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PUMA1: a novel protein that associates with the centrosomes, spindle and centromeres in the nematode *Parascaris*

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SUMMARY

We have identified a 227 kDa spindle- and centromere-associated protein in *Parascaris*, designated PUMA1 (*Parascaris univalens* mitotic apparatus), using a monoclonal antibody (mAb403) generated against *Parascaris* embryonic extracts. PUMA1 distribution was studied by immunofluorescence microscopy in mitotic and meiotic *Parascaris* cells, where centromere organization differs greatly. In mitosis, PUMA1 associates throughout cell division with the centrosomes and kinetochore-microtubules, and it concentrates at the continuous centromere region of the holocentric chromosomes. PUMA1 also localizes to the spindle mid-zone region during anaphase and at the midbody during telophase. In meiosis, PUMA1 associates with the centrosomes and with the discrete centromeric regions lacking kinetochore structures. The analysis of colchicine-treated embryos indicated that the association of PUMA1 with the centromeric region depends on microtubule integrity. mAb403 also recognizes spindle components in *Drosophila*. A series of overlapping cDNAs encoding the gene were isolated from a *Parascaris* embryonic expression library. Analysis of the nucleotide sequence identified an open reading frame capable of encoding a protein of 227 kDa. Analysis of the protein sequence indicated that PUMA1 is predicted to be a coiled-coil protein containing a large central α-helical domain flanked by nonhelical terminal domains. The structural features and cellular distribution of PUMA1 suggest that it may play a role in the organization of the spindle apparatus and in its interaction with the centromere in *Parascaris*.

Key words: Centromere, Spindle, Nematode, *Parascaris*

INTRODUCTION

The spindle apparatus, responsible for chromosome segregation, constitutes a biochemically complex structure that is assembled and maintained at each cell division. Microtubules, nucleated by the centrosomes, are the better characterized components of the spindle apparatus because of their essential and direct participation in chromosome movements. A subset of them, the kinetochore-microtubules which link the spindle poles and the centromere/kinetochore of each chromosome, are particularly involved in chromosome movement from prometaphase through anaphase (for review see Rieder, 1982; Mitchison, 1988). Several mechanisms have been proposed to explain chromosome movement along the kinetochore-microtubules including forces and energy generated by the assembly and disassembly of the microtubules at the kinetochores, and also forces generated by motor molecules acting at the centromere/kinetochore and along the microtubules bundles (for review see Koshland et al., 1988; Mitchison, 1988; Nicklas, 1989; Rieder and Salmon, 1994; Yen and Schaar, 1996). At the centromere/kinetochore of mammalian chromosomes several components have been identified. Some of them (CENP-A, B and C proteins for example), are known to be involved in the assembly and maintenance of the kinetochore structure (for review see Pluta et al., 1995). Three microtubule-dependent motor proteins have been found at the mammalian centromere/kinetochore (for review see Yen and Schaar, 1996). These are the cytoplasmic dynein (Pfarr et al., 1990; Steuer et al., 1990) and the kinesin-related proteins CENP-E (centromere protein E) (Yen et al., 1992) and MCAK (mitotic centromere associated kinesin) (Wordeman and Mitchison, 1995). The anaphase motors dynein and CENP-E are found at the fibrous corona and CENP-E is found in the outer kinetochore plate, the most external regions of the trilaminar kinetochore which are directly involved in microtubule binding (for review see Pluta et al., 1995). In addition to the kinetochore, dynein also distributes to the centrosomes and spindle fibers of mammalian cells (Pfarr et al., 1990; Steuer et al., 1990). CENP-E remains kinetochore-bound throughout mitosis but also associates with the spindle midzone fibers and midbody at anaphase and telophase, respectively (Yen et al., 1992; Yen and Schaar, 1996). CENP-F, a nuclear matrix protein, is also known to localize to the outer kinetochore plate of the mammalian centromere until early anaphase and to relocate then to the midzone and midbody by the end of mitosis (Rattner et al., 1993; Liao et al., 1995).

Several pieces of evidence suggest that, in addition to

microtubules, the structural framework of the spindle contains other nonmicrotubular proteins (for review see Steffen and Linck, 1992). Studies in sea urchin tubulin-depleted spindles revealed the existence of a nonmicrotubule component to which kinesin associates (Leslie et al., 1987). Moreover, immunofluorescence studies in CHO cells showed that the protein spoke defines a novel filamentous structure which apparently runs from near the mitotic spindle poles to each centromere coincident with the kinetochore microtubules (Paddy and Chelsky, 1991). Furthermore, in molluscan and mammalian cells the presence of a tektin-like filamentous spindle component have been reported (Steffen and Linck, 1992). The functional role of all these nonmicrotubular spindle components remains to be established. However, the existence of a spindle matrix that may provide a structural support for the mitotic spindle and may also be important for chromosome movement has already been proposed (for review see Pickett-Heaps et al., 1984; Steffen and Linck, 1992).

We have approached the identification of new components of the mitotic apparatus by generating monoclonal antibodies against total mitotic extracts of *Parascaris* embryos, and using immunofluorescence to isolate those antibodies that recognized the spindle and/or centromeres. We have chosen the nematode *Parascaris* since it is an excellent model to achieve detailed cytological characterization of new molecules during the cell cycle, both in the spindle apparatus and in the chromosomes. Most particularly since in *Parascaris* it is possible to analyze different cell types where structural and functional centromere organization greatly varies (for review see Pimpinelli and Goday, 1989). Previous studies on the ultrastructural organization of the centromere in *Parascaris* mitotic cells (gonial cells and embryonic cells) have revealed the existence of a continuous layered kinetochore plate along the chromatids ('ladder-like' kinetochore) (Goday et al., 1985, 1992). In mitotic gonial cells, this type of kinetochore structure extends the whole length of the chromatids and interacts regularly with numerous parallel microtubules (holocentric chromosomal organization) (Goday et al., 1985). In early embryonic cells, a similar kinetochore structure spans the central euchromatic region of the chromosomes while the heterochromatic chromosomal ends are devoid of centromeric structures (Goday et al., 1992). Studies on this particular type of centromere organization have demonstrated its involvement in the process of heterochromatin elimination in early presomatic blastomeres (Goday and Pimpinelli, 1993). By contrast, in meiotic cells, centromeric activity is restricted to the heterochromatic tips of the chromosomes where microtubules converge and interact with chromatin in the absence of kinetochore structures (Goday and Pimpinelli, 1989).

In this paper, we describe the cellular distribution of a protein recognized by a new monoclonal antibody (mAb403) in *Parascaris univalens* mitotic embryonic cells and meiotic cells. The results showed that the antigen recognized by mAb403, which we designate PUMA1 (for *Parascaris univalens* mitotic apparatus), associates with the centrosomes, kinetochore-microtubules and centromere regions throughout cell division. Moreover, PUMA1 localizes to the centromere regions both in mitotic and meiotic cells, irrespective of the presence of organized kinetochores. The analysis of colchicine-treated cells suggested that the presence of PUMA1 at the centromere is microtubule-dependent. We have also analyzed the spindle staining pattern of mAb403 in *Drosophila* cells. To further study the 403-antigen we have screened a *P. univalens* embryonic cDNA expression library and have cloned a gene encoding a putative 227 kDa protein. Polyclonal antibodies raised against the bacterially expressed fusion protein and against a synthetic peptide were generated to prove that the cloned gene encodes the 403 antigen. Our analysis predicts that PUMA1 contains a long central coiled-coil domain and shares sequence similarities with myosin heavy chains, the nematode protein Ant1 (Triteeraprapab et al., 1995), the mammalian proteins CENP-E (Yen et al., 1992), CENP-F (Rattner et al., 1993) and the nuclear mitotic apparatus protein NuMA (Lyderson and Pettijohn, 1980). Based on our data, we propose that PUMA1 constitutes a new coiled-coil protein involved in the spindle apparatus architecture and its interaction with centromeric regions in *Parascaris*.

MATERIALS AND METHODS

Live specimens of *P. univalens* were collected at the local abattoir along with horse intestinal contents. The worms were washed and maintained at 37°C in an isotonic salt solution (0.7% NaCl). As soon as possible after collection, the animals were dissected to remove the gonads.

