J. Cell Sci. 34, 289–301 (1978)
Printed in Great Britain © Company of Biologists Limited 1978

EVIDENCE FOR CENTRIOLAR REGION RNA FUNCTIONING IN SPINDLE FORMATION IN DIVIDING PTK_2 CELLS

SCOTT P. PETERSON AND MICHAEL W. BERNS
Developmental and Cell Biology, University of California at Irvine, Irvine, California 92717, U.S.A.

SUMMARY

The light-activated, nucleic acid-binding drugs, psoralens, were used in conjunction with a 365-nm laser microbeam to selectively bind to any nucleic acids in the centriolar region. 4′-aminomethyl-4,5′,8-trimethyl-psoralen (AMT) has a high affinity for both RNA and DNA and can be shown to cause mitotic abortion when centriolar regions of prophase PTK_2 cells are reacted with AMT and 365-nm laser light. Other psoralen derivatives which have a high affinity for DNA and a low affinity for RNA are not effective in blocking mitosis in dividing PTK_2 cells. Examination of psoralen-bound centriolar regions by single-cell electron microscopy shows that at various times after treatment, the number of microtubules associated with the irradiated poles is much lower than in normal, dividing cells. Light-activated psoralen binding of the centriolar regions does not seem to affect the condensation or structure of mitotic chromosomes. It is concluded that there is an RNA in the centriolar region that is responsible for the formation of the spindle in dividing cells.

INTRODUCTION

Psoralens are light-activated, nucleic acid-binding drugs. The reaction between these drugs and double-stranded nucleic acids (e.g., DNA, rRNA) begins with the intercalation of the planar psoralen molecule into the nucleic acid helix between pyrimidines on opposite strands of the molecule. The addition of near ultraviolet light to the intercalated psoralen molecule may result in the formation of a covalent bond between the psoralen and the nucleic acid (Ashwood-Smith & Grant, 1976). Interstrand psoralen crosslinks may be formed and have been shown to be stable under DNA denaturation conditions (Weisehahn, Hyde & Hearst, 1977).

Work by Isaacs, Shen, Hearst & Rappoport (1977) has shown that psoralens of slightly different structure have different affinities for RNA and DNA. AMT (4′-amino-methyl-4,5′,8-trimethylpsoralen) has been shown to have a high affinity for both RNA and DNA. Other psoralen derivatives have a much lower binding capacity for RNA while still being efficient DNA crosslinking drugs. Comparisons between the effects produced by these different psoralen derivatives when they are introduced to cells and bound to nucleic acids might be used to determine whether an observed effect is due to specific psoralen reaction with RNA or DNA.

Address correspondence to: Scott P. Peterson, Developmental and Cell Biology, University of California at Irvine, Irvine, California 92717, U.S.A.

The presence of nucleic acids in the centriolar region and in basal bodies has been shown by staining with acridine orange (Randall & Disbrey, 1965) and ethidium bromide (McGill, Highfield, Monahan & Brinkley, 1976). Recently, digestions of isolated basal bodies with RNase have suggested that RNA may be important in the nucleation of microtubules onto centriolar regions in dividing cells (Heidemann, Sander & Kirschner, 1977). In this study, we will investigate the effect of light-activated psoralen binding to the nucleic acid in the centriolar region while leaving the remainder of the cell unaffected.

Previous microirradiation experiments (Berns, 1974; Berns, Ohnuki, Rounds & Olson, 1970; Berns, Leonardson & Witter, 1976) relied upon the absorption of laser light by either naturally occurring cell molecules or dyes added to the cells in culture, which resulted in the production of small visible lesions. Laser lesions 0·25 μm in diameter have been produced by this technique and are most likely caused by heating. A near u.v. laser microbeam could be used to produce chemically, well defined binding between psoralens and nucleic acids rather than the more general heat effects produced by earlier techniques. An additional advantage of using the laser microbeam to bind psoralens to any nucleic acid that may be present in the centriolar region is that, unlike staining experiments, only the centriolar region is affected. Also, this technique may be carried out *in vivo* rather than *in vitro* as with RNase digestions of isolated basal bodies (Heidemann *et al.* 1977).

MATERIALS AND METHODS

Cell culture, irradiation and psoralen drugs

Potorous tridactylis, PTK_1 cells (American Type Culture Collection, CCL no. 56), were chosen for these experiments because they remain flat throughout mitosis. A particular clone of these cells, designated PTK_2W, has been isolated which has clearly visible centriolar regions during early prophase.

PTK_2W cells were maintained in Falcon T-25 flasks with Eagle's modified minimum essential medium and 10% heat-inactivated foetal calf serum (GIBCO). Cells were subcultured with 0·125% Viokase (GIBCO) and 0·1% EDTA and plated in Rose chambers 48 h before experiments were performed. At 37 °C, the cell cycle time is 30 h and mitosis lasts about 1 h.

Psoralen derivatives, generously supplied by Dr John E. Hearst of UC Berkeley, were dissolved in media and applied to cells 24 h before irradiations were performed. AMT (4′-aminomethyl-4,5′,8-trimethylpsoralen) was used at a concentration of 3·25 μg/ml; HMT (4′-hydroxymethyl-4,5′,8-trimethylpsoralen) and MMT (4′-methoxymethyl-4,5′,8-trimethylpsoralen) were both used at a concentration of 12 μg/ml. 4,5′,8-trimethylpsoralen (TMP) was used at a concentration of approximately 0·6 μg/ml (Isaacs *et al.* 1977). Cells have been shown to be permeable to all these psoralen derivatives (Isaacs *et al.* 1977; Weisehahn *et al.* 1977). After drug addition, chambers were maintained in the dark to prevent psoralen-nucleic acid reactions caused by room and incubator lights. Microscopic observations were all performed using phase-contrast optics with Ealing yellow filter no. 26-4358 with no transmission of light less than 540 nm to exclude near u.v. light from the chambers.

