Sister Chromatid Separation in Frog Egg Extracts Requires DNA Topoisomerase II Activity during Anaphase

Caroline E. Shamu* and Andrew W. Murray*‡

*Department of Biochemistry and Biophysics, and ‡Department of Physiology, University of California, San Francisco, California 94143

Abstract. We have produced metaphase spindles and induced them to enter anaphase in vitro. Sperm nuclei were added to frog egg extracts, allowed to replicate their DNA, and driven into metaphase by the addition of cytoplasm containing active maturation promoting factor (MPF) and cytostatic factor (CSF), an activity that stabilizes MPF. Addition of calcium induces the inactivation of MPF, sister chromatid separation and anaphase chromosome movement. DNA topoisomerase II inhibitors prevent chromosome segregation at anaphase, demonstrating that the chromatids are catenated at metaphase and that decatenation occurs at the start of anaphase. Topoisomerase II activity towards exogenous substrates does not increase at the metaphase to anaphase transition, showing that chromosome separation at anaphase is not triggered by a bulk activation of topoisomerase II.

ACCURATE chromosome segregation requires that the linkage between sister chromatids is regulated during the cell cycle. Sister chromatids must remain associated with each other from the time they are replicated until they have been correctly aligned on the metaphase plate, with the sister kinetochores attached to opposite poles of the mitotic spindle. At the onset of anaphase, the linkage between sisters must be promptly destroyed, allowing them to segregate to opposite poles of the spindle.

At metaphase, a polewards force is exerted on each kinetochore (McNeill and Berns, 1981; Rieder and Alexander, 1990). Because the sister chromatids are linked to each other, and attached to opposite poles of the spindle, these forces will tend to collapse the spindle and must be opposed by forces that tend to move the spindle poles apart (Cande and McDonald, 1985; Hiramoto and Nakano, 1988; Hyman and White, 1988). At anaphase, dissolution of the linkage between the sister chromatids destroys the opposition between the polewards force on the kinetochores and the forces tending to move the spindle poles apart. As a result, the forces in the spindle could move the chromosomes towards the poles (anaphase A) and the spindle poles apart from each other (anaphase B). In principle, breaking the linkage between sisters could initiate all the events of anaphase, without any change in the directions or magnitudes of the forces acting on components of the spindle. Indeed, measurements of the forces acting on kinetochores suggest that they do not change between metaphase and anaphase (Alexander and Rieder, 1991; Nicklas, 1988).

Recent advances have greatly increased our understanding of the regulation of the cell cycle. The entry into mitosis and the initiation of spindle assembly requires the activation of maturation promoting factor (MPF)[1] (for review see Murray and Kirschner, 1989b; Nurse, 1990). Active MPF is a complex between a catalytic subunit, $p34^{cdc2}$, and cyclin B. The onset of anaphase coincides with the destruction of cyclin B and with the consequent inactivation of MPF (Hunt and Ruderman, 1992; Lehner and O'Farrell, 1989; Murray et al., 1989). Unfertilized eggs of the frog *Xenopus laevis* are arrested in metaphase of meiosis II by cytostatic factor (CSF) (Masui and Markert, 1971) that is intimately related to the product of the c-*mos* proto-oncogene (Sagata et al., 1989), and stabilizes MPF activity by preventing cyclin degradation (Murray et al., 1989). Fertilization of the egg induces an increase in the intracellular concentration of calcium that leads to the destruction of cyclin, the inactivation of MPF and CSF (Lorca et al., 1991; Meyerhof and Masui, 1977; Watanabe et al., 1991), and the initiation of chromosome separation. CSF-arrested extracts are prepared from unfertilized frog eggs, and remain stably arrested with high levels of MPF. These extracts can be induced to inactivate MPF and progress to interphase by the addition of calcium (Lohka and Maller, 1985; Murray et al., 1989).

In contrast to our understanding of the activities that regulate progress through the cell cycle, we do not know what holds sister chromatids together, nor how this linkage is broken at anaphase. In mammalian cells, a number of proteins (inner centromere proteins [INCENP]) have been characterized that are located between sister chromatids during metaphase and that remain in the center of the spindle as chromosomes move towards the poles at anaphase (Cooke et

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1. *Abbreviations used in this paper*: CSF, cytostatic factor; kDNA, kinetoplast DNA; MPF, maturation promoting factor.

al., 1987). Sister chromatids may also be linked to each other by interlinking (catenation) of their DNA duplexes (Sundin and Varshavsky, 1980). Catenation arises from incomplete unwinding of the parental DNA duplex during DNA replication. Experiments in yeast and mammalian cells have established that the activity of type II DNA topoisomerases is required during mitosis to allow sister separation (DiNardo et al., 1984; Downes et al., 1991; Holm et al., 1985, 1989; Uemura et al., 1987), suggesting that sister chromatids are still catenated in mitosis. However, it is not known whether DNA catenation is sufficient or even necessary to maintain the linkage between sister chromatids, nor whether any of the identified INCENP proteins play a role in holding sisters together.

To investigate what holds sister chromatids together, we have developed a method for studying sister chromatid separation at anaphase in vitro. Sperm nuclei were allowed to undergo DNA replication in interphase frog egg extracts and induced to form metaphase spindles by the addition of CSF-arrested extract. Calcium was added to these extracts to inactivate MPF and initiate anaphase. We show here that chromosome separation follows the inactivation of MPF, and that the chromosomes separating from each other in these spindles are sister chromatids. The activity of type II topoisomerases is required at the metaphase–anaphase transition for successful sister chromatid separation, although topoisomerase II activity actually decreases during the progression from metaphase to anaphase.

Materials and Methods

Materials

Sperm nuclei were prepared as described (Murray, 1991) and stored at a concentration of $10^4/\mu l$ in small aliquots at $-80°C$. Before use, they were diluted in sperm dilution buffer (100 mM KCl, 1 mM MgCl$_2$, 150 mM sucrose) to a concentration of $\sim 10^3/\mu l$. Bovine brain tubulin was labeled, to one fluorochrome per tubulin dimer, with tetramethylrhodamine by the high pH labeling method (Hyman et al., 1990) and stored in small aliquots in injection buffer (50 mM K-glutamate, 0.5 mM glutamic acid, 0.5 mM MgCl$_2$). Aphidicolin (Sigma Chemical Company, St. Louis, MO) was dissolved at 20 mg/ml in DMSO. The topoisomerase II inhibitors VP-16 (demethylepipodophyllotoxin ethylidene-β-D-glucoside) and VM-26 (demethylepipodophyllotoxin thenylidene-β-D-glucoside) were obtained from Bristol-Myers Squibb (Wallingford, CT) and dissolved at 10 mM in DMSO; doxorubicin (Ben Venue Laboratories, Bedford, OH) was dissolved at 1 mg/ml in 0.45% NaCl; novobiocin (Sigma Chemical Company) was dissolved at 100 mg/ml in water. Inhibitors were stored in small aliquots at $-20°C$.

Preparation of Extracts

CSF-arrested extracts were made from freshly squeezed frog eggs as described previously (Murray, 1991; Murray et al., 1989) except that the crushing spin was carried out for 15 min at 10,000 g at 15°C and no clarifying spin was performed.

In Vitro Anaphase

CSF-arrested extracts were freshly prepared and rhodamine-labeled tubulin was added to 120 μg/ml. Extract was dispensed into microfuge tubes, diluted sperm nuclei were added to a concentration of 100/μl, and extract and sperm were incubated at room temperature (∼20°C) for 10 min. To drive the extracts into interphase and to start DNA replication, 4 mM CaCl$_2$ in sperm dilution buffer was added to each reaction to a final concentration of 0.4 mM. The reactions were incubated at room temperature for 80 min before adding 0.5 vol of CSF-arrested extract (which contained rhodamine-labeled tubulin, but no sperm nuclei) to induce nuclear envelope

breakdown and spindle assembly. We believe that the added CSF and MPF are stable because the calcium that was added at the start of the reaction has been sequestered. Metaphase spindles were allowed to assemble for 80 or 90 min. To induce anaphase, a fraction of the metaphase extract was placed in new microfuge tubes and calcium was added to 0.4 mM.

The morphology of the nuclei was determined by taking samples at various timepoints and fixing with formaldehyde in the presence of Hoechst 33342 (Murray and Kirschner, 1989a). Samples were examined by fluorescence and by phase contrast microscopy. Light micrographs were taken on a Nikon Microphot-FXA (Nikon Inc., Melville, NY) with 40× or 63× objectives, using TRI-X Pan or Technical Pan film (Eastman Kodak Co., Rochester, NY).

MPF Activity

To assay MPF activity, 1-μl aliquots of extract were frozen in liquid nitrogen at the desired time points and stored at $-80°C$ until assayed. MPF activity was assayed as H1 kinase activity as described by Murray (1991). H1 kinase activity was determined by scintillation counting slices of dried gels, and is expressed as arbitrary units.

