

polo encodes a protein kinase homolog required for mitosis in *Drosophila*

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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 polyploid 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*]

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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 Wolfner 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 Wolfner 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

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discs 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, *polo*², die as larvae (Sunkel and Glover 1988). The original weaker *polo*¹ 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 *polo*¹ 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 polo*¹ *on mitotic spindles*

Previously, we have described the effects of the *polo*¹ 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. 1), 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 *polo*² carried five additional P elements, it was necessary to perform three successive recombination 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 ($3-46.8 \pm 0.1$). 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 *polo*² mutation

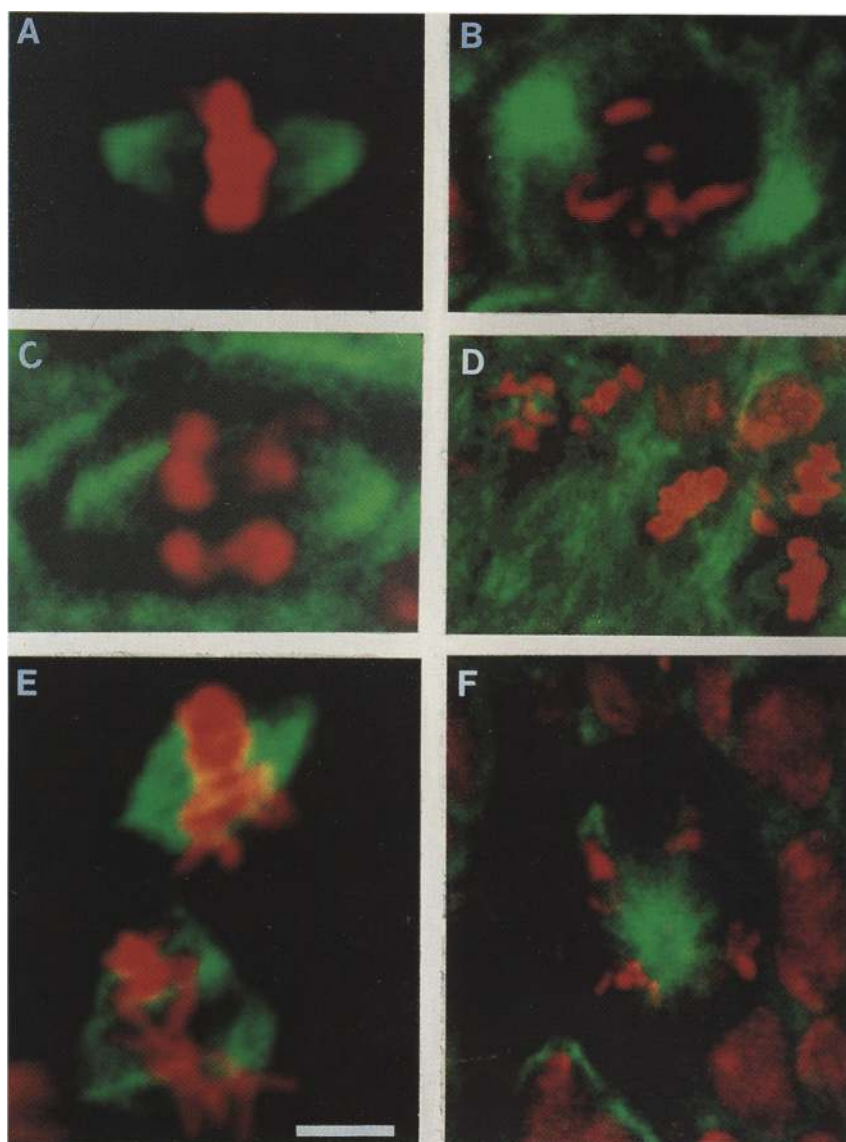


Figure 1. Mitotic figures in *polo* neuroblasts. Whole-mount preparations of third larval instar *polo* brains were prepared for immunostaining as described previously (Gonzalez et al. 1990). The preparations were stained to reveal DNA with propidium iodide, and microtubules, using as primary antibody 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 μ m. *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 μ m. 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 *Bam*HI fragment from the recombinant phage λ E1 (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*¹ and the lethality of *polo*² (Materials and methods), indicating that the *polo* gene is contained within this DNA segment.

