Microinjected Kinetochore Antibodies Interfere with Chromosome Movement in Meiotic and Mitotic Mouse Oocytes

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Abstract. Kinetochores may perform several functions at mitosis and meiosis including: (*a*) directing anaphase chromosome separation, (*b*) regulating prometaphase alignment of the chromosomes at the spindle equator (congression), and/or (*c*) capturing and stabilizing microtubules. To explore these functions in vivo, autoimmune sera against the centromere/kinetochore complex are microinjected into mouse oocytes during specific phases of first or second meiosis, or first mitosis. Serum E.K. crossreacts with an 80-kD protein in mouse cells and detects the centromere/kinetochore complex in permeabilized cells or when microinjected into living oocytes. Chromosome separation at anaphase is not blocked when these antibodies are microinjected into unfertilized oocytes naturally arrested at second meiotic metaphase, into eggs at first mitotic metaphase, or into immature oocytes at first meiotic metaphase. Microtubule capture and spindle reformation occur normally in microinjected unfertilized oocytes recovering from cold or microtubule disrupting drugs; the chromosomes segregate correctly after parthenogenetic activation. Prometaphase congression is dramatically influenced when antikinetochore/centromere antibodies are introduced during interphase or in prometaphase-stage meiotic or mitotic eggs. At metaphase, these oocytes have unaligned chromosomes scattered throughout the spindle with several remaining at the poles; anaphase is aberrant and, after division, karyomeres are found in the polar body and oocyte or daughter blastomeres. Neither nonimmune sera, diffuse scleroderma sera, nor sham microinjections affect either meiosis or mitosis. These results suggest that antikinetochore/centromere antibodies produced by CREST patients interfere with chromosome congression at prometaphase in vivo.

THE kinetochore, the site of spindle microtubule attachment to the centromeric region of the chromosome (reviewed by Rieder, 1982; Brinkley et al., 1989), has recently enjoyed increased attention as a dynamic structure responsible for critical activities at mitosis and meiosis (reviewed by Nicklas, 1988; Mitchison, 1988, 1989). It has been proposed to be responsible for: (*a*) microtubule capture and stabilization in vitro (Mitchison and Kirschner, 1985*b*), (*b*) congression, the alignment of the chromosomes at the spindle equator during prometaphase (Mitchison and Kirschner, 1985*b*), and/or (*c*) chromosome segregation at anaphase (Gorbsky et al., 1987; Mitchison, 1989; Nicklas, 1989).

The molecular characterization of the centromere/kinetochore complex has benefitted significantly from the use of autoimmune sera from patients with calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia (CREST)[1] scleroderma (Moroi et al., 1980, 1981; Brenner et al., 1981). Earnshaw and Rothfield (1985) have identified three proteins associated with the centromere/kinetochore complex: CENP-A (18 kD), CENP-B (80 kD), and CENP-C (140 kD). CENP-B has been cloned, sequenced (Earnshaw et al., 1987), and localized to the centromere/kinetochore region (Earnshaw et al., 1987; Earnshaw, 1989). Balczon and Brinkley (1987) demonstrate the crosslinking of an 80-kD centromeric protein to microtubules, and propose that CENP-B may be involved in spindle microtubule attachment to the chromosome.

To explore the possible functions of kinetochore proteins in vivo, we have microinjected mouse oocytes with CREST sera during first and second meiosis and first mitosis. The mouse egg represents a unique system to test the functions of the kinetochore in vivo since it is ovulated arrested at meiotic metaphase II, permitting an analysis of anaphase chromosome segregation independent from the events leading to metaphase chromosome alignment. In addition, immature germinal-vesicle stage oocytes will mature spontaneously in vitro and nuclear progression during meiotic maturation can be blocked at stage-specific sites (Wassarman et al., 1976); this allows for the examination of the events of prometaphase congression of chromosomes without the complications of anaphase onset. Finally, the events at fertil-

1. *Abbreviation used in this paper*: CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia.

ization and first mitosis can be explored. In this report, antikinetochore/centromere antibodies are found to interfere with prometaphase congression during meiosis I and II and mitosis; microtubule capture, spindle formation, and chromosome segregation at anaphase appear unaffected.

Materials and Methods

Antisera

CREST serum E.K., previously shown to recognize the centromere/kinetochore complexes in vertebrates (Balczon and Brinkley, 1987), invertebrates (Janicke, M. A., R. D. Balczon, B. R. Brinkley, and J. R. Fountain, Jr. *J. Cell Biol.* 107:456a.) and plants (Mole-Bajer, J., B. R. Brinkley, A. S. Bajer, and R. D. Balczon. *J. Cell Biol.* 107:454a.), was selected for microinjection following the demonstration of cross-reactivity with interphase and mitotic centromeres by indirect immunofluorescence microscopy (Schatten et al., 1988*a*). Diffuse autoimmune scleroderma serum, nonimmune human sera, IgG-depleted E.K. serum and sham microinjections were performed as controls in examining the effects of antisera on chromosomal congression and segregation. Treated and control oocytes were microinjected with ~4% of the egg volume. A second autoimmune CREST scleroderma serum, designated S.H. (and which also localizes the centromere/kinetochore complex in unfertilized mouse oocytes) was employed to confirm the observations reported for E.K. serum.

Gamete Collection and Microinjection

Immature oocytes from outbred ICR mice (Sprague-Dawley, Indianapolis, IN) were collected from minced ovaries and the cumulus cells were removed by pipetting. Fully grown oocytes were maintained in M-16 culture media with 100 μg/ml dibutyryl cyclic AMP (dbcAMP; Sigma Chemical Co.) to arrest spontaneous development (Wassarman et al., 1976). Meiotic maturation was initiated once the derivatized AMP was removed by rinsing in culture medium. Superovulation, in vivo fertilization, and collection of oviductal oocytes and embryos were performed as described by Schatten et al. (1985).

Micropipettes were front-loaded with serum from a small droplet under mineral oil juxtaposed to the culture medium containing the oocytes. Microinjection was performed by puncturing zona-intact oocytes with a 1-μm beveled micropipette (Sutter Instruments, Novato, CA), sucking in a small amount of cytoplasm, and expelling the serum and cytoplasm (Thadani, 1980; Uehara et al., 1977).

Cold Recovery, Cytoskeletal Drug Treatment, and Parthenogenetic Activation

5 μM nocodazole and 5 μM cytochalasin B were prepared in DMSO and diluted to the final concentration in culture medium. To investigate microtubule recovery in vivo after serum microinjection into metaphase-arrested unfertilized oocytes, injected eggs were incubated for two hours in ice-cold culture medium to depolymerize the microtubules; the oocytes were fixed at various intervals after recovery in warm medium (Schatten et al., 1988*b*). Anaphase was induced in metaphase-arrested unfertilized oocytes by activation with 7% ethanol in M-16 culture medium for 7 min (Kaufman, 1983).

Immunofluorescence Microscopy

The oocytes were permeabilized, fixed, and processed for the immunocytochemical detection of centromere/kinetochore complex as described previously (Schatten et al., 1988*a*).

To localize the centromere/kinetochore complex in oocytes microinjected with serum, the cells were labeled with biotin-avidin secondary antibodies after fixation. Biotinylated antihuman antibody (10 μg/ml in PBS; Vector Laboratories, Burlingame, CA) was applied for 40 min at 37°C and, after a brief rinse in PBS-Triton, the centromere/kinetochore complexes were visualized by the addition of fluorescein streptavidin (30 μg/ml in PBS; Jackson Immunoresearch Laboratories, West Grove, PA) applied under identical conditions. Uninjected control oocytes first were stained with a 1:50 dilution of the primary human serum for 40 min at 37°C before adding secondary antibodies. Application of biotinylated antihuman and fluorescein-streptavidin without microinjection or staining with nonimmune sera, sera from patients with diffuse scleroderma or immunodepleted CREST sera did not result in kinetochore detection. Microtubules were detected by application of a rabbit affinity-purified antitubulin antibody and a rhodamine-conjugated antirabbit secondary antibody as previously described (Schatten et al., 1985). The penultimate rinse in PBS-TX contained 5 μg/ml DAPI for fluorescence DNA localization. Epifluorescent microscopy and photography were performed on a Zeiss Axiophot equipped with appropriate filters for all three fluorochromes.

PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed as described by Balczon and Brinkley (1987). Immunoblots were performed using mouse 3T3 cells as the starting material.

Statistical Analysis

Statistical comparisons between the means of the controls and microinjected oocytes were performed by the *t* test. A minimum of three independent trials were run for each group examined and differences were considered statistically significant when $P < 0.01$.