Selected centriolar regions in dividing cells were exposed to light of 365 nm from an argon laser microbeam system (Berns, 1971). After irradiation, cells were observed by both light and electron microscopy. Cells were prepared for electron microscopy as previously reported (Rattner & Berns, 1974). They were sectioned with glass knives on an LKB Ultratome III, and silver sections were viewed on a Siemens 1A operating at 60 kV.

Radioactivity determinations and nucleic acid isolation

A tritiated derivative of AMT was used to determine the possible binding of psoralens to proteins. ^{3}H-AMT (3·25 μg/ml) was added to the cells 24 h before irradiation. Cells were removed from their flasks and suspended in ^{3}H-AMT medium and centrifuged to form a pellet. The pellet was resuspended in 0·5 ml of ^{3}H-AMT medium and then exposed to 365-nm laser light. After exposure to 25 pulses of laser light (2000 J m^{-2}), nucleic acids were extracted from approximately 10^8 cells by the procedure of Marmur (1961). Fractions containing nucleic acids and proteins were placed in scintillation vials with 10 ml of solution consisting of toluene PPO-Triton X-100. After removal of nucleic acids by winding on a glass rod, the aqueous phase was digested with both RNase and DNase for 45 min at 37 °C. Trichloroacetic acid (TCA) was used to collect a small amount of precipitate, and this was placed in a scintillation vial. In addition, calf thymus histone protein and cytochrome c were suspended in ^{3}H-AMT medium and exposed to 25 pulses of 365-nm laser light. Proteins were precipitated with TCA, washed 3 times in TCA solution and placed in scintillation vials. Total radioactivity was determined with a Beckman model CPM-100 scintillation counter.

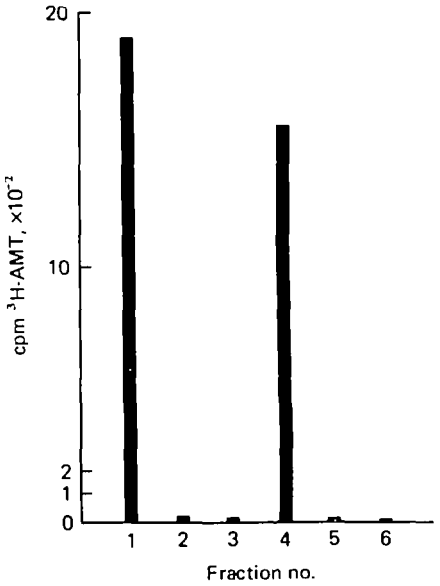


Fig. 1. Distribution of psoralen (^{3}H-AMT) in fractions from a DNA isolation after irradiation from a 365-nm argon laser. Background of 86 cpm is included in average counts and in graph. Fractions represent average of 6 isolations: (1) total radioactivity of 10^8 cells after washing 3 times with isotonic saline containing 5 μg/ml non-radioactive AMT (1900 cpm average); (2) total radioactivity of CCl_4 fraction from DNA isolation (92 cpm average); (3) total radioactivity of TCA precipitate from aqueous phase after digestion with RNase and DNase (98 cpm average); (4) radioactivity of nucleic acid fraction including both DNA and RNA (1600 cpm average); (5) photoreaction of ^{3}H-AMT with histone proteins dissolved in isotonic saline; protein was precipitated from solution with TCA, washed and counted (100 cpm average); (6) cytochrome c photoreacted with ^{3}H-AMT in solution and precipitated with TCA, washed and counted (95 cpm average).

RESULTS

Fig. 1 shows the presence of ^{3}H-AMT in various nucleic acid and protein fractions. The majority of the radioactive counts are found in the nucleic acid fraction (4). The protein fractions (2, 3, 5, 6) contain only a small amount of ^{3}H-AMT with the greatest radioactivity in the TCA-precipitate fraction (3).

Table 1. *Irradiation of both centriolar duplexes in the presence and absence of various derivatives in dividing PTK_2 cells*

Stage of mitosis	15 pulses u.v. alone	AMT	HMT	MMT	TMP
Prophase and prometaphase	0/56	53/73	1/30	0/26	2/32
Metaphase	0/12	2/10	0/14	0/8	1/18
Anaphase and telophase	0/18	0/25	0/9	0/5	0/12

Fraction represents the number of cells that aborted mitosis after irradiation with 1100 J m^{-2} of laser light.

Table 2. *Microirradiation of various cellular components in prophase with 15 pulses of 365-nm laser light in presence of 3·25 μg/ml AMT*

Area irradiated	Prophase and prometaphase	Metaphase
1 chromosome	0/8	0/4
Spindle	0/12	0/7
Cytoplasm	0/4	0/8

Fraction represents the number of cells that aborted mitosis after irradiation with 1100 J m^{-2} of laser light.

