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Expression and behaviour of CENP-E at kinetochores during mouse spermatogenesis

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Abstract Centromere protein E (CENP-E) is a microtubule motor protein localised in the outer kinetochore plate and in the fibrous corona that relocalises to the midzone in early anaphase. While its expression in somatic cells has been widely analysed, an accurate description of its behaviour during the two meiotic divisions has not yet been reported. We have carefully analysed by immunofluorescence the subcellular distribution of CENP-E during mouse spermatogenesis. CENP-E first appears during late diakinesis/early prometaphase I as very bright C-shaped or "crescent" signals at each homologous centromere. These crescent CENP-E signals are also observed in unaligned prometaphase I bivalents that are not attached to spindle microtubules, while in bioriented metaphase I bivalents two kinds of fainter signals are observed. Thus, some bivalents present a plate-like signal while others show a pair of spots representing sister kinetochores at each homologous centromere. Double labelling of CENP-E with CENP-G and an anticentromere serum indicates that in meiosis CENP-E is also located at the outer kinetochore plate and the fibrous corona. During early anaphase I CENP-E relocalises from kinetochores to the midzone where it is detected as fibrous strands, although some residual labelling persists at kinetochores until telophase I. During this stage CENP-E is detected as two parallel plates at the intercellular bridge. The general pattern of labelling during meiosis II is similar to that found during meiosis I. Our results suggest that CENP-E is implicated in the spindle checkpoint, and in chromosome alignment, during the two meiotic divisions in vertebrate males. We also demonstrate that the centromere changes its structure once alignment of all bivalents at the metaphase I plate has been reached.

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Introduction

The kinetochore is a proteinaceous structure located on the external surface of the centromere that interacts with the spindle microtubules (Rieder and Salmon 1998; Craig et al. 1999). Electron microscopy reveals that the vertebrate kinetochore is composed of three plates: an inner dense plate closely associated with the centromeric chromatin, a middle electron-lucent plate, and an outer dense plate (Rieder 1982). Additionally, in prometaphase chromosomes a fibrous corona is observed associated with the outer plate. The mitotic spindle checkpoint proteins Mad1, Mad2, Bub1, Bub3, and BubR1, which are involved in the regulation of the metaphase/anaphase transition, are located in the outer dense plate and fibrous corona (for review see Skibbens and Hieter 1998). Interestingly, the microtubule motor proteins cytoplasmic dynein, and centromeric protein E (CENP-E), which are involved in chromosome alignment during prometaphase and chromatid segregation at anaphase, are also localised in these kinetochore subdomains (for review see Banks and Heald 2001).

CENP-E is an M_r 312,000 kinesin-related microtubule motor protein (Yen et al. 1991, 1992) that has been ultrastructurally located in the outer kinetochore plate and fibrous corona (Cooke et al. 1997; Yao et al. 1997). Exam-

ination of native CENP-E suggested that it was a minus-end motor protein (Lombillo et al. 1995; Thrower et al. 1995). However, recombinant CENP-E motor domain moves in vitro towards the plus-ends of microtubules like classical kinesin (Wood et al. 1997). The subcellular distribution of CENP-E has been studied in different kinds of vertebrate somatic cells. It has been observed that it accumulates in the cytoplasm during interphase, and that it first appears at the kinetochore fibrous corona in early prometaphase, just after nuclear envelope breakdown, and before microtubule attachment (Yen et al. 1991; Cooke et al. 1997). During prometaphase congression, unattached kinetochores show a crescent CENP-E signal while attached kinetochores present a small plate-like signal (Thrower et al. 1996). These plate-like signals appear on all kinetochores when chromosomes are properly aligned at the metaphase plate. Shortly after the metaphase/anaphase transition, labelling at kinetochores decreases while strong labelling is detected at interzonal microtubules (Yen et al. 1991). CENP-E is still associated with kinetochores even as late as telophase but most of it is concentrated at the spindle midbody (Brown et al. 1996). Then, CENP-E is rapidly degraded when cells exit mitosis (Yen et al. 1992; Brown et al. 1994).

CENP-E function has been studied with different methodologies. Injection of antibodies into mammalian somatic cells (Yen et al. 1991; Schaar et al. 1997) and mouse oocytes (Duesbery et al. 1997) prevents correct chromosome alignment, thus promoting metaphase arrest. Antibodies against CENP-E inhibit poleward chromosome movements powered by microtubule disassembly in vitro (Lombillo et al. 1995). Additionally, inhibition of CENP-E function by immunodepletion from *Xenopus* egg extracts (Wood et al. 1997), or by antisense oligonucleotide suppression of its synthesis in mammalian cells (Yao et al. 2000), leads to failure to establish bi-orientation during prometaphase congression. Altogether, these studies demonstrate that CENP-E is required for chromosome congression at the metaphase plate, and for chromatid segregation at anaphase. On the other hand, CENP-E interacts with the mitotic spindle checkpoint kinase hBubR1 (Chan et al. 1998, 1999; Yao et al. 2000). Moreover, using *Xenopus* egg extracts, it has been suggested that CENP-E is required for establishing and maintaining the mitotic spindle checkpoint in vitro (Abrieu et al. 2000). In this regard, it has been suggested that CENP-E forms a link between spindle microtubule capture by the kinetochore and the mitotic spindle checkpoint (Yao et al. 2000). Recently, it has been shown that the kinetochore-associated Mps1 checkpoint kinase recruits active CENP-E at kinetochores (Abrieu et al. 2001), which in turn is necessary for the association of the checkpoint components Mad1 and Mad2 at unattached kinetochores (Abrieu et al. 2000). Thus, in addition to its motor activity, CENP-E also acts at an early signalling step in the checkpoint pathway although its exact role in this pathway is unknown.

