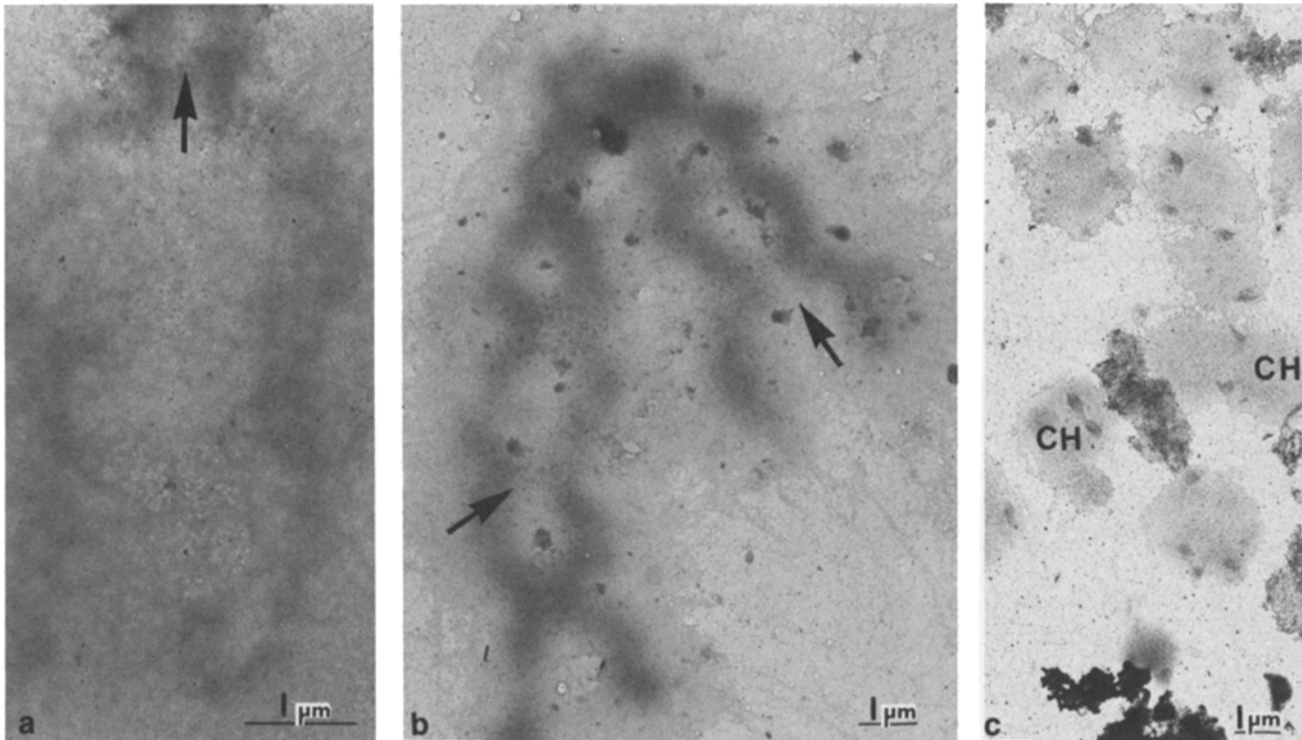
Figure 6. Electron micrographs of restriction endonuclease–digested chromosomes. L929 chromosomes were deposited on EM grids, air dried, and then digested with AvaII (*a*) and (*b*), or AluI (*c*). Arrows in *a* and *b* mark sites where material has been digested between sister chromatids. The marker chromosome in *b* shows two of these sites. *CH*, structurally intact centromeric heterochromatin masses.

chromosomes immobilized by drying onto EM grids and then digested with restriction enzymes. Fig. 6 illustrates the results of digestion of chromosomes with an enzyme which cleaves within the satellite repeat (AvaII, Fig. 6, *a* and *b*) or an enzyme which does not digest satellite (AluI, Fig. 6 *c*). Although much chromosomal DNA remained adsorbed to the grid, a region between sister chromatids appears to be depleted of heterochromatin when AvaII is used (Fig. 6 *a*). Fig. 6 *b* shows a marker chromosome after AvaII digestion; chromatin has been solubilized at two sites where sister chromatids are in contact. Fig. 6 *c* shows a more extensive digest of immobilized chromosomes with AluI which does not cleave in the satellite repeat. In this case, chromosome arms were totally digested. Despite the greater degree of digestion compared to Fig. 6, *a* and *b*, numerous centromere regions are visible which are ultrastructurally indistinguishable from those of undigested chromosomes. This observation supports the immunofluorescent data in Fig. 4, *b* and *c*, that enzymes which do not cleave satellite do not digest DNA crucial for the maintenance of sister chromatid contact. In addition, these results confirm those of Kaebling et al. (1984) who reported that BstN1 and AvaII digestion of fixed mouse chromosomes resulted in reduced C-band staining near the centromere and, in addition, support the notion that satellite sequences always occur where sister chromatids are paired. We cannot eliminate the possibility that the patterns of chromosome digestion observed are more reflective of the digestion properties of the different restriction enzymes than of chromosome organization per se. However, there is a direct correlation between the ability of several different restriction enzymes to digest satellite DNA and to cause the same struc-

tural alteration of metaphase chromosomes. This correspondence argues in favor of our interpretation of the data.

Conclusions

One major conclusion from the work described is that the association of kinetochores with centromeric regions of mouse chromosomes is not simply due to the presence of mouse satellite DNA sequences. However, mouse satellite DNA does appear to play a crucial role in the maintenance of contact between sister chromatids during metaphase. The following observations support the first conclusion. Kinetochores retain their ability to interact with an anti-kinetochore serum after digestion of much of the satellite DNA but they are not present at secondary constrictions, despite the presence of some satellite DNA. In addition, kinetochores are not found at the primary constrictions of undercondensed chromosomes which result from Hoechst-33258 treatment of mouse cells. The second conclusion is supported by our observations that satellite DNA is present at every location where metaphase chromatids are held in juxtaposition, and that destruction of satellite DNA can disrupt the contact between sister chromatids at the centromere as measured by increases in sister kinetochore distances and the appearance of spaces between sister chromatids at these sites.

Vig and Zinkowski (1985) demonstrated a direct relationship between the amount of C-banding of mouse chromosomes (presumably a reflection of the amount of mouse satellite DNA) and the timing of sister chromatid separation. Although C-band negative, the mouse Y chromosome ap-

pears to possess a small amount of satellite DNA (Narayanswami, S., and B. A. Hamkalo, preliminary observations) and separates first, followed by the separation of chromosomes with increasing amounts of C-banded material. Our observation that intact satellite DNA appears to be required for the maintenance of sister chromatid contact implies that the events that immediately precede separation must involve this DNA. These reactions probably include a small amount of DNA replication and/or topological resolution. Since satellite-containing chromatin is highly condensed, it may be less accessible to the enzymes involved and, therefore, the time required for completion of these reactions could be directly related to the amount of satellite DNA in a centromere region. Further work on the biochemical characterization of centromeric components offers an opportunity to investigate the molecular basis of the observations reported here.

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