DNA Replication

To monitor DNA synthesis, 5-μl samples of extract were removed from the reaction at the desired time points, added to 1 μl of 100 mM KCl, 1 mM MgCl$_2$ that contained 0.25 μCi of α-^{32}P-dCTP, and incubated for 10 min at room temperature. Reactions were stopped by the addition of 300 mM sucrose, 10 mM EDTA, pH 8.0, 50 μg/ml RNase A and incubated at room temperature for 10 min. 5 μl of freshly prepared 3× TAE loading buffer (120 mM Tris-Acetate, pH 8.0, 33 mM EDTA, 30% wt/vol glycerol, 1% SDS) was added to each reaction, the reactions were heated to 65°C for 10 min, and then run on a 0.7% agarose-TAE gel until the dye front had run ∼5 cm into the gel. Under these conditions, the sperm DNA remained in the wells of the gel. Gels were washed twice, for 1 h each, in 1× TAE, dried at 60°C onto paper, and autoradiographed. To inhibit DNA synthesis, aphidicolin (a specific inhibitor of DNA polymerase-α) (Ikegami et al., 1978) was added to a final concentration of 20 μg/ml, yielding a final DMSO concentration of 0.1%. Aphidicolin inhibited DNA synthesis by >95% (see Fig. 2 B).

Topoisomerase Inhibition

To test their effects on chromosome segregation, topoisomerase inhibitors were used in fresh extracts and were added after the extracts had reached metaphase. The final concentration of DMSO in the extract never exceeded 0.3%; this concentration has no effect on chromosome or spindle morphology (data not shown). The same lot of DMSO was used both to dissolve the topoisomerase inhibitors and for controls for the effect of added DMSO.

The ability of inhibitors to interfere with action of type II DNA topoisomerase was monitored by measuring the rate at which kinetoplast DNA was decatenated in the presence and absence of inhibitors. These assays were carried out in CSF-arrested extracts that had been frozen at $-80°C$ after the addition of sucrose to 200 mM. Topoisomerase activity is identical in fresh and frozen extracts (data not shown). Extracts were diluted 50-fold into dilution buffer (100 mM KCl, 2 mM MgCl$_2$, 10 mM Hepes, pH 7.7, 1 mM ATP, 8 mM creatine phosphate, 1 mM DTT, 200 mM sucrose) containing 5 ng/ml kinetoplast DNA (kDNA) from *Crithidia fasciculata*, (kindly provided by V. Klein and P. Englund, Johns Hopkins University School of Medicine, Baltimore, MD). Reactions were incubated at room temperature and at each time point, 25-μl samples were added to 0.25 ml of digestion buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.5% (wt/vol) SDS, 0.5 mg/ml proteinase K) and incubated at 37°C for 1 h to digest the proteinaceous component of any DNA-topoisomerase complexes. After digestion the samples were successively extracted with phenol and chloroform, ethanol precipitated, redissolved, and run on 0.7% agarose-TAE gels in the absence of ethidium bromide. Gels were stained with ethidium bromide and photographed. For some inhibitors a simpler assay, using undiluted extracts and in which the samples were not proteinase K digested before phenol extraction, was used.

To determine the level of topoisomerase II activity during metaphase and anaphase, we measured the rate of kDNA decatenation in fresh extracts that contained sperm nuclei and rhodamine-labeled tubulin. 80-μl samples of an extract were removed at metaphase or during anaphase, and added to 80 μl of ice-cold decatenation buffer (100 mM KCl, 1 mM MgCl$_2$, 10 mM K-Hepes, pH 7.7, 50 mM sucrose, 1 mM DTT, and 1.25 mM ATP) and 40 μl of ice-cold kDNA diluted to 24 μg/ml in decatenation buffer. Decatena-

tion reactions were performed on ice to prevent cell cycle progression during the course of the assay, and 25-μl samples were withdrawn at various times and processed for electrophoresis as described above, with the exception that they were not digested with proteinase K. H1 kinase activity was assayed at the beginning and end of the reaction to confirm that no cell cycle progression had occurred during the decatenation assay.

Electron Microscopy

100 μl of extract containing metaphase or anaphase spindles was diluted with 3.8 ml of BRB 80 (80 mM KPipes, pH 6.8, 1 mM $MgCl_2$, 6 mM EGTA) containing 0.3% Triton X-100 and 30% glycerol, and allowed to incubate at room temperature for 2.5 min. 1.3 ml of EM-grade 8% glutaraldehyde (Ted Pella Inc., Redding, CA) was then added, the reactions mixed gently, and then layered onto a 3-ml cushion of BRB 80, containing 40% glycerol, in Corex tubes that had been modified to hold a 12-mm-round acid-washed coverslip at the bottom (Evans et al., 1985). The tubes were spun at 13,000 g at 20°C for 1 h in a swinging bucket rotor. The spindles that had affixed to the coverslips were washed in 0.05 M Millonig's phosphate buffer (Hayat, 1970) in a humid chamber at 4°C overnight. The spindles and coverslips were then dehydrated and flat embedded in Epon/Araldite (Hayat, 1970). Sections 0.15–0.2 μm thick were cut on an ultramicrotome (Ultracut-E; Reichert Jung, Vienna) using a glass knife and placed on formvar-coated copper mesh grids. Sections were stained with 5% uranyl acetate at 60°C for 1 h and then destained with 0.2 M EDTA at room temperature for 0–15 min. Sections were then poststained for 10 min at room temperature with 0.6% lead citrate and examined at 100 kV on an electron microscope (model 100C, JEOL USA, Inc., Peabody, MA).

Results

We used frog egg extracts to prepare metaphase spindles that could be induced to enter anaphase (Fig. 1). Sperm nuclei were added to CSF-arrested extracts that were then induced, by the addition of calcium, to enter interphase and replicate their DNA. To induce entry into mitosis and the formation of metaphase spindles, CSF-arrested extract was added to the reaction 80 min after the initial calcium addition. To inactivate MPF and to induce the metaphase spindles to undergo anaphase, a second calcium addition was made 170 min after the initial calcium treatment. Samples were removed throughout the reaction. Nuclear morphology, and the distribution of DNA and microtubules were monitored by phase contrast and fluorescence microscopy. DNA replication was monitored by the incorporation of labeled nucleotides and MPF activity was assayed as histone H1 kinase activity.

The time course of DNA replication, H1 kinase activity and spindle morphology in a typical experiment are shown in Fig. 2. 35 min after the first calcium addition, H1 kinase activity was low, and the nuclei had decondensed and acquired nuclear envelopes. DNA synthesis typically began 30–40 min after calcium addition and continued for 20–40 min. The DNA synthesis inhibitor, aphidicolin (Ikegami, 1978), prevented DNA replication (Fig. 2 B), but did not affect progression of the cell cycle as monitored by the activity of H1 kinase (data not shown). No organized microtubule

arrays were seen associated with interphase nuclei. This may simply reflect the instability of interphase microtubule arrays under our fixation conditions. In all the experiments shown in this paper, mitosis was induced by adding CSF-arrested extract 80 min after the initial calcium addition. After this addition, no further DNA replication was detected (data not shown). Spindles were allowed to assemble for 90 min after the addition of CSF arrested extract, and by the end of this period, the extract contained numerous well organized bipolar spindles with the chromosomes aligned on a metaphase plate, equidistant from the two spindle poles (Fig. 2 C). Depending on the experiment, between 40 and 75% of the nuclei gave rise to bipolar spindles. Once assembled, these spindles were stable for several hours and MPF activity remained high (Fig. 2 A, and data not shown).

Anaphase was induced by the addition of calcium to the spindle containing extracts, 170 min after the initial calcium addition. Within 10 min of the second calcium addition, MPF was inactivated and anaphase A occurred: the chromosomes separated and moved towards the spindle poles (Fig. 2 C). We have not reproducibly observed anaphase B in these extracts. During anaphase the density of astral microtubules increased, while that of microtubules in the center of the spindle decreased. Telophase typically started between 190 and 200 min and was marked by the disappearance of spindle microtubules and by the decondensation of individual chromosomes and nuclear envelope formation around them to form karyomeres. DNA replication was detected again starting between 200 and 210 min (data not shown), and the fusion of karyomeres to form interphase nuclei was completed between 210 and 230 min. In all experiments, the fraction of bipolar spindles that underwent anaphase and progressed to form daughter interphase nuclei after the second calcium addition exceeded 90%. Thus, we have shown that the formation of metaphase spindles and the induction of anaphase can be reproducibly obtained in vitro.