Most studies of CENP-E have been carried out in somatic cells. CENP-E has been detected at kinetochores in mouse and rat spermatocytes; however, it was used as a centromere marker in a study of spindle morphogenesis during meiosis I (Kallio et al. 1998). On the other hand, Duesbery et al. (1997) analysed the involvement of this protein during arrest at metaphase II in mouse and *Xenopus* oocytes. However, in spite of these studies, a careful analysis of its subcellular distribution at kinetochores and interzonal microtubules during the two meiotic divisions has not been carried out. In this study we examined in detail the expression pattern and behaviour of CENP-E during mouse spermatogenesis by means of immunofluorescence on squashed cells. We used this squashing technique since it unambiguously allows differentiation of all meiotic stages, and does not affect chromosome condensation and positioning in dividing spermatocytes (Page et al. 1998; Prieto et al. 2001). We analysed the changes in morphology and brightness of CENP-E signals at kinetochores of congressing bivalents in relation to the attachment of spindle microtubules. In addition, we simultaneously analysed the colocalisation of CENP-E, CENP-G, a marker of the inner kinetochore plate (He et al. 1998), and proteins recognised with an anti-centromere (ACA) autoimmune serum.

Materials and methods

Immunofluorescence microscopy

Testes from adult male C57BL/6 mice were removed and then fixed for immunofluorescence as described by Page et al. (1998). Briefly, testes were fixed in freshly prepared 2% formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) containing 0.1% Triton X-100 (Sigma). After 5 min several seminiferous tubules were placed on a slide coated with 1 mg/ml poly-L-lysine (Sigma) with a small drop of fixative, and the tubules were gently minced with tweezers. The tubules were then squashed and the coverslip removed after freezing in liquid nitrogen. The slides were rinsed three times for 5 min each time in PBS and incubated for 45 min at room temperature with primary antibody. To detect CENP-E we used pAb1.6, a polyclonal rabbit serum that recognises the neck region (amino acids 256–817) of human CENP-E (Lombillo et al. 1995), at a 1:100 dilution in PBS. CENP-G was detected with EJ autoimmune human serum at a 1:250 dilution (He et al. 1998). To detect the centromere we employed an autoimmune human serum from a CREST patient at a 1:100 dilution (kindly provided by Dr. Chantal Andre). To detect microtubules we used the monoclonal mouse anti-β-tubulin N-357 antibody (Amersham) at a 1:100 dilution. In double-labelling experiments, primary antibodies were incubated simultaneously. Following three washes in PBS, the slides were

Fig. 1 Immunoblot analysis of mouse testes extracts probed with pAb1.6 serum. The positions of molecular mass markers are indicated by *numbers* and the *arrowhead* indicates the CENP-E band