Table 1 summarizes the cellular effects of laser irradiation (1100 J m^{-2}) in the presence and absence of various psoralen derivatives. Control irradiation of the centriolar region with near u.v. light of 365 nm does not seem to affect mitosis; cells divide normally after treatment. Near-u.v. irradiation in combination with psoralen derivatives with low RNA affinity (HMT, MMT and TMP) is not effective in blocking mitosis. The psoralen derivative with the high RNA affinity (AMT) is effective at blocking cell division. AMT reaction with the centriolar region causes cessation of mitosis most often in the early mitotic stages. As division proceeds into the later stages of mitosis (metaphase and anaphase), there is a drop in the number of cells that abort mitosis after centriolar region irradiation in the presence of AMT. Irradiation of the spindle, chromosomes or cytoplasm in the presence of AMT did not cause the cells to stop division (Table 2).

Examination of irradiated centriolar regions by electron microscopy immediately after light-induced psoralen binding shows that they are not different from non-

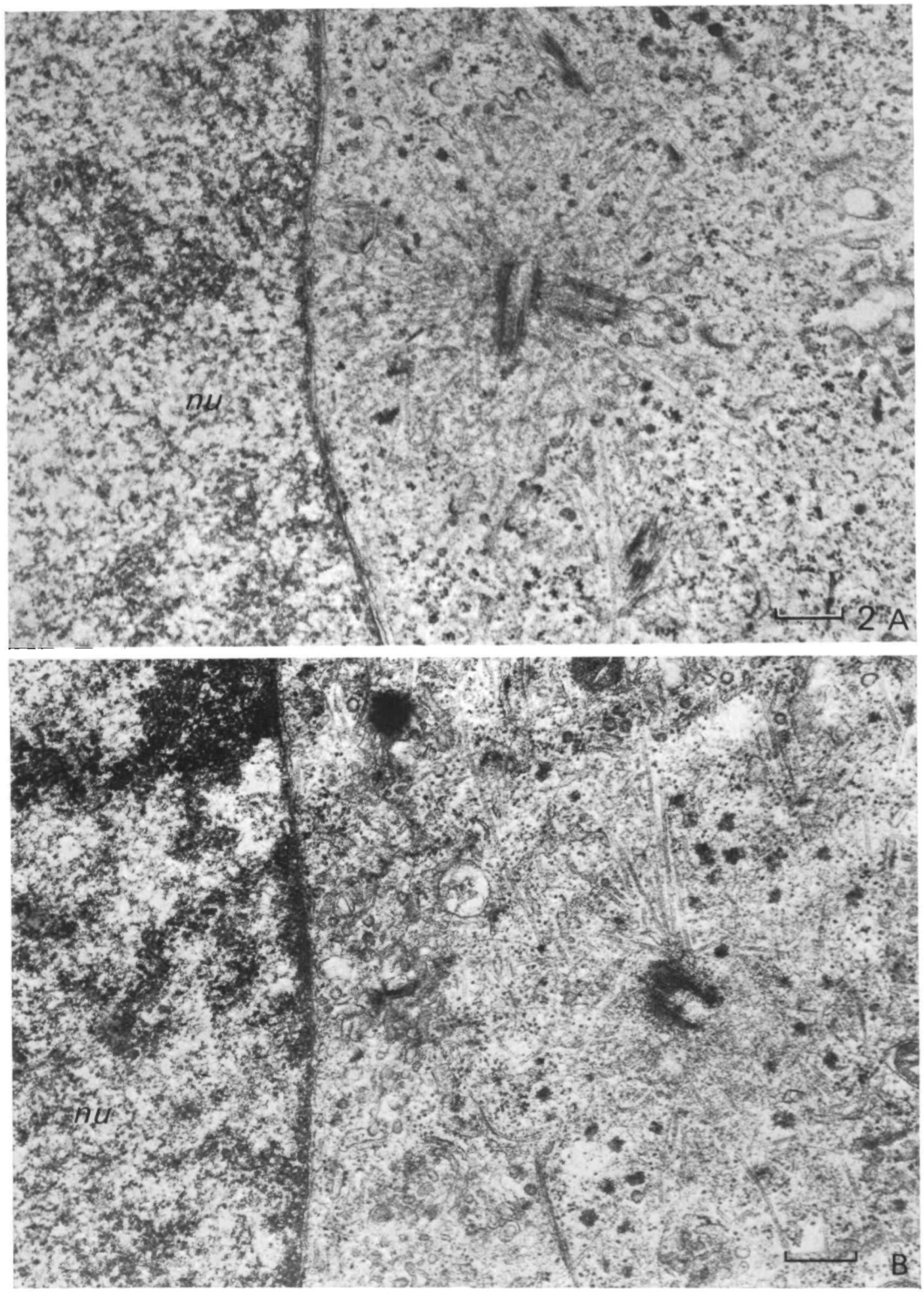
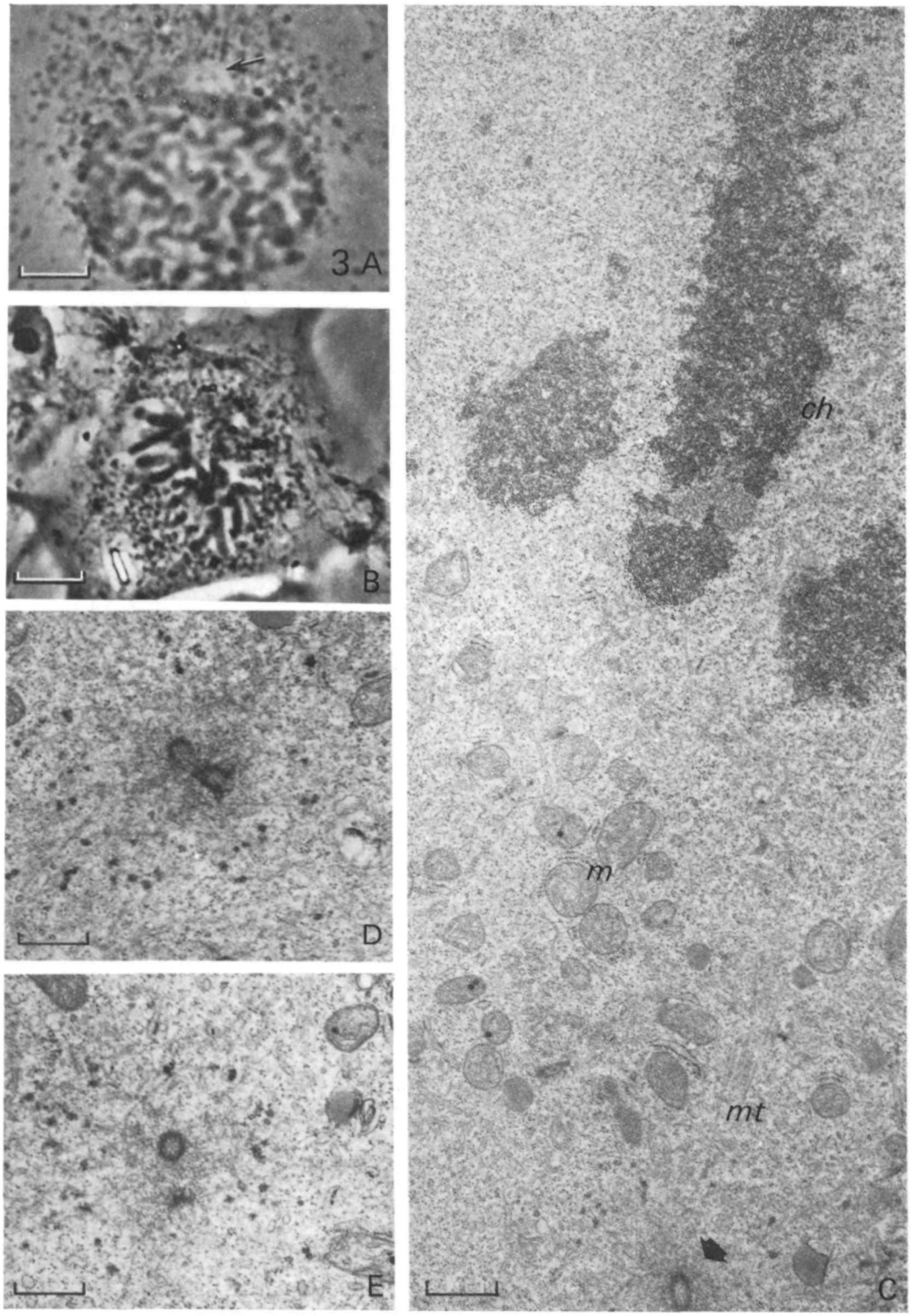