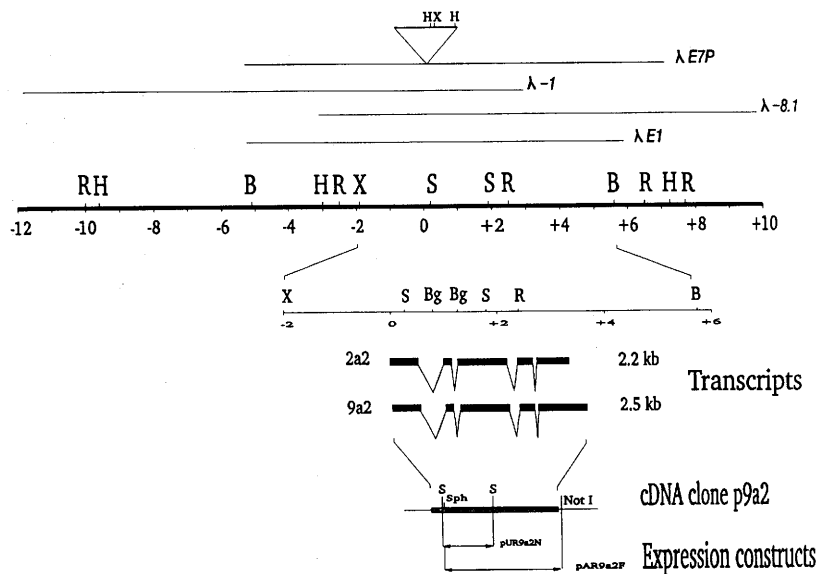
polo transcripts are abundant during stages of development at which there is extensive mitosis

To assess whether the developmental pattern of *polo* ex-

pression corresponded with that expected of a gene required for mitosis, we first carried out a Northern blot using the insert from the phage λ E1 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*² mutant DNA carried in phage λ E7P. 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.