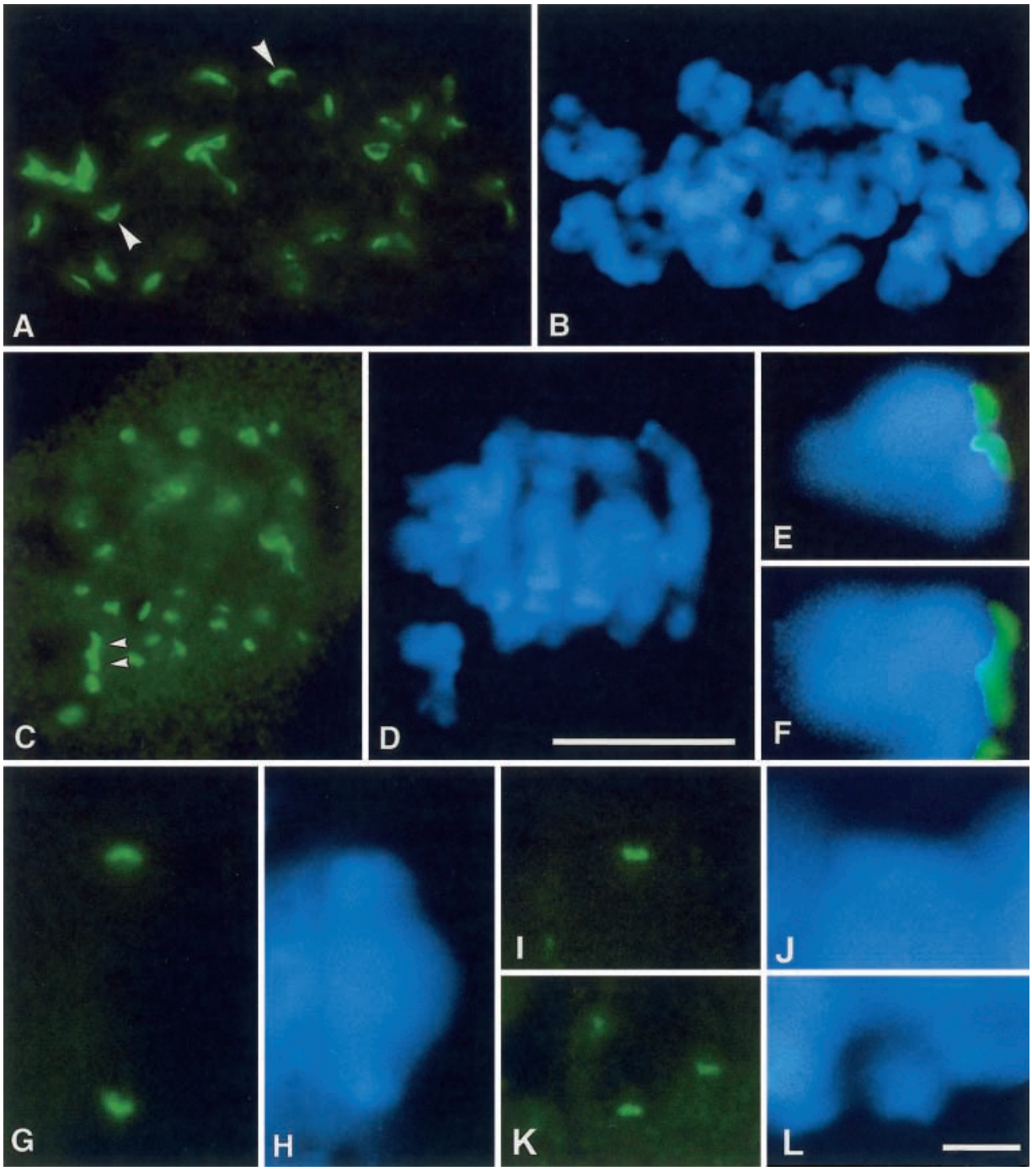

Fig. 2 Distribution of CENP-E at late diakinesis/early prometaphase I (**A**, **B**), in late prometaphase I spermatocytes (**C**, **D**) and bivalents (**E**, **F**), and in a selected bivalent (**G**, **H**), and at homologous centromeres (**I–L**) in metaphase I. **A**, **C** Partial projections of three focal planes showing CENP-E labelling. **G**, **I**, **K** CENP-E labelling. **E**, **F** Merged image of CENP-E labelling (*green*) and 4′, 6-diamidino-2-phenylindole (DAPI) counterstaining (*blue*). **B**, **D**, **H**, **J**, **L** DAPI counterstaining. In late diakinesis/early prometaphase I CENP-E appears as strongly labelled C-shaped or crescent structures (*arrowheads* in **A**) at homologous centromeres in almost all bivalents. In late prometaphase I the CENP-E signal at each homologous centromere in unaligned bivalents is intense and presents a crescent morphology (*arrowheads* in **C**, enlarged in **F**, and **E**). In aligned bivalents the signal at centromeres is smaller and fainter. In metaphase I CENP-E may appear as a slightly curved plate (**G**), or as two associated small dots at each homologous centromere (**I**, **K**). **A–D** *Bar* in **D** represents 10 µm. **E–L** *Bar* in **L** represents 2 µm

incubated for 30 min at room temperature with either a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson) secondary antibody at a 1:150 dilution in PBS, a Texas Red-conjugated goat anti-human IgG (Jackson) at a 1:150 dilution in PBS, or a Texas Red-conjugated goat anti-mouse IgG (Jackson) at a 1:150 dilution in PBS. In double-labelling experiments, secondary antibodies were incubated simultaneously. The slides were subsequently rinsed in PBS and counterstained for 3 min with 5 µg/ml DAPI (4′, 6-diamidino-2-phenylindole). After a final rinse in distilled water, the slides were mounted with Vectashield (Vector Laboratories).

Observations were performed using an Olympus BH-2 microscope equipped with epifluorescence optics, and the images were recorded on Kodak T-Max 100 or Agfa RSX_{II} 100 films. The negatives and colour slides were scanned in a Polaroid SprintScan 35 scanner, and the images were processed using Adobe Photoshop 6.0 software. The resulting final images were printed using a Fargo PrimeraPro Elite dye-sublimation printer.

Electrophoresis and immunoblotting

Testes from adult male C57BL/6 mice were removed and placed in 1 ml of SDS solubilisation solution (50 mM TRIS-HCl, pH 7.5, 5 mM EDTA, 1% SDS). Then the testes were homogenized on ice in a Potter homogenizer. The extract was placed in a boiling water bath for 5 min, briefly sonicated, and centrifuged to remove the insoluble material. The appropriate quantity of extract was diluted with 5×SDS sample buffer (62.5 mM TRIS-HCl, pH 6.8, 2% SDS, 5% β-mercaptoetanol, 10% glycerol, 0.005% bromophenol blue) and boiled for 5 min.

Electrophoresis was carried out in 6% SDS-polyacrylamide gels according to Laemmli (1970). Gels were electrically transferred to Problott sheets (Applied Biosystems) for 1.5 h at 4°C and 300 mA. Sheets were blocked for 1 h with 4% nonfat dry milk in PBS, followed by an overnight incubation at 4°C with pAb1.6 serum at 1:300 dilution in PBS. Immunoreactive bands were visualized by incubation for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Life Science) at a 1:5000 dilution in PBS, and subsequent development using an ECL (Enhanced Chemiluminescence) detection system (Amersham) according to the manufacturer's instructions.

