Abstract. In Parascaris the mitotic chromosomes of gonial germline cells are holocentric and possess a continuous kinetochore along their entire length. By contrast, in meiotic cells, the centromeric activity is restricted to the heterochromatic tips where direct insertion of spindle microtubules into chromatin without any kinetochore plate is seen. In the presomatic cells of early embryos, which undergo heterochromatin elimination, only euchromatin shows kinetic activity. After developing a technique to separate the very resistant egg shell from the embryos, we studied the cell divisions during early embryogenesis by immunochemical and EM approaches. The results reported here show that in presomatic cells microtubules bind only the euchromatin where a continuous kinetochore plate is present. We also report observations suggesting that the binding of the long kinetochores to the mitotic spindle initiates to a limited number of sites and extends along the entire length, during chromosome condensation. The existence of different centromere stages in different cell types, renders Parascaris chromosomes a very good model to study centromere organization.

THE classical studies of early embryonic divisions in the horse parasitic nematode Parascaris led to the discovery of the chromatin diminution process that occurs during separation of germ line and soma (Boveri, 1887, 1899). In germline cells, the genome of P. univalens is organized in two large holocentric chromosomes containing a central euchromatic region surrounded by two large blocks of heterochromatic AT-rich satellite DNA (~80% of total DNA) (Moritz and Roth, 1976; Roth, 1979; Goday and Pimpinelli, 1984). Chromatin diminution (or heterochromatin elimination) occurs only in presomatic blastomeres starting in the second or third embryonic division. In these cells, the chromosomes are fragmented, and all of the heterochromatin is eliminated. Thus, in P. univalens the two germinal chromosomes retain their integrity and maintain their heterochromatin, but somatic chromosomes are numerous (~60) and totally euchromatic.

The molecular mechanisms involved in chromatin diminution are still unknown, and the cytoplasmic factors involved in this process, whose existence in early embryos was proved by T. Boveri's studies (reviewed in Tobler, 1986), have not been identified.

Early cytological observations of first embryonic divisions in different Parascaris species suggest that, after chromosomes fragment, the heterochromatic regions are lost, because they lack kinetic activity and consequently are not capable of segregating (Boveri, 1887, 1889; Schrader, 1935). This was also supported by observing the kinetic behavior of chromosome fragments after x-ray treatment of early Parascaris embryos (White, 1936). This is an interesting point because it conflicts with cytological observations on the maintenance of apparently totally heterochromatic chromosomal fragments through Parascaris germline cell divisions (Moritz, 1967), and more recent data about the ultrastructural centromere organization of P. univalens chromosomes in germline cells. Mitotic germline chromosomes are holocentric and possess a continuous kinetochore plate that extends over the whole chromatid surface. The continuous kinetochore and the holocentric behavior imply kinetic activity of both euchromatic and heterochromatic regions. This particular layered kinetochore type (kt) ("ladder-like kt") is rather complex and contains structural elements similar to mammalian kinetochores (Goday et al., 1985). Moreover, during meiotic divisions, kinetic activity is highly modified and is restricted to terminal heterochromatic areas although no kinetochore structures are seen at the microtubule/chromatin binding points (Goday and Pimpinelli, 1989). Thus, in Parascaris germline chromosomes, two types of centromere activity have been found: one is continuous through both heterochromatin and euchromatin and the other is restricted to the terminal heterochromatic regions. The different behavior and organization of centromeres in mitotic and meiotic cells in the gonadal tissue of P. univalens, together with T. Boveri's data on the kinetic behavior of the chromosomes during the chromatin diminution process in the embryos, strongly suggests that a third type of centromere orga-
Materials and Methods

Live female specimens of *P. univalens* were collected at the local abattoir along with horse intestinal contents. The worms were washed and main-

Dechorionating *Parascaris* Eggs

Fertilized eggs were extracted from the uteri, transferred into a tube and held in 0.5 M NaOH for 1 h at room temperature. Eggs were then rinsed three times in distilled H2O and allowed to settle at 4°C. The supernatant was replaced with 0.4 M KOH and the eggs were held 30 min at room temperature. NaOCI (7% + 2% active Cl) was added to a 17% final concentra-

Obtaining *Parascaris* Embryos of Different Developmental Stages

Dechorionated fertilized eggs in M9 buffer were transferred to petri dishes, incubated at 37°C, and development was followed by phase contrast and Nomarski microscopic observations. Undiminished chromosomes of first and second mitotic divisions were obtained within the first 2 and the first 4 h, respectively. Embryos undergoing chromatid diminution were obtained between 4 and 17 h. After 17 h most embryos had completed the first five mitotic divisions (32 cell stage), the chromatin process had ceased and all divisions were somatic ones (see below). After 72 h most embryos had reached the first larval stage. In all cases, the suspensions of embryos were transferred to tubes, allowed to sediment, and fixed.