Results

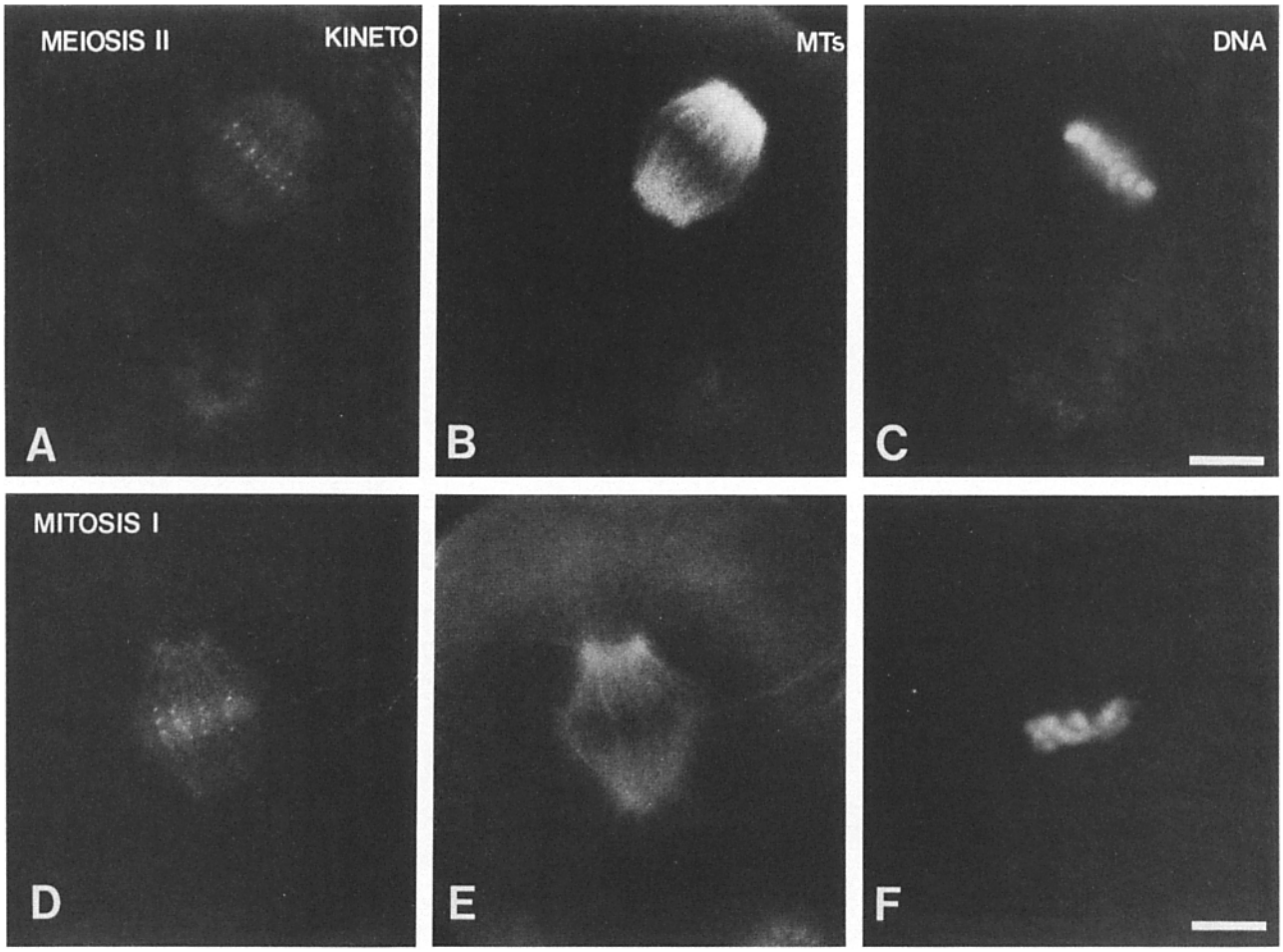
The results of microinjecting anti-kinetochore/centromere autoantibodies in ovulated unfertilized oocytes are presented first (Figs. 1–4), then the effects on fertilization and first division are demonstrated (Figs. 4–7) and finally the impact of antibody introduction on meiotic maturation in vitro is presented (Figs. 7–8).

Antikinetochore/Centromere Autoantibodies Recognize the Centromere/Kinetochore Complex In Vivo when Microinjected into Living Oocytes, and Cross-React with An 80-kD Protein

To determine if the centromere/kinetochore complex can be recognized within living cells, E.K. serum was microinjected into either unfertilized oocytes arrested at second meiotic metaphase (Fig. 1, *A–C*) or at first mitosis (Fig. 1, *D–F*). After labeling with only secondary human antibodies, the centromere/kinetochore complexes are detected exclusively with the microinjected antibodies as paired structures at the spindle equators (Fig. 1 *A*, second meiosis; Fig. 1 *D*, first mitosis). The centromere/kinetochore complexes are positioned between the spindle microtubules (Fig. 1, *B* and *E*) and the chromosomes are aligned at the metaphase plates (Fig. 1, *C* and *F*). This serum reacts with a single polypeptide of M_r 80,000 by Western blot analysis of mouse 3T3 cells (Fig. 1 *G*, lane *c*).

Microinjected Antikinetochore/Centromere Antibodies Do Not Prevent Anaphase Chromosome Separation in Unfertilized, Metaphase-arrested Oocytes

Anaphase, telophase and formation of the second polar body occur normally when metaphase-arrested unfertilized oocytes are microinjected with anti-kinetochore/centromere autoantibodies (Figs. 2, *A–C*, noninjected controls; Fig. 2, *D–F*; antibody microinjected). The completion of meiosis was initiated by activation with 7% ethanol. 30 min after ethanol treatment, the chromosomes have separated (Fig. 2 *C*, noninjected control; Fig. 2 *F*, antibody microinjected), interzonal microtubules persist (Fig. 2, *B* and *E*) and the centromere/kinetochore complexes are detected along the poleward faces of the chromosome masses (Fig. 2, *A* and *D*). Karyokinesis in microinjected oocytes at second meiosis occurs at nearly the same frequency as control oocytes (Fig. 2 *G*).



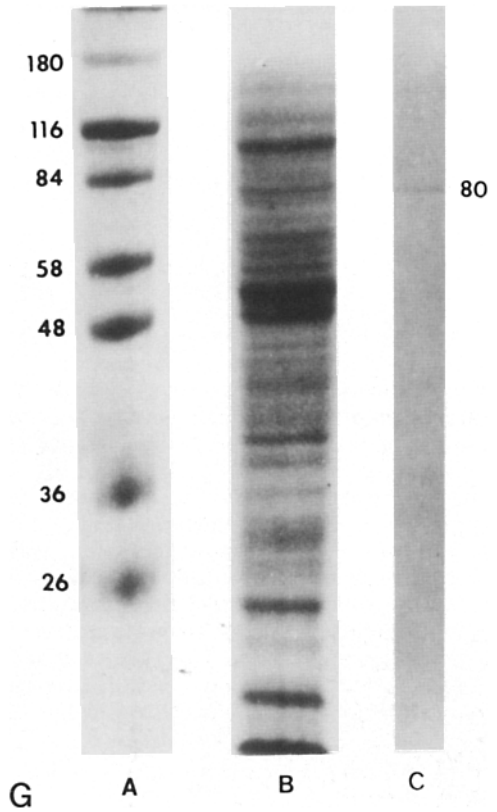
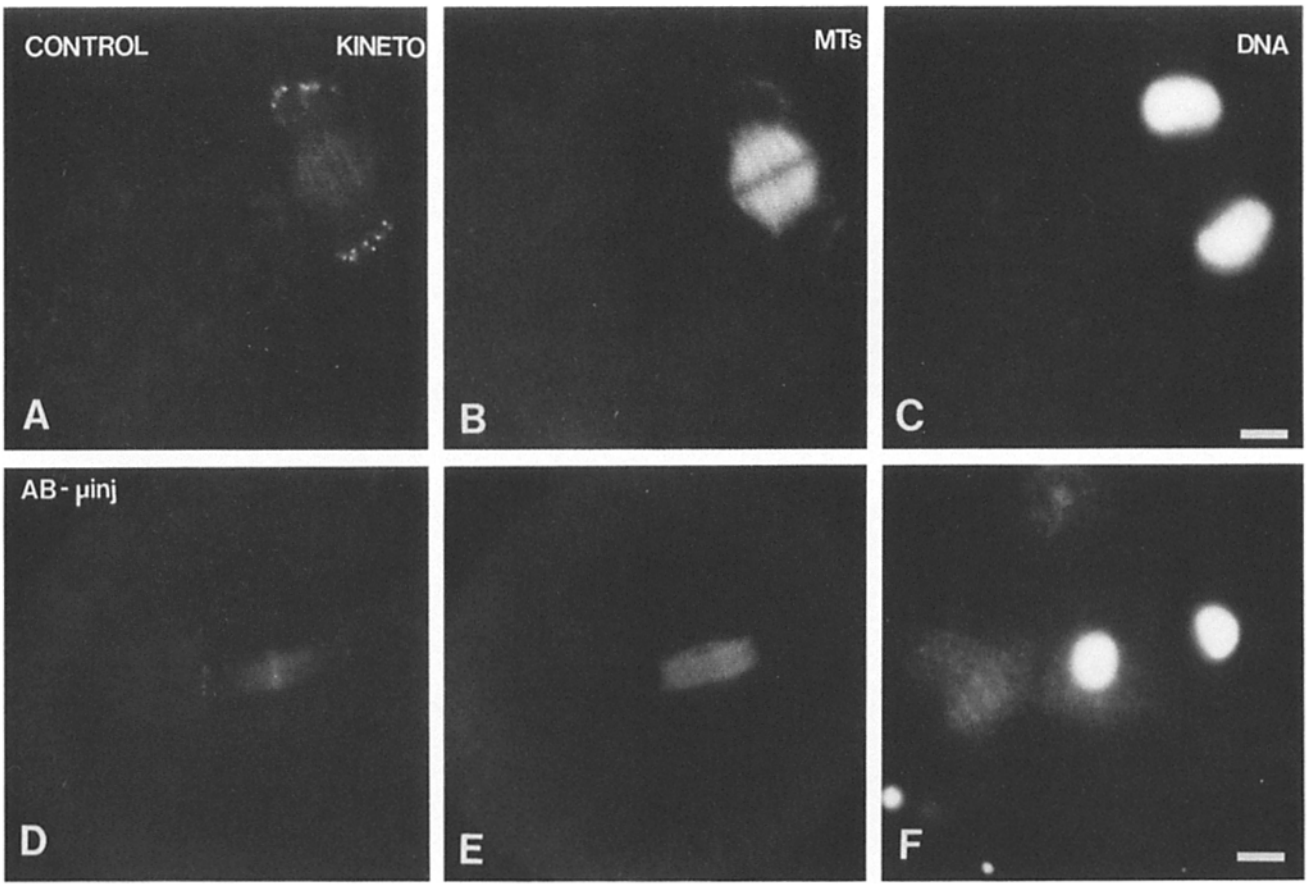