Results

Immunoblot assay

To test the specificity of the pAb1.6 serum we performed an immunoblot analysis with mouse testes extracts. We observed that this anti-CENP-E pAb specifically recognised a single protein band that migrated at a position representing approximately M_r 250,000 (Fig. 1). This band is similar to that found in HeLa extracts (Yen et al. 1992). Thus, pAb1.6 serum also recognises CENP-E in mouse spermatocytes.

Localisation of CENP-E during meiosis I

The squashing technique we employed to detect CENP-E allowed us to observe mitotic spermatogonial cells and spermatocytes at different developmental stages on the same slide. As expected, CENP-E was detected at sister kinetochores in spermatogonial metaphase chromosomes, and at the midzone and midbody during anaphase and telophase, respectively (data not shown). In prophase I spermatocytes, from leptotene up to diplotene, CENP-E was detected in the cytoplasm, but it was found in the nucleoplasm in late diplotene spermatocytes. CENP-E first appeared at centromeres in late diakinesis/early prometaphase I spermatocytes. During this stage

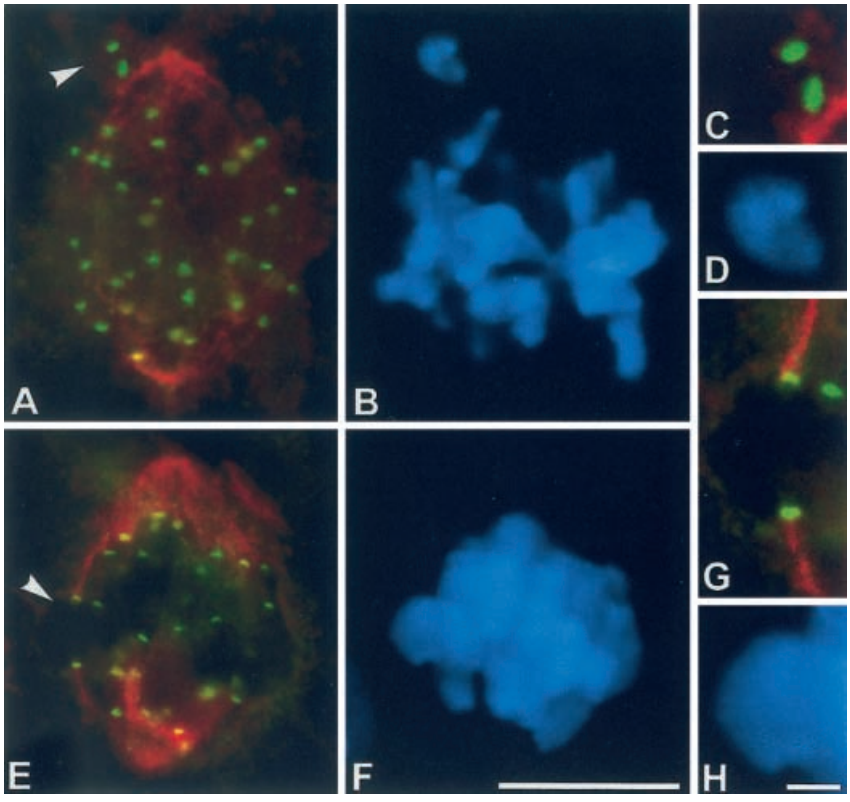

Fig. 3 Colocalisation of CENP-E and β-tubulin on prometaphase I (**A–D**) and metaphase I (**E–H**) spermatocytes. **A**, **C**, **E**, **G** Colocalisation of CENP-E (*green*) with β-tubulin (*red*). **B**, **D**, **F**, **H** DAPI counterstaining. **A**, **B** Projection of three focal planes throughout a prometaphase I spermatocyte. An unaligned bivalent shows two bright crescent CENP-E signals (*arrowhead* in **A**) corresponding to both homologous kinetochores. This bivalent has been enlarged (**C**, **D**) to show that homologous kinetochores are not attached to spindle microtubules. **E**, **F** Projection of three focal planes throughout a metaphase I spermatocyte. Most bivalents present a small curved CENP-E signal. **G**, **H** Enlarged bivalent (*arrowhead* in **E**) that shows a bundle of microtubules associated with both homologous kinetochores. **A**, **B**, **E**, **F** *Bar* in **F** represents 10 µm. **C**, **D**, **G**, **H** *Bar* in **H** represents 2 µm

the labelling at each homologous centromere was very bright, and usually appeared as a C-shaped signal (Fig. 2A, B).

In late prometaphase I spermatocytes, the centromere signals differed in morphology and brightness. It is worth noting that throughout meiosis I these two characteristics were similar for the two homologous centromeres. In those prometaphase I bivalents that were aligned at the equatorial plate and accurately bioriented, the labelling at each homologous centromere appeared as a small plate (Fig. 2C, D). In contrast, there were other unaligned bivalents, whose CENP-E signals at each homologous centromere showed a C-shaped or "crescent" morphology. These signals were brighter than those observed at homologous centromeres of properly aligned bivalents, and similar to those observed during late diakinesis/early prometaphase I (Fig. 2C, D). Interestingly, the crescent signals usually appeared in bichiasmate bivalents (arrowheads in Fig. 2C, and Fig. 2E, F). Similar crescent CENP-E signals were also observed at the centromeres of the sex chromosomes when they were occasionally observed as univalents during prometaphase I (data not shown).