Production of monoclonal antibody 403 (mAb403)

Fertilized eggs were extracted from the uteri, dechorionated and grown at 37°C to obtain embryos at first division stage as previously described (Goday et al., 1992). A total protein extract from approximately 2×10^5 embryos was prepared by sonication for 20 minutes on ice. The protein extract was emulsified in Freund's complete adjuvant and two mice were injected intraperitoneally. Protein extract for subsequent intraperitoneal boosts were emulsified in Freund's incomplete adjuvant. Finally, the mice were injected twice intravenously. 1 day after the final boost, the spleen was removed and fused for the production of monoclonal antisera as previously described (Köhler and Milstein, 1976). Hybridoma cells producing mAb403 were subcloned by limiting dilution and expanded. The isotypic characterization of mAb403 was performed by using the SBA Clonotyping System II kit (Southern Biotechnology Associates, Inc.) and ELISA technique. mAb403 purification was performed by gel filtration (Hiload Sephacryl HR, Pharmacia).

Indirect immunofluorescence

Fixation of embryos and male meiotic cells of *P. univalens*

Decorionated fertilized eggs were grown at 37ºC to obtain embryos at different early developmental stages and fixed in 100% methanol as previously described (Goday et al., 1992). Embryos were washed in PBS at room temperature (3× 5 minutes), treated with 1% Triton X-100 in PBS for 10 minutes and subsequently washed in PBS. Before incubation with antibodies, embryos were treated with 1% BSA (bovine serum albumin) in PBS for at least 30 minutes.

Meiotic cells were obtained by dissecting testes in 0.7% NaCl. After squashing, slides were frozen in liquid N_2 to remove coverslips and immediately fixed in 100% methanol at –20°C for 10 minutes or in 4% paraformaldehyde in PBS for 15 minutes at room temperature. The slides were washed in PBS (3× 5 minutes), treated with 1% Triton X-100 in PBS for 10 minutes at room temperature and subsequently washed in PBS. They were incubated for 30 minutes at room temperature with 3% nonfat dried milk in PBS, and then incubated with antibodies as described below.

Fixation of embryos and S2 culture cells of *D. melanogaster*

Wild-type Oregon embryos were collected for 2 hours to enrich for

the presence of syncytial blastoderm embryos. Embryos were dechorionated and fixed in methanol-EGTA as described (Warn and Warn, 1986). Following fixation embryos were washed in PBS containing 0.1% Triton X-100 (3× 15 minutes) and incubated with 1% BSA, 2% fetal calf serum, 0.3% Triton X-100 in PBS for 1 hour at room temperature.

Drosophila Schneider line 2 (S2) tissue culture cells were cytocentrifuged at 700 rpm for 10 minutes. Slides were fixed in 100% methanol at –20°C for 10 minutes and treated with acetone at –20°C for 1 minute. They were washed in PBS (3×15 minutes) and incubated in 1% BSA in PBS for 30 minutes at room temperature.

Antibody incubation

Primary and secondary antibodies were diluted in 3% BSA in PBS as follows: mAb403 (1:5), FITC-conjugated anti-mouse IgM (1:100) (Southern Biotechnology Associates, Inc.), anti-tubulin YL1/2 (1:100) (Sera-Lab), rhodamine-conjugated anti-rat (1:100) (Sigma), polyclonal antibodies polyI and poly2 (1:5), FITC-conjugated anti-mouse Ig (1:25) (Southern Biotechnology Associates, Inc.). Incubation with primary antibody was performed for 1-2 hours at room temperature or overnight at 4°C. Slides and embryo suspensions were washed three times in PBS and incubated with secondary antibody for 45 minutes at room temperature. They were washed in PBS and, finally, PBS was replaced by the staining solution. To perform double staining with a second primary antibody, the incubation process was repeated before staining. The staining solution consisted of 20 parts 1 mg/ml *p*-phenylenediamine in a 1:10 (v/v) mixture of PBS:glycerol; 1 part 2 μg/ml Hoechst 33258 in distilled water. The slides and embryos were stained for 10 minutes, coverslips were placed over the samples and the preparations were sealed with rubber cement.

Fluorescence microscopy observation and photography

Observations were made using a computer-controlled Zeiss Axioscope epifluorescence microscope equipped with a cooled charge coupled device (CCD) camera (Photometrics). FITC, Rhd and Hoechst fluorescence, detected by specific filter combinations, were recorded separately as gray scale images and when needed, digitally pseudocolored and merged.

Colchicine treatment of *P. univalens* embryos

Dechorionated fertilized eggs in M9 buffer, pH 7 (Brenner, 1974) were transferred to Petri dishes, incubated at 37°C for 2 hours to obtain embryos with two interphasic blastomeres. Colchicine (Sigma) was added to 2 mg/ml culture medium and embryos were incubated for 2-3 hours. Embryos were fixed and processed for immunofluorescence as described above.

Electrophoresis and western blot

Samples of total proteins from *P. univalens* embryos and gonads, *C. elegans* larvae and *D. melanogaster* larvae, were prepared by homogenization in sample buffer with 2 mM PMSF (phenylmethylsulfonyl fluoride) or RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) with 2 mM PMSF and 100 μg/ml of leupeptin, pepstatin, and aprotinin. SDS-polyacrylamide gels (10%) (Laemmli, 1970) were blotted onto nitrocellulose (Towbin et al., 1979), blocked with 3% BSA in PBS and probed either with mAb403 (1:100), polyI (1:500) or poly2 (1:500) for 3 hours at room temperature. After washing in 0.05% Tween-20 in PBS (TPBS), filters were incubated with the secondary antibody anti-mouse IgM conjugated to alkaline phosphatase (1:10,000) (Jackson Immunochemicals) or anti-mouse Ig conjugated to alkaline phosphatase (1:4,000) (Southern Biotechnology Associates, Inc.) for 90 minutes at room temperature. Filters were washed and developed with BCIP/NBT (bromochloroindolyl phosphate/nitro blue tetrazolium) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) as described previously (Harlow and Lane, 1988). Filters were photographed with Microfilm (Kodak).

cDNA library construction and identification of cDNA clones by expression library screening

cDNA complementary to poly(A)$^+$ mRNA of *P. univalens* extracted from gonads was synthesized and cloned in λ-ZAP II (Stratagene), according to protocols provided by the manufacturer. About 600,000 plaque-forming units (pfus) were plated and transferred to IPTG (isopropylthio-β-D-galactoside)-impregnated nitrocellulose filters. After blocking with 3% BSA in PBS, the filters were incubated with mAb403 (1:100) overnight at 4°C. They were washed in TPBS and incubated with the secondary antibody anti-mouse IgM conjugated to alkaline phosphatase (1:10,000) (Southern Biotechnology Associates, Inc.) for 90 minutes at room temperature. The filters were washed and developed as described for western blot. After plaque purification, the 2.5 kb cDNA insert corresponding to clone 403Pu-I was excised into pBluescript SKII plasmid in situ by superinfection with helper-phage (Short et al., 1988).

The 403Pu-I cDNA was then used to rescreen the same library by DNA hybridization using standard methods (Sambrook et al., 1989). The 5′ 600 bp portion of this cDNA was amplified by PCR and used as a probe for the library. The DNA probe was radiolabeled by random priming as described (Feinberg and Vogelstein, 1983). After hybridization, the filters were washed and exposed to Kodak X-OMAT S film at –80°C overnight. After the first DNA hybridization screening, clones 403Pu-4.2 and 403Pu-8.1 were isolated and excised. The 5′ 700 bp fragment of clone 403Pu-8.1 was amplified by PCR and used to rescreen again the same library. After the third screening, clones 403Pu-3.2 and 403Pu-4.1 were isolated and excised into pBluescript SKII.

Generation of polyclonal antibodies against the bacterially expressed protein and a synthetic peptide

The 403-I cDNA clone in pBluescript was transformed into SOLR *Escherichia coli* strain. Expression of the cDNA-encoded protein was induced by growing bacterial cultures to an OD_{600} of 0.2-0.5 and then adding IPTG to a final concentration of 20 mM. The cultures were grown for 6 hours, bacteria were harvested by centrifugation and resuspended in 9 volumes of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% sacarose, 1 mM PMSF). Lysozyme was then added to a final concentration of 1 mg/ml and the tubes were incubated for 20 minutes at room temperature. The lysates were centrifugated at 15,000 rpm for 15 minutes at 4°C. The pellet was resuspended in lysis buffer, $MgCl_2$ and DNAse I were added to a final concentration of 8 mM and 25 μg/ml, respectively. After incubation at 4°C for 2 hours and centrifuging, the pellets were washed twice with lysis buffer, resuspended in lysis buffer without sacarose, Triton X-100 was added to a final concentration of 1% and the purified fusion protein was immediately frozen. To generate polyclonal antibodies to the bacterially expressed protein, 20 μg of the purified protein extracts were mixed with Freund's Adjuvant and injected subcutaneously into Balb/C females mice at 7-day intervals. Serum were obtained by bleeding and used to perform immunocytochemistry and western blot analysis.