Figure 1. Serum E.K. detects the centromere/kinetochore complex when microinjected into mouse oocytes and crossreacts with an 80-kD protein in mouse cells. Microinjected E.K. serum binds to the centromere/kinetochore complex in oocytes naturally arrested at second meiotic metaphase (*A*) or at first mitotic metaphase (*D*); the cells are labeled with biotin–streptavidin secondary antibodies following fixation. The centromere/kinetochore complexes (*KINETO*: *A*, *D*) are detected as paired structures at the equator of the spindle detected with antitubulin immunofluorescence microscopy (*MTS*: *B*, *E*). DNA fluorescence (*DNA*: *C*, *F*). (*G*) E.K. serum (1:1,200) detects a polypeptide of 80 kD in mouse 3T3 cells. Lane *c*, immunoblot transfer. Lane *b*, Coomassie blue–stained 12.5% SDS-PAGE gel (125 μg of protein). Lane *a*, molecular weight standards in kilodaltons. Triple labeled images for kinetochores (*A* and *D*), microtubules (*B* and *E*), and DNA (*C* and *F*). Bar, 10 μm.



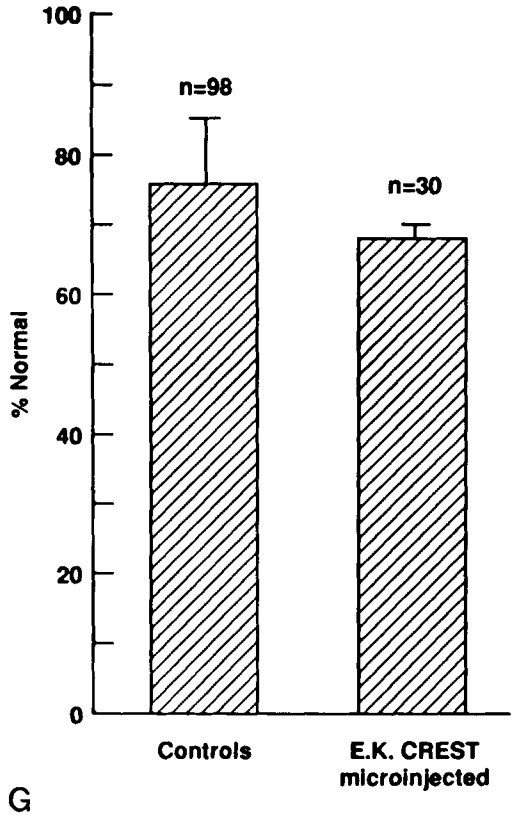


Figure 2. Microinjected antikinetochore/centromere autoantibodies do not prevent chromosome separation at anaphase in unfertilized, metaphase-arrested oocytes. Mature, ovulated oocytes arrested in second meiotic metaphase will complete meiosis II when artificially activated with 7% ethanol. (*A–C*) Noninjected control oocyte assayed 30 min after activation. The chromosome masses have segregated (*C*) and midbody microtubules persist (*B*). The centromere/kinetochore complexes are detected on the poleward faces of the reconstituting nuclei when stained with E.K. serum (*A*). (*D–F*) Microinjected oocyte. Chromosomal segregation (*F*) and midbody formation (*E*) occur normally in the presence of the antikinetochore/centromere antibody (*D*). (*G*) Karyokinesis at second meiotic metaphase occurs with a nearly identical frequency to that of controls cells when assayed 30 min after activation. Triple labeled for kinetochores (*A* and *D*), microtubules (*B* and *E*), and DNA (*C* and *F*). Bars, 10 μm.

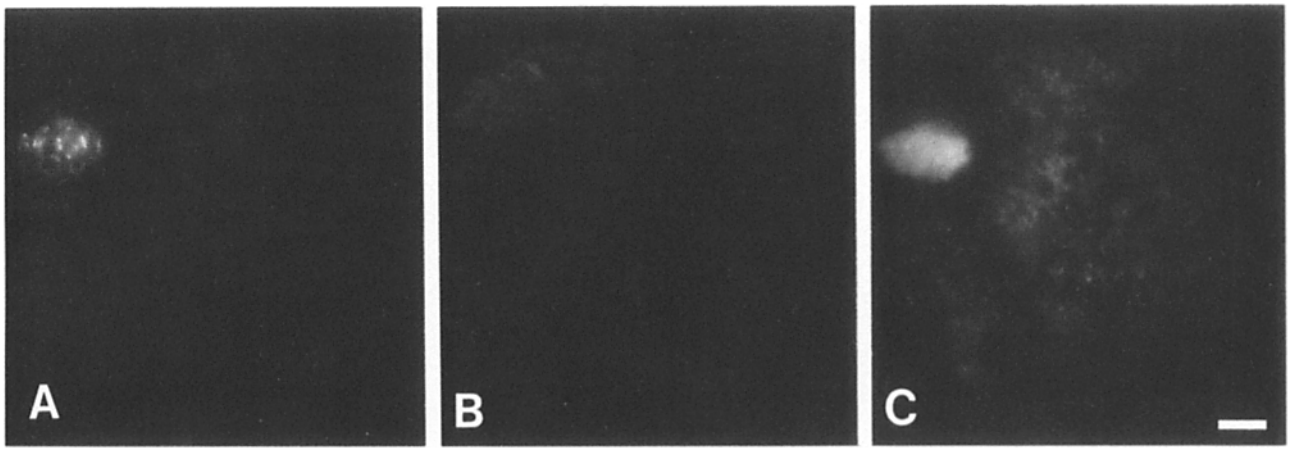


Figure 3. Cold disruption of metaphase II spindle microtubules. Oocytes arrested at second meiotic metaphase were microinjected with serum E.K. and placed in ice-cold culture medium for 2 h. When processed for indirect immunofluorescence without recovery, the kinetochores are slightly disordered (*A*) and microtubules are no longer detectable (*B*). Triple labeled for kinetochores (*A*), microtubules (*B*), and DNA (*C*). Bar, 10 μm.

