polo encodes a protein kinase homolog required for mitosis in Drosophila

S. Llamazares, A. Moreira, A. Tavares, C. Girdham, B.A. Spruce, C. Gonzalez, R.E. Karess, D.M. Glover, and C.E. Sunkel

Cancer Research Campaign, Cell Cycle Genetics Group, Department of Biochemistry, The University, Dundee DD1 4HN, Scotland; Laboratorio de Genetica Molecular, Centro de Citologia Experimental, Instituto Nacional de Investigação Cientifica, Universidade do Porto, 4100 Porto, Portugal; Department of Biochemistry, New York University Medical School, New York, New York 10016 USA

We show that mutation in polo leads to a variety of abnormal mitoses in Drosophila larval neuroblasts. These include otherwise normal looking mitotic spindles upon which chromosomes appear overcondensed; normal bipolar spindles with polyplody complements of chromosomes; bipolar spindles in which one pole can be unusually broad; and monopolar spindles. We have cloned the polo gene from a mutant allele carrying a P-element transposon and sequenced cDNAs corresponding to transcripts of the wild-type locus. The sequence shows that polo encodes a 577-amino-acid protein with an amino-terminal domain homologous to a serine-threonine protein kinase. polo transcripts are abundant in tissues and developmental stages in which there is extensive mitotic activity. The transcripts show no obvious spatial pattern of distribution in relation to the mitotic domains of cellularized embryos but are specifically concentrated in dividing cells in larval discs and brains. In the cell cycles of both syncytial and cellularized embryos, the polo kinase undergoes cell cycle-dependent changes in its distribution: It is predominantly cytoplasmic during interphase; it becomes associated with condensed chromosomes toward the end of prophase; and it remains associated with chromosomes until telophase, whereupon it becomes cytoplasmic.

[Key Words: Mitosis; Drosophila; protein kinase; polo]

Received August 19, 1991; accepted September 12, 1991.

Genes essential for mitosis in Drosophila have been identified by mutations that arrest development at stages in which this process is crucial. One such stage is the syncytial embryo in which there are 13 rounds of mitosis in the first 2 hr of development (Zalokar and Erk 1976; Foe and Alberts 1983). These cycles are dependent on maternally provided gene products (Szabad and Bryant 1982). Some of these maternally expressed genes, for example gnu (Freeman et al. 1986), abc (Underwood et al. 1990; Vessey et al. 1991), and fs(1)Ya (Lin and Wolnfr 1991), encode products that appear to be specifically required for the syncytial divisions. Females homozygous for the gnu mutation produce eggs that undergo many rounds of DNA synthesis in the absence of mitosis, to give giant nuclei (Freeman and Glover 1987). fs(1)Ya is required for the first and subsequent embryonic mitoses and encodes a cell cycle-dependent nuclear envelope component (Lin and Wolnfr 1991). Other maternally active genes encode products that persist only for the syncytial stages and are replaced by the zygotic product following cellularization during the fourteenth cycle. This cycle no longer takes place synchronously throughout the embryo but, instead, cells divide within a series of mitotic domains (Foe 1989). string (Edgar and O'Farrell 1989; 1990) exemplifies a gene whose products have to be synthesized de novo at this time, following the degradation of its maternally encoded gene products. Thus, embryos homozygous for mutations in these genes can undertake all of the syncytial mitoses utilizing wild-type protein provided by the heterozygous mother, and cell cycle progression is only prevented following cellularization. The string gene encodes a protein homologous to the cell cycle control gene cdc25 of fission yeast (Edgar and O'Farrell 1989, 1990; Jimenez et al. 1990), which activates cdc2. cdc2 encodes the catalytic subunit of a protein kinase essential for the G2-M transition in diverse organisms (for review, see Nurse 1990). Thus, the controlled expression of string is thought to regulate the length of the G2 phase within the mitotic domains of newly cellularized Drosophila embryos (Edgar and O'Farrell 1990). Mutations in the final category of genes can arrest development either in the embryo or at late larval stages of development in an allele-specific manner. In these cases, maternally provided wild-type proteins appear to persist until the larval stages of development, in which most cells undergo growth and polyploidization rather than proliferating. Thus, aberrant mitosis is first seen in proliferating cells of imaginal...
disks and the central nervous system in late larval development. This general phenotype was first recognized by observations of Gatti and co-workers (Gatti et al. 1983; Gatti and Baker 1989) on mutations resulting in late larval or early pupal lethality. polo is such a gene in which animals homozygous for a strong mutant allele, polo2, die as larvae [Sunkel and Glover 1988]. The original weaker polo1 allele was, however, first detected by its female sterile phenotype. Many females homozygous for this mutation do survive to adulthood but then produce embryos that are unable to undertake the syncytial mitotic cycles. Thus, the polo gene product is likely to be required at all proliferative stages of Drosophila development.

We showed previously that females homozygous for polo1 produce embryos that have complex networks of chromatin associated with branched and highly irregular arrays of microtubules [Sunkel and Glover 1988]. It is not possible to visualize centrosomes in these embryos by using antibodies against the centrosomal-associated antigen Bx63 [Frasch et al. 1985; Whitfield et al. 1988].

Orcein-stained preparations of larval neuroblasts showed a large number of polyploid cells, some of which appeared to have circular arrangements of condensed chromosomes. We were also able to detect abnormal chromosome disjunction in male meiosis, both by direct microscopical examination of living cells and by following the disjunction of marked chromosomes. In this paper we evaluate the mitotic phenotype shown in larval neuroblasts by applying recently developed immunostaining techniques. We show that the polo gene product encodes a putative serine–threonine protein kinase that undergoes cyclical changes in its distribution during embryonic mitoses.