The peptide KEAYRTSSTIKSSEGTTRE extending from amino acid 1,874 to 1,892 in the carboxy-terminal region of the predicted protein PUMA1 was synthesized and coupled to the carrier protein KLH (keyhole limpet hemacyanin) following the standard protocols of the manufacturer. To generate polyclonal antibodies, 100 μg of the peptide coupled to KLH were mixed with Freund's adjuvant and injected intraperitoneally into Balb/C females mice at 7-day intervals. Serum were obtained by bleeding and used to perform immunocytochemistry and western blot analysis.

DNA sequencing

Sequencing reactions were performed using the dideoxynucleotide termination method (Sanger et al., 1977) as modified for AmpliTaq

DNA Polymerase, FS (Amersham) by using the fluorescent dideoxy terminator method of cycle sequencing on an Applied Biosystems 377 automated DNA sequencer (Perkin Elmer). The reported nucleotide sequences were confirmed by obtaining the complete sequence of both strands of cDNAs. Database searches were performed with BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988). Prediction of secondary structure was performed using a program based on Garnier or Chou Fasman algorithms (Chou and Fasman, 1974; Garnier et al., 1978; Rost and Sander, 1993).

Northern blotting

50 μg of *Parascaris* total RNA was isolated from gonadal tissue as described (Sambrook et al., 1989). RNA was size-fractionated by electrophoresis in a formaldehyde agarose gel, transferred onto nitrocellulose and the filters were hybridized with the cDNA corresponding to clone 403Pu-I as a probe under the conditions described above.

RESULTS

Immunolocalization of mAb403 in *P. univalens* mitotic and meiotic divisions

Fig. 1 shows the distribution of mAb403 in embryos at the pronuclear stage (Fig. 1A, B) and at first prometaphase (Fig. 1C,D). At both stages the mAb403 associates to the pericentriolar domain of the centrosomes. At prometaphase, in addition to centrosomes, mAb403 also labels the cytoplasmic region where chromosomes lie (Fig. 1C,D). To analyze the staining pattern of mAb403 with respect to the spindle, triple staining with mAb403, anti-tubulin and Hoechst was performed in embryos from first metaphase to early telophase stages (Fig. 2). At metaphase (Fig. 2A), mAb403 decorates the poles and kinetochore-microtubules (kt-microtubules). In addition, a significant accumulation of the antibody signal is seen at the centromeric regions of the two chromosomes. As described elsewhere (Goday et al., 1992), the centromere in *Parascaris* blastomeres prior to chromosome diminution extends along the whole euchromatic central region of the chromosomes and contains a continuous layered kinetochore plate. The rest of the chromosome corresponds to heterochromatin and is devoid of centromere. Moreover, in contrast to mAb403, anti-tubulin antibodies do not react with the centromeric regions (Fig. 2B). This was clearly seen when the two staining patterns were superimposed (Fig. 2C). The association of the mAb403 to the spindle and to the centromeric regions of *Parascaris* chromosomes is maintained during anaphase movements (Fig. 2D,G). In some anaphase figures, as in Fig. 2G, where it is possible to observe the two chromatids reaching the pole separately, it is clear that mAb403 recognizes the centromeric region of each chromatid. In addition, from anaphase on, mAb403 also recognizes fibrillar-like structures at the equatorial region (Fig. 2D,G) where abundant interzonal microtubules were described to occur (Goday et al., 1992) as shown in Fig. 3. As chromosome segregation proceeds to telophase, mAb403 labeling at the midzone becomes progressively restricted to the midbody site (Fig. 2J). From these results, we concluded that the antigen recognized by mAb403 associates with the mitotic apparatus and the centromeric regions of *Parascaris* mitotic chromosomes. mAb403 cytological pattern was also investigated in more advanced embryos where presomatic blastomeres undergo chromosome fragmentation during the process of chromatin diminution (Fig. 4). Fig. 4A shows, again, that at metaphase in addition to the spindle, mAb403 decorates numerous discrete regions, devoid of anti-tubulin staining (Fig. 4B,C), which correspond to the centromeres of the newly formed small somatic chromosomes laying at the equatorial plate. These chromosomes at the EM level have been described to possess a continuous layered kinetochore plate along each chromatid and to segregate independently (Goday et al., 1992). At anaphase (Fig. 4D,E, F), the mAb403 labeling pattern at the centromeric regions resembles that observed for prediminuted intact chromosomes (Fig. 2D). That is, a single and extended centromeric fluorescent signal is observed. This undoubtedly reflects, as previously described (Goday et al., 1992), the parallel anaphase migration to the poles that exhibit the somatic group of chromatids. Therefore, our results indicate that the staining pattern of mAb403 is the same for presomatic and somatic cells.

Furthermore, we have investigated the distribution of mAb403 in *Parascaris* male meiotic cells, since during meiosis centromeric activity is restricted to the heterochromatic tips of the chromosomes, that are capable of binding microtubules despite their lack of kinetochore

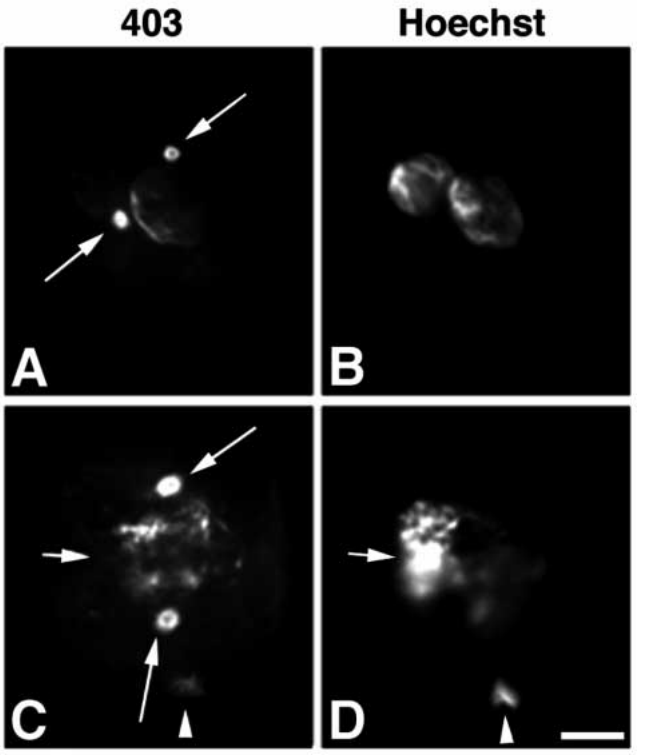


Fig. 1. Indirect immunofluorescence staining of mAb403 (A,C) and Hoechst 33258 chromosome staining (B,D) in *P. univalens* early embryos. (A,B) At pronuclear stage mAb403 decorates the pericentriolar domain of the centrosomes that initiate separation around the juxtaposed parental nuclei (long arrows); (C,D) first prometaphase. Centrosomes are already oriented along the embryo division axis; mAb403 labeling is seen at the centrosomes (long arrows) and also at the cytoplasmic region that corresponds to the condensing chromosomes (short arrows). Note that mAb403 also reacts with a discrete cytoplasmic region that colocalizes with the polar body (arrowheads). Bar, 15 μm.