Microtubule Capture and Spindle Formation Occurs Normally in Microinjected Oocytes

To determine if microinjected antibodies interfere with microtubule capture and/or spindle formation in vivo, microinjected metaphase-arrested oocytes (Fig. 3 *C*) were chilled on ice for 2 h to disassemble microtubules. Antitubulin immunofluorescence microscopy demonstrates the disruption of the spindle and cytastral microtubules (Fig. 3 *B*); the centromere/kinetochore complexes remain detectable after cold treatment (Fig. 3 *A*). Interestingly the meiotic chromosomes in cold-treated oocytes do not scatter along the cortex as is observed with microtubule-disrupting drugs (Maro et al., 1986; Schatten et al., 1986).

Upon warming, spindle microtubules reassemble and microtubule bundles attach to the kinetochores within the first 30 s of recovery in microinjected oocytes (Fig. 4 *A*). After an hour at 37°C, a normal barrel-shaped metaphase II spindle with aligned chromosomes is found in microinjected oocytes (Fig. 4 *B*). Activation of cold-recovered microinjected oocytes results in normal chromosome segregation and formation of the second polar body (Fig. 4 *C*).

Karyokinesis, But Not Cytokinesis, Is Prevented at First Mitosis

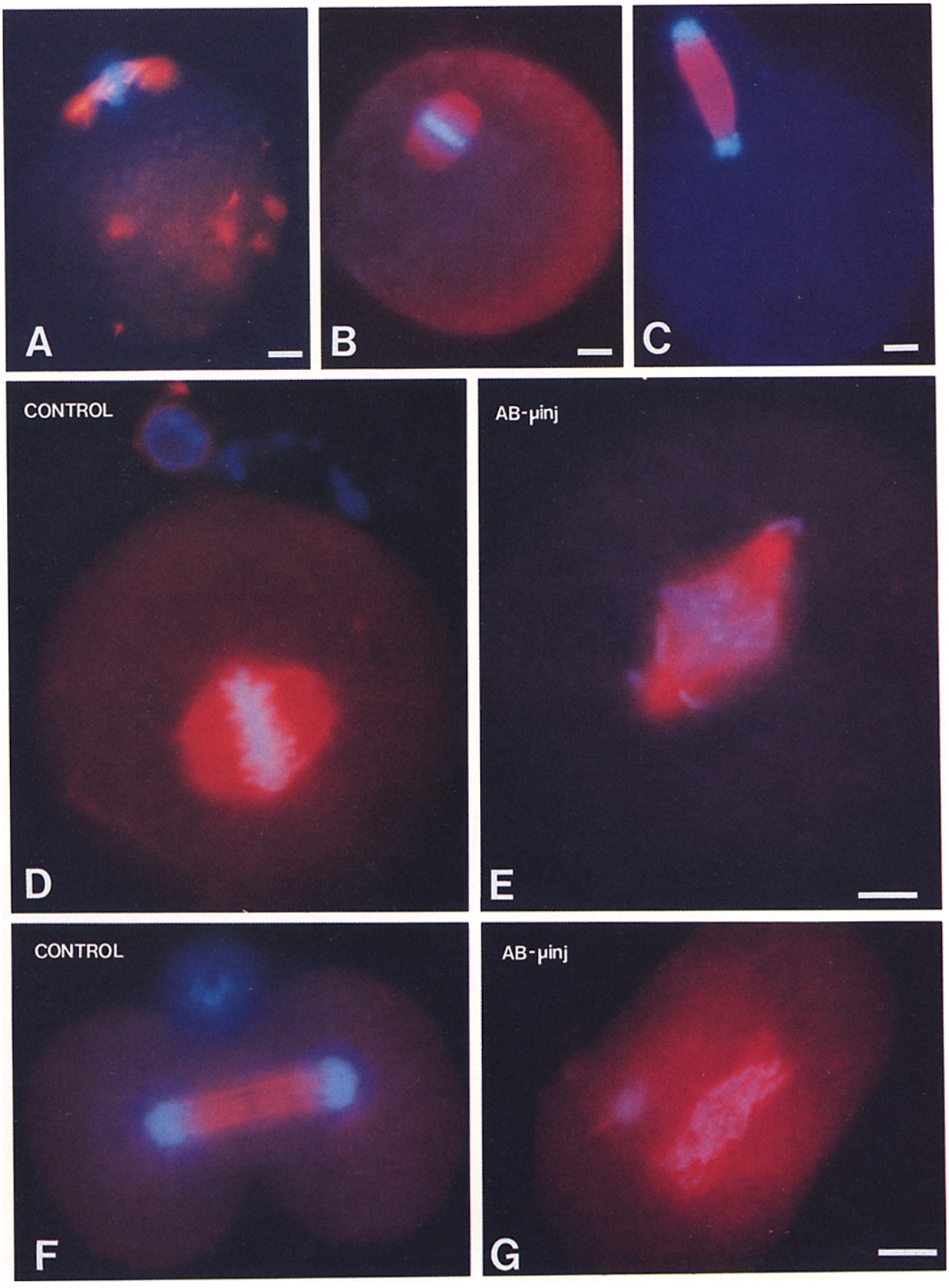
Antibody introduction at fertilization does not interfere with motility or pronuclear rearrangements. The first effect noted is at prometaphase of first mitosis. Microinjection into either the cytoplasm or pronuclei during first interphase prevents the normal alignment of the chromosomes on the mitotic metaphase plate (Fig. 4 *E*, microinjected vs. *D*, control). Whereas the majority of the chromosomes congress to the equator, the alignment at the plate is often disorganized and loosely arranged; some chromosomes remain near the spindle poles (Fig. 4 *E*). When injected before metaphase, anaphase chromosome separation is abnormal (Fig. 4 *G*), though this might well be an effect of separating the poorly aligned chromosomes. Anaphase occurs normally in sham microinjected cells (Fig. 4 *F*). First cleavage is normal in CREST-injected eggs, but karyomeres are found in both blastomeres separate from the major nuclear masses (Fig. 5 *A*). Fig. 5 *B* demonstrates that the effect of the antibody is on karyokinesis and not cytokinesis.

Antikinetochore/Centromere Autoantibodies Inhibit Congression when Injected Into Nocodazole-Arrested Mitotic Eggs

To determine if the observed effect on prometaphase alignment might be due to an impact on kinetochore maturation at the conclusion of interphase, rather than on prometaphase chromosome alignment, oocytes were arrested at first mitosis with nocodazole. Brenner et al. (1981) have shown that trilaminar kinetochores capable of interacting with spindle microtubules form on condensed chromosomes in cells arrested with microtubule inhibitors. This then permits an analysis of the effect of antikinetochore/centromere antibodies on structurally mature kinetochores as opposed to prekinetochores in interphase cells.

Pronucleate oocytes arrest in prometaphase in the presence of 5 μM nocodazole. Uninjected oocytes complete fertilization after drug removal by aligning chromosomes (Fig. 6 *A*) on the metaphase plate of the first mitotic spindle. Spindle elongation and chromosome separation (Fig. 6 *C*) occur normally at anaphase and the chromosomes are able to reach the mitotic poles (Fig. 6 *E*) in a synchronous fashion by telophase. Microinjection of the serum at prometaphase-arrest blocks normal chromosome congression (Fig. 6 *B*) and segregation (Fig. 6 *D*) after recovery, resulting in lagging chromosomes in the interzonal microtubules of the cleaving embryo (Fig. 6 *F*) as observed for microinjected interphase oocytes (see also Fig. 4, *E* and *G*).