Indirect Immunofluorescence

Embryos were fixed in 100% methanol at -20°C for 10 min, centrifuged for 3 min at 4,000 rpm and treated with aceton at -20°C for 1 min. They were then centrifuged and washed three times in PBS for 5 min at room temperature, treated with 1% Triton X-100 in PBS for 7 min and subsequently washed in PBS three times for 5 min each. Embryos were incubated for 1 h at room temperature with monoclonal anti-β-tubulin antibody (Amersham Corp., Arlington Heights, IL) at a 1:10,000 dilution in PBS. After three 7 min washes in PBS, embryos were incubated for 45 min at room tempera-

Electron Microscopy

Embryos were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 90 min at room temperature and for another 30 min at 37°C, then rinsed and postfixed in 1% OsO4 for 1 h at room temperature in the same buffer. The fixed embryos were then rinsed and incubated in 1% OsO4 for 1 h at room temperature in the same buffer. The fixed embryos were then incubated for 1 h at room temperature with monoclonal anti-β-tubulin antibody (Amersham Corp., Arlington Heights, IL) at a 1:1,000 dilution in PBS. After three 7 min washes in PBS, embryos were incubated for 45 min at room tempera-

Fluorescence Microscopy Observation

All observations were made under a microscope equipped with epifluores-
cence optics (Carl Zeiss, Inc., Thornwood, NY). Microphotographs were taken with Tri X film (Eastman Kodak Co., Rochester, NY).
Figure 1. First mitotic spindle formation in *P. univalens* embryos. Indirect immunofluorescence staining of tubulin (a–e); Hoechst 33258 staining (i); and CMA staining (c–e) chromosome staining. (a) *P.* zygote showing duplicated centrosomes (C) that are centrally located (a') and exhibit associated microtubules. (b) Centrosomes have separated and are already oriented along the embryo division axis. Note the microtubules connecting both centrosomes around the pronuclei (arrows) that contain prophase chromosomes (c). (c and d) First microtubule interactions with advanced prometaphase chromosomes after pronuclear membrane breakdown. The initial four kinetochore–microtubule groups (short arrows) that bind to discrete zones within the brightly CMA-stained central euchromatic regions (long arrows) are shown. Note the low number of kinetochore–microtubules. (e) In totally condensed metaphase chromosomes, kinetochore–microtubule interactions extend along the central euchromatic regions. These segments are brightly stained with CMA (long arrows) and they (i) correspond to the Hoechst-dull central regions (long arrows) of the same chromosomes. The short arrows point the most distal heterochromatic subregions. Although heterochromatic, these regions are CMA bright. Bar, 5 μm.

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Figure 2. First embryonic division of *P. univalens*. Indirect immunofluorescence staining of spindle tubulin (a–c) and Hoechst 33258 chromosome staining (i). In metaphase (a) kinetochore-microtubules (ktmts) (long arrow) are numerous and, in this figure, viewed perpendicular to long chromosome axis, it is still possible to distinguish the four kinetochore-microtubule groups that interact with the chromosomes from the non kt-microtubules. During anaphase movement (Fig. 2, b and c), kt-microtubules that are shorter and more compact, clearly interact exclusively with the central euchromatic (Hoechst-dull) regions that first initiate movement to the poles, while distal heterochromatic (Hoechst-bright) regions, free of microtubules, are still linked in early anaphase (Fig. 2 b) and only separate completely in late anaphase (Fig. 2 c). The heterochromatic segments therefore are the so-called “trailing arms” first described by T. Boveri (Boveri, 1887).

Moreover, as it has been described for the nematode *Caenorhabditis elegans* (Albertson, 1984), also in *Parascaris* during first embryonic anaphase the ventrally located half spindle is slightly smaller than the dorsal one and this results in size differences between ventral and dorsal blastomeres.