In metaphase I bivalents, no crescent signals were found, but CENP-E labelling appeared as a single slightly curved plate per homologous centromere (Fig. 2G, H), or as two spots, representing sister kinetochores, per centromere (Fig. 2I–L). Independently of their morphology, the signals did not occupy the entire external surface of the centromere as detected with DAPI, but they appeared centrally located (Fig. 2G–L). In addition to the CENP-E signals at kinetochores, some faint labelling was detected at spindle poles (data not shown), as has been reported during mitosis (Cooke et al. 1997).

To test whether the change in brightness and shape of CENP-E signals at homologous centromeres during congression was related to the presence or absence of kinetochore microtubules, we colocalised CENP-E and microtubules with an antibody recognising β-tubulin. We found that unaligned prometaphase I bivalents showing bright crescent signals were unattached to microtubules (Fig. 3A–D). In contrast, aligned metaphase I bivalents presenting a single CENP-E signal, or two spots per centromere, were always attached to microtubule bundles (Fig. 3E–H). Thus, crescent CENP-E signals were only detected in unaligned and unattached bivalents.

In early anaphase I, two tightly associated and faint CENP-E spots appeared at each centromere (insert in Fig. 4A). However, brighter CENP-E signals became evident as thin threads at the midzone between segregating chromosomes (Fig. 4A, B). In more advanced anaphase I spermatocytes, the labelling at centromeres decreased, and the number and brightness of the threads lying in the midzone increased (Fig. 4C, D). During telophase I the labelling at centromeres was still evident but barely visible (Fig. 4E, F). At this stage, the CENP-E strands previously observed were not more detectable, but two thin parallel plates perpendicular to the spindle axis were found at the intercellular bridge (Fig. 4E, F). This signal is probably located in zone 3 of the intercellular bridge

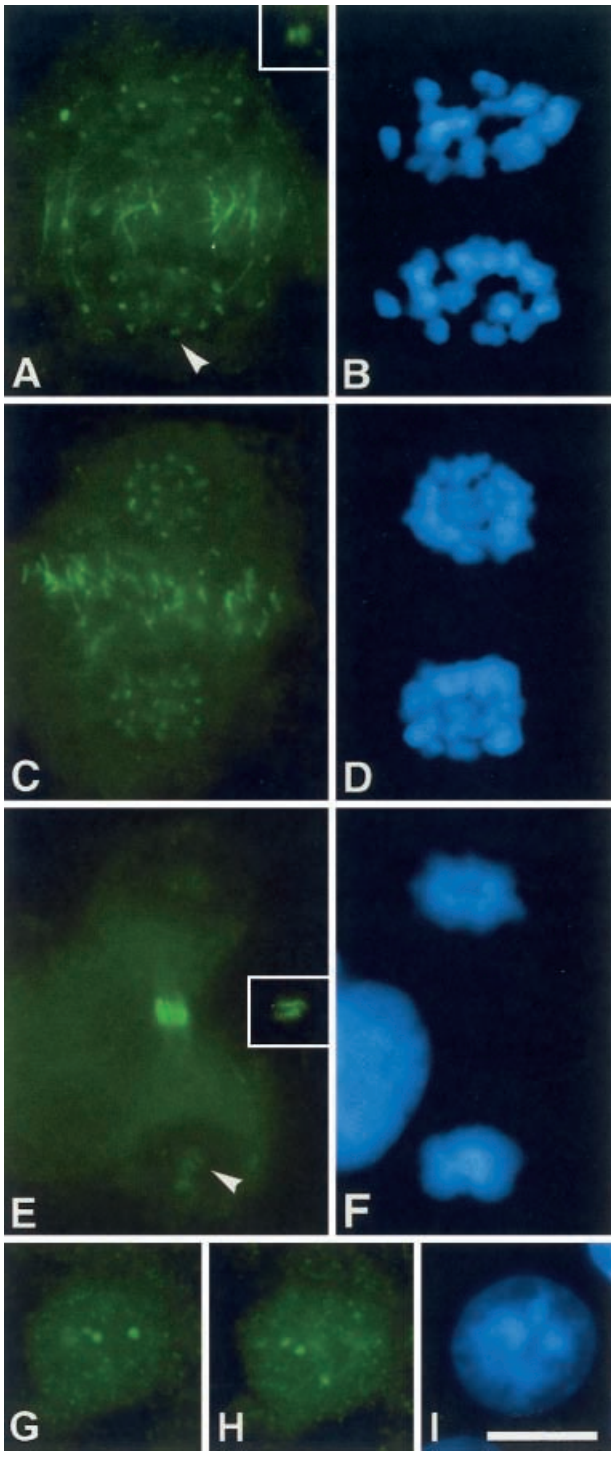

Fig. 4 Behaviour of CENP-E throughout anaphase I (**A–D**), telophase I (**E**, **F**) and interkinesis (**G–I**). **A**, **C**, **E**, **G**, **H** CENP-E labelling. **B**, **D**, **F**, **I** DAPI counterstaining. The labelling at centromeres decreases throughout anaphase I (**A**, **C**) but sister kinetochores are clearly detected (*arrowhead* in **A**, enlarged in *insert*). CENP-E signals at kinetochores are still faintly visible at telophase I (*arrowhead* in **E**). During early anaphase I some threads appear in the midzone (**A**). These threads become shorter as anaphase I progresses and occupy the entire width of the cell (**C**). In telophase I the signal at the midbody is very bright (**E**). When low exposure times are used, labelling at kinetochores is not apparent but in the midbody two small bright parallel lines are clearly delineated (insert in **E**). At interkinesis (**G–I**) some accumulations of CENP-E can be detected at the heterochromatic chromocentres. *Bar* represents 10 μm