ization 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

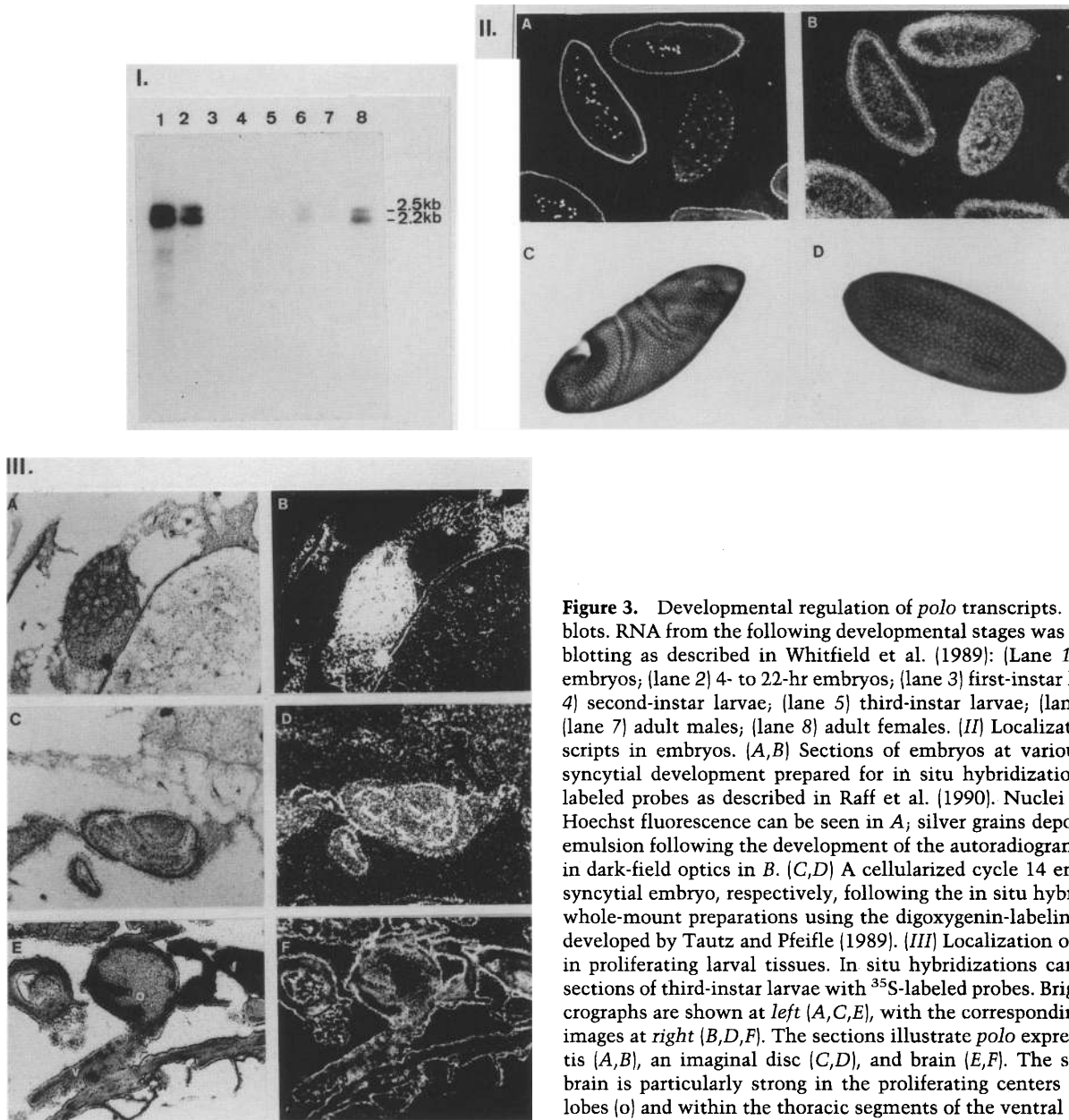


Figure 3. Developmental regulation of *polo* transcripts. (I) Northern blots. RNA from the following developmental stages was prepared for blotting as described in Whitfield et al. (1989): (Lane 1) 0- to 4-hr embryos; (lane 2) 4- to 22-hr embryos; (lane 3) first-instar larvae; (lane 4) second-instar 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 Raff 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).

indirect immunofluorescence. Different antibodies give equivalent staining patterns, examples of which are shown in Figure 7 illustrating the different stages of mitosis during the syncytial division cycle 13. During interphase, the *polo* protein is located predominantly in the cytoplasm (A). This cytoplasmic immunostaining becomes less pronounced during prophase (B), as the antigen begins to associate with condensing chromatin. Chromatin association is strongest at metaphase and anaphase (C,D), and during telophase the antigen appears to dissociate from chromosomes and cytoplasmic staining returns. This cyclical behavior is also seen in the newly cellularized embryo (Fig. 8). Figure 8 shows the anterior pole of an embryo that exhibits several of the

cycle 14 mitotic domains. The strongest staining is seen in interphase cells (i) in which the antigen is cytoplasmic. The staining becomes spread throughout cells as they progress through prophase (p), coming to associate with the condensed chromosomes during metaphase and anaphase (m/a).

