Chromosome Behavior after Laser Microirradiation of a Single Kinetochore in Mitotic PtK₂ Cells

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ABSTRACT The role of the kinetochore in chromosome movement was studied by 532-nm wavelength laser microirradiation of mitotic PtK₂ cells. When the kinetochore of a single chromatid is irradiated at mitotic prometaphase or metaphase, the whole chromosome moves towards the pole to which the unirradiated kinetochore is oriented, while the remaining chromosomes congregate on the metaphase plate. The chromatids of the irradiated chromosome remain attached to one another until anaphase, at which time they separate by a distance of 1 or 2 μm and remain parallel to each other, not undergoing any poleward separation. Electron microscopy shows that irradiated chromatids exhibit either no recognizable kinetochore structure or a typical inactive kinetochore in which the tri-layer structure is present but has no microtubules associated with it. Graphical analysis of the movement of the irradiated chromosome shows that the chromosome moves to the pole rapidly with a velocity of ~3 μm/min. If the chromosome is close to one pole at irradiation, and the kinetochore oriented towards that pole is irradiated, the chromosome moves across the spindle to the opposite pole. The chromosome is slowed down as it traverses the equatorial region, but the velocity in both half-spindles is approximately the same as the anaphase velocity of a single chromatid. Thus a single kinetochore moves twice the normal mass of chromatin (two chromatids) at the same velocity with which it moves a single chromatid, showing that the velocity with which a kinetochore moves is independent, within limits, of the mass associated with it.

It is now generally accepted that mitotic spindle formation and chromosome movement involve several structures, including microtubules, centrioles, pericentriolar regions, and the centromere regions of the chromosomes. There have been several investigations into the ultrastructure and chemical composition of the kinetochore, or attachment site of microtubules to the centromeric region (14, 30-32, 35). Electron microscope studies have revealed a relatively uniform morphology of the kinetochore region in many different eukaryotes (including PtK₂ cells [34]) consisting of a trilaminar structure 0.3–0.6 μm in diameter. Although the kinetochore has been demonstrated unequivocally to be a microtubule-organizing center in vitro (15, 22, 36, 38), very little biochemical information is available on it. However, there are indications that ribonucleoprotein is a component of the kinetochore (9, 32) and it appears to be associated with the inner plate of the trilaminar kinetochore in PtK₂ cells.

One of the earliest studies on the function of the centromere pertains to the mitotic behavior of x-ray-induced chromosome fragments lacking centromeres (11, 12). Although these fragments did not join the metaphase plate, there was some separation of the chromatids at anaphase. However, they lagged behind the rest of the chromosomes and were often enclosed in small accessory nuclei.

The first microirradiation studies of the centromere or kinetochore region of mitotic chromosomes were performed on newt cells by Uretz et al. (37) using a UV microbeam and by Bloom et al. (8) using a proton microbeam. These experiments showed that irradiation caused the chromosome to lose its ability to undergo directed movement. The chromosome did not congregate on the metaphase plate and was left behind in the interzone at anaphase, forming a micronucleus. Similar results were obtained by Izutsu (17) for UV irradiation of grasshopper meiotic chromosomes. However, later experiments by Bajer and Molé-Bajer (2) using a heterochromatic UV microbeam to irradiate Haemanthus kinetochores did not confirm these results. It was found that chromosomes with kinetochores irradiated during prophase, prometaphase, or metaphase did not show noticeable differences in their movements, as compared with nonirradiated chromosomes, until anaphase when the chromosome tended to remain in an equatorial position.
FIGURE 1 Series of phase-contrast micrographs of a PtK₂ cell in which a single kinetochore was irradiated in prometaphase. Time (in minutes) from irradiation is indicated in bottom right-hand corner of frames. × 2,560. (a) Prometaphase. Lower kinetochore of centromere indicated by arrow is about to be irradiated. (b) Immediately after irradiation. A visible phase-pale lesion is produced at the site of irradiation. (c and d) Chromosome is moving across the spindle towards the pole to which it still has an attachment. (e and f) Metaphase. The undamaged kinetochore is close to the pole. (g) Early anaphase. The irradiated chromosome has split but both chromatids remain close to the pole. (h) Anaphase. The unirradiated chromosomes are moving apart normally but the chromatids of the irradiated chromosome remain close to the pole, parallel, and separated by only a slight distance.
The introduction of the laser microbeam as a tool for experimental cytology by Bessis et al. (7) made it possible to use red light from a ruby laser to produce specific lesions at the subcellular level. The development of a tunable dye laser microbeam (4) provided a wide range of laser wavelengths that could be used to produce many different kinds of subcellular lesions. In particular, the laser microbeam system provides a more precise method than the earlier UV systems for selectively damaging specific regions of the chromosome because the irradiation times are much shorter and the beam can be focused to a smaller, diffraction-sized spot (down to \( \sim 0.2 \mu \text{m} \) in diameter).