Chromosomes Separate Soon after Histone H1 Kinase Levels Fall

To determine when MPF levels fall relative to the onset of anaphase, we compared spindle morphology and H1 kinase activity every 2 min after the second addition of calcium. We could distinguish seven morphological stages during the progression to interphase: (1) metaphase; (2) metaphase-anaphase transition, in which part of the chromosomes had moved towards the poles but no separation of DNA staining could be seen in the center of the spindle; (3) early anaphase, in which a separation between the groups of segregating chromosomes could be seen at the former site of the metaphase plate; (4) mid-anaphase, in which the chromosomes were approximately equidistant between the center of the
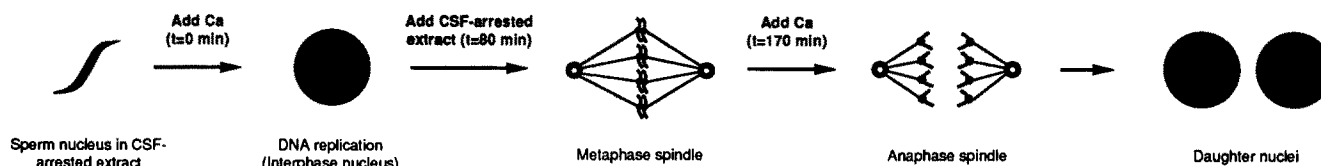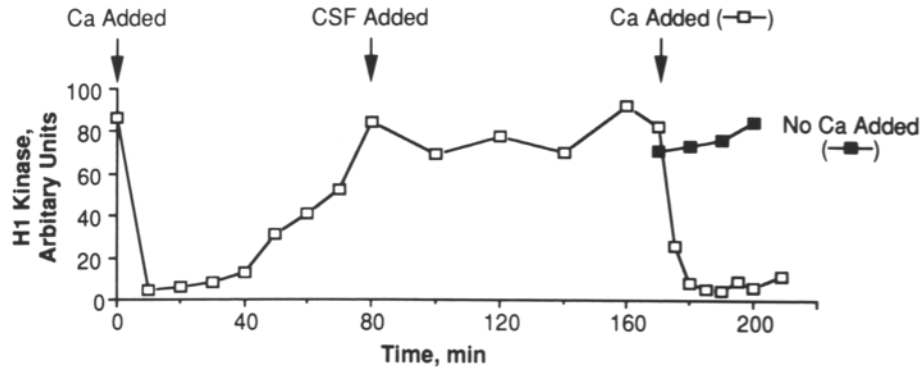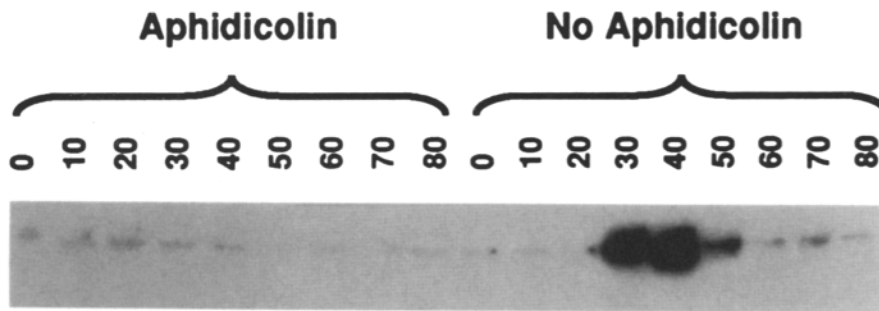

Figure 1. A schematic representation of the protocol used to produce anaphase in frog egg extracts. Times are relative to the time of first calcium addition.
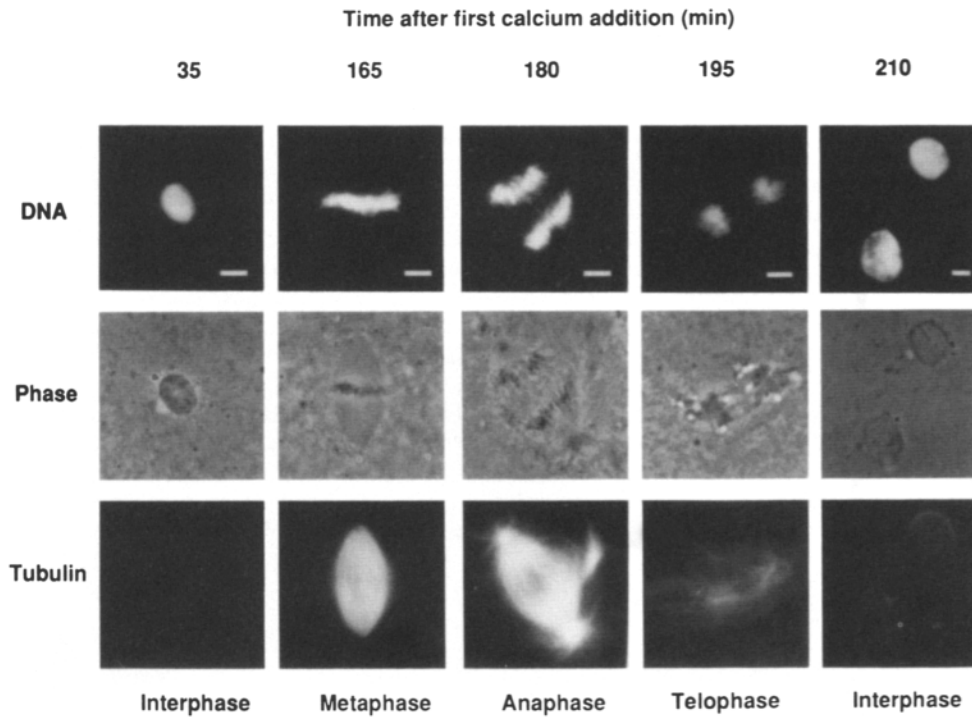
Figure 2. MPF activity and nuclear morphology during the course of a typical experiment. (*A*) MPF activity (measured as histone H1 kinase activity) graphed as a function of time ($\square$). If calcium addition at 170 min is omitted, MPF activity remains high ($\blacksquare$). (*B*) Timing of DNA replication during interphase and the inhibition of DNA replication by aphidicolin. Aphidicolin was added to a concentration of 20 μg/ml at the beginning of the experiment. Times are in minutes after the first addition of calcium. (*C*) Nuclear morphology at various time points; Note the change of magnification in 210 min time point. Data in *A* and *C* are from the same experiment, while that in *B* is from a different experiment. The time at which DNA replication begins and ends varies by ~15 min between experiments. Bars, 10 μm.
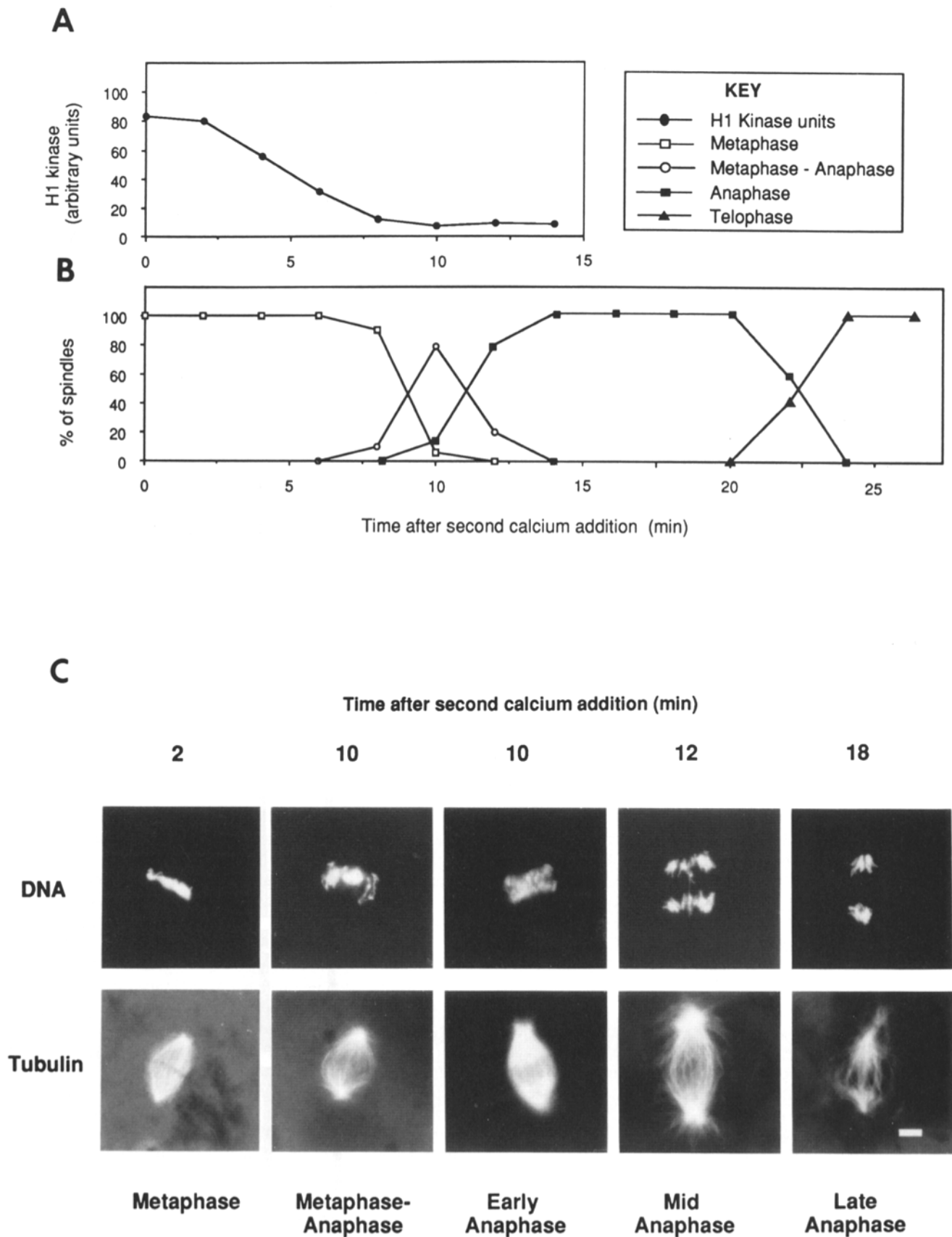
Figure 3. A detailed time course of MPF inactivation and nuclear morphology after the second calcium addition. Data from one of three experiments that demonstrated that MPF activity falls 2–4 min before anaphase chromosome movement begins. (*A*) MPF activity (measured as histone H1 kinase activity) graphed as a function of time after second calcium addition. (*B*) A quantitation of the number of spindles in each phase of the cell cycle at each time point. The categories of early, mid, and late anaphase, described in the text, have been expressed as a single category, anaphase. (*C*) Nuclear morphology in stages from metaphase to late anaphase. Bar, 10 μm.
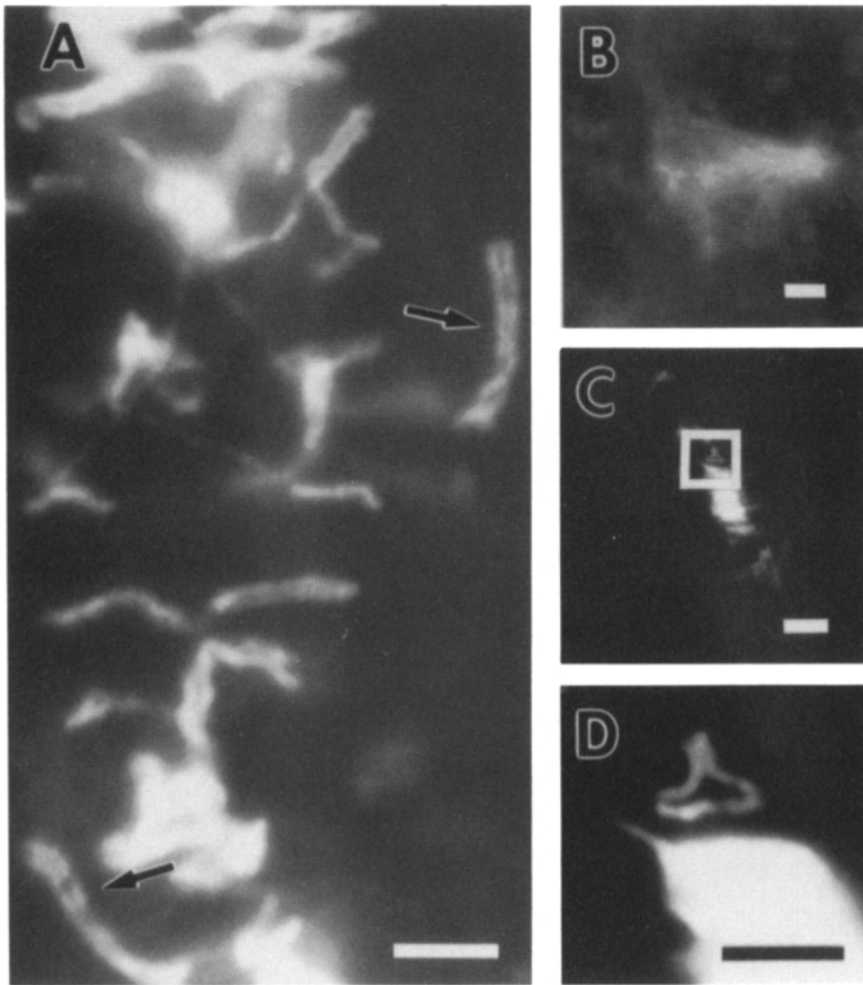
Figure 4. Metaphase and anaphase chromosomes in frog egg extracts. (*A*) Chromosomes at the metaphase plate of a large spindle; the spindle is not shown but is oriented horizontally. The arrows indicate chromosomes that are composed of paired chromatids of equal length. (*B*) A spindle (visualized with rhodamine tubulin), and (*C*) its chromosomes at the metaphase–anaphase transition. This spindle was from a sample taken 8 min after the second calcium addition in the experiment described in Fig. 3. (*D*) Enlargement of the boxed region of the spindle in *C*, showing a pair of chromatids separating from each other. Bars: (*A* and *D*) 5 μm; (*B* and *C*) 10 μm.