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

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0      GCTCCGCTTTGTGCTTGGTTTTCGTGCAATTCGATGGGCAAACCGAGATTTGATCGCCGCTTCTCTTGTCAACCGTGTGG
81     ATCTGATCTCCGTTTTCCGAGGCACCTTCAGCCGATCGCCGGTGAATTATAGGCTCAGAACGAGGAGTATCGCCGCAAAA
161    CTCCAGGCGAACGCAAACGCAAAGGGGCGAGTCCGTAGTAAAGAAGAAAGGAGAGCAAGATGGCCGCGAAGCCCGAGGAT
      M A A K P E D 7
241    AAGAGCACGGATATTCGGATCGCCTCGTCGACATCAACCGCGGAAAACCTACAAGCGCATGCGGTTCTTCGGCAAGGG
      K S T D I P D R L V D I N Q R K T Y K R M R F F G K G 34
321    CGGGTTTGCAAATGTTACGAGATCATCGATGTGAAACCGACGACGCTTCGCCGGCAAGATCGTATCCAAGAAGCTGA
      G F A K C Y E I I D V E T D D V F A G K I V S K K L 60
401    TGATCAAACACAATCAGAAGGAGAAGACCGCCAGGAGATAACTATTCACCGGAGCCTTAACCATCCGAACATTGTCAAG
      M I E K H N Q K E K T A Q E I T I H R S L N H P N I V K 87
481    TTTCAACTACTTTGAAGATTTCGAGAATATCTACATTGTGCTGGAGCTGTGCAAGAAAAGATCCATGATGGAGCTGCA
      F H N Y F E D S Q N I Y I V L E L C K K R S M M E L H 114
561    CAAACGTAGGAAGAAGCATTACGGAGTTCGAATCCGCTACTACATTACAGATAATCCAGGGCGTTAAGTACTTGCACG
      K R R K A S I T E R F E R K I T I H R S L N H P N I V K 140
641    ATAACCGCATTATCCATCGAGATCTGAAGCTGGCAATCTCTTCTCAACGATTTGTTGCACGTGAAGATCGGGGATTTC
      D N R I I H R D L K L G N L F L N D L L H V K I G D F 167
721    GGGTTGGCCACGCGCATTGAGTATGAGGGCGAGCGAAAAAGACCTTATCGGGAACCGCCAATATATAGCCCCGGAGAT
      G L E Y E K E K T A Q E I T I H R S L N H P N I V K 194
801    CCTACCAAGAAGGGCCACTCCTTCGAGGTGGACATCTGGTGCATTGGCTCGTTCATGTACACACTGCTTGTGGGCCAGC
      L T K K G H S F E V D I W S I G C V M Y T L L V G Q 220
881    CGCCGTTTCAAAACCAAGACTCTGAAGGATACGTACTCGAAAATCAAGAAGTGCAGTACCGCGTGCCACGCTACTTAAGG
      P P F C E A T K T L K E T A Q E I T I H R S L N H P N I V K 247
961    AAACCGCGCGGCGATATGGTTCATCGCCATGCTGCAGCCAAATCCGGAGAGCCGCCCGCAATTGGTCAGCTGCTGAACTT
      K P A A D M V I A M L Q P N P E S R P A I G Q L L N F 274
1041   TGAGTTCCTCAAGGGCTCAAAGGTGCCATGTTCTTGCCAGCTCTTGTCTGACAATGGCGCCGCTATCGGCAGCAACG
      E F L K G S K V P M F L P S C L T M A P R I G S N 300
1121   ACACCATCGAGGATTCGATGCACCGCAAGCCACTGATGGAGATGAACGGCATCAGGCCCGACGACACTCGTCTGGAGTCCG
      D T I E D S M H R K P L M E M N G I R P D D T R L E S 327
1201   ACCTTCTCAAGGCCAATCTGCACGACGCCATTACCGCGTCAGCGCAGGTGTGCCGCCACAGCGAGGACTATCGCAGCGA
      T F L H D A N L H D A I T A S A V Q V C R H S E D Y R S D 354
1281   TATCGAGAGCCTGTACCAGCAGCTCACTAATCTTATCAACGGAAAGCCGCAATTCTGCAAGCAATCTGGCGACGAGA
      I E S L Y Q Q L T N L I N G K P R I L Q G N L G D E 380
1361   ACACAGATCCTGCAGCGCAGCCGCTCTTCTGGATATCCAAGTGGGTTGACTACAGCGACAAGTACGGATTGGTTACCAG
      N A Q P L F W F I S K W V D Y S D K Y G F G S D 407
1441   CTGTGCGATGAGGGCATCGGCGTGTGTTCAACGACACCACAAGCTGATCCTGCTGCCGAATCAGATCAACGTACACTT
      L C D E G I G V M F N D T T K L I L L P N Q I N V H F 434
1521   CATCGACAAGGATGGCAAGGAGACGTACATGACCATTCCAGGATTACTGCAAGTCCGTTGACAAGAAGATGAAGCTGCTGT
      I D K D G K E T Y M T I T A S A V Q V C R H S E D Y R S D 460
1601   CGTACTTTAAGCGCTACATGATCGAGCACCTGGTGAAGGCAGGTGCCAACAAATGTGAACATTGAGAGCGATCAAATCTCG
      S Y F K R Y M I E H L V K A G A N N V N I E S D Q I S 487
1681   CGTATGCCCAHTTACACTCCTGGTTCGGTACAACATGTGCCGTAGTTATGCAATTTGACCAACGGTCTGTGCAGCTAAA
      R M P L H S W F R M T T C A V V M H L T N G S V Q L N 514
1761   CTCTCAGATCACATGAAGCTCATCCTCTGCCCGCATGAGTGCTATAACCTATATGGACCAGGAGAAGAAGCTCCGCA
      F S D H M K L I L C P R M S A I T Y M D Q E K N F R 540
1841   CCTACCGATTTTCGACCATTTGTGGAGAACGGCGTGTCTAAAGACTTGTACCAGAAGATCCGATATGCCAGGAGAAACTT
      T Y R F S T I V E N G V S K D V L Y Q K I R Y A Q E K L 567
1921   AGGAAAATGCTGGAGAAGATGTTACATAAGCGTAGCCAGCCCAACTATCATTATAAGGCCGAATGTTAGTTTAAAGCTAA
      R K M L E K M F T 576
2001   TTCACGAATGCCCTGGCCAACTTCATTTATAGCCAGAAAGTATCCTCTCCCATCATCTTTAAATTTGATGTTCCCGTT
2081   CAAATTGATTTGTTTCGATGTTTATAGAATTTATTTGTTTTGCCCCCTCCCTTCATATCGAAAATACTGTTAAGTTAT
2161   ATTCATCGTCAGTGTGGGCTCCCTCAAAGTAATTTAATATATCTGTTTAAATGGTTTTCGTACAGATCCGATCACTT
2241   AATGCATTTTAAAGAGATCAAATTAATGTTTAAACTAAGCAAACGTTTCGAAATGCCTATATTCACCGAGGTGACTG
2321   ATAACAAAATTTAATGCTGGATACATTATAAAGTAATAGTGAATATTGTGCGTTCGTAGTGCCTATAGCCCATTT
2401   AAAATAATACATAAGTTACAATACTGCTGCAAAGTGTAAAGTGTACAAGTATATTCAACTTTGGCCAGAAATATCTGTA
2481   GCTATAGGATACAATTTGTAATGCTTTTGAACATAAAGCGAATAATATAAAATTTAAT