**Results**

**The effect of polo1 on mitotic spindles**

Previously, we have described the effects of the polo1 mutation upon mitotic chromosomes in aceto-orcein-stained preparations of squashed third-instar larval brains. We were concerned that these techniques destroy the mitotic spindles and distort the three-dimensional arrangement of chromosomes within these cells and so chose to re-examine the larval neuroblast phenotype by using recently developed techniques for indirect immunofluorescence with confocal microscopy [Axton et al. 1990; Gonzalez et al. 1990; C. Gonzalez and D.M. Glover, in prep.]. We find a wide range of spindle morphologies within these cells [Fig. 1A], which include those with a normal complement of chromosomes aligned on the metaphase plate of spindles that appear wild type, such as the example shown in Figure 1A. The mitotic figure in Figure 1B, on the other hand, is unusual in that although the extent of chromosome condensation is more like that normally seen at metaphase, the distribution of chromosomes is typical of prophase, the chromosomes not being aligned on a metaphase plate. Chromosome condensation can be even more extreme, as though this process is continuing to occur even though other aspects of mitosis are delayed. This is evident in the anaphase figure shown in Figure 1C. In this case, the chromosomes show a similar state of overcondensation to those reported previously in orcein-stained preparations [Sunkel and Glover 1988]. The field shown at lower magnification in Figure 1D has five mitotic cells with condensed chromosomes, four of which are clearly at metaphase and have their chromosomes associated with bipolar spindles. Closer examination of such spindles often reveals abnormalities: the metaphase figures in Figure 1E are both polyploid, but whereas the spindle of the upper cell has a wild-type appearance, the spindle of the lower cell has one unusually broad pole. Finally, a proportion of the mitotic figures are monopolar structures in which chromosomes are arranged in a circle around a large aster of microtubules. When immunostained to reveal the Bx63 antigen [Frasch et al. 1985; Whitfield et al. 1989; not shown], such structures are seen to be nucleated from a central single centrosome.

**Molecular localization of polo**

To clone polo we have used a mutant allele tagged with the P-element transposon. This was identified within a collection of larval lethal mutations on the third chromosome induced by the mobilization of multiple P elements by P-M hybrid dysgenesis [R.E. Karess and D.M. Glover, unpubl.]. As the third chromosome of the K3.1 stock carrying polo2 carried five additional P elements, it was necessary to perform three successive recombinant experiments both to eliminate unwanted P elements and identify the element responsible for the mutation [for details, see Materials and methods]. In all stocks carrying either recombinant or revertant chromosomes, we found that the polo phenotype correlated with the presence of a P element at 77A3. The location of this P element is in good agreement with that expected of the polo locus from its mapping by meiotic recombination [3-46.8 ± 0.1]. Recombinant chromosome 3 appeared to contain only a single P element at 77A3 by in situ hybridization. We therefore constructed a genomic library of DNA from flies in which the recombinant chromosome 3 was balanced over TM3. Screening of this library with a P element probe identified two classes of recombinant phage that hybridized to wild-type polytene chromosomes at sites 77A3 and 82C. We believe that the second insertion at 82C is a defective P element detected more easily by filter hybridization to a phage library than by hybridization to polytene chromosomes. DNA fragments from the class of recombinant phage shown to originate from 77A were then used to screen a genomic library from the Canton-S wild-type strain. We isolated three such phage that correspond to overlapping DNA fragments from a 22-kb region of wild-type third chromosome DNA from 77A3 [Fig. 2]. A comparison of the restriction endonuclease cleavage map of the λ phage carrying DNA from this region of the recombinant chromosome 3, E7P [Fig. 2], suggests that the polo2 mutation
polo encodes a protein kinase homolog

Figure 1. Mitotic figures in polo neuroblasts. Whole-mount preparations of third larval instar polo brains were prepared for immunostaining as described previously (Gonzalez et al. 1990). The preparations were stained to reveal DNA with propidium iodide, and microtubules, using as primary antibody the rat monoclonal YL1/2, were detected with fluorescein-conjugated goat anti-rat antibody [Jackson Immunochemicals]. The confocal images in A, B, and C show a wild-type-like metaphase, an abnormal prophase, and an anaphase figure with overcondensed chromosomes, respectively. These three fields are at comparable magnification such that the bar length would correspond to 4 μm. D shows a larger field at about one-third of this magnification showing a high mitotic index. E shows two spindles, the lower of which has one normal pole and one broad pole. Bar, 6 μm. The field in F has a monopolar structure.

Results from the insertion of a defective 1.6-kb P element.

To show that the cloned sequences at the site of the P element at 77A were able to provide the wild-type polo function, we reintroduced this DNA into flies by P-element-mediated germ-line transformation (Materials and methods). We subcloned a 10-kb BamHI fragment from the recombinant phage λE1 (Fig. 2) into a P-element transformation vector. We established five lines of transformed flies and found that chromosomes carrying the transposon with DNA from 77A3 were able to rescue both the female sterility of polo and the lethality of polo2 (Materials and methods), indicating that the polo gene is contained within this DNA segment.

polo transcripts are abundant during stages of development at which there is extensive mitosis

To assess whether the developmental pattern of polo expression corresponded with that expected of a gene required for mitosis, we first carried out a Northern blot using the insert from the phage λE1 that contains the polo gene [Fig. 2] to probe RNA from the major stages of Drosophila development. Figure 3I shows that this DNA fragment detects two transcripts of 2.2 and 2.5 kb, whose temporal pattern of expression is indistinguishable. The transcripts are present throughout development, being particularly abundant in adult females and early embryos. This pattern of expression reflects the requirement for the synthesis of cell-cycle proteins during oogenesis, embryogenesis, and late larval and early pupal stages and has been observed previously for the Drosophila cell-cycle genes cyclins A and B (Lehner and O'Farrell 1989, 1990a; Whitfield et al. 1989, 1990), cdc2 [Jimenez et al. 1990; Lehner and O'Farrell 1990b], and string [Edgar and O'Farrell 1989, 1990; Jimenez et al. 1990].