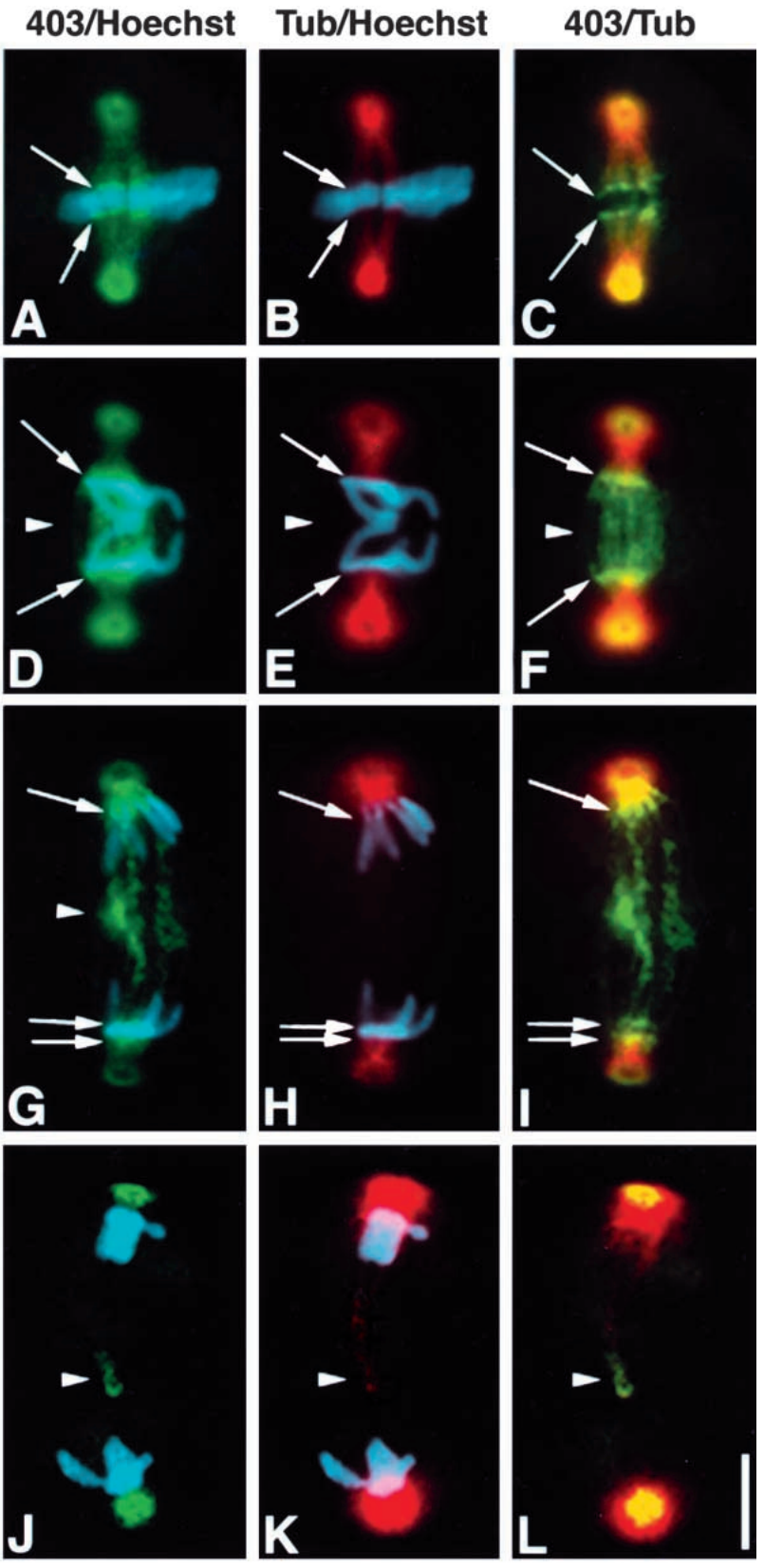


Fig. 2. First embryonic division of *P. univalens*. Triple staining with mAb403 (green), anti-tubulin antibodies (red) and Hoechst 33258 chromosome staining (blue). (A-C) Metaphase; mAb403 associates to the centrosomes, kinetochore-microtubules and concentrates at the centromeric regions of the two chromosomes (arrows in A). Note that the mAb403 stained centromeric regions are devoid of tubulin staining (arrows in C). (D-F) Anaphase; mAb403 associates with the centrosomes, kinetochore-microtubules, and the centromeric regions (arrows in D) that are tubulin-dull (arrows in E and F). mAb403 also recognizes fibrillar-like structures at the midzone region of the cell (arrowheads). (G-I) Late anaphase; mAb403 associates with the centromere region of each single chromatid (arrows in G) that is tubulin-dull and corresponds to the euchromatic central region of the chromosomes (arrows in H). (J-L) Telophase; mAb403 associates with the centrosomes and the midbody in coincidence with polar microtubules (arrowheads). Bar, 15 μm.

structures (Goday and Pimpinelli, 1989). Fig. 5 shows the localization of mAb403 in meiotic cells at metaphase (Fig. 5A) and at anaphase (Fig. 5D). At both stages mAb403 decorates brightly the centrosomes, faintly the kt-microtubules and, again, it concentrates at discrete regions that correspond to the centromeric regions of the meiotic chromosomes. As found in mitosis, the centromeric regions of the meiotic chromosomes are brightly labeled by mAb403 but

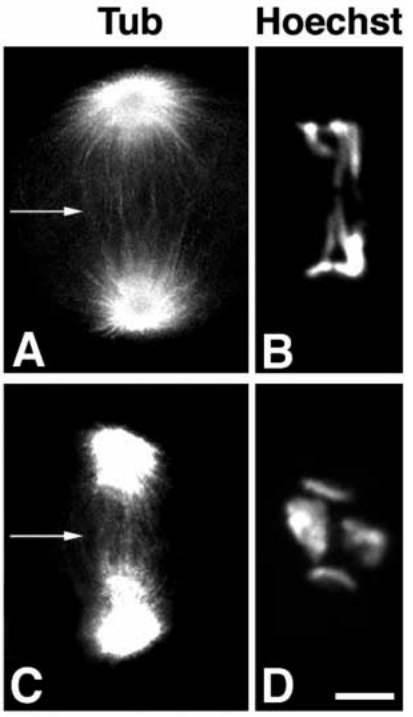


Fig. 3. Spindle staining with anti-tubulin antibodies (A,C) and Hoechst 33258 chromosome staining (B,D) of *P. univalens* embryos. (A,B) First embryonic division at anaphase, and (C,D) a blastomere undergoing chromatin diminution at anaphase. The arrows point to the mid-zone microtubules. Bar, 7 μm.

are lightly-stained by anti-tubulin antibodies (Fig. 5B,E). This was confirmed when the two staining patterns were superimposed (Fig. 5C,F). The cytological staining pattern of mAb403 described above for mitotic and meiotic cells was the same when higher dilutions of the antibody (1:100, 1:500) were used (data not shown). Taken together, our data indicated that the antigen recognized by mAb403 associates to both mitotic and meiotic spindle and to the centromeric regions of *Parascaris* chromosomes irrespective of their chromosomal location and ultrastructural organization.

In view of these results we have investigated whether the observed mAb403 pattern is dependent on the presence of intact microtubules. Disruption of spindle microtubules by a microtubule-destabilizing drug (colchicine) resulted in the displacement of mAb403 staining from the centromeric chromosomal sites to disperse sites in the cytoplasm (Fig. 6). This suggested that the cellular distribution of the mAb403 antigen during cell division is microtubule dependent.

Immunolocalization of mAb403 in *Drosophila*

Fig. 7 shows the distribution of mAb403 in *Drosophila* S_2 cells and in *Drosophila* embryos at the syncitial stage. In S_2 cells (Fig. 7A,B), mAb403 recognizes the centrosomes in dividing cells and apparently also in interphase cells. In addition, at telophase it is often possible to detect midbody staining (Fig. 7A). In embryonic mitotic cells, mAb403 associates with the centrosomes and the spindle microtubules (Fig. 7C,D). However, at this resolution it is not possible to discern whether centromeres of the chromosomes are specifically labeled by mAb403.

Western blot analysis of mAb403 in *P. univalens*, *C. elegans* and *Drosophila*

Fig. 8A shows that mAb403 in *P. univalens embryonic* total protein extracts (Pu) recognizes a major band with an apparent molecular mass of 220 kDa. The same result was obtained when a higher dilution of mAb403 (1:500) was used (data not shown). In *C. elegans* larvae total protein extracts (Ce), two bands of aprox. 230 and 106 kDa are stained by mAb403. A single polypeptide is identified in *D. melanogaster* (larvae total protein extracts, Dm) corresponding to aprox. 80 kDa.

Isolation and molecular characterization of the cDNA encoding the 403 antigen (PUMA1) in *P. univalens*