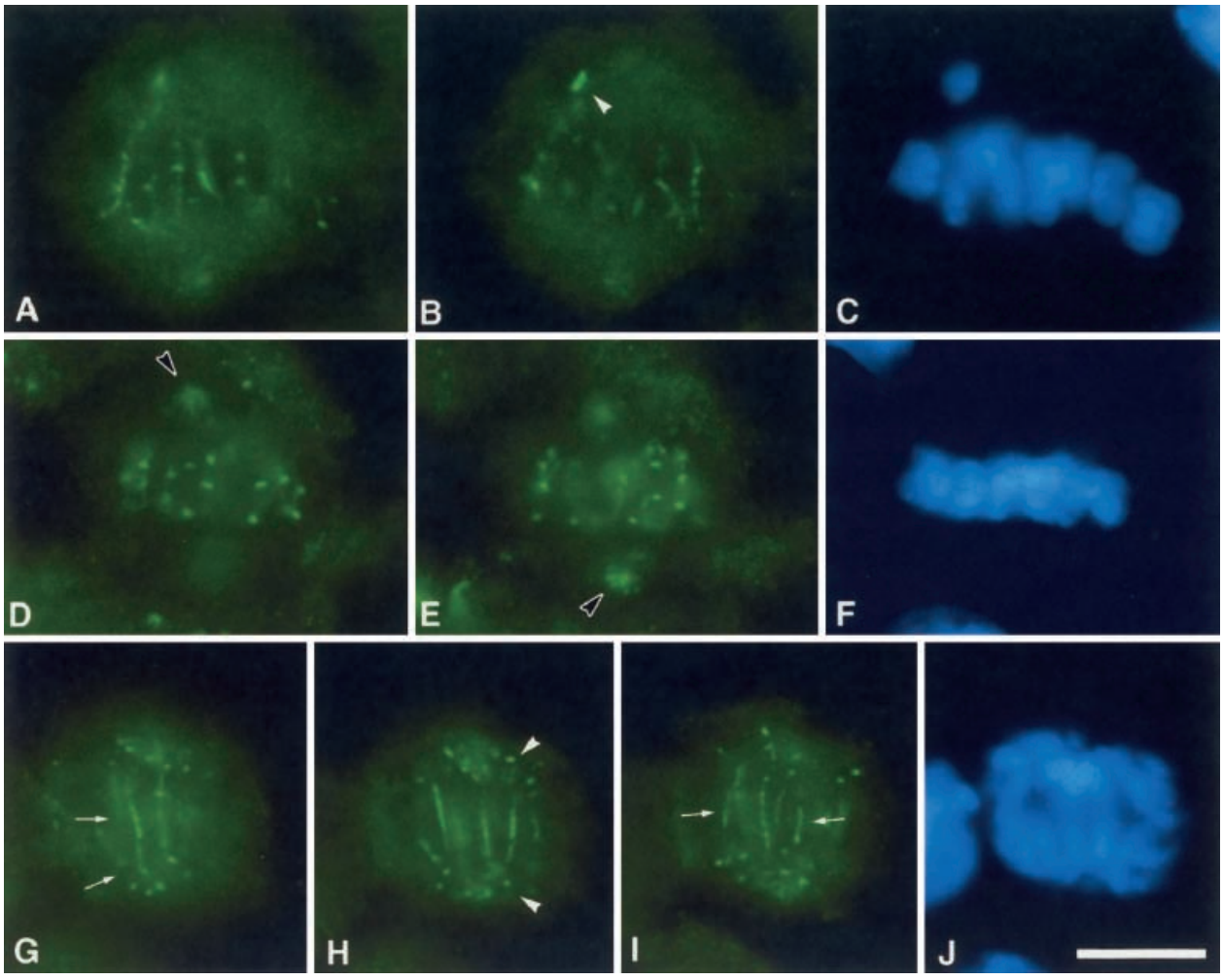

Fig. 5 Labelling of CENP-E during prometaphase II (**A–C**), metaphase II (**D–F**), and anaphase II (**G–J**). **A**, **B**, **D**, **E**, **G**, **H**, **I** CENP-E labelling. **C**, **F**, **J** DAPI counterstaining. **A–C** Two focal planes of a prometaphase II spermatocyte with an unaligned chromosome. The unaligned chromosome (*white arrowhead*) shows strong CENP-E labelling at the centromere. **D–F** Two focal planes of a metaphase II spermatocyte. CENP-E appears as small signals at sister kinetochores. Additionally, faint labelling is observed at cell poles (*black arrowheads*). **G–J** Three focal planes of an anaphase II spermatocyte. During this stage CENP-E signals at kinetochores are faint (*arrowheads* in **H**), while in the midzone some threads are detected (*arrows* in **G**, **I**). *Bar* represents 10 µm

according to Rattner (1992). These plates were so bright, by comparison with labelling at centromeres, that the exposure time had to be reduced to demonstrate them (insert in Fig. 4E).