Fig. 2. Comparison of prophase centriolar regions, untreated and photoreacted with AMT (*nu* = nucleus). A, untreated centriolar region. Note the presence of centrioles, pericentriolar cloud and satellites. Many microtubules may be seen entering the centriolar region. ×31000, bar = 0·33 μm. B, centriolar region after photoreaction with AMT. Cell was fixed immediately after irradiation for electron microscopy. Note similarity of this treated centriolar region to the untreated pole in A. Cloud material, satellites and microtubules may be seen associated with the centrioles. The second centriole of this duplex is in a different sectioned plane. ×42300, bar = 0·24 μm.


3 A
B
D
E
ch
m
mt
C

irradiated mitotic poles. Fig. 2 shows 2 prophase pole regions in different cells, one untreated and one irradiated with 365-nm laser light in the presence of AMT. Both poles appear normal, showing centrioles, electron-dense pericentriolar cloud material, pericentriolar satellites or virus particles, and many microtubules.

Cells fixed for electron microscopy 20–25 min after irradiation of both centriolar duplex regions in prophase (Fig. 3) show differences from normal pole structure. Very few microtubules were observed extending from the poles to the chromosomes. Mitochondria are seen in the region between the chromosomes and the poles where they are not usually found in dividing cells. High-power electron micrographs of the 2 centriolar regions in this cell (Fig. 3D, E) show that the centriolar regions appear normal with a dense cloud, pericentriolar satellites and a centriolar duplex but only a small number of microtubules.