In order to learn about the sequence encoding PUMA1, an

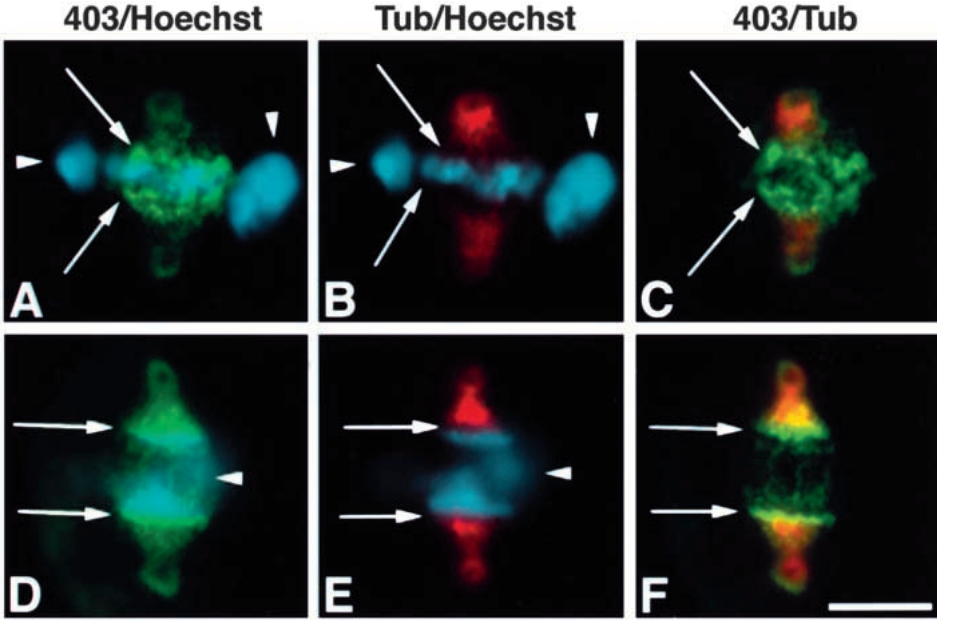


Fig. 4. Presomatic blastomeres of *P. univalens* embryos undergoing chromatin diminution. Triple staining with mAb403 (green), anti-tubulin antibodies (red) and Hoechst 33258 chromosome staining (blue). (A-C) Metaphase; mAb403 associates with the centrosomes, kinetochore microtubules and the centromeric regions of the newly formed small and numerous euchromatic chromosomes (arrows in A and C). Note that the bright mAb403 centromeric staining corresponds to tubulin-dull staining (arrows in B). Arrowheads in A and B point to the excised heterochromatic terminal blocks that will remain at the equatorial plate to be eliminated. (D-F) Anaphase showing the persistent mAb403 fluorescent signal at the centromeric regions (long arrows in D and F) corresponding to the alignment of each centromere of the somatic group of chromatids that migrate parallel to the poles. Note that the centromere labeling coincides with a dull-tubulin area (arrows in E). Arrowhead in D points to the fibrillar-like structures stained by mAb403 at the midzone region. Bar, 15 μm.

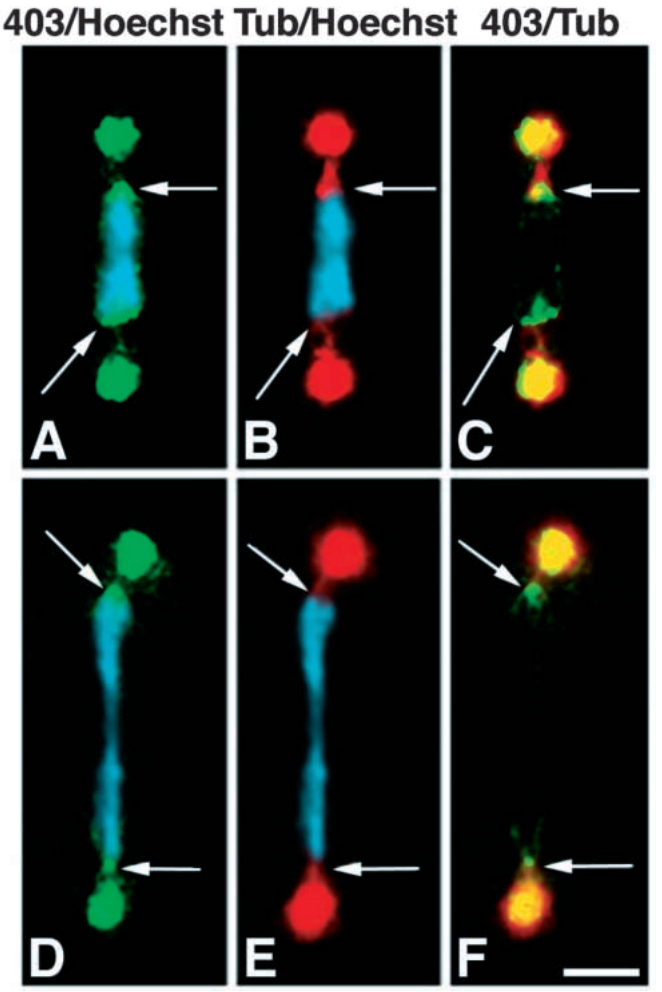


Fig. 5. (A-F) First male meiotic division of *P. univalens*. Triple staining with mAb403 (green), anti-tubulin antibodies (red) and Hoechst 33258 chromosome staining (blue). (A-C) Metaphase I; mAb403 associates with the centrosomes, kinetochore-microtubules and the terminal centromeric regions of the bivalent (arrows). Note that the accumulation of mAb403 staining at the centromere sites (arrows in A) corresponds to tubulin-dull staining (arrows in B). This is particularly clear in the superimposition of mAb403 and tubulin patterns shown in C. (D-F) Anaphase I showing a similar staining pattern. Arrows point to the centromeres. Bar, 15 μm.

immunoscreening of a *P. univalens* λ-ZAP expression library using mAb403 as a probe was performed. A positive clone (403Pu-I) was isolated from a total of 500,000 recombinant phage screened. Clone 403Pu-I contained a 2.5 kb insert. This cDNA was not full length and we used the bacterially expressed fusion protein to obtain polyclonal antibodies to establish that this clone partially encoded the authentic antigen to which the 403 antibody was directed. The obtained sera contained antibodies that in *P. univalens* total protein extracts specifically recognized a protein of >200 kDa (Fig. 8B, polyI) that was not detected by the preimmune sera (Fig. 8B, preI). The same result was obtained with a lower dilution of polyI and preimmune sera (1:100) (data not shown). This immunoreactive protein has the same apparent molecular mass as the protein detected by the mAb403 (Fig. 8A, Pu). The immunofluorescence staining patterns of the polyclonal antibodies in *P. univalens* embryos (Fig. 9A) were similar to the pattern observed with the mAb403, while preimmune sera failed to exhibit any such staining (not shown). These results indicated that 403Pu-I cDNA clone encodes an authentic segment of PUMA1.

In addition, based on the preliminary homology results obtained from the database searches with the partial predicted protein encoded by clone 403Pu-I (see below), we used a synthetic peptide located in the carboxyl-terminal region of the 403Pu-I predicted protein (KEAYRTSSTIKSSEGTTRE from amino acid 1,874 to 1,892, in Fig. 10B) to obtain polyclonal antibodies. As we report below, the terminal domain of the predicted protein differs considerably from the most related proteins in other nematodes. Thus, polyclonal antibodies raised against this part of the molecule should recognize preferentially PUMA1. Consistently, both cytological (Fig. 9C) and western blot analysis (Fig. 8B, poly2) using the obtained

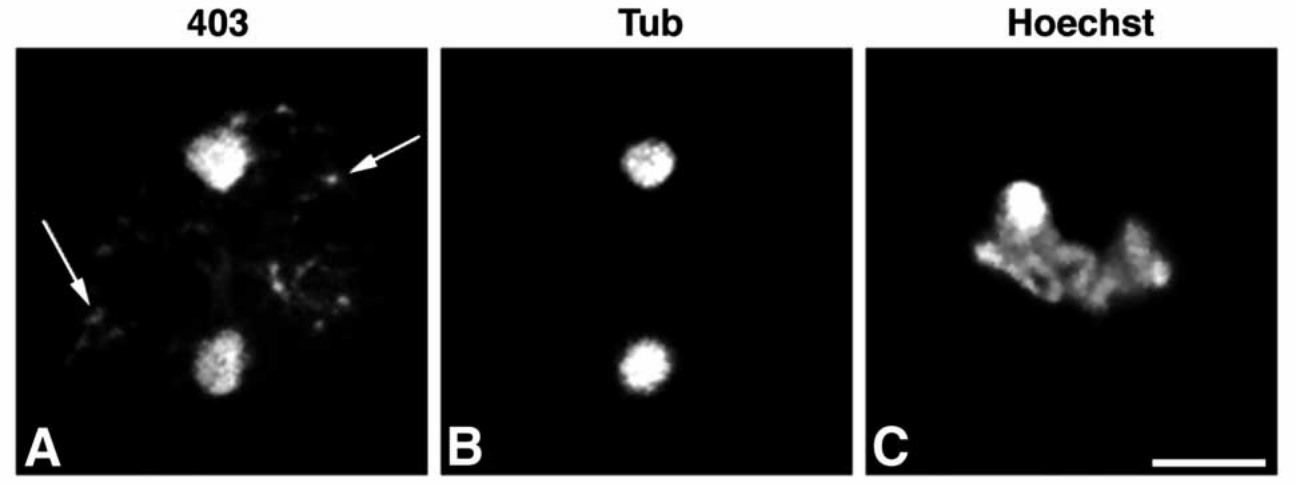


Fig. 6. *P. univalens* embryo treated with colchicine during the first embryonic division. Triple staining with mAb403 (A), anti-tubulin antibodies (B), and Hoechst 33258 chromosome staining (C). mAb403 decorates the centrosomes and disperse cytoplasmic sites (arrows in A). Note that no spindle microtubules are present (B) and chromosomes are not fully condensed (C). Bar, 15 μm.