Localization of the polo transcripts by in situ hybrid-
Figure 2. Molecular map of the polo locus at 77A. Top The relative positions of four recombinant bacteriophage containing DNA from 77A, with respect to the genomic map shown below them. The scale on this map is given in kilobases with respect to the site of the P-element insertion in the polo" mutant DNA carried in phage AE7P. The three other phage carry wild-type DNA. Transcripts have been positioned with respect to the genomic map by comparison of the cDNA clone sequences (Fig. 4) with the genomic sequence (A. Moreira, A. Tavares, and C.E. Sunkel, unpubl.). The indicated fragments of the cDNA clone p9a2 were inserted into expression vectors as described in the Materials and methods.

Expression shows that the embryonic transcripts are initially present throughout the syncytial embryo and come to occupy a cortical layer during blastoderm in a manner that resembles the distribution of cyclin A RNA (Whitfield et al. 1989; Raff et al. 1990) (Fig. 3II, A,B,D). Following cellularization, the distribution of polo RNA corresponds only broadly to those regions of the embryo undergoing division (Fig. 3II, C), and we do not see specific association of the transcripts with mitotic domains as is seen with the string gene (Edgar and O’Farrell 1989). polo transcripts are also abundant in larval tissues that contain diploid cells (Fig. 3III), such as the testis (A,B), discs (C,D), and brain (E,F). In these tissues, the RNA is most abundant within cell types undergoing proliferation. Thus, the strongest signals in the brain can be seen within the proliferating centers of the optic lobes (o) and within that part of the ventral ganglion (v) that gives rise to thoracic innervation. This region of the ventral ganglion has been shown to have greater numbers of dividing neuroblasts (Truman and Bate 1988).

polo encodes a protein kinase homolog

We have isolated and sequenced two cDNA clones, p2a2 and p9a2, which correspond to the 2.2- and 2.5-kb transcripts, respectively. Each of these contains an identical open reading frame encoding a 577-amino acid protein (Fig. 4). The longer cDNA differs from the shorter by additional nucleotides in both the 5’ and 3’ regions. The protein comprises two domains. The amino-terminal 277 amino acids show considerable identity with the catalytic domains of protein kinases. Hanks et al. (1988) have devised a phylogenetic tree of protein kinases based on the extent of sequence identity within 11 conserved regions within their catalytic domains. We have carried out a computer data base search that shows the polo kinase to be related most closely to a cluster of kinases that they identify encoded by the budding yeast genes SNF1, KIN1, and KIN2 and the fission yeast gene nim1, and is thus likely to phosphorylate serine and threonine residues (see discussion by Hanks et al. 1988). Within subdomain VIII of polo we find the sequence Gly-Thr-Ala-Asn-Tyr-Ile-Ala-Pro-Glu, which conforms to the consensus sequence described by Hanks et al. (1988) as typical of Ser-Thr protein kinases. The polo kinase is next most closely related to the cyclic nucleotide-dependent kinases. The sequence relationships between such kinases and protein kinases known to be essential for entry into mitosis are shown in Figure 5.

The polo kinase shows cyclical changes in its distribution during embryonic mitoses

To facilitate the characterization of the polo gene product in Drosophila cells, we have raised antibodies against the protein expressed in bacteria (see Materials and methods). To confirm that cDNAs representative of the two classes of transcripts encode the same protein as indicated from their DNA sequence and to determine the size of this protein, we took advantage of the directional cDNA cloning procedure that positions the cDNAs downstream of an SP6 promoter (Brown and Kafatos 1988). We transcribed the two cDNAs in vitro using SP6 polymerase, translated the product using a reticulocyte lysate, and subjected it to immunoprecipitation using rabbit antisera that had been preabsorbed against β-galactosidase. A 65-kD polypeptide was precipitated from the in vitro transcription/translation product of both cloned cDNAs (Fig. 6A). Antibodies against the bacterially expressed polo protein also specifically recognize a single polypeptide of 65 kD in extracts of Drosophila embryos subjected to immunoblotting (Fig. 6B).

We have used both polyclonal and monoclonal antibodies to follow the distribution of the polo protein throughout mitosis in syncytial blastoderm embryos by
polo encodes a protein kinase homolog

Developmental regulation of polo transcripts. (I) Northern blots. RNA from the following developmental stages was prepared for blotting as described in Whitfield et al. (1989): [Lane 1] 0- to 4-hr embryos; [lane 2] 4- to 22-hr embryos; [lane 3] first-instar larvae; [lane 4] second-instar larvae; [lane 5] third-instar larvae; [lane 6] pupae; [lane 7] adult males; [lane 8] adult females. (II) Localization of transcripts in embryos. (A,B) Sections of embryos at various stages of syncytial development prepared for in situ hybridization with 35S-labeled probes as described in Raft et al. [1990]. Nuclei revealed by Hoechst fluorescence can be seen in A; silver grains deposited in the emulsion following the development of the autoradiogram are shown in dark-field optics in B. (C,D) A cellularized cycle 14 embryo and a syncytial embryo, respectively, following the in situ hybridization of whole-mount preparations using the digoxigenin-labeling technique developed by Tautz and Pfeifle (1989). (III) Localization of transcripts in proliferating larval tissues. In situ hybridizations carried out on sections of third-instar larvae with 35S-labeled probes. Bright-field micrographs are shown at left (A,C,E), with the corresponding dark field images at right (B,D,F). The sections illustrate polo expression in testis [A,B], an imaginal disc [C,D], and brain [E,F]. The signal in the brain is particularly strong in the proliferating centers of the optic lobes [o] and within the thoracic segments of the ventral ganglion [v].