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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 *cdc2* gene itself encodes a 34-

kD protein kinase, p34^{cdc2} whose homolog is a component of mitosis promoting factor (MPF) in multicellular eukaryotes, and is thus part of a highly conserved mech-

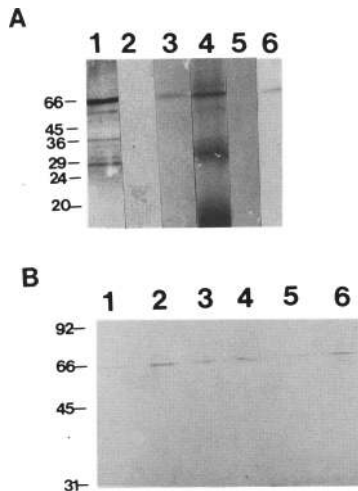


Figure 6. Detection of the putative *polo* kinase with antibodies. (A) Coupled in vitro transcription and translation of *polo* cDNAs was carried out as described in Materials and methods. The total protein products of such a reaction with cDNAs 2a2 and 9a2 are shown in lanes 1 and 4, respectively. The 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.

anism that regulates the G_2 -M transition (for review, see Nurse 1990). The active form of $p34^{cdc2}$ is associated with a mitotic cyclin, a protein that undergoes periodic degradation at each metaphase-anaphase transition in most mitotic cycles. The $p34^{cdc2}$ kinase is activated by dephosphorylation on tyrosine and threonine residues in its ATP-binding site and phosphorylation on a threonine residue. The enzymes responsible for these phosphorylation or dephosphorylation steps have not been identified, although dephosphorylation at the tyrosine residue is controlled in fission yeast by *cdc25*. *Drosophila* has a *cdc25* homolog, *string*, that has been shown to positively regulate the G_2 -M transition within the mitotic domains of the cellularized embryo (Edgar and O'Farrell 1989, 1990). $p34^{cdc2}$ kinase is under the negative regulation of *wee1*, which itself encodes a protein kinase (Rus-