T. Boveri initially reported that chromatin diminution in the presomatic S blastomere occurs during the second mitotic division. Later observations (Herla, 1895; Boveri, 1910; Goday and Pimpinelli, 1986) indicate that the start of this process can be delayed until the third division. In many embryos that we analyzed for the present study diminution began at the third division. Independently of the initiation stage, diminution invariably ceases at the 32 cell stage where the two blastomeres that are the primordial germ cells, retain nondiminished chromosomes. We think it is of interest to note that the pregerminai P blastomere initiates second cleavage before the presomatic dorsal S blastomere, but it is S; that concludes the division first. S; gives rise to two dorsal blastomeres S1a and S1b that reach interphase while P1 is still in metaphase or anaphase (Fig. 3). This timing of the P1 and S1 cell divisions is independent of whether diminution has already begun or is delayed until the next, third division. Cytological analysis of the spindle and chromosomes in presomatic blastomeres of four-cell embryos undergoing the process of heterochromatin elimination is shown in Fig. 4. Chromatin diminution results in separation of the distal, Hoechst-bright, heterochromatic blocks, and fragmentation of the euchromatin. From prometaphase to late anaphase (Fig. 4, a–d) the spindle microtubules interact only with the newly formed euchromatic chromosomes, while the heterochromatic material does not associate with microtubules and remains at the equatorial plate. In all cases, we have ob-
Figure 3. Second embryonic division of *P. univalens*. Indirect immunofluorescence staining of tubulin (a–c) and Hoechst 33258 chromatin staining (i). (a) Two-cell embryo showing interphase nuclei (arrows) and dispersed tubulin in the cytoplasm. (b) Embryo showing the presomatic *S*₁ blastomere in metaphase and the pregerminal *P*₁ blastomere in anaphase. (c) Three-cell embryo where it is possible to see two presomatic blastomeres (*S*₁ and *S*₁ₐ) already in interphase, while the pregerminal *P*₁ blastomere is still in anaphase. Note that chromatin diminution has not occurred at this stage. Bar, 5 μm.

Figure 4. Heterochromatin elimination in presomatic blastomeres of four-cell *P. univalens* embryos. Indirect immunofluorescence staining of spindle tubulin (a–d) and Hoechst 33258 chromosome staining (i). (a) Prometaphase showing that spindle microtubules interact only with Hoechst-dull euchromatic chromosomal fragments, while Hoechst-bright and split terminal heterochromatic regions remain out of the spindle interaction area (arrows). (b) Lateral view of a metaphase where all euchromatic small chromosomes are grouped at the equatorial plate and show microtubule interactions, while condensed blocks of heterochromatin remain separated (arrows). (c) Early anaphase showing the initial parallel separation of the two chromatid groups. Arrows point to the fused heterochromatic blocks. (d) Late anaphase; note the slightly bent appearance of the chromatid groups that correspond to Hoechst-dull spindle areas (short arrows) and the heterochromatic blocks that remain in the equatorial region (long arrows). Bar, 5 μm.
Electron Microscope Analysis of the Centromere in P. univalens Presomatic and Somatic Chromosomes

In longitudinal sections, first mitotic metaphase chromosomes of P. univalens embryos show continuous kinetochore structures running along the chromosomes' surface in close association with the chromatin (Fig. 5). Numerous microtubules are seen distributed regularly along the length of the kinetochore plates and they associate individually with the kinetochore (Fig. 5, a and b). In some longitudinal sections, as well as in cross sections, two kinetochore structures, corresponding to each chromatin, are seen on each poleward face of the metaphase chromosome (Fig. 5, b and c). The kinetochore profile of these embryonic chromosomes is similar to that described in mitotic germline Parascaris chromosomes (ladder-like kinetochore) (Goday et al., 1985). Moreover, it does not possess regular interruptions along its length, as would be expected if a polycentric organization were present in the euchromatic central regions of presomatic chromosomes. From our observations in different sections, the longest longitudinally sectioned kinetochore profile measured 4.2 μm and the longest distance between the edges of kinetochore plates observed in the same chromosome was 6 μm. The latter corresponds approximately to the estimated length of the euchromatic central regions of these chromosomes. From transverse sections (Fig. 5 c), we have estimated that the entire width of individual kinetochore plates is about 0.5 μm. In addition, in the chromatin regions where no associated microtubules are seen, we have not seen any type of kinetochore structure (Fig. 5 d). These are the same regions that are heterochromatic in fluorescence analysis and that lack any association with the spindle microtubules. This observation is more clearly documented in Fig. 5 e where a reconstructed anaphase chromosome from partial serial sections of an embryo at pre-elimination stage is shown. As it has been reported (Goday and Pimpinelli, 1986), heterochromatic regions of Parascaris chromosomes are highly polymorphic; in our observations of individual longitudinal sections their lengths ranged from 7.2 to 9 μm.