Discussion

The sequence of the polo protein shows it to have at least two domains: the amino-terminal, which is most closely related to the catalytic domain of a family of serine/threonine protein kinases represented by the budding yeast genes SNF1, KIN1, and KIN2, and the fission yeast
Figure 4. Nucleotide sequence of polo cDNAs. The sequence of the longer cDNA in the plasmid p9a2 is shown. The nucleotide sequence is enumerated at left; numbers at right refer to the amino acid sequence encoded by the longest open reading frame. The ends of the shorter cDNA sequence from p2a2 are indicated by arrowheads. The polyadenylation signal AATATAT is found upstream of the 3' ends of each transcript at nucleotides 2201 and 2523. As many protein kinases are themselves regulated by phosphorylation, we have searched for putative phosphorylation sites within the polo protein. We find potential sites for the following kinases (numbers refer to the last amino acid of the motif): (casein kinase I) 10; (casein kinase II) 113, 123, 357; (calmodulin kinase II) 245, 290, 328; (myosin heavy-chain kinase) 25, 58, 110, 202, 462, 500; (cAMP-dependent kinase) 120; (cGMP-dependent kinase) 489 (Kemp and Pearson 1990).

gene nim1 [Hanks et al. 1988]. The fission yeast member of this family acts as an inducer of mitosis in the cdc2 regulatory pathway. The polo gene itself encodes a 34-kD protein kinase, p34cdc2 whose homolog is a component of mitosis promoting factor (MPF) in multicellular eukaryotes, and is thus a part of a highly conserved mech-
polo encodes a protein kinase homolog

Figure 5. (See following page for legend.)
Figure 6. Detection of the putative polo kinase with antibodies. [A] Coupled in vitro transcription and translation of polo cDNAs was carried out as described in Materials and methods. The total protein products of such a reaction with cDNAs 2a2 and 9a2 are shown in lanes 1 and 4, respectively. The preimmune serum fails to precipitate any radiolabeled protein specified by either 2a2 (lane 2) or 9a2 (lane 5) cDNAs. The anti-polo rabbit serum Rb287 precipitates a single 65-kD protein encoded by both 2a2 (lane 3) and 9a2 (lane 6). Molecular mass markers [in kD] are shown at left. [B] Immunoblot of embryo extracts with the monoclonal anti-polo antibody MA75. Two loadings are shown of extracts from 2- to 4-hr embryos (lanes 1, 2); 4- to 8-hr embryos (lanes 3, 4); and 8- to 24-hr embryos (lanes 5, 6). Molecular mass markers [in kD] are shown at left.

Figure 7. Cyclical distribution of polo kinase during syncytial mitotic cycles. Embryos were prepared to visualize DNA with propidium dioide [left], and the polo protein by indirect immunofluorescence [right] by using the techniques described previously by Whitfield et al. [1990] for confocal microscopy. The micrographs represent the following mitotic phases: [A] interphase; [B] prophase; [C] metaphase; [D] anaphase; [E] late telophase. E shows a gradient of telophase nuclei; those nuclei at top right are at earlier telophase stages.) Bar, 10 μm.
Deletion of this fission yeast gene causes cells to enter mitosis at half the size of wild-type cells. The polo kinase has been shown to catalyze its autophosphorylation on both tyrosine and serine residues (Featherstone and Russell 1991), and its coexpression with cdc2 and cyclin in a baculoviral expression system suggests that it may phosphorylate the tyrosine residue that regulates kinase activity (Parker et al. 1991). The nim1 protein kinase has been postulated, on the simplest interpretation of genetic interactions, to have its inducing effect upon mitosis by inhibiting polo, though more complex interpretations are possible. Is the polo kinase the counterpart of nim1 or just a family member? Until functional tests have been carried out, this remains an open question. polo kinase differs from the nim1 kinase by having a longer carboxy-terminal domain. Furthermore, the catalytic domain shows only 29% identity to that of nim1. This compares to a 59% identity between fission yeast and fission yeast. The activation of cdc2 kinase is part of a cascade of phosphorylation events required for the transit of the cell into and through mitosis. Other putative protein kinases have been described that are essential for these events, but their precise role is uncertain. The nimA gene of Aspergillus, for example, appears to have analogous functions to cdc2 of fission yeast and may represent another component of this regulatory pathway. Recessive mutations in nimA result in a cell-cycle block late in G2 (Morris 1976; Oakley and Morris 1983; Osmani et al. 1987), whereas inducible overexpression of the gene causes mitotic induction in which chromatin is maintained in a condensed state (Osmani et al. 1988). It is clear from its sequence that the nimA kinase is homologous to neither of the kinases encoded by cdc2, wee1, or nim1 of fission yeast nor the polo kinase. Another protein kinase has been identified in mammalian cells that may act to maintain the mitotic state since when it is overexpressed in CHO cells, it appears to result in a delay in the progression through telophase and in the subsequent entry into S (Bunnell et al. 1990). Many of the rounded-up cells that result from overexpression of this kinase contain tubulin mid-bodies similar to those seen in the telophase spindle. Not only is a complex network of protein phosphorylation and dephosphorylation involved in regulating the entry into mitosis, but there is also a major requirement for protein phosphorylation in the mitotic events of nuclear membrane breakdown (e.g., Gerace and Blobel 1980), chromosome condensation (e.g., Bradbury et al. 1974), spindle formation, and the activation of microtubule-organizing centers (Piras and Piras 1975; Vandre et al. 1984; Verde et al. 1990). Although some of these phosphorylation events have been postulated to be mediated directly by p34cdc2 kinase (Peter et al. 1990; Ward and Kirschner 1990), the phosphorylation of such a diverse set of substrates may well require a number of protein kinases.