The chromosome imbalance found at the two-cell stage is most likely a result of a compromised prometaphase stage caused by the anti-kinetochore/centromere sera. To test this theory, E.K. serum was microinjected into mitotic metaphase eggs. Because synchrony is not precise in the mouse, an enrichment for metaphase eggs was obtained after a 1-h recovery from nocodazole-arrested prometaphase stage. Eggs microinjected at the time calculated for metaphase (Fig. 7,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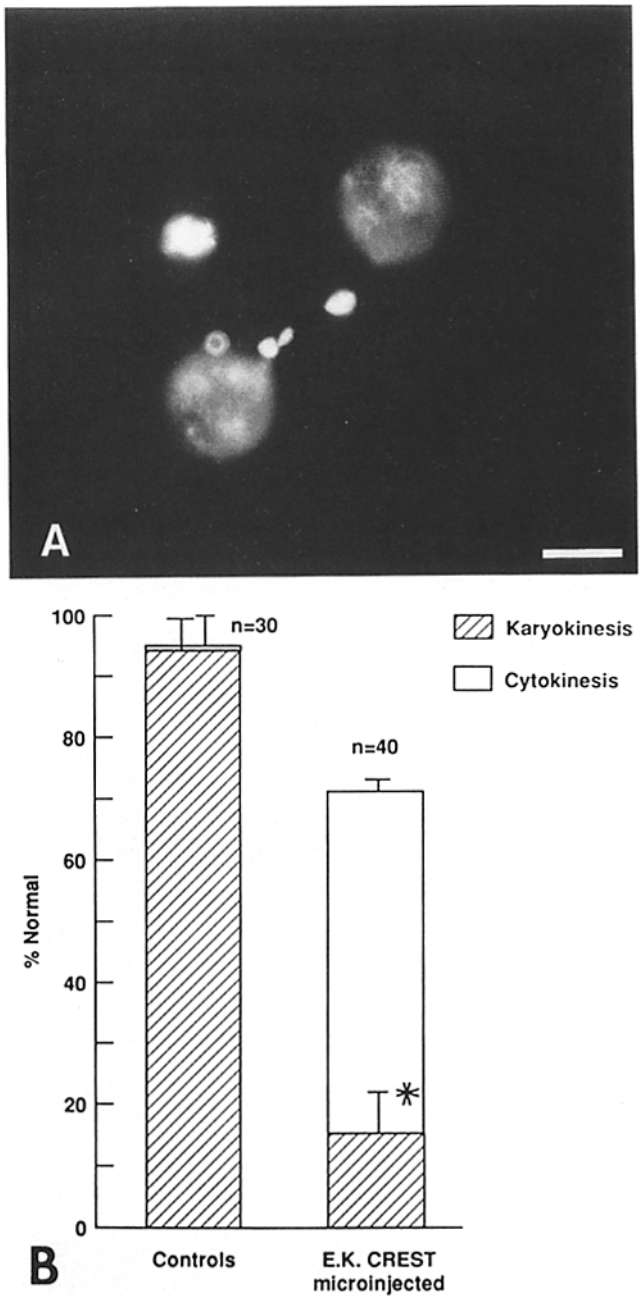


Figure 5. Microinjected serum interferes with karyokinesis, but not cytokinesis, at first division. Pronucleate oocytes were microinjected and assayed after first cleavage. (*A*) DNA fluorescence demonstrating that several karyomeres decondense separate from the major nuclear masses in the sibling blastomeres after first cleavage. Bar, 10 μm. (*B*) Cytokinesis is not significantly affected in the presence of microinjected antikinetochore/centromere serum (*B, open bars*), whereas chromosome segregation is significantly impaired (*stippled bars*). *Significant difference with uninjected controls ($P < 0.01$).

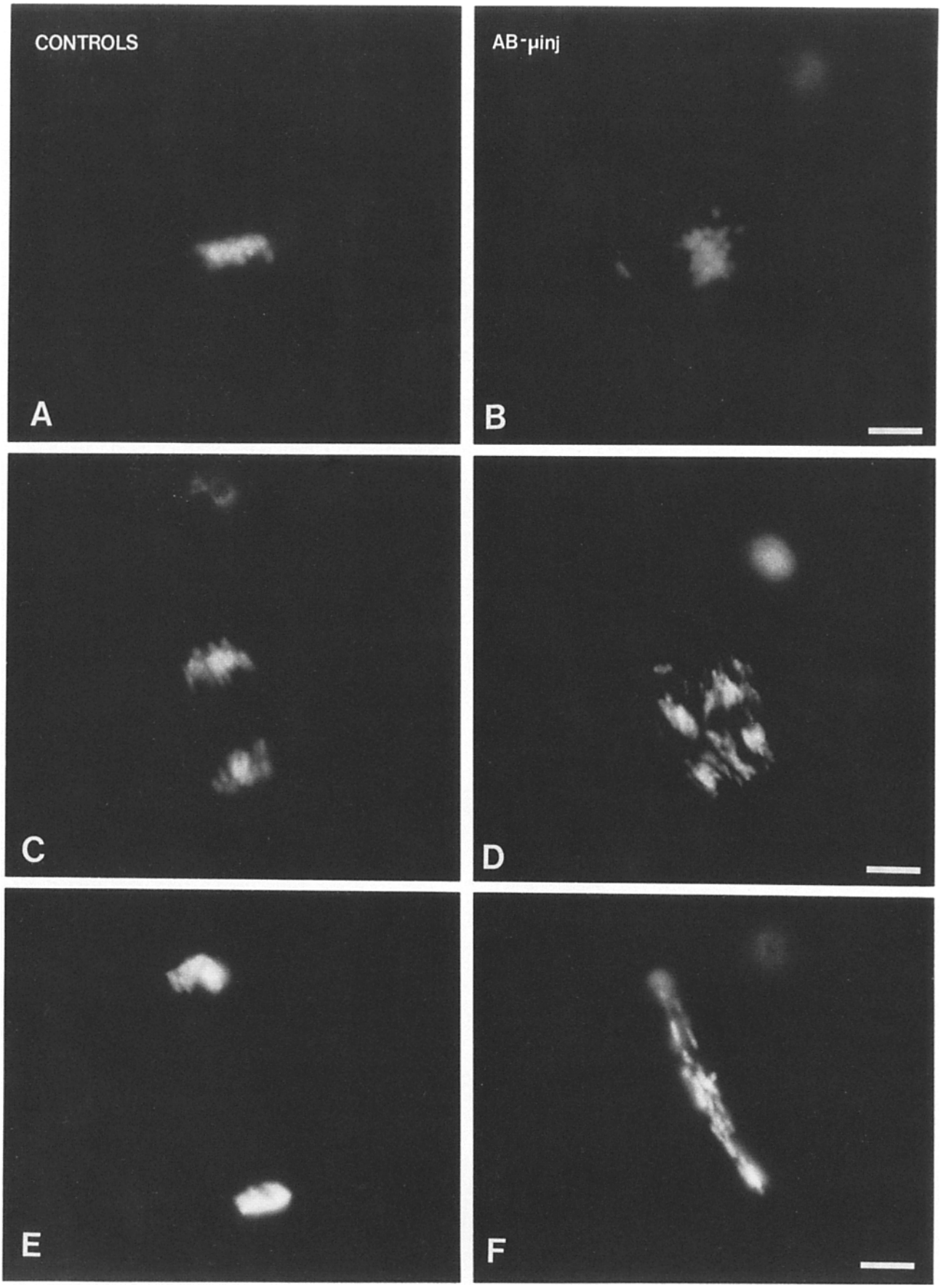
C and *D*) were able to segregate chromosomes at anaphase similar to control cells (Fig. 7, *A* and *B*), supporting the results found in unfertilized oocytes arrested at metaphase of second meiosis. Fig. 7 *E* demonstrates that the chromosomes ultimately decondense into single nuclear masses in each blastomere; karyomeres are not observed when eggs at first mitotic metaphase are microinjected with antikinetochore/centromere autoantibody. Similar results are observed when oocytes collected at first meiotic metaphase are injected and assayed after the formation of the first polar body; the anaphase segregation of the chromosomes is not arrested and the chromatin is located exclusively in the two daughter nuclei (Fig. 7, *F* and *G*).

Chromosomal Congression, But Not First Polar Body Formation, Is Prevented during Meiotic Maturation In Vitro in the Presence of Antikinetochore/Centromere Antibodies

Immature oocytes will mature spontaneously in culture and arrest at second meiotic metaphase; dbcAMP and cytoskeletal inhibitors arrest developing immature oocytes at specific stages of nuclear development (Wassarman et al., 1976), permitting an analysis of the impact of anti-kinetochore/centromere serum on the events leading to metaphase alignment of the chromosomes (Fig. 8 *A*).

When oocytes are microinjected with E.K. serum into either the germinal vesicle or the cytoplasm and allowed to mature spontaneously, the chromosomes do not align properly on the metaphase II plate; numerous chromosomes are found near the spindle poles and throughout the meiotic apparatus (Fig. 8 *C*; *B* is control). Similar observations were found when nocodazole-arrested prometaphase I oocytes were microinjected and allowed to mature in vitro, reinforcing the finding that neither kinetochore maturation nor microtubule capture is affected by the presence of the antikinetochore/centromere serum (Fig. 8 *D*). While prometaphase congression of the chromosomes is retarded by the antikinetochore/centromere serum (Fig. 9 *A*), first polar body formation was not prevented (Fig. 9 *B*).