On the other hand, the EM analysis of newly formed P. univalens somatic chromosomes in advanced embryos, where all blastomeres are already diminished (Fig. 6), shows that individual chromosomes contain continuous kinetochore structures in both chromatids. In these, microtubules attach individually and in most cases they seem to end in the outermost kinetochore region (Fig. 6, a, c, and d). In some sections (Fig. 6 b) it is possible to observe nonkinetochore microtubules passing between adjacent chromosomes. In all cases, the kinetochore structures seen in longitudinal sections are apparently different from those observed in undiminished chromosomes. They appear more fuzzy and less electron dense (Fig. 6 d). From our observations on longitudinal sections, the mean kinetochore length is 0.25 μm, but we have also observed kinetochore plates up to 0.6 μm long suggesting that somatic chromosomes may differ in size, or may aggregate during segregation.

Discussion

The present work has permitted us to overcome the impermeability of Parascaris eggs to most fixatives. It has therefore been possible to analyze the interactions of the mitotic spindle and chromosomes during the prediminution, diminution, and postdiminution mitotic divisions that occur during embryonic development by immunocytochemical light microscopy methods. From the first embryonic division on, the cytological analysis demonstrates that only the euchromatin of P. univalens chromosomes is capable of binding spindle microtubules while heterochromatin, as it has been classically claimed (Boveri, 1887; Schrader, 1935), lacks centromeric activity. Heterochromatic segments are therefore lost after chromosome fragmentation in presomatic blastomeres. As shown in Fig. 7, where a diagrammatic representation of our previous and present results is reported, although heterochromatin lacks kinetic activity in embryonic cells, two different forms of centromeric activity and structure are induced in the heterochromatin during postembryonic germ-line development. In mitotic gonial cells, a continuous laminar kinetochore (ladder-like kt) spanning both the euchromatin and heterochromatin is present (Goday et al., 1985). In meiotic cells, which lack visible kinetochore structures, kinetic activity is achieved by "direct insertion" of microtubules into the heterochromatic tips (Goday and Pimpinelli, 1989). The absence of kinetic activity of heterochromatin in embryos implies existence of yet a third form of centromere organization. Our EM data demonstrates that, before chromosome fragmentation, the euchromatic chromosomal regions maintain the kinetochore organization described for the holocentric mitotic gonial chromosomes. The heterochromatic terminal regions, however, do not form kinetochore plates. On the other hand, once presomatic chromosomes have fragmented during the chromatin diminution process and enter metaphase, it is clear that the newly formed somatic chromosomes segregate and bind individually to spindle microtubules. At the EM level, these small chromosomes are also holocentric because the spindle microtubules regularly attach along the whole chromatid surface, which exhibits a continuous kinetochore structural specialization.

Figure 5. Longitudinal sections (a, b and d) and transverse section (c) of P. univalens undiminished metaphase chromosomes of two-cell stage embryos; (e) reconstruction of partial serial sections of P. univalens undiminished anaphase chromosome. (a) Chromosome section (CH) showing the continuous kinetochore (K) structure with associated microtubules (arrows) corresponding to the central euchromatin region. Microtubules run parallel to each other and do not form bundles. In (b) it is possible to see the kinetochore profile (K) on both poleward faces. (c) Transverse section of euchromatic regions showing the entire width of the kinetochore profile (K) on both sides of the chromosomes. (d) Partial section showing a chromosome region that corresponds to distal heterochromatin (thick arrows) and lacks kinetochore structures as well as associated microtubules. In e the kinetochore profile (thick line pointed at by arrowheads) with associated microtubules (small arrows) is restricted to euchromatin (E). Heterochromatic trailing arm (Het) lacks kinetochore plate and associated microtubules. A single chromatid arm is seen due to the sections orientation that is not exactly parallel to the longitudinal anaphase plane. Bars: (a, b, and c) 0.25 μm; (d) 0.5 μm.
Figure 6. Longitudinal sections of *P. univalens* metaphase–early anaphase somatic chromosomes of post-32-cell stage embryos. (a) Individual chromosomes (CH) with continuous kinetochore structures (K) and associated microtubules (arrows) that do not form bundles and that run parallel to each other. (b) Partial section showing non-kinetochore microtubules (arrows) passing between adjacent chromosomes. (c) Section where it is possible to see less electron-dense kinetochore plates (K) on both poleward faces of chromosomes. Note the slightly different lengths of kinetochores among chromosomes. (d) A higher magnification partial section of the same chromosomes showing the fuzzy structural appearance of the kinetochore plate (K) and the individual microtubule interactions with the outermost kinetochore regions (arrows). Bars: (a and c) 0.5 μm; (b and d) 0.25 μm.