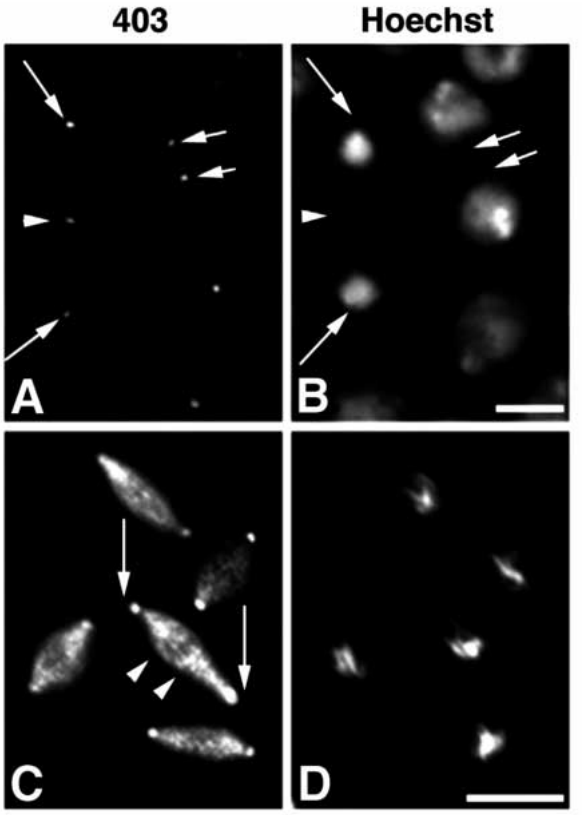


Fig. 7. *D. melanogaster* S_2 cells (A,B) and an embryo at syncitial stage (C,D) stained with mAb403 (A,C) and Hoechst 33258 (B,D). In S_2 cells in telophase, mAb403 reveals the centrosomes (long arrows in A and B) and the midbody (arrowhead in A and B). At interphase, fluorescent signals apparently corresponding to centrosomes can be observed (short arrows in A and B). In embryos at metaphase (C,D) mAb403 decorates the centrosomes (long arrows) and the spindle microtubules (arrowheads). Bars, 25 μm.

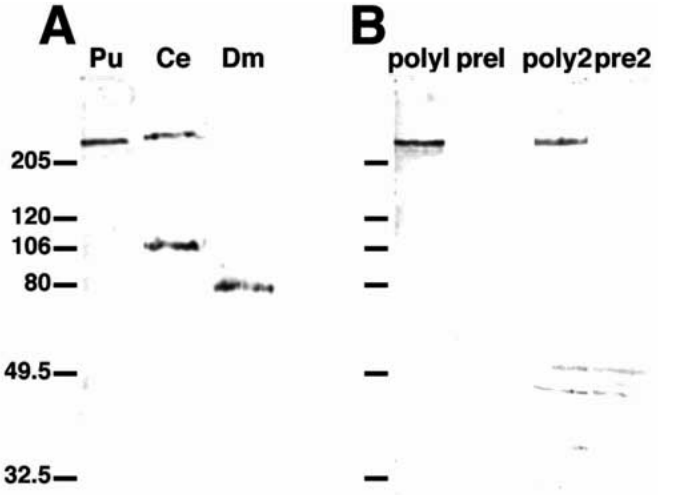


Fig. 8. (A) Western blot of total protein extracts from *P. univalens* embryos (Pu) *Caenorhabditis elegans* larvae (Ce) and *Drosophila* larvae (Dm) immunostained with mAb403. (B) Western blot of total protein extracts from *P. univalens* embryos immunostained with polyclonal antibodies raised against the 403Pu-I fusion protein (polyI) and preimmune serum I (preI) and polyclonal antibodies against a synthetic peptide located at the carboxyl terminus of 403Pu-I protein (poly2) and preimmune serum (pre2). Positions of molecular mass standards (kDa) are indicated.

sera were equivalent to those obtained with polyclonal antibodies against the 403Pu-I fusion protein. Therefore, we concluded that the clone 403Pu-I undoubtedly encoded PUMA1 partially.

The cDNA of clone 403Pu-I was then used to rescreen the same cDNA library by DNA hybridization. Two successive 'walking' experiments (see Materials and Methods) were needed to isolate clones that allowed us to obtain the full coding sequence for PUMA1 (Fig. 10A). Fig. 10B shows the cDNA encoding PUMA1 and the deduced amino acid sequence. The putative ATG initiation codon is located at nucleotide 39 and is preceded by two in-frame translation terminators. The nucleotides at this position fulfilled the criteria for a strong ribosomal initiation site that should contain A/G at position −3 and G at position +4 (Kozak, 1986). An in-frame TAG termination codon is found at position 5,904, giving an open reading frame of 5,865 base pairs, encoding 1,955 amino acids. The calculated molecular mass of the protein is 226,964 Da, which is in good agreement with the apparent molecular mass for PUMA1 in *P. univalens* determined from SDS-PAGE. Several stop codons in all reading frames are contained in the 3′-noncoding region. The large size of the 403 mRNA is consistent with the aproximately 6 kb single transcript observed by RNA blot analysis of *Parascaris* gonadal RNA using the 2.5 kb probe of clone 403Pu-I (data not shown).

Features of the deduced PUMA1 sequence

The predicted amino acid sequence of PUMA1 revealed a high content of positively and negatively charged amino acids. About 19% are positively charged and 20% are negatively charged and they are distributed mostly along the central region of the molecule. Secondary structure predictions (Chou and Fasman, 1974; Garnier et al., 1978; Rost and Sander, 1993) indicated that PUMA1 is predicted to have three structural domains, with two terminal globular segments separated by a discontinuous ~1,750 amino acid α-helical region (drawn schematically in Fig. 10C). Using the BLAST and FASTA algorithms (Pearson and Lipman, 1988; Altschul et al., 1990) to search the EMBL and GenBank databases for DNA or protein sequences similar to PUMA1, a 55% identity at the DNA level was found with the *ovt1* gene of the nematode *Onchocerca volvulus* (Triteeraprapab et al., 1995) along the central part of the PUMA1 cDNA. At the protein level, both proteins (PUMA1 and the myosin-like Ant1 encoded by the *ovt1* gene) share a 41% identity and 65% similarity. PUMA1 also shares a 40% identity and 62% similarity with a *Caenorhabditis elegans* predicted protein (EMBL: U64862; SPTREMBL: Q23081) that also shows a strong similarity to *O. volvulus* Ant1. These results suggest that we have cloned in *P. univalens* a protein similar to the myosin-like Ant1 in *O. volvulus*. Comparative sequence analysis of the three proteins (data not shown), revealed that the central regions of the molecules share the strongest identity and similarity but the terminal domains differ considerably. PUMA1 predicted protein also shows similarity to proteins such as myosins,

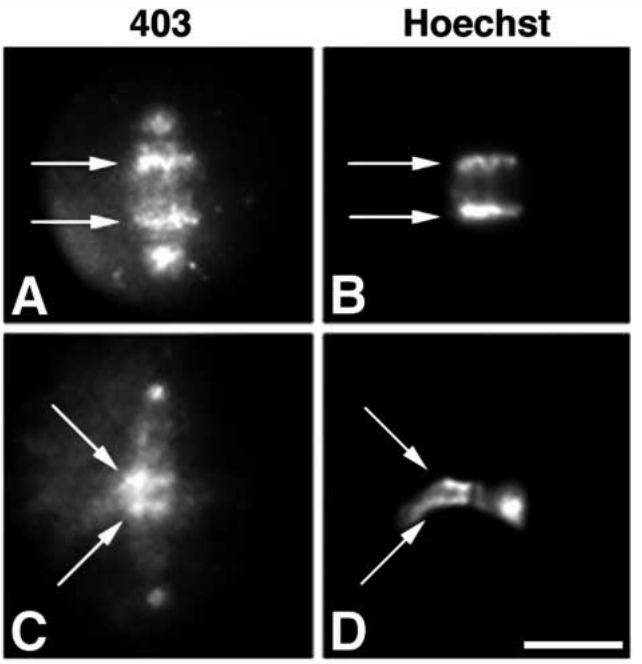


Fig. 9. *Parascaris* embryos stained with a polyclonal antibody (polyI) raised against the 403Pu-I fusion protein (A), and a polyclonal antibody (poly2) raised against a synthetic peptide (C), and simultaneous Hoechst 33258 chromosome staining (B and D). (A,B) Somatic mitotic division in an isolated blastomere after chromatin diminution occurrence. polyI recognizes the centrosomes and concentrates at the centromeric regions of the small chromosomes that migrate parallel to the poles (arrows); kinetochore-microtubules are faintly stained. (C,D) First embryonic division. Metaphase showing the association of poly2 with the centrosomes, kinetochore-microtubules and the accumulation at central centromeric regions of the two chromosomes (arrows). Bar, 15 μm.