Specific regions of chromosomes were first deleted by laser microbeam irradiation in 1969 (6) when it was shown that cell...
FIGURE 5  Electron micrographs of serial sections through the irradiated chromosome shown in Fig. 1. x 25,000. (a) Section through the kinetochore region (arrow) of the unirradiated chromatid. Note that the kinetochore is close to the centriole (C). (b) Section through the edge of the kinetochore (arrow) of the unirradiated chromatid. Numerous microtubules are associated with this region. (c) Section passing through the kinetochore region of the irradiated chromatid. No kinetochore structure is visible in this or adjacent sections.
viability could be maintained for several days after irradiation. Later experiments involving deletion of the nucleolar organizer region showed that the deficiency is heritable in the daughter cells (5). In similar experiments on the function of the centromere region, both sister kinetochores of a chromosome were irradiated at prometaphase with a laser microbeam (10). The results confirmed the earlier finding of Uretz et al. (37), Bloom et al. (8), and Izutsu (17) that the chromosome detaches from the spindle and undergoes no further directed movement for the duration of mitosis. In addition, it was shown that the irradiated chromatids, which were frequently enclosed in a micronucleus at telophase, retain their capacity to replicate their DNA and condense at the next mitosis but do not reattach to the spindle.

In the present study, a single kinetochore of a double-chromatid chromosome was irradiated at prometaphase or metaphase of mitosis in order to investigate the function of the kinetochore in chromosome movement. It was of interest to see what effect the irradiation of a single kinetochore would have on the orientation of the remaining kinetochore and on the subsequent movement of the chromosome and chromatids. It was thought possible that such irradiation would produce behavior similar to that of a univalent in meiosis in which one or more reorientations of the univalent occur followed by nondisjunction (25).

MATERIALS AND METHODS

Cell Culture

The experiments described here were all performed on PtK2 cells, an established line obtained from the American Type Culture Collection. This is an epithelial line from the rat kangaroo kidney and is characterized by the fact that the cells remain flat during division, permitting clear visualization of the chromosomes. The cells were grown as monolayer cultures in T25 flasks in a modified Eagle's medium containing 0.85 g/liter of NaHCO₃, supplemented with 10% fetal
calf serum; no antibiotics were used. Cells were subcultured once a week using a 0.125% solution of Pancreatin 4, N.F. from Grand Island Biological Co. (Grand Island, New York) with 0.1% EDTA. 2 or 3 days before an experiment, cells harvested from the stock cultures were seeded into Rose chambers for irradiation. As visible wavelengths were to be used, the Rose chambers were assembled with standard No. 1 thickness uncoated glass covers.