Localisation of CENP-E during meiosis II

During interkinesis CENP-E appeared as dots colocalising with the heterochromatic chromocentres (Fig. 4G–I). In some prometaphase II spermatocytes, unaligned chromosomes showed a single bright CENP-E signal, suggesting that sister kinetochores had not individualised (Fig. 5A–C). Those chromosomes show a crescent CENP-E signal (arrowhead in Fig. 5B) like the signals found at homologous kinetochores in unaligned bivalents in prometaphase I. In metaphase II spermatocytes the spindle poles appeared faintly labelled, and sister kinetochores were detected as small plates (Fig. 5D–F).

Throughout anaphase II, as occurred during anaphase I, kinetochore signals became fainter and thin threads appeared in the midzone (Fig. 5G–J). These threads shortened as sister chromatids separated to opposite poles. In late telophase II labelling at kinetochores decreased dramatically, and two parallel plates similar to those found during telophase I, but shorter, appeared in the intercellular bridge (data not shown). CENP-E was not detected in nuclei of spermatids at different developmental stages.

Subcellular localisation of CENP-E at kinetochores

In order to accurately localise CENP-E in the centromere we performed double colocalisation studies using a CREST ACA serum, or an antibody against CENP-G, a protein located at the inner kinetochore plate. In all cases, CENP-E appeared above and slightly overlapping with the centromere signal obtained with the ACA serum (Fig. 6A–I). On the other hand, CENP-E was detected

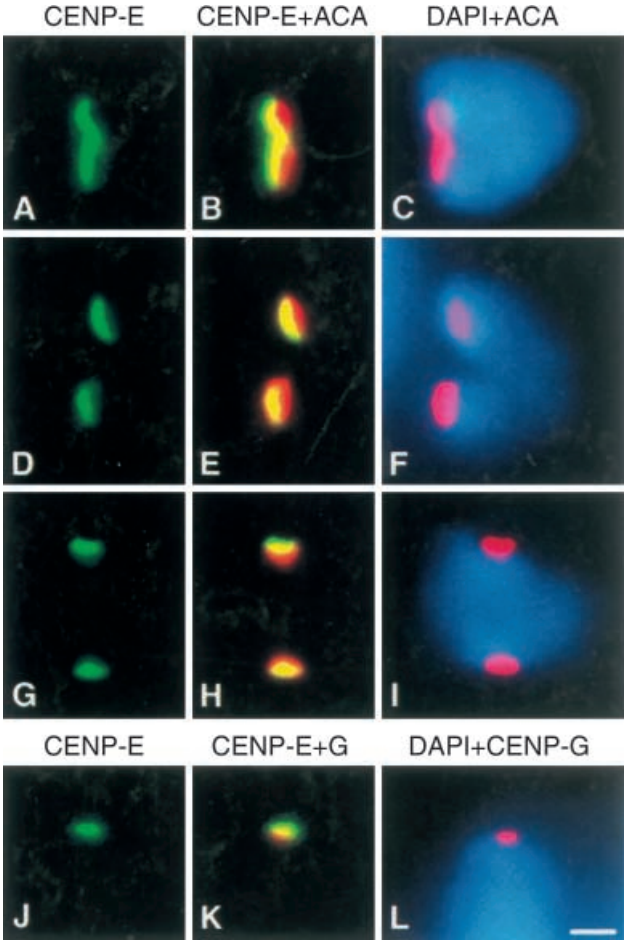

Fig. 6 Colocalisation of CENP-E with centromere proteins recognised by an anticentromere antigen (ACA) serum (**A–I**) and CENP-G (**J–L**) on prometaphase (**A–C**) and metaphase I bivalents (**D–L**). **A**, **D**, **G**, **J** CENP-E labelling. **B**, **E**, **H** Colocalisation of CENP-E (*green*) with ACA proteins (*red*). **K** Colocalisation of CENP-E (*green*) with CENP-G (*red*). **C**, **F**, **I** Colocalisation of chromatin revealed with DAPI (*blue*) with ACA proteins (*red*). **L** Colocalisation of chromatin revealed with DAPI (*blue*) with CENP-G (*red*). In unaligned prometaphase I bivalents, as in aligned metaphase I bivalents, CENP-E always appears above either ACA proteins or CENP-G, although there is a region showing colocalisation (*yellow*). *Bar* represents 2 μm

above the CENP-G signal (Fig. 6J–L). These results suggest that CENP-E may be located around the outer kinetochore plate as has been shown for kinetochores in somatic cells (Cooke et al. 1997; Yao et al. 1997).