spindle and the poles; (*5*) late anaphase, in which the chromosomes were still condensed but located at or near the spindle poles; (*6*) telophase, in which the clustered chromosomes had begun to form karyomeres that fused with each other; and (*7*) interphase, in which fusion had been completed to yield spherical interphase nuclei bounded by membranes (Figs. 2 *C* and 3 *C*). In Fig. 3 *B* early, mid-, and late anaphase spindles are presented as a single class. In this experiment, H1 kinase activity begins to decline 4 min after the second calcium addition, and has reached interphase levels by 8 min. The metaphase–anaphase transition was first observed at 8 min, and clear chromosome separation (early anaphase) was first observed at 10 min, demonstrating that H1 kinase activity begins to fall before the morphological transition from metaphase to anaphase. Nuclei proceeded through the pathway from metaphase to interphase synchronously: at any time point, the majority of nuclei were in the same phase of mitosis (Fig. 3 *B*).

Sister Chromatids Separate during Anaphase in vitro

Having demonstrated that we could induce anaphase chromosome movement in vitro, we next asked whether the chromosomes that were segregating from each other were indeed sisters. The behavior of spindles as observed by light microscopy is entirely consistent with sister chromatid segregation. In metaphase spindles, we often observed chromosomes

composed of two paired chromatids of equal length (Fig. 4 *A*) (see also Sawin and Mitchison, 1991). After the second addition of calcium, equal amounts of DNA moved to each pole, as judged by the intensity of Hoechst staining (e.g., Fig. 2 *C* and 3 *C*). The individual chromosome arms that could be seen at this point were about the width of one member of the paired chromatids that we had observed at metaphase. In extracts to which no calcium was added, the width of the chromosomes did not decrease, indicating that this decrease was not the result of progressive chromosome condensation with increasing length of time in mitosis. In addition, some chromosomes showed mirror symmetry about the metaphase plate as they were being pulled away (Fig. 4 *B–D* and Fig. 2 *C*, 180 min), suggesting that they were sister chromatids in the act of separating.