Figure 4. Effects of microinjected anti-kinetochore/centromere antibody on meiosis II (*A–C*) or first mitosis (*D–G*). (*A–C*) Kinetochore microtubule bundles (*A*), normal spindle reformation (*B*), and correct chromosomal segregation occur in microinjected oocytes recovering from cold-induced microtubule disruption (see Fig. 3). (*A*) 30 s after warming, cytoplasmic and spindle microtubules reform in oocytes previously microinjected with antikinetochore/centromere antibodies; microtubule bundles terminate at kinetochores (detected in a third wavelength not shown in double exposure). (*B*) At an hour after recovery, a normal barrel-shaped spindle is observed in an oocyte previously microinjected with antikinetochore/centromere autoantibody. (*C*) Fully recovered spindles complete meiosis correctly after activation in the presence of the kinetochore antibody. (*D–G*) Microinjection into interphase oocytes impairs chromosome alignment at first mitotic metaphase as well as segregation at first mitotic anaphase. (*D*) First mitotic metaphase control. (*E*) Kinetochore antibodies prevent the proper prometaphase alignment of the chromosomes when injected into the cytoplasm (in *E*) or pronuclei (not shown) of zygotes at first interphase. Nonaligned chromosomes are found at the poles and throughout the spindle. (*F*) Sham microinjected control after first mitosis. (*G*) If the serum is introduced before metaphase, chromosome separation at anaphase is irregular and chromosomes are scattered throughout the spindle. Double labeled for DNA (*blue*) and microtubules (*red*). Bars, 10 μm.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Chromosomal congression after microinjection of E.K. serum can be specifically investigated using cytochalasin B to arrest spontaneous development at metaphase I (Fig. 8 *E*); this allows for the events at prometaphase to be investigated separate from anaphase. Primary oocytes microinjected with anti-kinetochore/centromere serum and cultured in cytochalasin B do not properly align their chromosomes on the first metaphase equator (Fig. 8 *F*). Likewise, when nocodazole-arrested prometaphase I oocytes are microinjected with CREST serum and allowed to mature in vitro in the presence of cytochalasin B, normal chromosome congression at metaphase I is prevented; numerous chromosomes are scattered throughout the spindle microtubules (Fig. 8 *G*).

Nonimmune Human Sera, S.H. CREST Antisera, Diffuse Scleroderma Serum, and Sham Microinjection Controls

Neither sham microinjection, accomplished by puncturing the vitelline membrane of mouse oocytes and then withdrawing the micropipette, nor microinjection of human sera from patients who do not exhibit the CREST syndrome (diffuse scleroderma) affected chromosomal congression or segregation when examined at first mitosis and cleavage (data not shown). Human nonimmune serum microinjected into the cytoplasm of primary oocytes that were allowed to mature spontaneously did not prevent either chromosome alignment at the equator of the second metaphase spindle (Fig. 9 *A*) or first polar body formation (Fig. 9 *B*). However, microinjection of S.H. CREST serum did block normal chromosomal congression and segregation in meiotic and mitotic oocytes in an identical manner as described for E.K. serum, supporting the observation that microinjected CREST antikinetochore/centromere autoantibodies prevent proper prometaphase chromosome alignment but do not block anaphase A chromosome separation (data not shown).

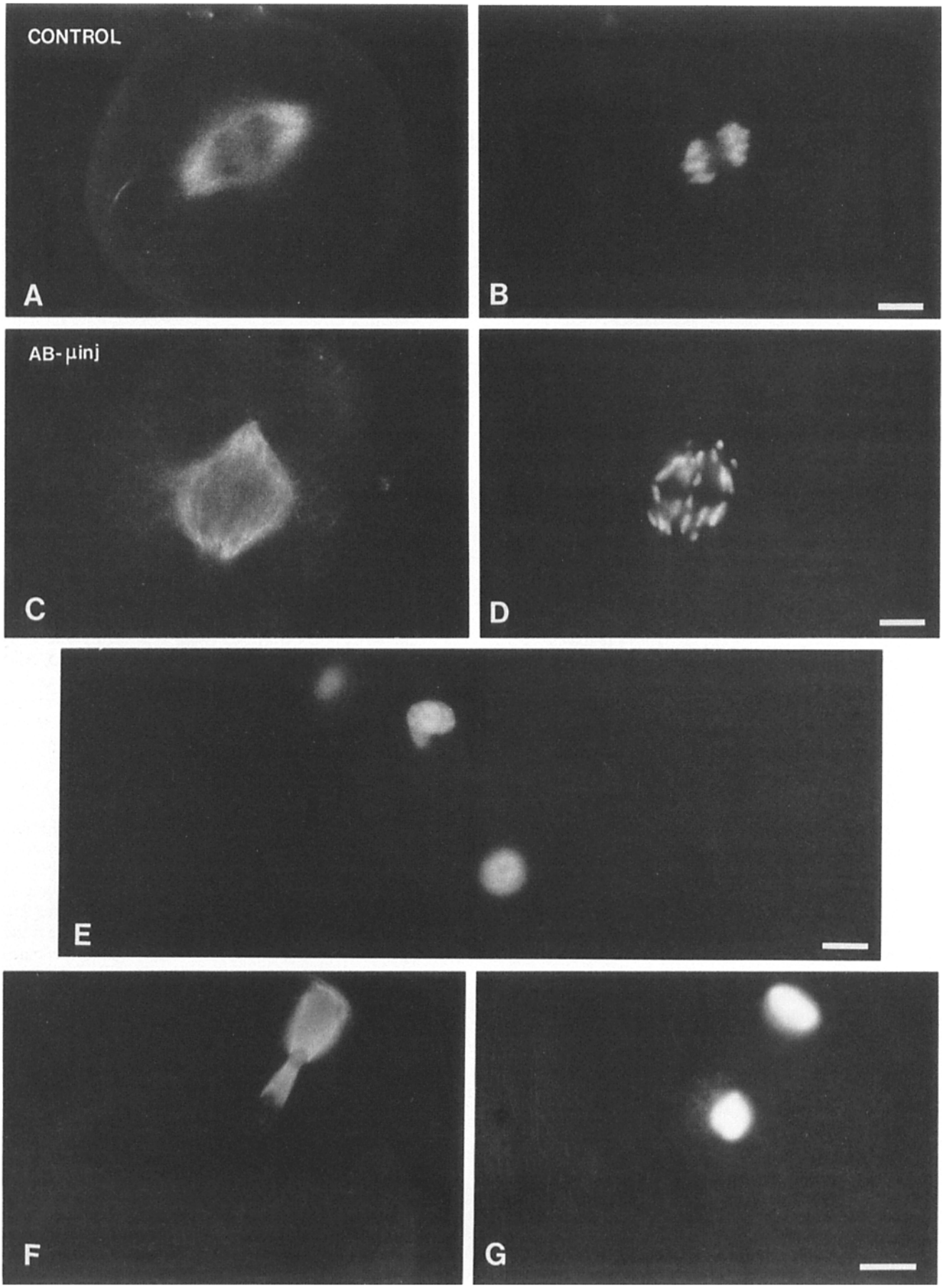
Discussion

The term "motile kinetochore" has been coined recently by Nicklas (1989) to highlight its possible roles during chromosome separation and alignment. Since antikinetochore/centromere autoantibodies have been so essential in the molecular characterization of this organelle (Earnshaw and Rothfield, 1985; Earnshaw et al., 1987), this investigation was undertaken to explore the cellular activities of the CREST antigens in vivo. The mouse oocyte provides a unique combination of natural and drug-induced arrest sites for studying cell division events: it is ovulated blocked at metaphase II, permitting an analysis of anaphase distinct from prometaphase events, and immature oocytes can be halted at specific stages during meiosis I (Wassarman et al., 1976). Microinjection of an antibody binding to an 80-kD protein found at the centromere/kinetochore complex, perhaps CENP-B, always interferes with the prometaphase alignment of the chromosomes at first and second meiosis and at first mitosis; neither microtubule attachment nor the anaphase chromosome separation is grossly affected in this system.

Autoimmune sera have been used in these experiments and questions might be posed about reagent specificity. The sera used here (E.K., S.H.) detect centromere/kinetochore complexes in a variety of mammalian, invertebrate, and plant cells (Janicke, M. A., R. D. Balczon, B. R. Brinkley, and J. R. La Fountain, Jr. 1989. *J. Cell Biol.* 107:456a; Mole-Bajer et al., 1990; Schatten, H., et al., unpublished observation) and E.K. serum recognizes a single 80-kD antigen in mouse cells by immunoblotting (Fig. 1 *C*) and localizes specifically to the centromere/kinetochore complex in either microinjected (Fig. 2, *A* and *D*) or permeabilized mouse oocytes. Notwithstanding these observations, we cannot exclude the possibility that the sera contains undetectable titers of other kinetochore antibodies or different antibodies that influence prometaphase in vivo. CREST sera are rarely monospecific for a single centromere protein (Earnshaw et al., 1986, 1989) and so it might be premature to conclude that only the recognized 80-kD antigen is specifically targeted in vivo by E.K. autoantibody. Additionally, since processing protocols could remove weakly bound spindle antigens, it is possible other autoantibodies are also present. However, Tan (1989) notes that one of the distinguishing characteristics of the CREST variety of scleroderma is the homogeneity of autoantibody in the serum; up to 85% of the patients contain centromere antigens exclusively. Microinjection of non-CREST sera (diffuse scleroderma and nonimmune human serum which do not recognize kinetochores) does not influence chromosome congression or segregation at meiosis or mitosis. Serum S.H., another high titer probe which localizes the centromere/kinetochore complex as well as microtubule caps on *Tetrahymena* cilia (Miller et al., 1990), produces identical results.