Do the phenotypes resulting from mutation in polo give any clues to its point of action? This question is difficult to address with the existing alleles because polo mutants, in common with other Drosophila mitotic mutants, have been selected for their developmental phenotype. These mutations are therefore unlike the cell-cycle mutations in the yeasts and in Aspergillus that have been selected on the basis of a conditional lethal phenotype, usually temperature sensitivity, and in which it is possible to follow the effect of mutation in all cells following a temperature shift. The embryos of females homozygous for polo fail to develop, as a consequence of totally aberrant mitotic events throughout the syncytial stages (Sunkel and Glover 1988). However, chromatin,

---

**Figure 8.** Distribution of polo kinase with respect to mitotic domains in cycle 14. Shown is the anterior of a cellularized embryo prepared for immunofluorescence as in Fig. 7. Several mitotic domains can be seen as distinct regions containing condensed chromatin. The three labeled areas exemplify cells at different cell cycle stages: (m/a) one of the mitotic domains with chromosomes in metaphase and anaphase showing staining with the anti-polo antibody; (i) a region of interphase cells showing strong cytoplasmic anti-polo staining; (p) a region of prophase cells with diffuse anti-polo staining. Bar, 50 μm.
which is highly disorganized, does appear to undergo condensation and decondensation, and DNA replication continues to produce many polyploid nuclei. Such a phenotype appears to be a common feature of many maternal-effect mutations that affect mitosis in the syncytial embryo. In such cases when one disrupts the nuclear cycle, centrosomes may break free from nuclei and undergo autonomous replication reflecting the loose coupling of the nuclear and cytoplasmic events of mitosis at this developmental stage. Thus, free centrosomes are seen in embryos of females homozygous for gnu, in which nuclear division is prevented leading to giant nuclei [Freeman et al. 1986]; abnormal spindle [Gonzalez et al. 1990]; lodestar, which leads to lagging anaphase chromosomes [Girdham and Glover 1991]; aurora, which leads to spindle branching and polyploidy [M. Leibowitz and D.M. Glover, in prep.]; fs(l)jy [Lin and Wolffner 1991], in which there are few if any early nuclear divisions; and abc [Vessey et al. 1991]. Centrosomes will also dissociate from nuclei and replicate autonomously following the injection of aphidicolin to inhibit DNA replication [Raff and Glover 1988]. However, not only can free centrosomes not be seen in polo embryos but the disorganized chromatin is associated with highly branched microtubules that do not appear to be nucleated by organized centrosomes. Although the Bx63 centrosomal antigen is present within these embryos, it only aggregates into punctate bodies visible by immunofluorescence later in the aberrant development of these embryos. These bodies are associated with the polyploid nuclei [Sunkel and Glover 1988]. The lack of centrosomes in polo embryos is therefore unusual among the maternal-effect mitotic mutations that have been described to date and may point toward the polo kinase having a role in regulating both nuclear and cytoplasmic aspects of the mitotic cycle.

We also see an effect of the polo1 mutation on the mitotic apparatus in larval neuroblasts. Previously, we had observed a high frequency of polyploid cells in orcein-stained squashed preparations of larval neuroblasts. Many of these appeared to be in a circular arrangement leading us to speculate that they might be associated with a single spindle pole [Sunkel and Glover 1988]. In this paper we present evidence for such monopolar structures within polo brains. The mitotic phenotypes of the recessive polo1 mutation are seen in homozygous mutant larvae as the maternal wild-type protein is gradually eliminated and replaced by mutant protein. Sufficient cells survive the effects of the mutation such that adults are produced. This points to the protein produced by the polo1 allele as having some residual function because maternal protein in larval homozygous for the polo2 allele, disrupted by a P-element insertion, only enables their survival to second instar, a point at which we have been unable to examine the mitotic phenotype. Our recent observations on larval neuroblasts in third-instar larvae homozygous for polo1 indicate a broad pattern of mitotic defects. Not only does the mutation affect the organization of spindle poles but it also results in polyploidy and in cells in which aspects of the mitotic cycle are delayed, often leading to overcondensation of chromosomes. In this respect, the effects of polo2 differ from the effects of drugs such as colchicine, or mutations such as asp, which arrest the mitotic cycle of neuroblasts at clearly defined points [Gonzalez et al. 1990; Whitfield et al. 1990]. The pleiotropy of the polo1 mutation is much more similar to that of the null allele of the protein phosphatase 1 locus at 87B [Axton et al. 1990]. Such pleiotropic effects may indicate either that these enzymes act upon a wide range of substrates or that they act in the upper echelons of the mitotic regulatory hierarchy. We are now in a position to begin to ask questions relating to the substrate specificity of the polo kinase and also to study its interaction with the growing collection of mitotic mutants of Drosophila to study its role in mitotic regulation.