To extend these observations, we examined sections of metaphase and anaphase spindles by EM. We could see paired chromatids of equal length apparently attached to the metaphase spindle (Fig. 5 *A*). At the interface between the microtubules and chromosomes, we did not observe the trilaminar structure characteristic of many kinetochores. Sawin and Mitchison (1991) were also unable to find trilaminar kinetochores by EM in metaphase spindles assembled in *Xenopus* egg extracts. In anaphase spindles, we observed individual chromosomes fixed in the process of moving toward the poles and each of these chromosomes was one-half the
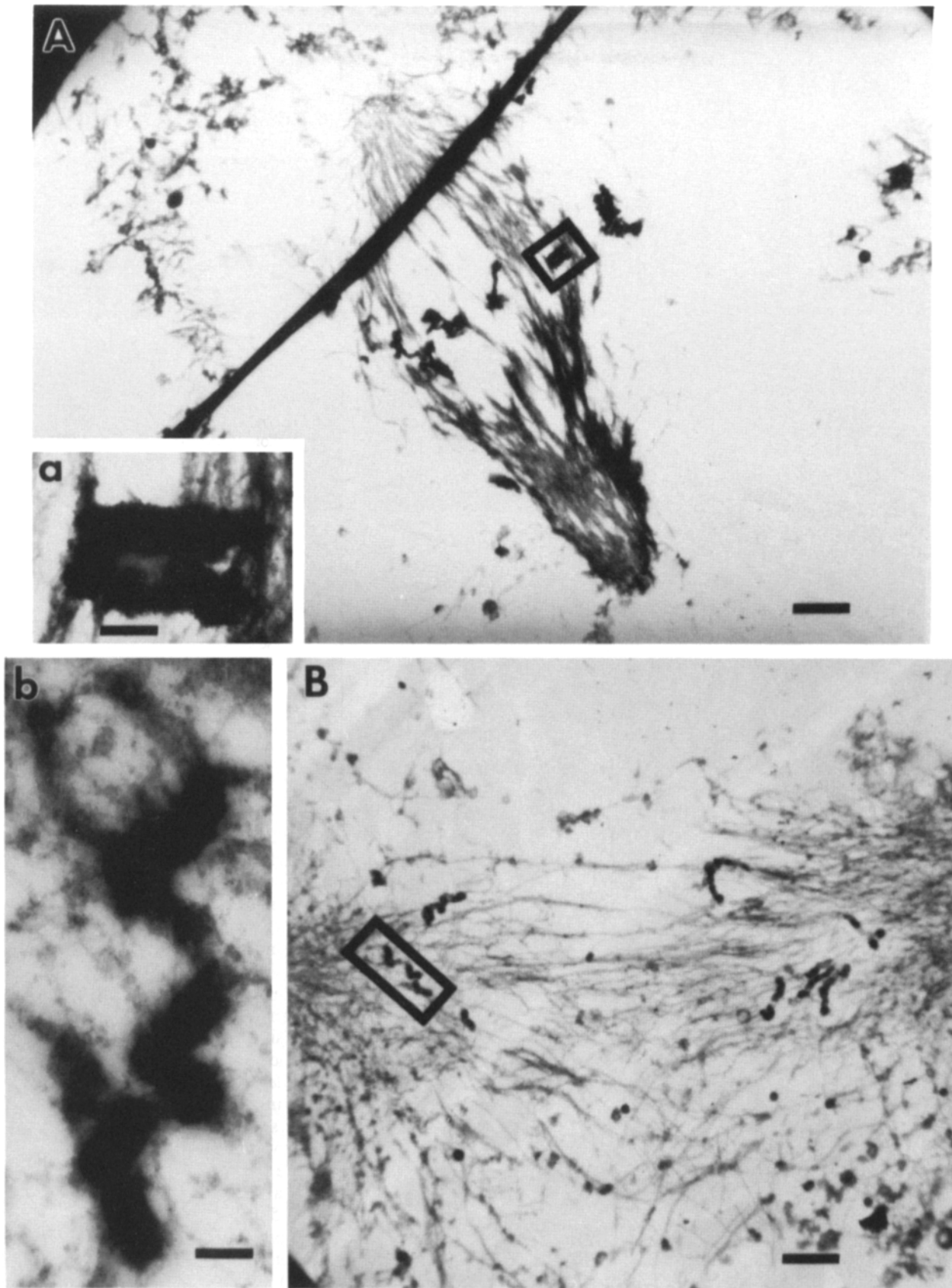
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Figure 5. Electron micrographs of metaphase and anaphase spindles. (*A*) A metaphase spindle. The dark bar running across the spindle is a fold in the section. (*a*) An enlargement of the area inside the box in *A* showing a pair of chromatids at the metaphase plate. (*B*) A late anaphase spindle. (*b*) An enlargement of the area inside the box in *B* showing chromosomes segregating to the pole at anaphase. Note that the width of each chromosome in *b* is one-half that of the chromosome in *a*. Bars: (*A* and *B*) 2.9 μm; (*a* and *b*) 0.4 μm.
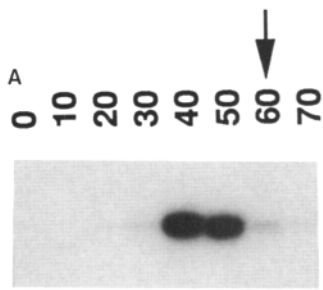
Figure 6. DNA replication and nuclear morphology in the presence and absence of aphidicolin. (*A*) DNA replication in a reaction without aphidicolin. The times listed above each well correspond to the number of minutes after the first calcium addition. The arrow indicates the time at which aphidicolin was added to a parallel reaction, whose spindles are shown in *C*. (*B*) Nuclear morphology in a control reaction and (*C*) in a reaction to which aphidicolin had been added to 20 µg/ml 60 min after the first calcium addition (as indicated in *A*). Metaphase spindles were fixed immediately before calcium addition. Anaphase spindles were fixed 22 min after the second calcium addition and interphase samples were taken 52 min after the second calcium addition. Bars, 10 µm.
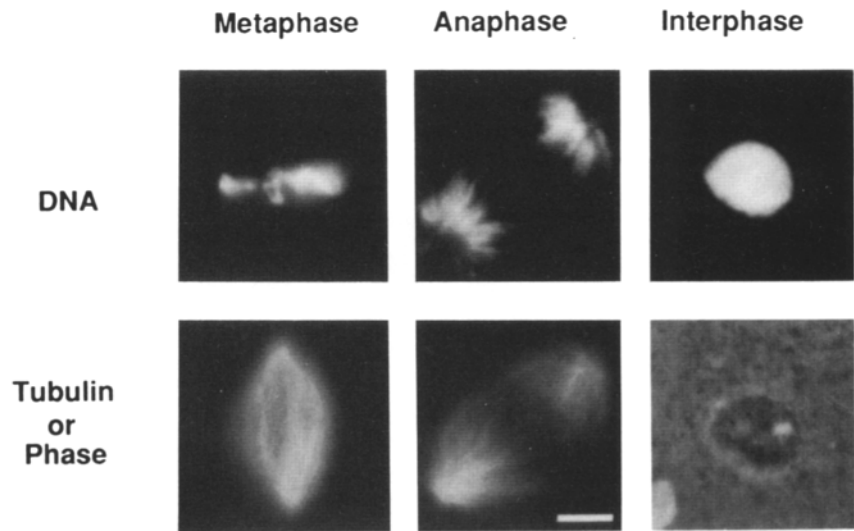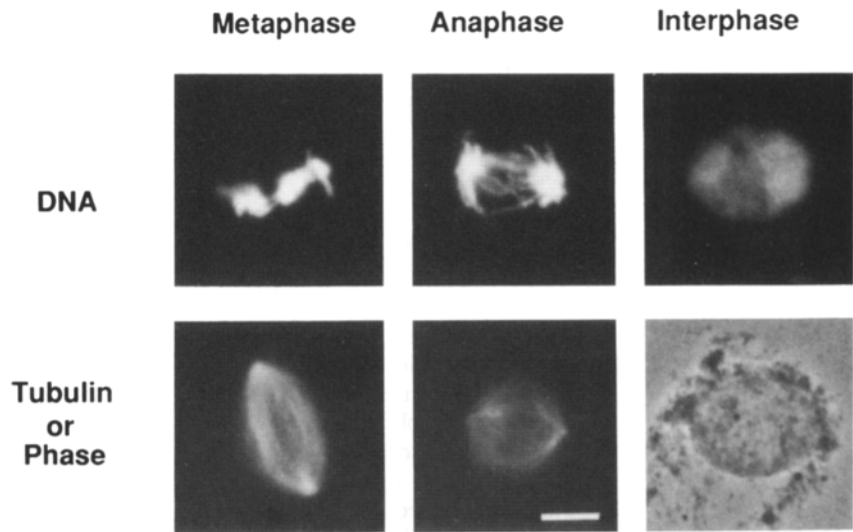
width of a metaphase chromosome pair. In some sections, the chromosomes had a V-shaped morphology, as though they were being pulled to the spindle poles by their kinetochores (Fig. 5 *B*). The simplest interpretation of these observations is that the paired chromosomes seen at metaphase are indeed sister chromatids and that the sisters separate from each other at anaphase.

To confirm that the segregating entities were sister chromatids, we devised a treatment that would interfere with chromosome separation only if the chromosomes were sisters. We added aphidicolin, an inhibitor of DNA replication, late during DNA replication to create pairs of sister chro-

matids that would be mostly replicated, but held together by some unreplicated regions. In these regions sister chromatids are held together by base pairing between DNA strands, as well as by the normal linkage between sisters, and cannot be easily separated from each other. Therefore, the separation of sister chromatids will be prevented by inhibiting the completion of chromosome replication, while that of homologs, or randomly associated chromosomes will not.

We found that extracts to which aphidicolin had been added late in S phase (60 min after the initial calcium addition) (Fig. 6 *A*) formed morphologically normal metaphase spindles. However, when these spindles were induced to en-

A No Aphidicolin B Aphidicolin late in S phase C Aphidicolin in metaphase

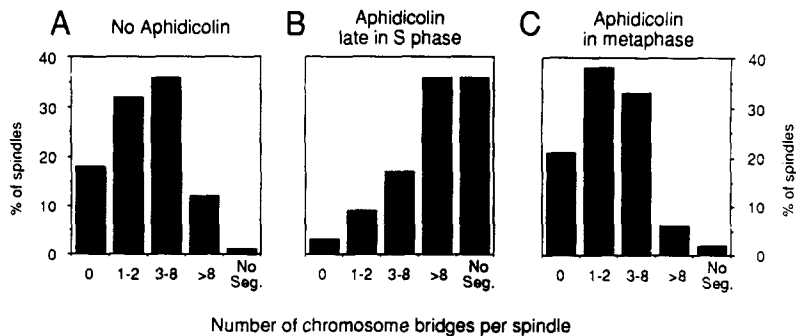Number of chromosome bridges per spindle

Figure 7. The distribution of chromosome bridges on spindles in the absence and presence of aphidicolin. The number of cross-bridges per spindle was scored for spindles fixed at mid-anaphase and the percent of the total number of spindles in each category was determined. The spindles in the "no segregation" (*No Seg.*) category appeared not to segregate their chromosomes. Chromosome bridges stretched all over these spindles and the bulk of chromatin was in the middle of the spindle, not near the poles. The data presented here are compiled from four separate experiments. (*A*) No aphidicolin added; 136 spindles scored. (*B*) Aphidicolin added late in S phase, as determined by DNA replication assays; 70 spindles scored. (*C*) Aphidicolin added at the time of second calcium addition, after DNA replication had been completed; 48 spindles scored.

ter anaphase, many chromosome bridges, representing incompletely separated chromosomes, were seen (Fig. 6 *C*), compared to spindles in which DNA synthesis had not been inhibited (Fig. 6 *B*). To quantify this effect, we compared the number of bridges present in anaphase in aphidicolin-treated extracts with the number in untreated extracts. A substantial increase in both the number of bridges per spindle and in the number of affected spindles was seen after aphidicolin treatment (Fig. 7, *A* and *B*). However, the timing of MPF inactivation, chromosome decondensation, and nuclear envelope formation was identical in aphidicolin-treated and untreated extracts (Fig. 6 and data not shown).