paramyosins, MLP1, CENP-E, CENP-F and NuMa. PUMA1 shares the highest sequence similarities with myosin heavy chains, e.g. when compared to human myosin heavy chain from fast skeletal muscle, the PUMA1 sequence exhibited 23% identity over a 1,135 amino acid region. A 20% identity was found with CENP-E, CENP-F and NuMa. All these proteins have been shown or predicted to form long coiled-coils. The similarity of PUMA1 with all of them may be due to the presence of coiled-coil domains in PUMA1. In a coiled-coil structure, long α-helices of two molecules dimerize through hydrophobic interactions based on heptad periodicity (Cohen and Parry, 1986, 1990). The study of the central part of the PUMA1 sequence reveals that it is organized in heptad repeats suggesting that PUMA1 is a coiled-coil protein. The periodicity of the heptad repeats is interrupted by short segments containing prolines. Since prolines usually disrupt an α-helix, the former region of PUMA1 may be divided into five α-helical segments (Fig. 10C). In addition, in the deduced protein sequence of PUMA1, three repeats of the hypothetical leucine zipper sequence are present at positions 744-765, 1,610-1,630 and 1,715-1,736, respectively.

Many protein kinase recognition sequence motifs are also present. The most interesting of these is a potential phosphorylation site for $p34^{cdc2}$ kinase (S/TPXZ; where X is a polar amino acid and Z is generally basic; Moreno and Nurse, 1990) located at the amino-terminal globular domain (amino acids 16-19). This suggests that PUMA1 may be a cell cycle-dependent phosphorylation substrate.

An additional feature of the PUMA1 amino terminus is a potential GTP-binding site (amino acids 62-69) (Robinshaw et al., 1986). A potential nuclear localization signal, KRLK, is also located at position 532-535 (Chelsky et al., 1989).

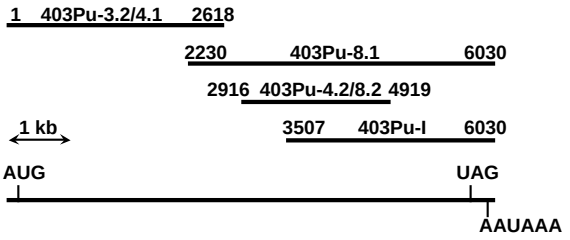
DISCUSSION

We have identified a novel nonmicrotubular protein which we have named PUMA1 that associates with the spindle apparatus and with the centromere in *Parascaris*. Analysis of the 6 kb PUMA1 cDNA showed that it encodes a protein of 1,955 amino acids with a calculated molecular mass of 227 kDa. From our sequence analysis PUMA1 is a new member of a large group of myosin-like proteins that share a high content of potential coiled-coil structures similar to myosin and other fibrous proteins such as tropomyosins, cytokeratins and intermediate filaments.

From our cytological analysis, PUMA1 is a cytoplasmic protein that colocalizes with the mitotic apparatus of *Parascaris* coincident with its assembly following nuclear membrane breakdown. An interesting feature is that PUMA1 associates with the centrosomes, kinetochore-microtubules and centromeric region throughout mitosis. We note that most of the previously described spindle- and centromere-associated antigens display a transitory cellular distribution; that is, they localize to different regions of the mitotic apparatus at specific stages of mitosis (for review see Earnshaw and Bernat, 1991; Kalt and Schliwa, 1993; Rattner et al., 1993). With respect to the centromeric localization of PUMA1, the present data revealed a notable accumulation of PUMA1 at the plus-end of the kinetochore-microtubules in the centromeric regions of *Parascaris* mitotic cells. This leads us to question whether PUMA1 might become a structural component of the mature kinetochore plate once the nuclear membrane is disassembled. In this respect, the fact that PUMA1 centromeric staining is abolished in cells treated with mitotic inhibitors suggests that PUMA1 is not a structural component of the kinetochore itself. Instead, its presence at/on the centromere/kinetochore site apparently depends on the existence of kinetochore-microtubule interactions. This view is further supported by the analysis of PUMA1 distribution in *Parascaris* meiotic cells, where the centromeres lack kinetochore plates and centromeric activity is achieved by ‘direct insertion’ of microtubules into chromatin (Goday and Pimpinelli, 1989). In these cells, we have found, again, that PUMA1 accumulates at the plus-end of microtubules at the centromere sites. Taken together, the data strongly suggest that, in *Parascaris*, the association of PUMA1 with the centromere sites is correlated to its attachment to the spindle microtubules. Thus, it does not depend on the type of centromere organization but, most probably, on its functional capacities.

Clues as to the possible functions of PUMA1 can be inferred from its subcellular distribution during the cell cycle and comparison with other proteins which associate with the mitotic apparatus, as well as from relevant features of the predicted PUMA1 protein sequence. Since PUMA1, in

A



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B

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                                                                                                  oooooooooo

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                                                                                                  ======================

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1138 N  E  K  I  E  K  L  R  I  E  H  D  H  L  E  R  D  Y  R  E  K  T  K  E  V  D  R  L  K  E  V  E  K  T  F  E  L  K  V  N  R  A  R  Q  E  L  D  E  F  S

3601 AGAAGTTGATAGTCACAGAAACGGAGCGTAATGCTATTAGTGGAGAAGCACAAAAATTGGATAAGGAGGTGCAATTGGTGAAGGAGCAACTGCAATACAAGAGTGATGAATTCCATAAGGCGCTAGATGAACTCGCGAACGCTCATCGAA
1188 K  K  L  I  V  T  E  T  E  R  N  A  I  S  G  E  A  Q  K  L  D  K  E  V  Q  L  V  K  E  Q  L  Q  Y  K  S  D  E  F  H  K  A  L  D  E  L  A  N  A  H  R