Similar microinjection experiments have been performed on dividing cultured cells (LLCPK) in the laboratories of Drs. W. Earnshaw and G. Borisy (Bernat et al., 1990), and that collaboration demonstrates that chromosome movements are compromised when autoimmune IgGs are microinjected. It is of interest that their monoclonal antibodies produced against CENP-B (Bernat et al., 1990) do not arrest chromosome movement. Perhaps those monoclonal reagents, which are directed against single epitopes, are unable to impair kinetochore function in vivo. This is consistent with the ideas developed by Tan (1989) that an autoimmune reaction is in response to subcellular complexes and not individual

Figure 6. Antikinetochore/centromere antibodies microinjected into zygotes at first mitosis interfere with prometaphase congression. To explore the possibility that the antibodies were affecting kinetochore maturation at the interphase: mitotic transition versus congression, E.K. was microinjected into mitotic oocytes arrested at prometaphase with 5 μM nocodazole. (*A*, *C*, and *E*) Uninjected controls recovering from nocodazole block for an hour. (*B*, *D*, and *F*) Recovery in the presence of anti-kinetochore/centromere antibodies. While control cells can undergo proper chromosome alignment at metaphase (*A*), microinjected oocytes are unable to complete congression (*B*). At anaphase controls segregate their chromosomes in an orderly fashion (*C*), whereas microinjected cells do not (*D*). After first mitosis, controls display two well-separated nuclei (*E*) while the injected cells have many chromosomes which remain trapped in the interzonal area of the mitotic spindle (*F*). Double labeled for DNA (shown) and microtubules (not shown). Bars, 10 μm.


CONTROL
A
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proteins; introduction of serum might then be expected to recognize a functional native moiety.

The functions of kinetochore proteins in vivo are not understood. CENP-B is a major antigen of the centromere/kinetochore complex in isolated chromosome scaffolds (Earnshaw et al., 1989) and may represent a microtubule binding protein (Balczon and Brinkley, 1987). Analysis of the amino acid sequence of a cDNA encoding 95% of CENP-B reveals the presence of two highly acidic regions on this protein which may function to bind histones and destabilize higher order chromatin (Earnshaw et al., 1987) and it has been reported to interact with alphoid DNA (Masumoto et al., 1989). In addition the sequence of CENP-B has homologies with other microtubule-associated proteins (Paschal et al., 1989) and has been speculated to be the molecule responsible for attaching the chromosome to microtubules (Brinkley, 1990). It is possible, but not certain that the 80-kD antigen recognized in this study on murine material is analogous to CENP-B identified in human and other cell types (Earnshaw and Rothfield, 1985).

The observations that the prometaphase alignment of the chromosomes are impaired by antibody introduction are consistent with conclusions drawn from analyses of kinetochore activity in vitro (Mitchison and Kirschner, 1985*b*). Perhaps the antibody binds directly to a molecule essential for pushing the kinetochore toward the growing or plus end of the microtubule. Alternatively the effect of the antibody might well be indirect and its binding to the centromere/kinetochore complex prevents congression.

The attachment of microtubule bundles to the kinetochore appears normal in the presence of kinetochore serum. The appearance of the meiotic and mitotic spindles are typical and on schedule in microinjected oocytes, and spindle reformation is seemingly unaffected after recovery from microtubule-disrupting drugs and cold treatment. During the initial phase of recovery microtubule bundles can be observed attached to the kinetochores, and microtubule bundles terminate at the kinetochores even when the chromosomes are unable to align at the metaphase plate. In vitro studies with isolated chromosomes demonstrate tubulin subunit exchange occurs at kinetochores and microtubule dynamic instability could provide the mechanism responsible for maintaining metaphase alignment (reviewed by Mitchison and Kirschner, 1984; Rieder et al., 1986; Mitchison, 1988). Microtubules in the meiotic spindle in the metaphase-arrested unfertilized mouse oocyte also turn over rapidly (Gorbsky et al., 1990).

Recent evidence suggests that the kinetochore may possess motor activity at anaphase during the translocating of the chromosome toward the centrosome (Mitchison, 1987; Gorbsky et al., 1988; Nicklas, 1989; Salmon, 1989). The antibody used in this study does not appear to affect anaphase during first or second meiosis or first mitosis unless prometaphase has been compromised first. Therefore it appears that the motions at prometaphase are more sensitive to the microinjected antibody than those at anaphase: perhaps there are greater demands on proper kinetochore functions at prometaphase when the spindle microtubules are being captured and when the chromosomes are aligning at the spindle equator. Misalignment of the metaphase chromosomes due to an aberrant prometaphase might well be expected to result in an imperfect chromosomal segregation at anaphase (i.e., Figs. 4 *G* and 6 *F*). Perhaps the recognized 80-kD antigen is not involved in this motion, perhaps there is sufficient redundancy so that partial interference does not impact on separation, or perhaps the density of structures in and around the anaphase kinetochore prevents antibody accessibility. The differences in prometaphase versus anaphase could also be due to differences in the modes of microtubule–kinetochore interactions, studied recently by Rieder and Alexander (1990).

Since kinetochores change shape during interphase to mitosis/meiosis transition (Rieder, 1982; Brinkley et al., 1985), perhaps these results could be interpreted as affecting kinetochore maturation rather than kinetochore activity. Kinetochore maturation into the trilaminar plate occurs normally in the presence of microtubule inhibitors (Brenner et al., 1981), and this finding has been used here to separate the antibody effects on maturation from effects on congression. When nocodazole-arrested oocytes, expected to have mature kinetochores, or synchronized oocytes after nuclear breakdown are microinjected with antikinetochore/centromere autoantibodies congression is impaired. This has been observed during first (Fig. 8 *G*) and second (Fig. 8 *D*) meiotic prometaphase and during first mitotic prometaphase (Fig. 6, *B–F*). In addition, microinjection into oocytes expected to have mature kinetochores, such as those at first meiotic telophase, results in an impaired congression at second meiotic prometaphase (data not shown). While these results imply that the observed effects are not due to kinetochore maturation, but rather prometaphase congression, the possibility remains that maturation occurs atypically during oogenesis or that it might have even begun before germinal vesicle breakdown. Also the site of microinjection during interphase does not appear to affect the experimental outcome: introduction into either the cytoplasm or the germinal vesicle or pronuclei impair prometaphase alignment.

In summary, an autoantibody to an 80 kD centromere/kinetochore antigen interferes with the prometaphase chromosome alignment in vivo but not anaphase separation or microtubule capture. This observation provides a crucial link between the molecular characterization of the centromere/kinetochore complex, their associated proteins, and kinetochore function during mitosis and meiosis.

Figure 7. Antikinetochore/centromere antibodies do not prevent the anaphase separation of the chromosomes at first meiosis or mitosis when introduced at metaphase. (*A* and *B*) Uninjected controls undergoing first mitotic anaphase after recovery from nocodazole arrest. (*C–E*) Zygotes microinjected at first metaphase, successfully separate their chromosomes at anaphase (*C* and *D*) and produce two sibling blastomere nuclei at first cleavage (*E*). (*F* and *G*) Karyokinesis at first meiosis is also normal if antibody is introduced at metaphase I. Chromosomes at first meiotic metaphase segregate normally to the first polar body and the oocyte, and no karyomeres are observed (*G*, DNA fluorescence, *F*, midbody microtubules). Double labeled for microtubules (*A*, *C*, and *F*) and DNA (*B*, *D*, and *G*). (*E*) DNA fluorescence. Bars, 10 μm.