As a control for the effects of the drug, we added aphidicolin at the time of second calcium addition, long after DNA replication had been completed. This did not interfere with anaphase, demonstrating that aphidicolin blocks chromosome separation by interfering with DNA replication rather than by inhibiting activities involved in destroying the linkage between fully replicated chromosomes (Fig. 7 *C*). These data strongly suggest that the chromosomes that segregate at anaphase in our extracts are indeed sisters.
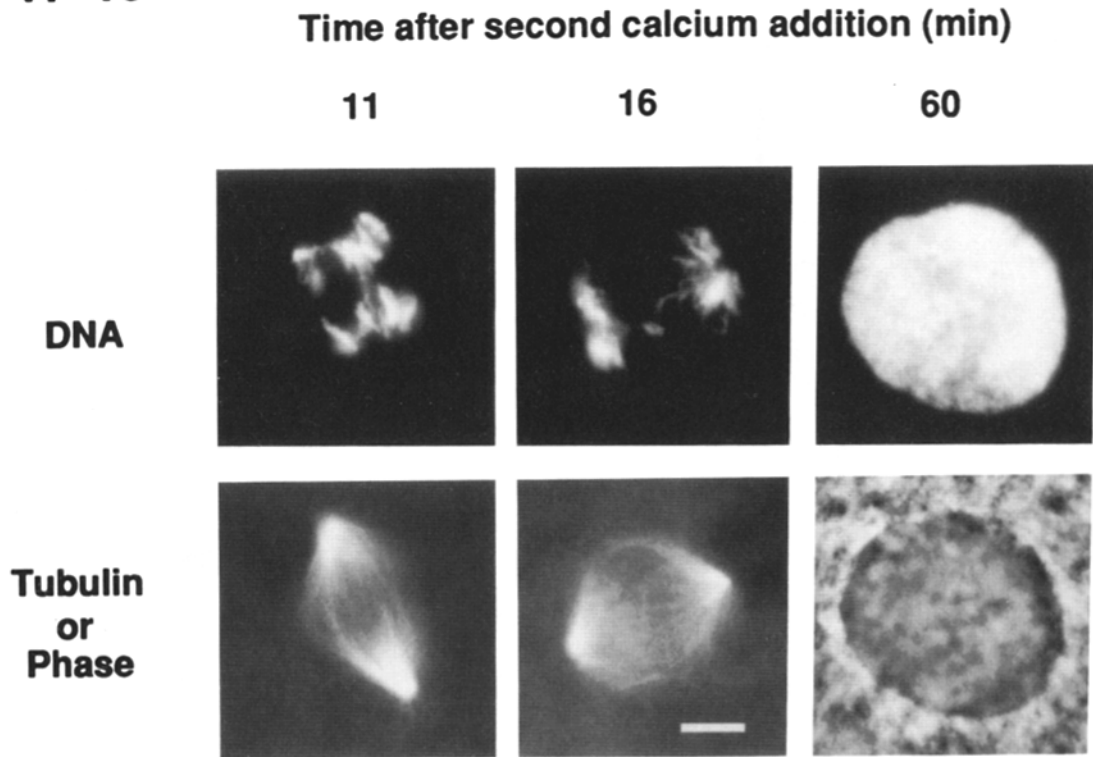
Topoisomerase II Activity Is Required for Sister Chromatid Separation

To examine the role of DNA topoisomerase II at anaphase, we tested the ability of topoisomerase inhibitors to block chromosome segregation. Metaphase spindles were formed, and topoisomerase II inhibitors were added immediately before the calcium addition that induces anaphase. The addition of 10 μM VP-16, a potent inhibitor of topoisomerase II (Chen et al., 1984), had no effect on the morphology of metaphase spindles but grossly disrupted chromosome segregation when anaphase was induced (Fig. 8). In VP-16 treated extracts, although some chromosome movement occurred, no clear chromosome segregation had occurred by the time that control spindles had reached mid-anaphase (Fig. 8, *A* and *B*). The addition of DMSO from the same lot that had been used to dissolve the VP-16 had no effect on chromosome segregation (data not shown). The amount of chromosome movement was further reduced at higher concentrations of VP-16 (30 μM). By the time that control spindles had reached late anaphase, chromosomes had moved away from the metaphase plate in VP-16-treated extracts but were still associated by multiple chromosome bridges (Fig. 8 *B*), suggesting that the linkage between sister chromatids could not be completely dissolved in these extracts. Delays
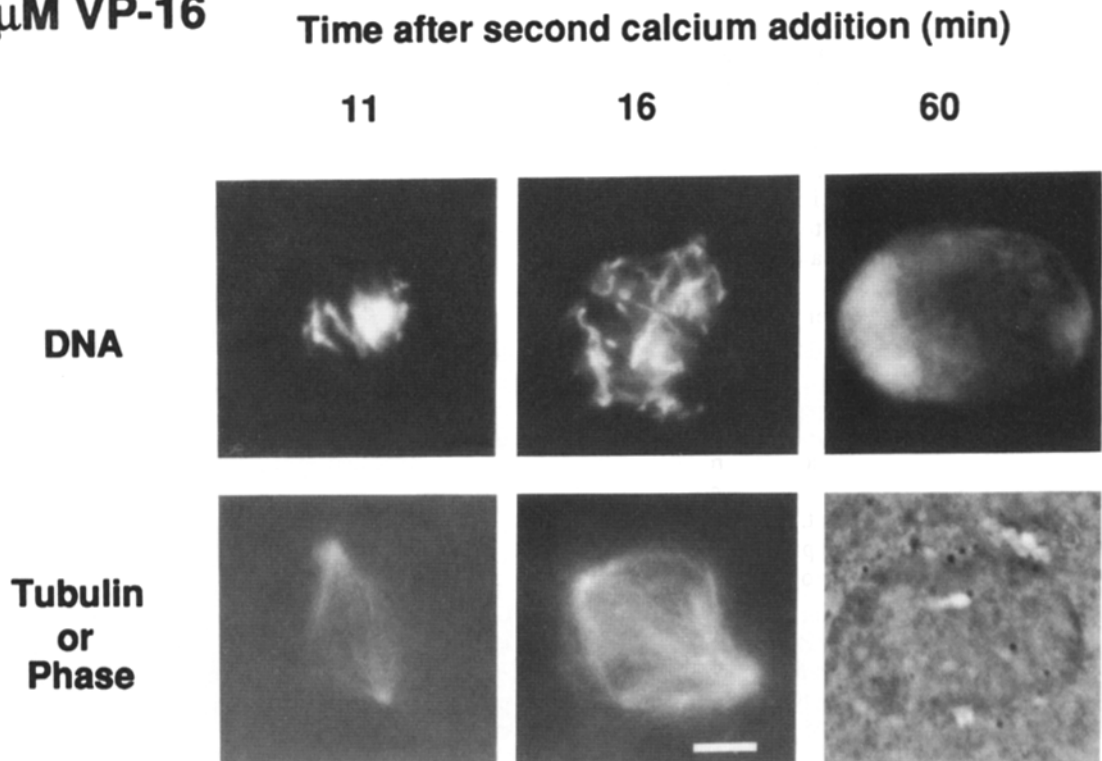
in chromosome movement and the subsequent formation of chromosome bridges were observed even when metaphase spindles were incubated for 45 min before VP-16 addition (data not shown). VP-16-treated extracts still carried out other aspects of the progression to interphase: MPF inactivation, chromosome decondensation, and interphase nuclei formation were initiated at the same time in untreated and in VP-16-treated extracts (Fig. 8). The addition of three other compounds that have been reported to inhibit topoisomerase II, VM-26 (10 μM or 30 μM) (Chen, 1984), doxorubicin (1 μg/ml) (Tewey et al., 1984), or novobiocin (250 μg/ml) (Hsieh and Brutlag, 1980; Osheroff et al., 1983) had essentially the same effect on chromosome movement as VP-16 (data not shown). The aberrations in chromosome morphology seen with topoisomerase inhibitors are more severe than those seen with late inhibition of DNA replication (Fig. 6). We believe this reflects the fact that most DNA was allowed to replicate in the experiments with inhibitors of DNA replication, while the topoisomerase inhibitors were added before the beginning of chromosome separation. Therefore, the number of points at which sister chromatids remain attached to each other may be smaller in the experiments where DNA replication was inhibited than it is in those where topoisomerase inhibitors were added.