Discussion

Our results show that CENP-E first becomes detectable at homologous centromeres during late diakinesis/early prometaphase I, while it relocalises to the spindle midzone during early anaphase I, and appears at the midbody during telophase I. We observed that the pattern of expression of CENP-E during meiosis II is similar to that observed during meiosis I. Our colocalisation data demonstrate that most CENP-E lies outside the site of CENP-G labelling although some degree of colocalisation was observed. Since CENP-G is found at the inner kinetochore plate (He et al. 1998), this colocalisation pattern suggests that CENP-E is located at the outer kinetochore plate and fibrous corona. Thus, the subcellular localisation of CENP-E at kinetochores, and the general pattern of expression of CENP-E during the two meiotic divisions, is similar to that found previously in somatic cells (Yen et al. 1991; Brown et al. 1996; Cooke et al. 1997; Yao et al. 1997).

Our most interesting result is the change in morphology and brightness of CENP-E signals at homologous centromeres during congression to the metaphase I plate. We found that in late diakinesis/early prometaphase I, and in unaligned late prometaphase I bivalents, CENP-E appears as bright crescents at both homologous kinetochores. In contrast, in aligned bivalents, CENP-E signals at each homologous centromere are fainter and may appear either as a single plate-like signal or as a pair of spots corresponding to sister kinetochores. The appearance of crescent CENP-E signals at homologous kinetochores has not been previously reported in studies on rat and mouse spermatocytes (Kallio et al. 1998), and mouse and *Xenopus* oocytes (Duesbery et al. 1997). Nevertheless, bright CENP-E crescents have been reported at sister kinetochores on unaligned prometaphase mitotic chromosomes, and on all kinetochores when mitotic cells are arrested in a prometaphase-like stage by microtubule depolymerising drugs (Thrower et al. 1996; Yao et al. 1997; Hoffman et al. 2001). Moreover, the microtubule motor protein cytoplasmic dynein (Hoffman et al. 2001), and the mitotic spindle checkpoint proteins Bub3 (Martinez-Exposito et al. 1999), and BubR1 and Mad2 (Hoffman et al. 2001), are also detected as bright crescents in unattached mitotic kinetochores. It has been shown by electron microscopy that the crescent CENP-E signals observed by immunofluorescence on unaligned mitotic chromosomes correspond with labelling at the fibrous corona that curls in around the ends of the outer kinetochore plate. Accordingly, it has been suggested that the crescent morphology shown by unattached mitotic kinetochores may substantially increase its surface for recruiting, and then capturing spindle microtubules (Cooke et al. 1997; Hoffman et al. 2001). Taking into account these results on mitotic kinetochores, our observation of bright CENP-E crescents at the kinetochores of unaligned bivalents during prometaphase I, and unaligned chromosomes during prometaphase II, may similarly indicate that meiotic vertebrate kinetochores also increase their surface to facilitate microtubule capture. Our results support this possibility since unaligned bivalents with crescent CENP-E signals are not attached to spindle microtubules (see Fig. 3).

The decrease in intensity of CENP-E fluorescence at kinetochores during congression at the two meiotic divisions also merits some comment. A similar decrease in brightness at kinetochores has been reported for CENP-E (Thrower et al. 1996; Hoffman et al. 2001), and the mitotic spindle checkpoint proteins Bub1, BubR1, Bub3,

and Mad2 (Jablonski et al. 1998; Martinez-Exposito et al. 1999; Hoffman et al. 2001), during congression in human and mouse somatic cells. Since CENP-E may form a link between spindle microtubule capture by the kinetochore and the mitotic spindle checkpoint (Yao et al. 2000; Abrieu et al. 2001), we suggest that a spindle checkpoint also operates during meiosis in male vertebrates, as has recently been demonstrated in budding (Shonn et al. 2000), and fission yeast meiosis (Bernard et al. 2001).

Our data also reveal that the number of homologous centromeres showing a pair of CENP-E spots in aligned bivalents instead of a single plate-like signal increases throughout congression to the metaphase I plate. Thus, a gradual change in the structure of the meiotic centromere occurs during meiosis in males from nuclear envelope breakdown until accurate biorientation at the metaphase I plate is reached. First, bright crescent CENP-E signals at homologous centromeres are observed during capture and stabilisation of spindle microtubules. Second, when biorientation is achieved, fainter plate-like signals are detected in congressing bivalents. Finally, a pair of faint CENP-E spots representing sister kinetochores are discerned after all bivalents are properly arranged at the metaphase I plate. Since there are previous light (for references see Rufas et al. 1989), immunofluorescence (Suja et al. 1999), and electron microscopy (Müller 1972; Goldstein 1981; Rufas et al. 1994; Lee et al. 2000) studies where the individualisation of sister kinetochores has been reported, we suggest that the change in centromere structure is an event that takes place specifically during meiosis I. Moreover, our results suggest that the tight association of sister kinetochores, appearing as a single plate-like signal during congression, is a mechanism for ensuring that a bivalent will possess only two points of interaction with the spindle microtubules, thus allowing its correct biorientation and congression.

In summary, our results on the distribution and behaviour of CENP-E at kinetochores during the two meiotic divisions, particularly the appearance of crescent signals, demonstrate that the contribution of this motor protein in chromosome alignment during prometaphase is similar in mitosis, and meiosis in male vertebrates. The changes in brightness of CENP-E at homologous kinetochores during congression also suggest that a spindle checkpoint may function during vertebrate male meiosis. Finally, we demonstrate that in meiosis I vertebrate males the centromere changes its structure once alignment of all bivalents at the metaphase I plate has been reached.

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