The chromosome plasticity observed in *Parascaris* gives support to "the repeat subunit model" for the centromere–kinetochore complex that has been recently proposed (Zinkowski et al., 1991). According to this model, along the DNA fiber, short microtubule-binding segments are discontinuously repeated. Thus, the kinetochore is considered to be an assembly of repeated subunits and its morphology is a direct consequence of chromatin condensation. As the authors say, this is the first model that can account for the structural and evolutionary diversity of the kinetochore and its relationship to the centromere of eucaryotic chromosomes of different species. In the present case, this model offers a very good formal explanation for the kinetochore variations in *Parascaris*. In mitotic gonial cells the presence of a continuous kinetochore along the whole chromatid length suggests that centromeric DNA sequences should be scattered along the chromosome. On the other hand, in meiotic chromosomes, where kinetic activity is restricted to telomeric heterochromatin, no kinetochore plates have been observed and, in presomatic chromosomes the kinetochore is present only in the euchromatin. That is, the different centromere organization found in different cell types, where
chromosomes are not modified in their DNA content, strongly suggests that centromeric DNA sequences scattered along the chromosomes are probably differently available to build up a kinetochore along the chromosomes. This implies a specific chromosomal organization in the different cell types that would explain not only the regional restrictions of the kinetochores between mitotic gonial and presomatic embryonic cells, but also the lack of kinetochore structures in meiotic cells. The presence or absence of a kinetochore plate in a specific region could be the direct consequence of differential kinetochore subunit assembly because of a cell-specific chromosomal organization.

In certain ciliates and insects (Eichenlaub-Ritter and Ruthmann, 1982; Ruthmann and Permantier, 1973; Rieder et al., 1990b) that have a high number of small chromosomes, it has been reported that they associate as "compound" chromosomes during anaphase movement. These observations have been interpreted as a possible mechanism to assure correct segregation of the chromatids in these organisms. Moreover, in the ciliates Nyctotherus ovalis and N. cordiformis, ultrastructural analysis has revealed the existence of kinetochore plates shaded by the anaphase associated chromatids (Eichenlaub-Ritter and Ruthmann, 1982). In the hemipteran Agallia constricta the holokinetic anaphase behavior of polycentric metaphase chromatin is due to the aggregation of smaller prometaphase chromosomes that contain a single diffuse kinetochore (Rieder et al., 1990b). In the case of Parascaris, the cytological picture of late anaphase somatic chromosomes is not informative, but the present EM observations of metaphase and early anaphase chromosomes indicate that kinetochore length varies among them and that nonkinetochore microtubules frequently pass between chromosomes. This can be interpreted in two ways: (a) either some chromosomes may "aggregate"; or (b) there are unequally spaced cleavage sites during presomatic chromosome fragmentation. Preliminary data from colchicine-treated embryos favors the latter view. The fact that in early anaphase Parascaris somatic chromosomes segregate independently does not exclude that in late anaphase they could aggregate as found in Agallia. From our previous EM work (Goday et al., 1985) and the present data, it is now clear that holocentric germline and presomatic chromosomes of Parascaris are not the result of smaller chromosome assembly, and we think that as Rieder et al. (1990b) have discussed, the existence of polycentric chromosomes as an assembly of smaller monocentric ones is questionable.

Mechanisms of kinetochore fiber formation and chromosome transport are now being intensively investigated. Recently, in monocentric chromosomes of newt pneumocytes, it was shown that initial attachment of a prometaphase chromosome to the spindle resulted from an interaction between a single microtubule and one of the kinetochores of the chromosome (Rieder et al., 1990a). Our cytological observations during the formation of first mitotic embryonic spindle in Parascaris permitted us to note that immediately after the promonuclear membranes breakdown, when the chromosomes are still not totally condensed, the first microtubules/chromatin interactions occur apparently in discrete zones within the euchromatic regions, and that a progressive binding of microtubules occurs in all euchromatic kinetically active regions along with chromosome condensation. The mechanism of microtubule binding in holocentric chromosomes with long kinetochore plates may be similar to that described for monocentric chromosomes. If this is the case, the observation that the heterochromatic regions lacking kinetochore plates do not show affinity for microtubules might assume particular significance regarding the proposition that microtubule turnover is stabilized by mitotic chromatin, rather than by a specific kinetochore activity (Karsenti et al., 1984a, b; Nicklas and Gordon, 1985; Sawin and Mitchison, 1991). In fact, from our results the microtubule stabilizing effect of chromatin might be applicable only to euchromatin. We are now analyzing the microtubule-chromatin interactions in Parascaris first cleavage spindle in more detail.

In conclusion, the present results coupled with those of our previous work clearly show that in Parascaris, under physiological conditions, there is a regulation of kinetochore organization that produces different centromere states in different cell types. Thus, this organelle differs in location, size, and structure in gonial mitotic, meiotic, and presomatic cells. Parascaris chromosomes, therefore, provide a particularly good model for studying centromere organization.

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