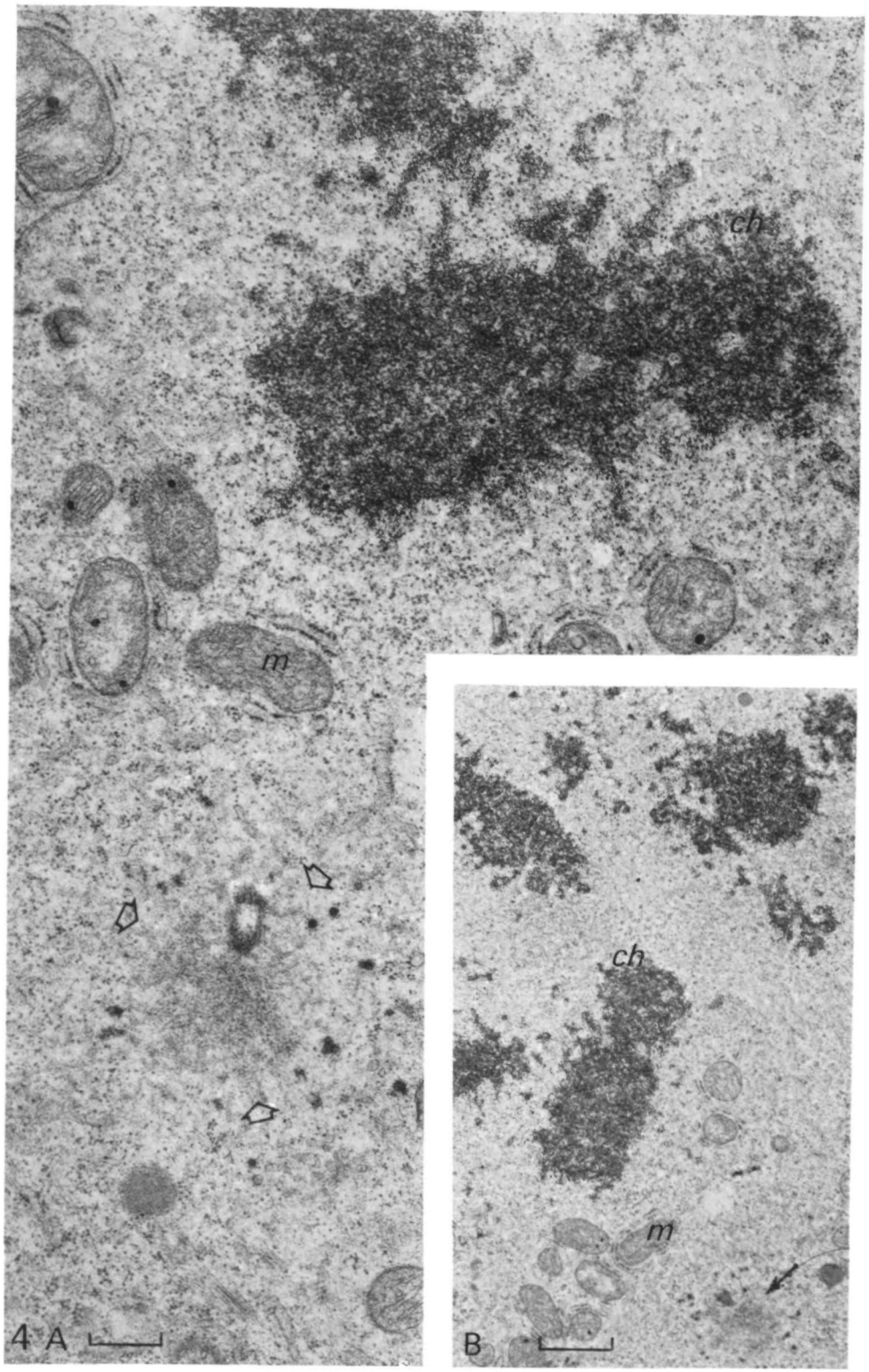
Cells fixed 30–40 min after prophase centriolar-region irradiation in the presence of AMT (Fig. 4) lack microtubules extending from the poles to the chromosomes. Mitochondria are evident in a region from which they are normally excluded in dividing cells. Prophase irradiations of the pole regions in the presence of AMT seem to interfere with the formation of the spindle but not the normal condensation of chromosomes.

Fig. 5 shows a cell fixed for electron microscopy 1 h and 10 min after prophase irradiation. Phase-contrast micrographs of this cell 30 min after irradiation (Fig. 5A) show condensed mitotic chromosomes but no clear spindle region. At 70 min after irradiation (Fig. 5B), there has still been no cytokinesis or karyokinesis, and the chromosomes have lost their mitotic appearance and formed a dense mass in the centre of the cell. Electron-microscopic examination of this cell shows the centrioles with no microtubules associated with them and a very dense nucleus (Fig. 5C).

Fig. 6 is a dividing cell where only a single pole was irradiated in prophase. This cell was fixed for electron microscopy 35 min after irradiation. Both centriolar regions were located and are illustrated in Fig. 6D, E. One pole has many microtubules associated with it, and a lower-magnification picture (Fig. 6C) of the same pole shows that there are many tubules extending into the chromatin mass. Notice that there are no mitochondria in this region. The second pole shows very few microtubules

---

Fig. 3. Both poles irradiated in prophase in the presence of AMT. After irradiation, cell continued mitosis for 20 min and was then fixed for electron microscopy. A, prophase cell at time of irradiation. Arrow indicates the 2 centriolar regions at the edge of the nucleus. ×5000, bar = 2 μm. B, cell 20 min after photoreaction when it was fixed for electron microscopy. Chromosomes have condensed normally, but there is no spindle region. ×3000, bar = 3·3 μm. C, electron micrograph of cell in Fig. 3B. Centriolar region (arrow) still appears normal except for the lack of microtubules extending into the pole. Mitochrondria (*m*) may be seen in a region normally occupied by the spindle. Only a few microtubules (*mt*) may be seen in the field. Chromosomes (*ch*) seem to be normal. ×23000, bar = 0·47 μm. D, one pole in adjacent section to Fig. 3C. Note the cloud and satellites and the few microtubules associated with the centriolar region. ×29000, bar = 0·3 μm. E, second pole in same cell. Again, few microtubules are associated with the centriolar region but cloud and satellites are still present. ×32000, bar = 0·3 μm.


ch
m
4 A
ch
m
B

associated with the centriolar region. The reaction of the AMT psoralen derivative with the centriolar region is specific for only the pole that is exposed to 365-nm laser light.