Materials and methods
Molecular genetics of polo
The polo2 allele was identified in a collection of larval and pupal lethal mutations induced by crossing females homozygous for a third chromosome marked with red e with P males. Stocks were established from single-pair matings of the grandsons of the dysgenic cross with females carrying balancer chromosomes. The third chromosomes from these stocks were tested for their ability to complement the polo1 allele [Sunkel and Glover 1988]. Several isogenic lines were established from the original stock, K3.1, that carries polo 2. In situ hybridization of a P-element probe to polytene chromosomes showed that the third chromosome carried six P transposons. Recombinant chromosomes were then generated between the P-element-carrying chromosome and a multiply marked chromosome carrying the recessive alleles ru h th st cu sr ca. Recombinant chromosomes in the st–cu interval, in which polo had been reported to map, were tested for polo2. Recombinants showing the polo phenotype were found by in situ hybridization to have a P element at 77A, whereas all polo+ recombinants lacked this P element. This experiment also revealed two unrelated lethal mutations in the th–cu interval on the K3.1 chromosome. As one of these lethal mutations [P] was still present on the recombinant chromosome rec1, a second round of recombination was undertaken with a chromosome marked with ru h th. Unfortunately, recombinants in the th–cu interval that carried polo2 also carried P. One such recombinant chromosome [rec2: ru h th lethal1 polo cu sr ca] was therefore subjected to further recombination with a chromosome marked with st in ri eag Ki. st polo cu sr ca recombinants were collected that had lost the flanking lethal mutation in the recombination process but still carried the P element inserted in 77A as revealed by in situ hybridization. We refer to this chromosome as rec3.

To confirm that the P element inserted at 77A3 was responsible for the polo phenotype in polo2, the rec2 chromosome was subjected to hybrid dysgenesis to select for revertants of the polo2 mutation. Males of a line carrying rec2/TM6 were crossed to TM1/TM3 M females. Four fully viable and fertile polo revertants were studied in a total of 11,359 chromosomes screened. In situ hybridizations showed that all had lost the P element at 77A while maintaining various combinations of other P elements present on the chromosome. Southern blotting analysis indicated that a 6-kb EcoRI fragment present on the rec2 chromosome was no longer present but was replaced by a fragment that was 1.6 kb shorter (not shown).
Molecular cloning of polo

DNA was extracted from flies carrying the rec3 chromosome balanced over TM3, partially digested with Sau3A, and used to construct a genomic library in λEMBL4. Recombinants carrying P elements and their flanking genomic sequences were selected by hybridization with an 0.8-kb fragment from the p25.1 plasmid (O′Hare and Rubin 1983). Nineteen positives were isolated, which fell into two classes by the criteria of their restriction cleavage maps and by in situ hybridization to sites 82C and 77A3 on wild-type polytene chromosomes. DNAs prepared from the class of phage hybridizing to 77A3 were used to probe libraries of genomic DNA from the wild-type strain Canton-S in EMBL4 and in λ-Dash.

P-element-mediated germ-line transformation

Transformations were carried out as described by Axton et al. [1990]. The 10-kb BamHI fragment from phage E-1 [Fig. 2] was introduced into pW8 [Klemenz et al. 1987] and injected together with the helper element, pUCls-2.3, into embryos homozygous for w118. Lines were established in which the insertions were carried on either the X or second chromosomes. w118 Flies of the genotype w ~8 P[w pol]/w~8; ru st pol e ca/TM3 females and males from lines carrying X insertions were crossed to virgin w118, ru st pol1 e ca/TM3 females. Individuals carrying the transposon were selected by their white + phenotype, and the chromosome carrying pol1 was identified by its segregation from TM3. Flies of the genotype w118 P[w pol] ru st pol1 e ca were backcrossed against w118; ru st pol1 e ca/TM3 females and female offspring of the genotype w118 P[w pol]/w118, ru st pol e ca/st pol1 e ca were selected and checked for fertility. All were found to be fertile, giving rise to embryos that developed to produce adults. An analogous procedure was followed by using the polo2 allele, in which case both lethality and fertility were fully complemented.

Generation of antibodies

Generation of antibodies, Western blotting, and the fixing and immunostaining of embryos were all carried out as described previously [Whitfield et al. 1990]. A SalI fragment of the cDNA clone p9a2 encoding the amino-terminal part of the protein [Fig. 2] was subcloned into the expression vector pUR292 [Ruther and Muller-Hill 1983] to give the plasmid pUR9a2N in which it is expressed as a fusion protein with Escherichia coli β-galactosidase. A Sphi–NotI fragment was also subcloned into the pAR expression vector [Studier and Moffatt 1986]. This construct encodes a fusion protein in which the 28 amino-terminal amino acids of polo is essential for mitosis, Cell 63: 33–46. Bradbury, E.M., R.J. Inglis, and H.R. Matthews. 1989. Control of cell division by very rich lysine (F1) phosphorylation. Nature 349: 808–811. Featherstone, C. and P. Russell. 1991. Fission yeast p107 mitotic inhibitor is a tyrosine/serine kinase. Nature 349: 808–811.

In vitro transcription, translation, and immunoprecipitation

In vitro transcription was performed by using an SP6 transcription kit from Amersham. Two micrograms of either p2a2 or p9a2 cDNA was linearized with NhoI and incubated with 10 units of SP6 polymerase and 0.025 units of RNase inhibitor in the recommended buffer at 37°C for 1 hr. Plasmid DNA was digested with RNase-free DNase and the RNA isolated by phenol–chloroform extraction followed by precipitation in 5 M ammonium acetate. In vitro translation was performed on 200 ng of RNA incubated with 17.5 μCi of [35S]methionine [800–1200 Ci/mole, Amersham] in 20 μl of rabbit reticulocyte lysate [Amersham] for 1 hr at 37°C. Before boiling for 3 min, 6.5 μl of the reaction was prepared for SDS-PAGE by incubation with an equal volume of RNase (100 μg/ml in 50 mM EDTA) for 15 min at 37°C, followed by the addition of a further volume of SDS–gel-loading buffer. Immunoprecipitation was carried out by diluting the remaining 20 μl to 1 ml in NET [50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide] containing 1 mM PMSF. This was divided into two microcentrifuge tubes for centrifugation for 15 min at 4°C in a microcentrifuge. Either preimmune or Rb287 serum (5 μl) was added, and the mixture was incubated for 15 min at 4°C. IgGsorb [500 ng] was added, and the incubation continued for an additional 15 min at 4°C. Immunoprecipitates were collected by centrifugation and washed several times before being resuspended in 10 μl of SDS–gel-loading buffer. Following electrophoresis, the gel was fixed in methanol, and dried for autoradiography.