3751 TCTCCGAAGAGGGACGTGTTAATGCGATTCATCAACTAGAAGCTAGAAGATTCGAAATTGATGATTTGAAGTCGCGTCTTGAGAACTCCGAGCAACGTCTGGCAACACTGCAACAAGAATACGTGAATGCCGATAAAGAACGAGGCGCTT
1238 I  S  E  E  G  R  V  N  A  I  H  Q  L  E  A  R  R  F  E  I  D  D  L  K  S  R  L  E  N  S  E  Q  R  L  A  T  L  Q  Q  E  Y  V  N  A  D  K  E  R  G  A
```

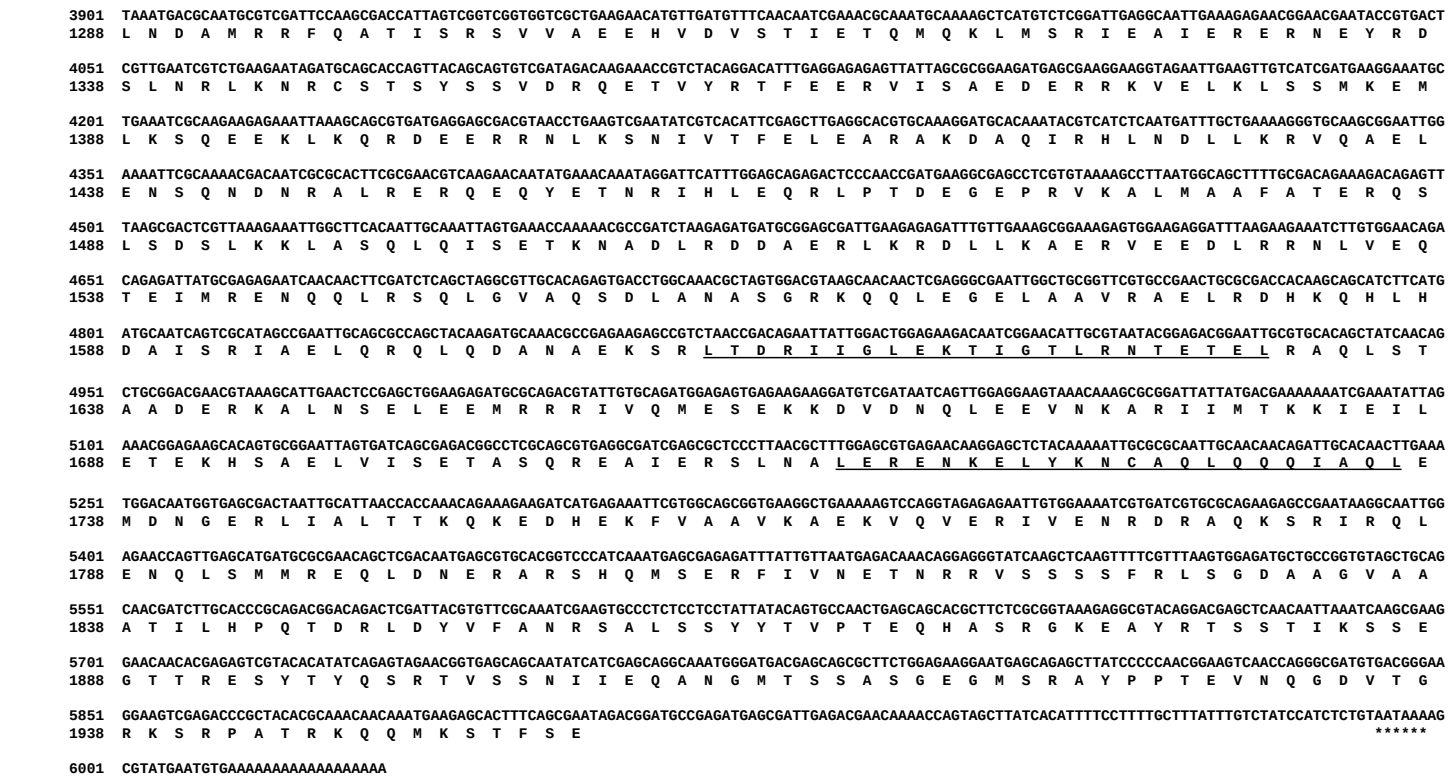

Fig. 10. Schematic summary of the 403 cDNA clones, the cDNA nucleotide and deduced amino acid sequences and the predicted secondary structure of PUMA1. (A) Schematic drawing of each of the six overlapping cDNA clones that span the entire 6,030 basepair length of the 403 mRNA transcript. The positions of the translation start (AUG) and stop (UAG) codons, and the polyadenylation signal sequence (AAUAAA) are indicated. (B) Composite nucleotide and predicted amino acid sequence of PUMA1 were determined by sequencing the overlapping cDNAs shown in A. The 3′polyadenylation signal is marked by asterisks. Leucine zipper domains are underlined. GTP binding domain is double-underlined and, circles (o) mark putative sites for a consensus phosphorylation site for $p34^{cdc2}$ kinase (residues 16-19) and a nuclear localization signal (residues 532-535). These sequence data are available from GenBank/EMBL/DDBJ under accession number AF009623. (C) Schematic diagram of PUMA1 protein predicted secondary structure indicating the positions of the two globular domains (dark gray dotted regions) and the α-helical coiled-coil domains (light gray dotted regions). Interruptions of the helical domain by proline (P) residues are indicated by open spaces.

addition to kinetochore-microtubules and the centromeric region, also associates with centrosomes, midzone, and midbody sites, it shares common cellular distribution features with most of the numerous described antigens that associate to some of the mitotic apparatus components. However, we think that it is of interest to note here the cellular distribution of the 120 kDa spoke antigen (Paddy and Chelsky, 1991). Like PUMA1, antispoke immunofluorescence staining during metaphase and anaphase in CHO cells revealed a structure with many of the attributes of kinetochore microtubules. Moreover, simultaneous anti-tubulin and CREST serum centromere staining indicated that spoke localization extends from the spindle poles and terminates at, or very near, the centromere of each chromosome (Paddy and Chelsky, 1991). Like PUMA1, spoke distribution is disrupted in cells treated with microtubule inhibitors. Unfortunately, to our knowledge, neither the molecular nature of spoke nor its putative functional role has been further studied.

PUMA1 shows 65% of similarity to the 234 kDa myosin-like protein Ant1 that is encoded by the gene *ovt1* in the nematode *O. volvulus* (Triteeraprapab et al., 1995). *ovt1* has been reported to be expressed during embryonic development but the subcellular localization of Ant1 protein, as well as its possible functional role, has not been studied. PUMA1 also shows similarities to myosin heavy chains, the myosin-like yeast protein MLP1 (Kolling et al., 1993), the mammalian centromeric proteins CENP-E (Yen et al., 1992), CENP-F (Rattner et al., 1993), and the nuclear mitotic apparatus protein NuMA (Lyderson and Pettijohn, 1980). A common feature of all of them is that they contain potential coiled-coil structures capable of forming filaments. NuMA, is a high molecular protein (235 kDa) that during mitosis accumulates at the mitotic spindle poles (Lyderson and Pettijohn, 1980). In addition to its role in nuclear matrix organization, the participation of NuMA in the organization and stabilization of the spindle microtubules has been reported (Kallajoki et al., 1991; Yang and Snyder, 1992; Compton and Luo, 1995; Gaglio et al., 1995). Similarly to NuMA (Yang et al., 1992), PUMA1 is predicted to have a three domain structure containing an unusually long α-helical central domain flanked by nonhelical ends. However, the 1,737 amino acid central coiled-coil region of PUMA1 is longer than that of NuMA (1,485 residues long) considered the longest coiled-coil region so far described (Yang et al., 1992). Based on the molecular features of PUMA1 it is possible that, as described for other coiled-coil proteins (Cohen and Parry, 1986; Cohen

and Parry, 1990), PUMA1 might form coiled-coil dimers due to hydrophobic interactions between their α-helical domains. Moreover, the central domain in PUMA1 is interrupted by five short amino acid stretches containing prolines. Thus, as suggested for other coiled-coil proteins including NuMA (see Yang et al., 1992), these nonhelical segments could be involved in the interactions with other proteins, as well as in forming higher order structures of PUMA1 dimers. In addition, three leucine zippers are present at the central α-helical region of PUMA1. Similarly to Ant1 (Triteeraprapab et al., 1995), no basic charged regions critical for DNA-binding function are located up-stream of the zipper sequences. Thus, it is more likely that the leucine zippers could represent a region facilitating protein dimerization as already described (Landschulz et al., 1988).

Many consensus sequences for phosphorylation by different kinases are found to be dispersed through the PUMA1 protein. This is consistent with reported data on the importance of phosphorylation in the assembly of fibrous coiled-coil proteins such as myosins and intermediate filaments (for review see Yang et al., 1992). A potential phosphorylation site for $p34^{cdc2}$ kinase located at the amino-terminal globular domain suggests that PUMA1 may be phosphorylated in a mitosis-specific fashion. In this respect, it is noteworthy that mutations of the predicted $p34^{cdc2}$ phosphorylation sites in the carboxy-terminal tail of NuMA resulted in the abolishment of its capacity to associate with the pericentrosomal region of the mitotic spindle, blocking mitosis without altering the association of NuMA to the interphase nucleus (Compton and Luo, 1995).

Although PUMA1 associates with the spindle apparatus, it does not appear to possess any described microtubule-binding motif characteristic of the conventional microtubule-associated proteins (MAPs). However, as said above, the molecular features of PUMA1 support the idea that it could interact with other proteins. Therefore, one might imagine, that PUMA1 may bind to microtubules via an unknown mechanism, or may be part of a complex of proteins capable of binding to microtubules. Further specific analyses are required to elucidate this point.

As shown in this paper, PUMA1 also associates with the spindle midzone region (and midbody) where the two half spindles are interdigitated at anaphase. A dense fibrogranular matrix has been described within these zones coating antiparallel microtubules (Buck and Tisdale, 1962; McIntosh and Landis, 1971). Interestingly, proteins like the CHO1 antigen and the INCENPs, suggested to play a role in the structural reinforcement of the two half spindle, show primary sequences similarity to the coiled-coil domain of myosin, tropomyosin and kinesins (Nislow et al., 1992; Mackay and Earnshaw, 1993).

Taken together, our data lead us to envisage PUMA1 as a spindle structural component that may be involved in the assembly and/or maintenance of the spindle architecture during cell division. Moreover, since PUMA1 concentrates both at the centrosomes and at centromeres, its function has to be compatible with the microtubule turnover (polymerization and depolymerization) both at the minus- and plus-end of microtubules. In addition, as said above, the accumulation of PUMA1 at the plus-end of the microtubules at/on the centromeric regions in *Parascaris* does not depend on the presence of organized kinetochore structures. This could reflect, at least in *Parascaris*, the existence of a specific framework or 'centromeric matrix' that may provide a proper environment for centromeric motor activity, either at or on the kinetochore structures, as well as at chromosomal sites capable of binding microtubules.

Finally, mAb403 also recognizes spindle components in *Drosophila* cells suggesting the presence of a protein containing epitopes structurally related to PUMA1. We have now undertaken the study of this related antigen to learn about its conservation and possible functional role during cell division in an organism amenable to genetic analysis.

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