These experiments indicate that the AMT reaction with the centriolar region interferes with spindle formation. Few microtubules form from poles to chromosomes after treatment of prophase centriolar regions with this psoralen derivative and 365-nm laser light.

DISCUSSION

The data presented here indicate that there is no apparent binding between ^{3}H-AMT and protein after irradiation with 365-nm laser light. This means that the observed effects on cell division and pole structure are due to psoralen reaction with nucleic acids rather than to non-specific protein binding.

Of the psoralen derivatives examined in this work, AMT has the highest affinity for RNA (Isaacs *et al.* 1977). AMT is also the most effective derivative in blocking mitosis and the formation of the spindle. This suggests that the abortion of mitosis in cells with psoralen-reacted centriolar regions may be due to a modification of an RNA needed in the formation of the mitotic spindle. If this effect were due to psoralen reaction with DNA, the other psoralen derivatives should have been more effective in blocking mitosis. In addition, DNase digestion of isolated basal bodies has no effect on the ability of these structures to nucleate microtubules (Heidemann *et al.* 1977).

Psoralen reaction of the centriolar region affects spindle formation but not anaphase chromosome movements. When poles are irradiated in metaphase, there is no effect on chromosome separation. Centriolar region RNA, as detected by reaction with psoralen, is probably necessary to the formation of the spindle but not to its function after the microtubules have been established. Though the present findings do not allow us to characterize precisely the centriolar region RNA, certain judgments can be made about its structure. In order for AMT to be effective in binding and causing mitotic abortion, centriolar-region RNA must have some secondary structure. Previous experiments with high concentrations of cycloheximide have shown that cells can divide in the absence of protein synthesis (Peterson & Berns, 1978). Nucleation of microtubules onto centrioles *in vitro* can proceed without the need for transcription or translation precursors (Snyder & McIntosh, 1975). These data indicate

---

Fig. 4. Centriolar regions irradiated in prophase in the presence of AMT and allowed to continue mitosis for 35 min. Cell was then fixed for electron microscopy. A, micrograph showing mitotic pole (arrow). Mitochondria (*m*) are seen in a region normally occupied by the spindle. Few microtubules are seen in this cell extending from the pole to the chromosomes (*ch*). ×43500, bar = 0·24 μm. B, adjacent section of cell in Fig. 4A. Note mitochondria (*m*) in region normally occupied by spindle and mitotic chromosomes (*ch*). Centriolar region is indicated by arrow. ×19000, bar = 0·4 μm.