**Laser Irradiation**

The microbeam system utilized a Chromatix No. 1000 pulsed neodymium-YAG laser with an output of 5 kW at the second harmonic wavelength of 532 nm and a pulse duration of 180 ns. After attenuation with neutral density filters, the energy density of the focused spot varied from 800 to 1,200 μJ/μm². The laser beam was deflected through a Zeiss photomicroscope system and focussed using a Zeiss Neofluar ×100 objective to give a spot diameter of ~0.25 μm. The laser beam energy was monitored with a calibrated Eppley thermopile No. 14011 attached to a Hewlett-Packard No. 419 voltmeter (Hewlett-Packard Co., Palo Alto, Calif.). The cells were maintained at 37°C using a Sage air curtain incubator (Sage Instruments Div., Oron Research Inc., Cambridge, Mass.). Chromosomes for irradiation were chosen when a clear centromeric constriction could be seen and the two chromatids were lying side by side such that both kinetochore regions could be distinguished. Three successive pulses were usually needed to produce a visible lesion although occasionally two were sufficient. After irradiation, the cells were monitored behaviorally using a GYYR model DAS 300 time-lapse video recorder and/or were fixed for electron microscopy.

**Electron Microscopy**

Irradiated cells were fixed at a selected time by perfusion through the Rose chamber of 3% glutaraldehyde made up in serum-free culture medium buffered to pH 7.4 with Millonig's phosphate buffer. The initial fixation took place at room temperature (~23°C) but the chambers were refrigerated for the remaining fixation time of 1-72 h. The cells were postfixed at room temperature in 1% osmium tetroxide made up in the same buffer, dehydrated through an ethanol series followed by hydroxypropyl methacrylate, and embedded in Epon. The fixation time of 1-72 h. The cells were postfixed at room temperature in 1% osmium tetroxide made up in the same buffer, dehydrated through an ethanol series followed by hydroxypropyl methacrylate, and embedded in Epon. For irradiation, a distinct lesion consisting of clumps of electron-dense material can be seen at the site of the irradiated kinetochore. As visible wavelengths were to be used, the Rose chambers were assembled with standard No. 1 thickness uncoated glass covers.

**RESULTS**

Prometaphase cells are usually chosen for irradiation as the individual chromosomes are more easily distinguished than in metaphase. A chromosome is selected when both chromatids are lying side by side and the primary constriction can be seen clearly. A total of 92 kinetochores were irradiated in 65 cells. Of these, a single kinetochore was successfully irradiated in 42 cases, resulting in movement of the chromosome to the pole. 18 of these were examined in the electron microscope at varying time periods after irradiation, and 24 were allowed to progress completely through division. In 11 of the 92 cases the whole centromere region was damaged and behavior similar to that described by Brenner et al. (10) occurred. 20 of the 92 irradiations produced no effect and were presumably below threshold. In the remaining experiments the cells rounded up so that the irradiated chromosome was no longer visible, or the cell died immediately after irradiation. 25 of the irradiations were performed on metaphase cells, the rest during prometaphase. When the data are tabulated with respect to either the time interval between irradiation and anaphase initiation (a range from 1 to 73 min), or the division stage as determined by observation, no pattern of behavior can be seen. The occurrence of damage to a single kinetochore, the whole centromere region, lack of any effect, or cell death appears to be completely random with respect to the previously mentioned timing or mitotic stage parameters.

Immediately after irradiation of one of the kinetochores, the chromosome moves rapidly towards the pole to which it has an undamaged connection, i.e., the pole to which the nonirradiated kinetochore is oriented, while the remaining chromosomes continue to congregate on the metaphase plate (Fig. 1). A pale lesion is usually visible in phase contrast for a brief period after irradiation (Fig. 1b). In most experiments, the chromosome approaches very close to the pole and remains there throughout prometaphase and metaphase. At anaphase, the chromatids of the irradiated chromosome separate simultaneously with the rest of the chromosomes to a distance of 1 or 2 μm. The damaged chromatid does not move towards the opposite pole but remains parallel to, and slightly separated from, the undamaged chromatid, resulting in nondisjunction of the irradiated chromosome.