Acknowledgments

We thank the Cancer Research Campaign of Great Britain and the Instituto de Investigacao Cientifica and the Junta Nacional de Investigacion Cientifica e Tecnologica (grant 67/676) of Portugal for supporting this work. We also thank Jordan Raft for carrying out some of the initial in situ hybridization experiments to localize polo transcripts in embryos and Ines Chavez for the screening of polo revertants. We thank Kevin O′Hare and Andrew Tomlinson for providing useful bits of P elements.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References

Axton, J.M., V. Dombradi, P.T.W. Cohen, and D.M. Glover. 1990. One of the protein phosphatase 1 isoenzymes in Drosophila is essential for mitosis, Cell 63: 33–46. Bradbury, E.M., R.J. Inglis, and H.R. Matthews. 1989. Control of cell division by very rich lysine (F1) phosphorylation. Nature 349: 257–261. Brown, N.H. and F.C. Kafatos. 1988. Functional cDNA libraries from Drosophila embryos. J. Mol. Biol. 203: 425–437. Bunnel, B.A., L.S. Heath, D.E. Adams, J.M. Lahti, and V.J. Kid&. 1990. Increased expression of a 58kD protein kinase that leads to changes in the CHO cell cycle. Proc. Natl. Acad. Sci. 87: 7467–7471. Edgar, B.A. and P.H. O′Farrell. 1989. Genetic control of cell division patterns in the Drosophila embryo. Cell 57: 177–187. Erdner, S. and V. Abcouwer. 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. Cell 62: 469–480. Featherstone, C. and P. Russell. 1991. Fission yeast p107 mitotic inhibitor is a tyrosine/serine kinase. Nature 349: 808–811.
Foe, V.E. 1989. Mitotic domains reveal early commitment of cells in Drosophila embryos. Development **107**: 1-22.

Foe, V. and B.M. Alberts. 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis. *J. Cell Sci.* **61**: 31-70.

Frasch, M., D.M. Glover, and H. Sauwweber. 1985. Nuclear antigens follow different pathways into daughter nuclei during mitosis in Drosophila embryos. *J. Cell Sci.* **82**: 115-172.

Freedman, M., C. Nüsslein-Volhard, and D.M. Glover. 1986. The dissociation of nuclear and centrosomal division in gnu, a mutation causing giant nuclei in Drosophila. *Cell* **46**: 457-468.

Gatti, M. and B.S. Baker. 1989. Genes controlling essential cell cycle functions in Drosophila melanogaster *Genes & Dev.* **3**: 438-453.

Gatti, M., D.A. Smith, and B.S. Baker. 1983. A gene controlling the condensation of heterochromatin in Drosophila melanogaster. *Science* **221**: 83-85.

Gerace, L. and G. Blobel. 1980. The nuclear envelope lamina is reversibly polymerized during mitosis. *Cell* **19**: 277-287.

Girdham, C.H. and D.M. Glover. 1991. Chromosome tangle and breakage at anaphase result from mutations in *lodestar*, a Drosophila gene encoding a putative nucleoside triphosphate-binding protein. *Gene & Dev.* **5**: 1786-1799.

Gonzalez, C., R.D.C. Saunders, J. Casal, I. Molina, M. Carmena, P. Ripoll, and D.M. Glover. 1990. Mutations at the asp locus lead to multiple free centrosomes in syncytial embryos, but restrict centrosome duplication in larval neuroblastas. *J. Cell Sci.* **96**: 605-616.

Hanks, S.K., A.M. Quinn, and T. Hunter. 1988. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241**: 42-52.

Harlow, E. and D. Lane. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Jimenez, J., L. Alphey, P. Nurse, and D.M. Glover. 1990. Complementation of fission yeast cdc25" and cdc25" mutants identifies two cell cycle genes from Drosophila, a cdc2 homologue and string. *EMBO J.* **9**: 3565-3571.

Kalderon, D. and G. Rubin. 1989. cGMP dependent protein kinase genes in Drosophila. *J. Biol. Chem.* **264**: 10738-10748.

Klemenz, R., U. Weber, and W. Gehring. 1987. The white gene as a marker in a P-element vector for gene transformation in Drosophila. *Nucleic Acids Res.* **15**: 3947-3959.

Lee, M.G. and P. Nurse. 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature* **327**: 31-35.

Lehner, C.F. and P.H. O'Farrell. 1989. Expression and function of Drosophila cyclin A during embryonic cell cycle progression. *Cell* **56**: 957-968.

—. 1990. The roles of cyclins A and B in mitotic control. *Cell* **61**: 535-547.

—. 1990b. Drosophila cdc2 homologues: A functional homologue is coexpressed with a cognate variant. *EMBO J.* **9**: 3573-3581.

Lin, H. and M.F. WoIfnier. 1991. The Drosophila maternal effect gene fs(1)Ya encodes a cell cycle dependent nuclear envelope component required for embryonic mitoses. *Cell* **64**: 49-62.