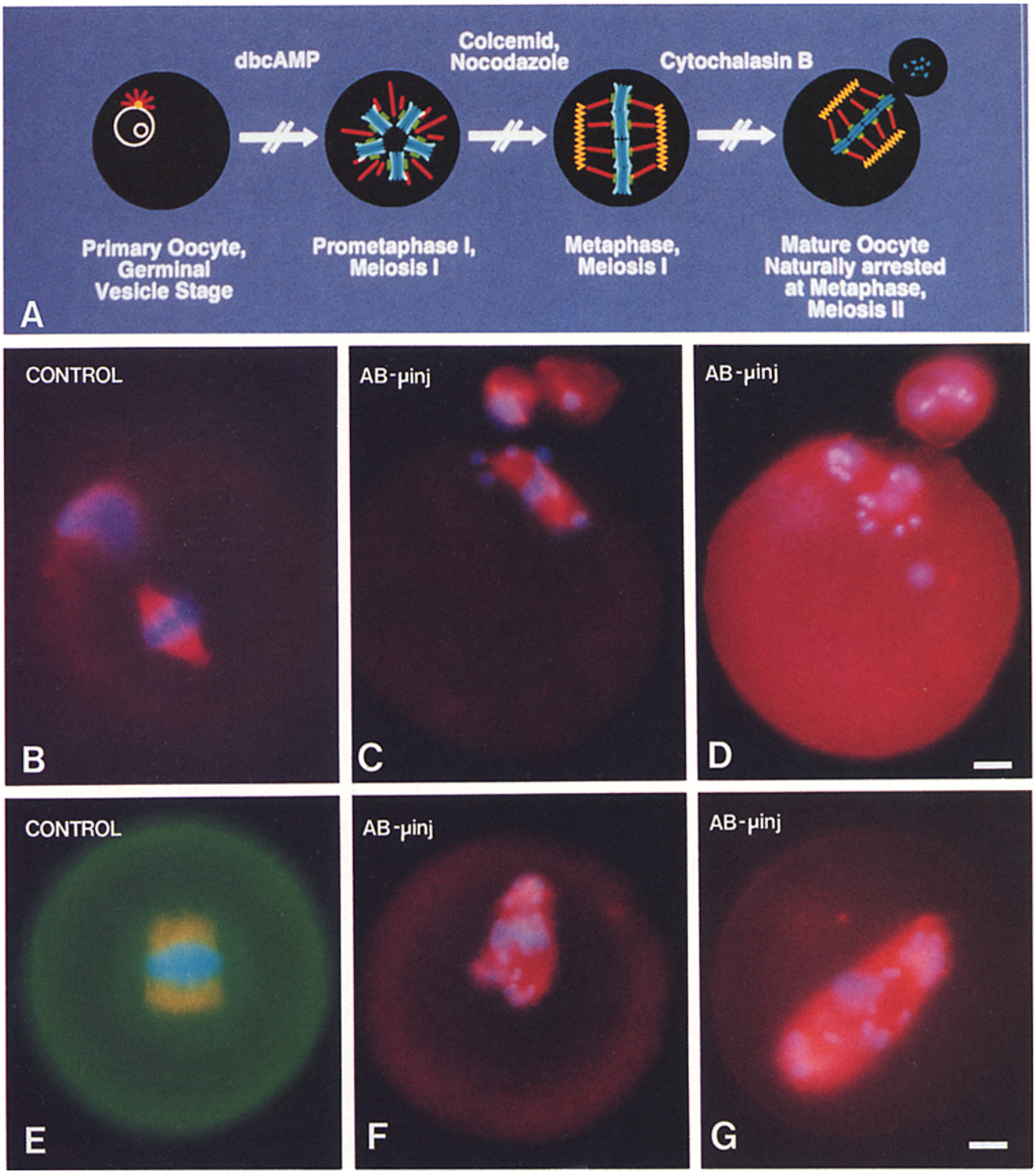

Figure 8. Microinjected anti-kinetochore/centromere antibodies impair congression during meiotic maturation of primary oocytes matured in vitro. (*A*) Germinal vesicle-stage oocytes mature spontaneous in the absence of dbcAMP and arrest naturally at metaphase II. Microtubule inhibitors permit germinal vesicle breakdown (*GVBD*) but reversibly arrest development at prometaphase I. Cytochalasin B (*CB*) allows primary oocytes to undergo GVBD but, curiously, the oocytes arrest terminally at metaphase I (according to Wassarman et al., 1976; DNA: *blue*; kinetochores: *green*; microtubules: *red*). (*B*) Control in vitro matured oocyte arrested spontaneously at metaphase II. (*C*) Chromosomes are misaligned during maturation in vitro with kinetochore antibodies introduced at the germinal vesicle stage. First polar body formation is unaffected. (*D*) Congression at prometaphase I is impaired when microinjected nocodazole-arrested oocytes are permitted to recover to metaphase II. (*E–G*) Effects on first meiotic congression. (*E*) Primary oocytes cultured for 12 h in 5 μM CB arrest at first meiotic metaphase; bivalents align properly on the first metaphase spindle. (*F*) Antibody introduction permits an examination of congression separate from anaphase. The microinjected serum prevents the normal alignment of chromosomes during prometaphase I. (*G*) Microinjected nocodazole-arrested prometaphase I oocytes cultured to metaphase I in the presence of CB cannot undergo normal congression. Triple-labeled for microtubules (*red*), DNA (*blue*), and kinetochores (not shown except in *E* as *green*).

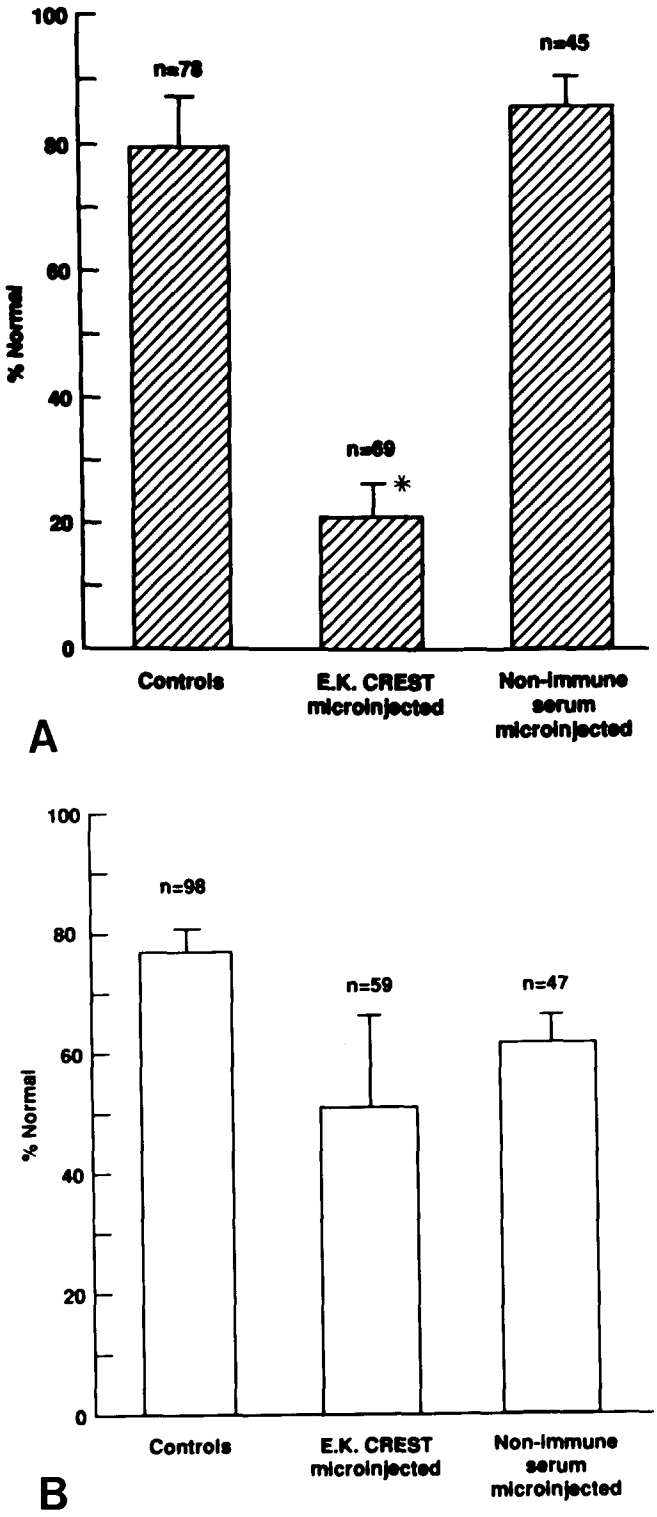

Figure 9. Karyokinetic (*top*), but not cytokinetic (*bottom*) events are affected by microinjected anti-kinetochore/centromere antibodies during meiotic maturation. (*A*) Microinjection of E.K. serum (middle bar), but not nonimmune serum (*right bar*), prevents the proper alignment of metaphase II chromosomes at the spindle equator. (*B*) Microinjection of E.K. serum (*middle bar*) or a nonimmune sera (*right bar*) into primary oocytes does not prevent the formation of the first polar body. *Significant difference with uninjected control ($P < 0.01$).

This paper is dedicated to the memory of our friend and colleague Professor Walter S. Plaut.

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