A graphical representation of the movement of the chromosome in Fig. 1 is shown in Fig. 2 together with the movement of the poles. If the movement of the chromosome (the nonirradiated kinetochore) is plotted with respect to the pole to which it moves (Fig. 3), a clearer picture is presented of the movement caused by shortening of the kinetochore fiber without the component due to whole spindle elongation or shortening. This shows the chromosome approaching the pole to within 1 μm with a velocity of 2.5 μm/min and then remaining stationary throughout prometaphase and metaphase.

If the cell is fixed for electron microscopy immediately after irradiation, a distinct lesion consisting of clumps of electron-dense material can be seen at the site of the irradiated kineto-
FIGURE 8  Electron micrographs of serial sections through the irradiated chromosome shown in Fig. 7. × 25,000. (a) Section through the kinetochore of the unirradiated chromatid (arrow). The typical layered structure is seen associated with many microtubules. (b) Section through the irradiated kinetochore (arrow). The trilaminar structure of the kinetochore, which is facing away from both poles, is still apparent but few microtubules are associated with it. The undamaged chromatid is very close to the centriole (C). (c) Adjacent section through the irradiated kinetochore (arrow). Again, the layered structure is visible but there are no microtubules linking it to either pole.
chore (Fig. 4). The unirradiated chromatid exhibits a normal kinetochore structure in adjacent sections (not depicted in Fig. 4). However, if the cell is fixed 2 or more min after irradiation, no such electron-dense material is observed, and a lesion is not apparent. Nothing resembling a kinetochore is found in serial sections through the irradiated chromatid (Fig. 5). If irradiation takes place late in metaphase, the chromosome frequently does not reach the pole before the initial separation of the chromatids occurs. In this case, the undamaged chromatid moves to the pole during anaphase while the irradiated chromatid remains in the interzone or is dragged part of the way across the spindle by the other chromosomes. If it is still in the interzone at the end of anaphase, the chromatid is passively distributed to one or the other of the daughter cells during cleavage. In this case, the irradiated chromatid may be incorporated in a micronucleus if it is far from the other chromosomes. If such a cell is followed through to the next mitosis, one double-chromatid chromosome condenses in the micronucleus although the long trailing arms of the chromosome suggest that it is not fully condensed. The chromosome does not move onto the metaphase plate, but at anaphase the chromatids separate slightly and once again are passively distributed to the daughter cells.

The chromosome depicted in Figs. 6 and 7 was chosen for irradiation because initially it was close to one pole. The kinetochore that was oriented to that pole was irradiated, with the result that the chromosome was pulled across the whole spindle. Fig. 7 shows that the chromosome moves rapidly through the half-spindles but is dramatically slowed down as it traverses the equatorial region. The ultrastructure of this chromosome is shown in Fig. 8. Again, the undamaged kinetochore approaches very close to the pole, being within 0.5 μm of the centriole. In contrast to the irradiated chromatid shown in Fig. 5 in which no kinetochore structure was apparent, serial sections of the irradiated chromatid from this cell show a typical inactive kinetochore in which the tri-layer structure is present but is not oriented towards either pole and has few microtubules associated with it. The nonirradiated sister chromatid exhibits a normal kinetochore.

Of the 18 irradiated chromosomes which were examined by electron microscopy, only the two that were fixed within 1 min of irradiation show electron-dense lesion material. Three kinetochores exhibit the tri-layer structure but have very few microtubules associated with them while another three are partly damaged and have the kinetochore plates pulled out of the chromosome (P. A. McNeill and M. W. Berns, manuscript in preparation). On the remaining irradiated chromosomes, no kinetochore structure can be detected. There is no correlation between the appearance of the irradiated kinetochore and the division stage at which irradiation occurs.

Analysis of the movement of chromosomes that had one kinetochore irradiated shows that the chromosome moves to the pole with a velocity of ~3 μm/min, although occasionally velocities as high as 7 μm/min have been recorded. The rate of anaphase movement in control cells is 2.5–3 μm/min. If the chromosome must traverse the whole spindle, it is slowed down in the region of the metaphase plate, but the velocity in both half-spindles is approximately the same and is of the same magnitude as the anaphase velocity of a single chromatid.