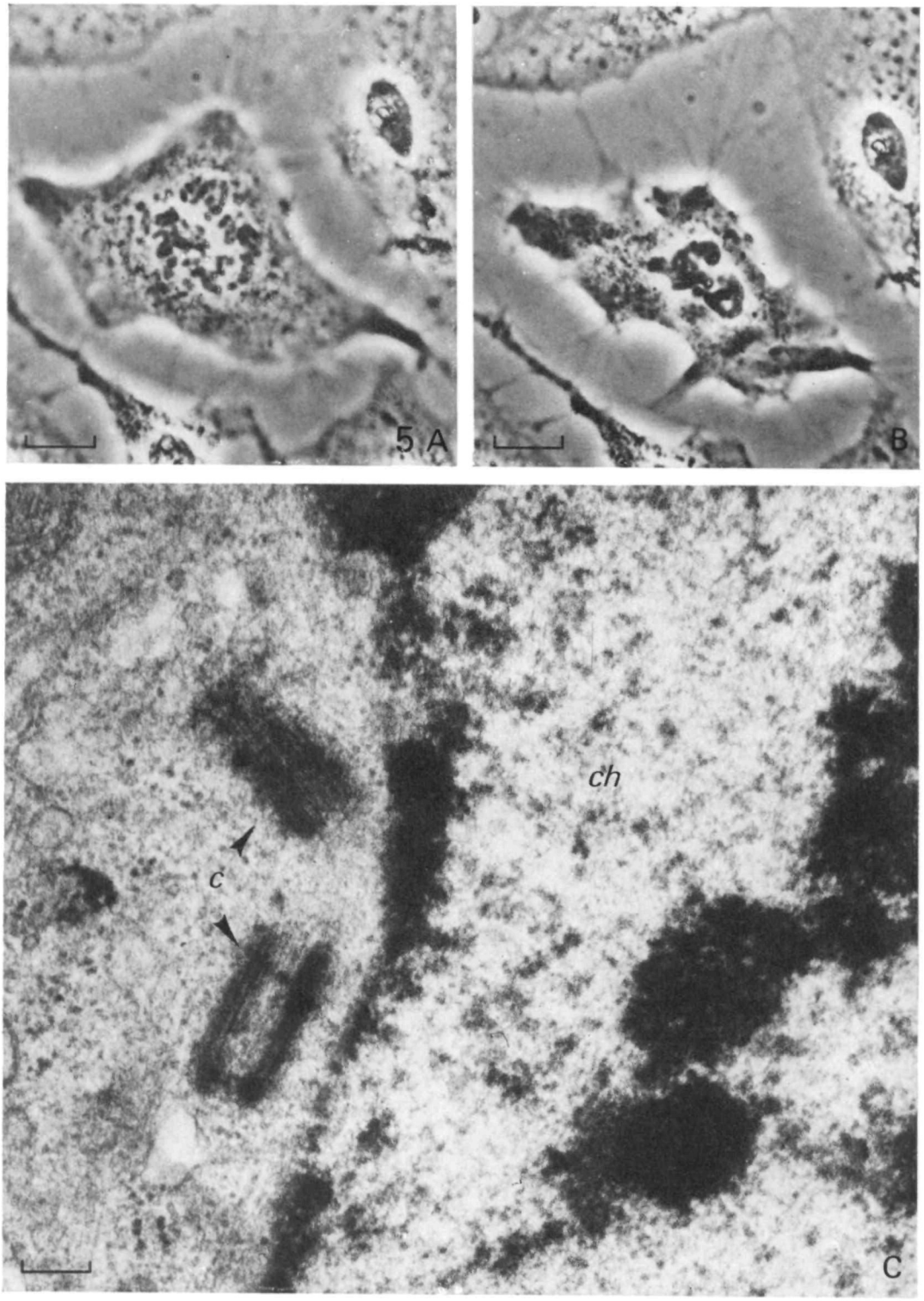


Fig. 5. Irradiated cell 1 h and 10 min after photoreaction with AMT in prophase centriolar region. A, cell 35 min after prophase photoreaction of both centriolar regions with AMT. Note random arrangement of mitotic chromosomes and lack of metaphase plate. ×3100, bar = 3·2 μm. B, photoreacted cell 1 h and 10 min after irradiation. No cytokinesis or karyokinesis occurred in this cell. Chromosomes have lost mitotic appearance and have formed a phase-dense mass in the centre of the cell. ×3100, bar = 3·2 μm. C, electron micrograph of cell in Fig. 5B. No microtubules were seen associated with the centriolar region (*c*) in this cell. ×92100, bar = 0·1 μm.

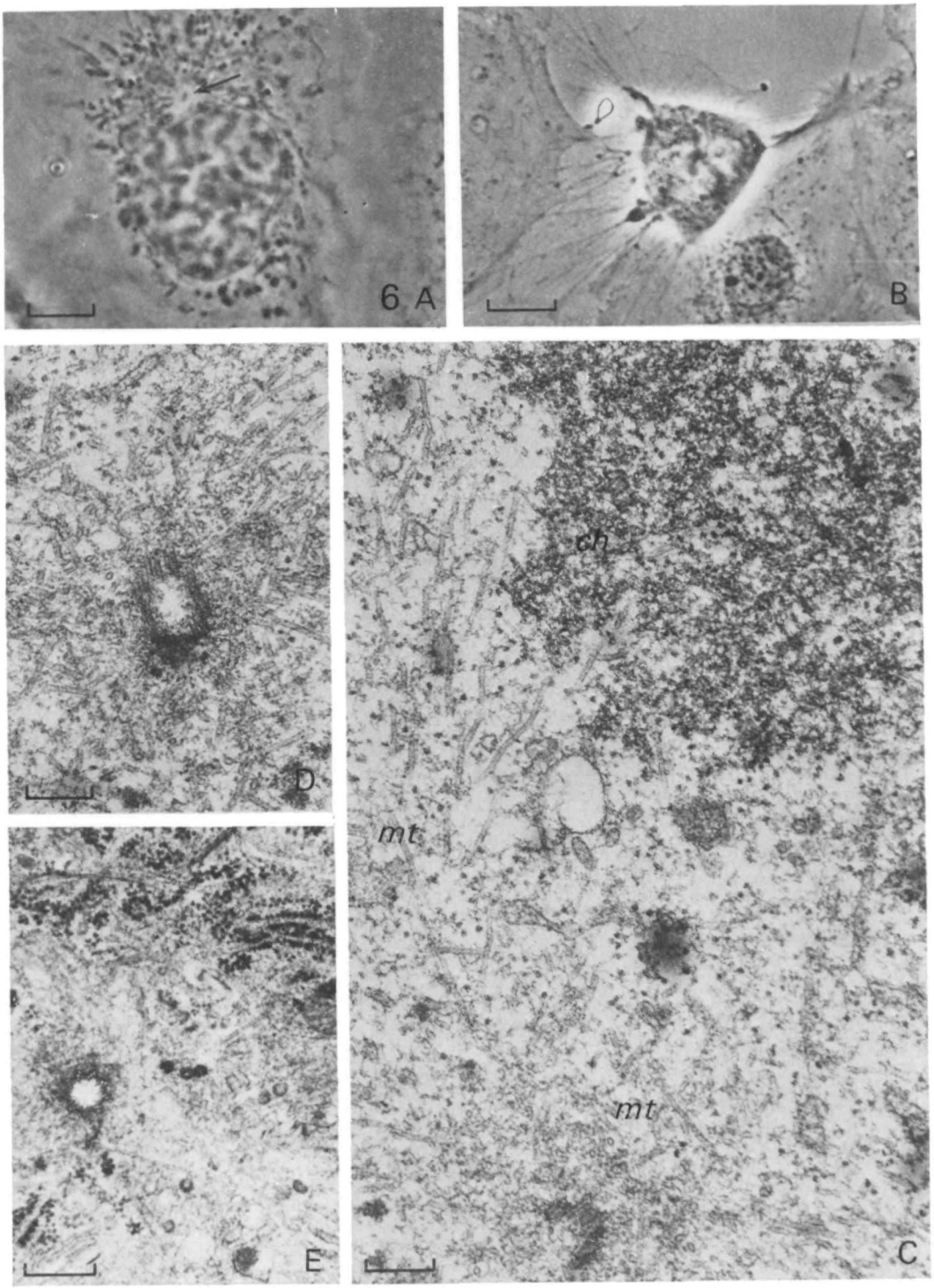


Fig. 6A–E. For legend see p. 300.

that the RNA involved in spindle formation in the centriolar region is probably not mRNA.