Lisiewicz, J.A., H. Godany, H. Forster, and H. Kuntzel. 1987. Isolation and nucleotide sequence of a Saccharomyces cerevisiae protein kinase gene suppressing the cell cycle start mutation cdc25. *J. Biol. Chem.* **262**: 2549-2553.

Llorinaz, A.T. and S.I. Reed. 1984. Primary structure homology between the product of yeast cell division gene CDC28 and vertebrate onco genes. *Nature* **307**: 183-185.

Morris, N.R. 1976. Mitotic mutants of Aspergillus nidulans *Genet. Res.* **26**: 237-254.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature* **344**: 503-507.

Oakley, B.R. and N.R. Morris. 1983. A mutation in Aspergillus that blocks the transition from interphase to prophase. *J. Cell Biol.* **96**: 1155-1158.

O’Hare, K. and G. Rubin. 1983. Structures of transposable elements and their sites of insertion and excision in the Drosophila melanogaster genome. *Cell* **34**: 25-35.

Osmani, S.A., G.S. May, and N.R. Morris. 1987. Regulation of the mRNA levels of nimA, a gene required for the G2-M transition in Aspergillus nidulans. *J. Cell. Biol.* **104**: 1495-1504.

Osmani, S.A., R.T. Pu, and N.R. Morris. 1988. Mitotic induction and maintenance by overexpression of a G2 specific gene that encodes a potential protein kinase. *Cell* **53**: 237-244.

Parker, L.L., S. Atherton-Fessler, M.S. Lee, S. Ogg, J.L. Falk, K.I. Swenson, and H. Pwnica-Worms. 1991. Cyclin promotes the tyrosine phosphorylation of p 34cdc2 in a wee 1" dependent manner. *EMBO J.* **10**: 1255-1263.

Peter, M., J. Nakagawa, M. Doree, J.C. Labbe, and E.A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M-phase specific phosphorylation of lamins by cdc2 kinase. *Cell* **61**: 591-692.

Piras, R. and M.M. Piras. 1975. Changes in microtubule phosphorylation during cell cycle of HeLa cells. *Proc. Natl. Acad. Sci.* **72**: 1161-1165.

Raff, J.W. and D.M. Glover. 1988. Nuclear and cytoplasmic mitotic cycles continue in Drosophila embryos in which DNA synthesis is inhibited with aphidicolin. *J. Cell Biol.* **107**: 2009-2019.

Raff, J., W.G.F. Whitfield, and D.M. Glover. 1990. Two distinct mechanisms localise cyclin B transcripts in syncytial Drosophila embryos. *Development* **110**: 1249-1261.

Rosenthal, A., L. Rhee, R. Yadeghani, R. Paro, A. Ullrich, and D.V. Goeddel. 1987. Structure and sequence of a Drosophila melanogaster protein kinase C gene. *EMBO J.* **6**: 443-441.

Russell, P. and P. Nurse. 1987a. Negative regulation of mitosis by wee 1", a gene encoding a protein kinase homolog. *Cell* **49**: 599-567.

—. 1987b. The mitotic inducer nim1" functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49**: 5689-5696.

Rüther, U. and B. Müller-Hill. 1983. Early identification of cDNA clones. *EMBO J.* **2**: 1791-1794.

Studier, F.W. and B.A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. *J. Mol. Biol.* **189**: 113-130.

Sunkel, C.E. and D.M. Glover. 1988. polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. *J. Cell. Sci.* **89**: 25-38.

Szabad, J. and P.F. Bryant. 1982. The mode of action of discluse mutations in Drosophila melanogaster. *Dev. Biol.* **93**: 240-256.

Tautz, D. and C. Pfeifle. 1989. A non radioactive in situ hybridisation method for the localisation of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**: 81-85.

Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler. 1987. Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell*...
polo encodes a protein kinase homolog

Truman, J.W. and M. Bate. 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. Dev. Biol. 125: 145–157.

Underwood, E.M., A.S. Briot, K.Z. Doll, R.L. Ludwiczak, D.C. Otteson, J. Tower, K.B. Vessey, and K. Yu. 1990. Genetics of 51D-52A, a region containing several maternal-effect genes and 2 maternal-specific transcripts in Drosophila. Genetics 126: 639–650.

Vandre, D.D., F.M. Davis, P.N. Rao, and G.G. Borisy. 1984. Phosphoproteins are components of mitotic microtubule organizing centers. Proc. Natl. Acad. Sci. 81: 4439–4443.

Verde, F., J.C. Labbe, M. Doree, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. Nature 343: 233–238.

Vessey, K.B., R.L. Ludawiczak, A.S. Briot, and E.M. Underwood. 1991. abnormal chromatin, a maternal effect locus in Drosophila melanogaster. J. Cell. Sci. 98: 233–243.

Ward, G.E. and M.W. Kirschner. 1990. Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. Cell 61: 561–577.

Whitfield, W.G.F., S.E. Millar, H. Saumweber, M. Frasch, and D.M. Glover. 1988. Cloning of a gene encoding an antigen associated with the centrosome in Drosophila. J. Cell. Sci. 89: 467–480.

Whitfield, W.G.F., C. Gonzalez, E. Sanchez-Herrera, and D.M. Glover. 1989. Transcripts of one of two Drosophila cyclin genes become localized in pole cells during embryogenesis. Nature 338: 337–340.

Whitfield, W.G.F., C. Gonzalez, G. Maldonado-Codina, and D.M. Glover. 1990. The A- and B-type cyclins of Drosophila are accumulated and destroyed in temporally distinct events that define separate phases of the G2-M transition. EMBO J. 9: 2563–2572.

Zalokar, M. and I. Erk. 1976. Division and migration of nuclei during early embryogenesis of Drosophila melanogaster. J. Microbiol. Cell 25: 97–106.