In some experiments, for example in the cell shown in Fig. 9, the irradiated chromosome approaches the pole but is frequently jerked back towards the other pole, although the chromosome does not move onto the metaphase plate. This erratic movement can be seen more clearly when represented graphically (Fig. 10). In this case, the velocities of the individual jerks are similar in magnitude to those observed when the chromosome moves directly to the pole.

**DISCUSSION**

Although electron microscope studies show that irradiated kinetochores have been inactivated, their morphology varies considerably. One factor affecting the structure is the time elapsing between irradiation and fixation. If this time is very brief (1 min or less), electron-dense lesion material is found at the irradiation site and it obscures the remaining structure. In cells that have been fixed later than 1 min after irradiation, the difference in structure observed may be caused by slight differences in the lesion location. Although very few microtubules are associated with the damaged kinetochore shown in Fig. 5, it has a structure that is similar to the inactive kinetochores described by Roos (34) for colcemid-treated PtK₂ cells, the inner dense layer of the normal trilaminar structure not being apparent. Although the findings on the actual site of attachment of microtubules to the kinetochore are somewhat contradictory, there is good evidence that, at least in PtK₂ cells, the

**FIGURE 9** Series of phase-contrast micrographs of a PtK₂ cell in which a single kinetochore was irradiated during prometaphase. Time (in minutes) from irradiation is shown in bottom right-hand corner of frames. X 1,664. (a and b) Prometaphase. Right-hand kinetochore of chromosome indicated by arrow was irradiated. (c and d) Irradiated chromosome moves towards the pole while the remaining chromosomes congregate on the metaphase plate. (f) The irradiated chromosome again moves towards the pole. (g and h) The chromosome rotates slightly and begins moving towards the equatorial region again. (i and j) The chromosome moves almost onto the metaphase plate. Note the change in shape indicating a force acting on the centromere region towards the right-hand pole. (k) The chromosome returns to the pole while the remaining chromosomes are aligned on the metaphase plate. (l) Early anaphase. The chromatids of the irradiated chromosome have separated (arrow) but are not undergoing any poleward movement.

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**FIGURE 10** Movement of the irradiated chromosome shown in Fig. 9 relative to the pole to which it moves. The chromosome is frequently pulled back towards the opposite pole, indicating that there is some linkage between the centromere region and both poles. The velocity is approximately the same in both directions.
microtubules originate in the outer layer of the trilaminar kinetochore (34, 35). Although this outer layer appears intact in Fig. 8, the fact that microtubules can no longer be nucleated there suggests that irradiation damage has occurred at least to this outer layer.

The behavior of the irradiated chromatid at the second division after irradiation is similar to that described by Brenner et al. (10) for whole centromere irradiation. Although the chromosome appears to replicate normally, we must conclude that there is no kinetochore repair.

The fact that anaphase is a complex process has been known for many years. As summarized by Nicklas (27), anaphase motion has three components: the initial chromatid separation occurring in the absence of spindle attachments (11), poleward movement of the chromosomes, and chromosome separation caused by spindle elongation (33). If we disregard the component because of spindle elongation, we can consider "chromosomal anaphase" as a two-stage event. The evidence for this is clear. Cells treated with colchicine exhibit chromatid separation but no poleward movement of the chromosomes in the absence of a spindle (18, 19, 24, 29) as do unfertilized sea urchin eggs treated with NH4OH (21) which induces the chromosome cycle but no spindle formation. Chromosomes in which both kinetochores have been destroyed by microirradiation also exhibit initial chromatid separation with no subsequent poleward movement (10). In the experiments described here, only one kinetochore lacks a spindle attachment and, as a result, there is no bipolar tension between the sister kinetochores. The observations that chromatid separation occurs at the beginning of anaphase in the absence of the bipolar tension associated with two functional kinetochores suggests that the initial separation is not dependent on the kinetochore region and is not caused by microtubule-mediated forces.