To demonstrate that VP-16 was acting to inhibit type II topoisomerase in our experiments, we assayed topoisomerase activity in the presence and absence of the drug. To measure topoisomerase II activity, we examined the ability of our extracts to release small DNA circles from kinetoplast DNA (kDNA) (Marini et al., 1980), the highly catenated networks of DNA isolated from the kinetoplasts of hemoflagellate protozoans. Topoisomerase activity was inhibited by the following compounds: VP-16 (10 μM, Fig. 9), VM-26 (10 μM, data not shown), doxorubicin (1 μg/ml, data not shown), novobiocin (250 μg/ml) (data not shown). The observation that these compounds block topoisomerase II activity at the same concentration at which they prevent chromosome segregation, strongly suggests that the topoisomerase II-mediated decatenation of sister chromatids is required for their separation. In vivo metaphase lasts less than 10 min during the early embryonic cell cycle. The ability of topoisomerase II inhibitors to block chromosome segregation, even when added after spindles have spent as much as 45 min in metaphase, demonstrates that sister chromatid decatenation cannot be completed during metaphase, but requires the events that induce anaphase.

A
No VP-16

Time after second calcium addition (min)

11   16   60

DNA

Tubulin
or
Phase

B
10 μM VP-16

Time after second calcium addition (min)

11   16   60
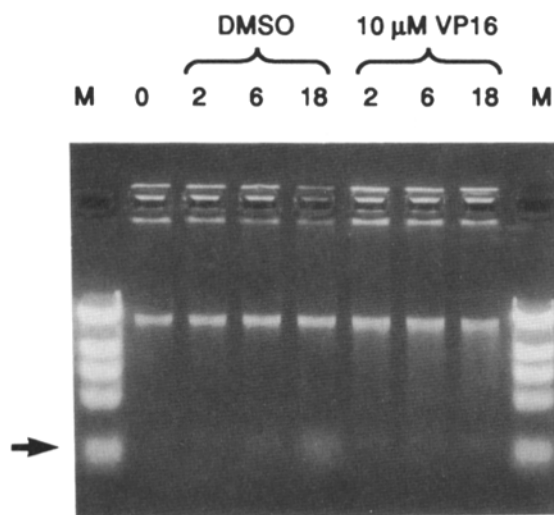
DNA

Tubulin
or
Phase

Figure 9. VP-16 inhibits topoisomerase II activity in frog egg extracts. Decatenation assays were carried out using frozen CSF-arrested extracts with kinetoplast DNA as a substrate, as described in Materials and Methods. Samples were taken at the indicated times (in min) and run on 0.7% agarose gels. The position at which the released mini-circles run is indicated by an arrow, while the catenated DNA networks are retained in the wells of the gel. Reactions contained either 1% (vol/vol) DMSO or 10 μM VP-16 (final DMSO concentration 1% (vol/vol). The markers (M) are a HindIII digest of lambda DNA.

Topoisomerase II Activity Does Not Increase at the Metaphase-Anaphase Transition

Does topoisomerase II activity increase at the metaphase to anaphase transition to cause sister chromatid separation? We assayed topoisomerase II activity by measuring the rate of kDNA decatenation. Topoisomerase II activity and spindle morphology were determined at metaphase and at three different times after the induction of anaphase: just before the earliest visible metaphase–anaphase transition; in mid-anaphase, soon after sister chromosomes had separated; and in late anaphase, well after sister chromosomes had separated. We found that the rate of kDNA decatenation was highest in samples taken from metaphase, slightly lower in the first two time points after calcium addition, and lowest in late anaphase (Fig. 10). Thus, topoisomerase II activity appears to decrease as extracts leave mitosis, although we cannot exclude the possibility that we have failed to detect a transient increase in topoisomerase activity during the metaphase–anaphase transition.

Discussion

We have assembled metaphase spindles in frog egg extracts and shown that these spindles can be induced to undergo anaphase. Sister chromatid separation is prevented by incomplete DNA replication, or the presence of topoisomerase II inhibitors during the induction of anaphase.

Anaphase In Vitro Resembles Anaphase In Vivo

To what extent does the anaphase we observe in extracts resemble anaphase in intact cells? We have used CSF, a natural inhibitor of the exit from mitosis, to arrest extracts with high levels of MPF and allow sufficient time for spindle assembly. Sawin and Mitchison (1991) have shown, using conditions similar to ours, that each bipolar metaphase spindle forms from a single nucleus. The length of the spindles assembled in the frog egg extracts ($\sim$30 μm) is similar to that seen in intact frog eggs (Karsenti et al., 1984). There are no direct measurements of the rate of anaphase chromosome movement in frog embryos, but the observation that spindle size and the duration of anaphase are not grossly different between embryos and extracts (A. W. Murray, unpublished observations) argues that the rate of anaphase chromosome movement is similar in these two systems.

Because CSF-arrested extracts are made from cells arrested in metaphase of meiosis II, the spindles that we have assembled by adding CSF-arrested extracts to our reactions might be regarded as meiotic rather than mitotic spindles. Whether these spindles are truly in mitosis or meiosis II is irrelevant to studies on the mechanism of sister chromatid segregation, because sister chromatids separate from each other in both mitotic and meiosis II anaphases. When induced to enter anaphase, spindles formed in extracts undergo morphological changes similar to those seen in cells: as chromosomes move to the poles, astral microtubules become more prominent and microtubule density at the center of the spindle decreases.

If frog egg extracts are to be used to study the nature of sister chromatid linkage and the mechanism of sister chromatid segregation, it is crucial to demonstrate that the segregating chromosomes are indeed sisters. A number of observations demonstrate that sister chromatid segregation occurs during the in vitro anaphase. When examined by light or by electron microscopy, spindles assembled in vitro contain chromatid pairs aligned at the metaphase plate; the two members of each pair have identical lengths and morphologies. In early anaphase these pairs are observed to split and their two members are pulled towards opposite poles of the spindle. When the DNA polymerase inhibitor aphidicolin is added to extracts late in DNA replication, pairs of chromatids held together by unreplicated regions are formed. When spindles formed under these conditions are induced to undergo anaphase, chromatin bridges are formed that stretch between the segregating chromosomes, demonstrating that the chromosomes attempting to segregate from each other are indeed sisters.

The following considerations confirm that the chromosomes whose segregation we observe must be sisters. One alternative possibility is that the paired chromosomes that separate are not sisters but nonhomologous chromosomes that are being paired and segregated by a distributive disjunction mechanism. Such a system operates in meiosis I to segregate chromosomes that have failed to undergo mitotic recombination (Grell, 1962; Dawson et al., 1986). Recent observations on

Figure 8. The effect of inhibiting topoisomerase II on nuclear morphology at anaphase. (*A*) Control anaphase. (*B*) Anaphase in the presence of the topoisomerase II inhibitor VP-16. VP-16 was added to the extract to 10 μM, just before the second calcium addition. Bars, 10 μm.
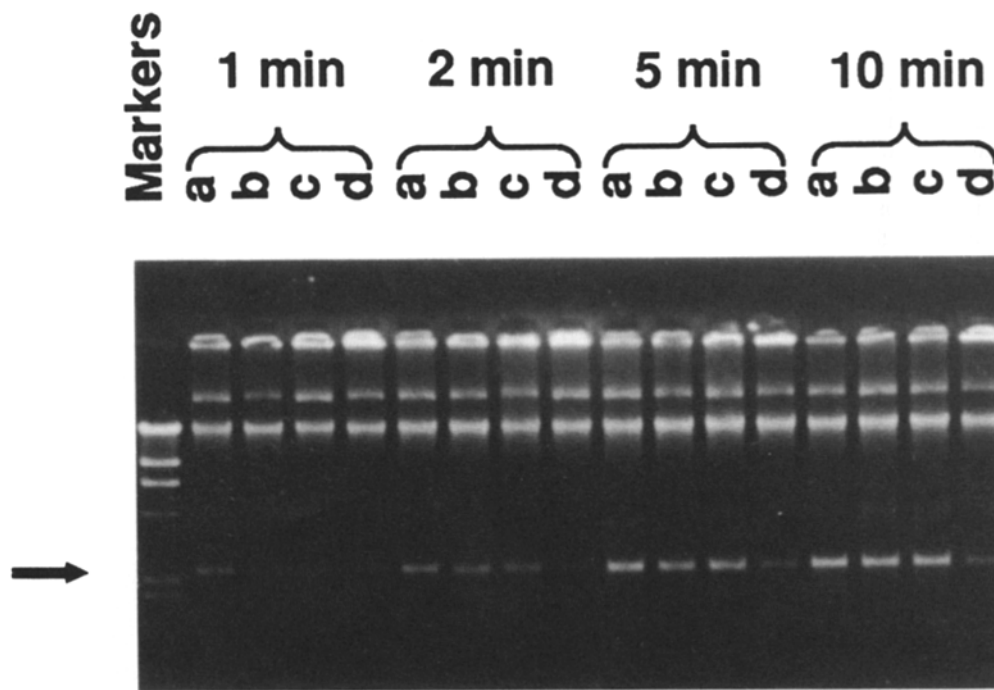
Figure 10. Topoisomerase II activity falls after the metaphase to anaphase transition. Topoisomerase II activity was assayed in fresh extract at four different time points: (*a*) metaphase; (*b*) after the induction of anaphase before chromosome movement had begun (8 min after second calcium addition); (*c*) in mid-anaphase (13 min after second calcium addition); and (*d*) in late anaphase (18 min after second calcium addition). A sample of extract from each time point was mixed with kDNA to begin decatenation reaction. Reactions were carried out on ice to halt progression through the cell cycle and to slow the rate of decatenation. Topoisomerase activity was monitored as the extent of decatenation that had occurred after 1, 2, 5, and 10 min. The markers are a HindIII digest of lambda DNA. The arrow indicates the position in the gel to which decatenated minicircles migrate.