Treatment of the centriolar regions with psoralen-u.v. leads to a conclusion concerning the independence of spindle poles from each other. Irradiation of single centriolar regions in dividing cells in the presence of AMT only affects the irradiated pole. The non-treated centriolar region is still capable of nucleating microtubules and forming a partial spindle.

Chromosome condensation and decondensation appear to be independent of the formation of the spindle. Even though there is no spindle formed after prophase psoralen reaction with the centriolar regions, chromosomes condense normally. Similar results have been obtained for a long period of time when cells were treated with colcemid to block mitosis but still retain the metaphase chromosome appearance for use in karyotyping.

Presently, psoralen reaction with the centriolar region is being used to investigate the function of interphase centrioles and the mechanism of centriolar duplication. The possible roles of DNA and RNA in division and centriolar duplication could provide information about the mechanics and control of mitosis.

The authors would like to thank Dr Kenneth Strahs for critically reading this manuscript and Ms Elaine Kato for her assistance in manuscript preparation. This research was supported by NIH grants HL15740, GM22754, GM23445, and grant AFOSR-77-3136 from the U.S. Air Force. This work has been conducted in partial fulfillment for the Ph.D. by S.P.P.

REFERENCES

ASHWOOD-SMITH, M. J. & GRANT, E. (1976). Conversion of psoralen-DNA monoadducts in *E. coli* to interstrand DNA cross-links by near UV light (320–360 nm): Inability of angelicin to form cross-links, *in vivo*. *Experientia* **33**, 384–386.

BERNS, M. W. (1971). A simple and versatile argon laser microbeam. *Expl Cell Res.* **65**, 470–473.

BERNS, M. W. (1974). Laser microirradiation of chromosomes. *Cold Spring Harb. Symp. quant. Biol.* **38**, 165–174.

BERNS, M. W., OHNUKI, Y., ROUNDS, D. E. & OLSON, R. S. (1970). Modification of nuclear expression following laser micro-irradiation of chromosomes. *Expl Cell Res.* **60**, 133–138.

BERNS, M. W., LEONARDSON, K. & WITTER, M. (1976). Laser microbeam irradiation of rat kangaroo cells (PTK_2) following selective sensitization with bromodeoxyuridine and ethidium bromide. *J. Morph.* **149**, 327–338.

Fig. 6. Single pole irradiated in prophase cell in the presence of AMT. Cell continued mitosis for 35 min and was then fixed for electron microscopy. A, prophase cell at time of irradiation. Arrow indicates centriolar region at edge of nucleus. ×3100, bar = 3·2 μm. B, photoreacted cell at time of fixation for electron microscopy. Chromosomes have formed mitotic appearance. ×1300, bar = 7·8 μm. C, electron micrograph of cell in Fig. 6B showing non-irradiated pole. Note microtubules (*mt*) and chromosomes (*ch*) in cell. Mitochondria are not found in the spindle region. Arrow indicates pole region. ×56000, bar = 0·18 μm. D, non-irradiated pole, adjacent section to Fig. 6C. Many microtubules may be seen associated with this centriolar region. ×60000, bar = 0·16 μm. E, irradiated pole in cell of Fig. 6B. Note lack of microtubules in centriolar region when compared to non-irradiated pole (Fig. 6D). ×30700, bar = 0·33 μm.

HEIDEMANN, S. R., SANDER, G. & KIRSCHNER, M. W. (1977). Evidence for a functional role of RNA in centrioles. *Cell* **10**, 337–350.

ISAACS, S. T., SHEN, C.-K. J., HEARST, J. E. & RAPPOPORT, H. (1977) Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA. *Biochemistry, N.Y.* **16**, 1058–1064.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3**, 208–218.

MCGILL, M., HIGHFIELD, D. P., MONAHAN, T. M. & BRINKLEY, B. R. (1976). Effects of nucleic acid specific dyes on centrioles of mammalian cells. *J. Ultrastruct. Res.* **57**, 43–53.

PETERSON, S. P. & BERNS, M. W. (1978). Chromatin influence on the function and formation of the nuclear envelope shown by laser-induced psoralen photoreaction. *J. Cell Sci.* **32**, 197–213.

RANDALL, SIR JOHN & DISBREY, C. (1965). Evidence for the presence of DNA at basal body sites in *Tetrahymena pyriformis*. *Proc. R. Soc.* B **162**, 473–491.

RATTNER, J. B. & BERNS, M. W. (1974). Light and electron microscopy of laser microirradiated chromosomes. *J. Cell Biol.* **62**, 526–533.

SNYDER, J. A. & MCINTOSH, J. R. (1975). Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* **67**, 744–760.

WEISEHAHN, G. P., HYDE, J. E. & HEARST, J. E. (1977). The photoaddition of trimethylpsoralen to *Drosophila melanogaster* nuclei: a probe for chromatin structure. *Biochemistry, N.Y.* **16**, 925–932.

(*Received* 17 *April* 1978)