The question of chromosome reorientation is still not resolved. It was suspected that inactivation of one of the kinetochores of a chromosome would lead to successive reorientations similar to those undergone by univalents in meiosis (25). The oscillating movement exhibited by some irradiated chromosomes (e.g., Figs. 9 and 10) may be caused by two different factors: either by reorientation of the undamaged kinetochore or by the fact that the irradiated kinetochore is only partly damaged and still retains some connection with the opposite pole. We do have electron microscope evidence to support the latter possibility (P. A. McNeill and M. W. Berns, manuscript in preparation) but not the former. It seems that reorientation occurs infrequently in mitosis when one kinetochore is inactivated. The low frequency compared with that of meiotic univalents may be caused by the much shorter time-course of prometaphase and metaphase in mitosis as compared with meiosis. There may also be differences in behavior because of the fact that only a single kinetochore would be involved in reorientation of an irradiated mitotic chromosome, whereas a pair of kinetochores are present side by side on a meiotic univalent. Clearly, more studies should be conducted on the phenomenon of chromosome reorientation.

The question of why chromosomes of vastly different sizes within the same cell move at the same rate in anaphase has puzzled investigators for many years (see reviews of Nicklas [27] and Bajer and Molé-Bajer [3]). There is a range of anaphase velocities among different species from 0.2 μm/min to 8 μm/min (20), but it is not yet clear whether this range is correlated with chromosome size. There has also been considerable speculation on the relationship between chromosome size and the number of microtubules attached to the kinetochore. From the review of Fuge (14) it seems that there may be some general correlation, at least between species. Dietz, in the discussion following the paper by Fuge (13), postulates that there is a correlation between chromosome size and the number of kinetochore microtubules in anaphase. This suggests that the force required to move different chromosomes in a given cell with the same velocity at anaphase is determined by the number of nucleating sites in a given kinetochore. However, Moens (23) in a comparative study of mitosis and meiosis of three species shows that the numbers of kinetochore microtubules cannot be predicted from chromosome size alone. Nicklas (26) found that the velocity of the sex univalent in Melanoplus spermatoocytes remains constant when the chromosome is suddenly shortened to one-quarter of its length. In the opposite situation our studies show that one kinetochore can move two chromatids at the same velocity (~3 μm/min) with which it normally moves a single chromatid. If we assume that the number of microtubule-nucleating centers in a given kinetochore is constant, then this number can move twice the normal mass with the same velocity. The experiments of Bajer (1) on the behavior of chromosome bridges and our observation that the chromosome is slowed down when traversing the mass of chromosomes in the equatorial region show that there are limits to the force exerted by the microtubules. However, we can conclude, within limits, that the velocity with which a kinetochore moves is independent of mass.

It has been suggested that spindle fibers may not pull continuously and evenly on the kinetochore from prometaphase to anaphase and that there may be a reduction in the force during late prometaphase and metaphase (16, 28) but our findings on PtK2 cells in vitro clearly conflict with this idea as similar velocities are found throughout prometaphase, metaphase, and anaphase.

Our results are consistent with the idea that the rate of chromosome movement is controlled solely by the rate of depolymerization of the microtubules and that the depolymerization rate remains constant from prometaphase to anaphase, even through the force transmitted by the microtubules may change considerably.

In summary, we may conclude that:
(a) Two functional kinetochores (and, therefore, bipolar tension) are necessary for the alignment of a chromosome on the metaphase plate and for normal anaphase movement.
(b) Irradiation and inactivation of one kinetochore lead to nondisjunction of the irradiated chromosome.
(c) The initial separation of the chromatids occurs at anaphase in the absence of two functional kinetochores.
(d) The initial separation of the chromatids at anaphase is not caused by microtubule-mediated forces.
(e) Chromatids with irradiated kinetochores retain their ability to replicate but are unable to repair the damaged kinetochore region.
(f) Within limits, the velocity with which a kinetochore moves is independent of the mass associated with it.

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