meiotic spindles in *Drosophila* by Theurkauf and Hawley (1992) show that the chromosomes segregated by the distributive pairing mechanism, lie not on the metaphase plate but in between the plate and the spindle poles. We do not observe chromosomes in this position. In addition, inhibition of DNA replication, or type II topoisomerase activity should have no effect on the segregation of nonhomologous chromosomes. The possibility that the segregating chromosomes are homologous chromosomes that have become paired with and linked to each other by meiotic recombination can also be eliminated. Using conditions similar to ours, Sawin and Mitchison (1991) demonstrated that each spindle is formed from a single sperm nucleus. Since sperm nuclei are haploid no chromosome will have a homolog with which it could pair.

In summary, by a variety of criteria, the anaphase that we have observed in vitro resembles anaphase in vivo, strongly suggesting that anaphase in vitro proceeds by the same mechanism as anaphase in vivo. We believe that the extracts described here will be valuable for studying the mechanisms of sister chromatid separation and anaphase chromosome movement.

The Role of DNA Catenation in Sister Chromatid Linkage

Are sister chromatids at metaphase linked by DNA catenation? Incomplete unwinding of DNA duplexes during replication produces replicated DNA molecules that are linked by catenation (Sundin and Varshavsky, 1980, 1981), and that can only be separated from each other by the action of type II DNA topoisomerases. Experiments in budding and fission yeast and, more recently, in mammalian cells, show that type

II DNA topoisomerase activity is required during mitosis for successful chromatid separation (DiNardo et al., 1984; Downes et al., 1991; Holm et al., 1989; Uemura et al., 1987). This suggests that, in vivo, sister chromatids are catenated and that this catenation must be resolved in mitosis to allow them to segregate from each other. However, none of these experiments address the question of when, in a normal spindle, topoisomerase activity is required to ensure complete sister chromatid segregation. In both mammalian cells and yeasts, the metaphase–anaphase transition cannot be experimentally regulated without depolymerizing spindle microtubules. In such cells, chromosomes do not experience normal metaphase forces. On the other hand, without experimentally controlling the metaphase–anaphase transition, it is not possible to determine whether topoisomerase activity is inhibited before or after the induction of anaphase. We have used CSF, a physiological regulator, to control the transition between metaphase and anaphase without affecting spindle function. We find that topoisomerase activity is required during anaphase, even in spindles that have been arrested in metaphase before the addition of topoisomerase inhibitors, unequivocally demonstrating that topoisomerase activity is required during anaphase for sister chromatid segregation.

The observation that topoisomerase II inhibitors block sister chromatid segregation strongly suggests that sister chromatids are catenated at metaphase. We cannot exclude the possibility that topoisomerase II has a role in chromatid segregation other than decatenating sister chromatids and that we have inhibited this activity. However, because the topoisomerase inhibitors used in this study have different mechanisms of action, we feel that this possibility is unlikely (Osheroff et al., 1983; for review see Liu, 1989).

It has been difficult to establish the role that DNA catenation plays in the linkage between sister chromatids. On one hand it has been proposed that the primary linkage between sister chromatids is DNA catenation (Murray and Szostak, 1985). On the other, experiments on the segregation of mini-chromosomes in yeast suggest that although catenation may be a prerequisite for bipolar attachment of chromosomes to the mitotic spindle, it need not be continuously maintained to ensure accurate chromosome segregation (Koshland and Hartwell, 1987). Because of the inability to visualize individual chromosomes in budding yeast, it is impossible to exclude the possibility that mini-chromosomes segregate as soon as they are decatenated, even though the natural chromosomes are still on the metaphase plate.

If catenation is the only linkage between sister chromatids, then sister chromatid segregation could be induced by the activation of topoisomerase II at the onset of anaphase. However, by monitoring the decatenation of an exogenous substrate (kDNA), we find that there is no increase in topoisomerase II activity at the metaphase–anaphase transition. Thus, an increase in bulk topoisomerase activity at anaphase cannot be the trigger for chromosome segregation. This observation can be explained in a number of ways. The first is that there are two populations of topoisomerase II: one which is soluble and a second, in the nuclear scaffold, which is closely associated with the chromosomes (Earnshaw et al., 1985). The soluble pool, whose activity we have assayed using an exogenously added substrate (kDNA), may be regulated differently from the chromosome-associated pool. The second possibility is that, during metaphase, the catenation between sister chromatids is protected from topoisomerase activity, and that the protecting factors are removed when anaphase is induced. Finally, catenation may not be the only entity holding sister chromatids together. Instead there might exist another linkage, involving unknown components, whose dissolution is the primary trigger for sister chromatid separation. This linkage would maintain a high local concentration of DNA, so that strand passages catalyzed by topoisomerase II would be as likely to increase catenation as to decrease it. At anaphase the destruction of the other linkage would allow sister chromatids to move apart, decreasing the local DNA concentration and favoring decatenation (Holm et al., 1989). In this view, decatenation would be triggered by anaphase and would be a consequence rather than a cause of chromosome segregation and would not require an increase in topoisomerase activity. Candidates for a proteinaceous linkage between the chromosomes must be present along the chromosome arms as well as the kinetochore, since acentric sister chromatids remain paired throughout metaphase (Bajer and Mole-Bajer, 1972). The INCENP proteins that are localized between sister chromatids along their length are candidates for such a linkage. However, the recent observation that the association of these proteins with chromosomes is abolished considerably before sister chromatid separation and the onset of anaphase (Earnshaw and Cooke, 1991), suggests that they cannot be the only components holding sister chromatids together.

Models for chromosome segregation in which chromosomes are held together only by catenation of the sister chromatids, and separation is triggered by an increase in the force acting at the kinetochores can be eliminated. Earlier studies on mitosis demonstrate that the initial separation of sister chromatids does not require kinetochore activity. In cells arrested in mitosis by colchicine, the sister chromatids eventually separate from each other in the absence of spindle microtubules, although there is no directed movement of the kinetochores towards the spindle poles (Mole-Bajer, 1958; Sluder, 1979). In these mitoses, the kinetochores often remain linked at the centromeres after the arms have separated, suggesting that there may be special components that hold sister kinetochores together in addition to the components that hold the chromosome arms together. In normal mitoses careful time-lapse cinematography has shown that the initial separation of the sisters occurs synchronously all along the length of a chromosome, rather than beginning at the kinetochore and then moving towards the poles, as would be expected if the sisters had to be pulled apart by their kinetochores (Bajer and Mole-Bajer, 1972). Acentric sister chromatids do separate from each other at anaphase, although after their initial separation they fail to show directed movements towards the spindle poles (Bajer and Mole-Bajer, 1972). Finally, experimental measurements suggest that force acting on kinetochores does not increase in anaphase (Alexander and Rieder, 1991; Nicklas, 1988).

The event that triggers sister chromatid separation is unknown. As a first step towards identifying this event we have examined the timing of sister chromatid separation relative to the inactivation of MPF. The first stages of sister separation are seen 4 min after H1 kinase activity begins to fall. It is tempting to conclude from this temporal correlation that the inactivation of H1 kinase is a prerequisite for sister chromatid separation, as it is for chromosome decondensation and nuclear envelope assembly (Murray et al., 1989). However, it is also possible that sister separation is triggered, not by the inactivation of MPF, but by the events that trigger cyclin B degradation and that the proteolytic system that degrades cyclin B also degrades proteins involved in holding sisters together. The existence of nondegradable forms of cyclin should make it possible to distinguish between these possibilities (Murray, 1989). Anaphase in meiosis II of frog oocytes, and in our in vitro extracts, is induced by an increase in the intracellular calcium concentration (Kline, 1988; Meyerhof and Masui, 1977). Although a role for calcium in inducing mitosis in other systems has been reported (Hepler, 1983; Izant, 1983; Poenie et al., 1986; Schollmeyer, 1988; Tombes and Borisy, 1989), it is not clear whether an increase in the intracellular calcium levels is a universal prerequisite for the induction of anaphase (Tombes and Borisy, 1989).

The ability to induce chromosome segregation in vitro will facilitate studies both on the initial separation of sister chromatids at the metaphase–anaphase transition, and on the anaphase movement of the chromosomes towards the poles. In vitro studies will help to clarify the role of DNA catenation, and the INCENP proteins in holding sister chromatids together. In addition, they will allow a biochemical dissection of the linkage between sister chromatids and of the mechanism by which this linkage is destroyed at anaphase.

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