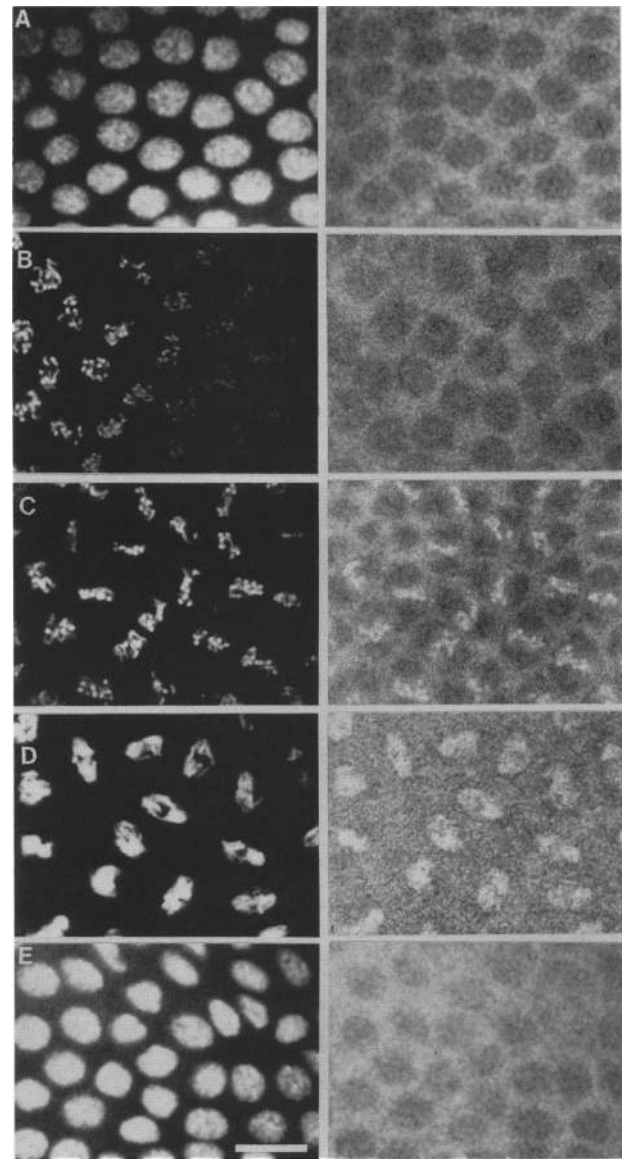


Figure 7. Cyclical distribution of *polo* kinase during syncytial mitotic cycles. Embryos were prepared to visualize DNA with propidium diode (left), and the *polo* protein by indirect immunofluorescence (right) by using the techniques described previously by Whitfield et al. (1990) for confocal microscopy. 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 μ m.

Figure 5. Comparison of the catalytic domain of the putative *polo* kinase with other kinases. The catalytic domains of *polo* and other kinases have been aligned with reference to the 11 sub-domains (I-XI) described by Hanks et al. (1988), indicating with asterisks (*) the most highly conserved residues noted by these investigators. These and all other conserved amino acids have been boxed. The kinases used for comparison are *nim1* of *Schizosaccharomyces pombe* (Russell and Nurse 1987b); cGMP, cGMP-dependent protein kinase of *Drosophila* (Kalderon and Rubin 1989); TPK1, cAMP-dependent protein kinase of *Saccharomyces cerevisiae* (Toda et al. 1987; Lisiewicz et al. 1987); DPKC, protein kinase C-related gene product of *Drosophila* (Rosenthal et al. 1987); CDC28, *cdc28* kinase of *S. cerevisiae* (Lorincz and Reed 1984); CDC2HS, functional homolog of *S. pombe cdc2* from human (Lee and Nurse 1987); *wee1*⁺, *wee1*⁺ kinase of *S. pombe* (Russell and Nurse 1987a); *NIMA*, never in mitosis gene of *Aspergillus* (Osmani et al. 1988). The number of the amino acid beginning the catalytic domain comparison is indicated on the first line of each sequence, and the amino acid final number is on the last line. Finally, percent identity is indicated in brackets.

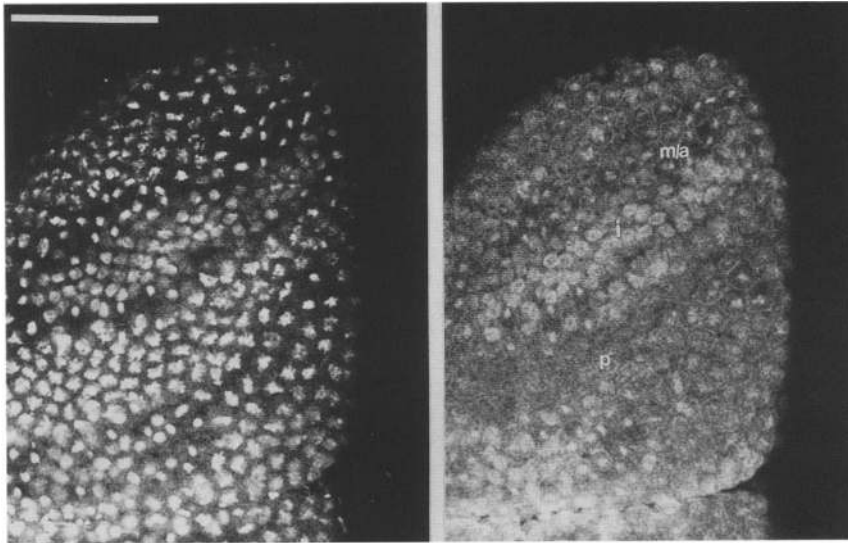


Figure 8. Distribution of *polo* kinase with respect to mitotic domains in cycle 14. Shown is the anterior of a cellularized embryo prepared for immunofluorescence as in Fig. 7. Several mitotic domains can be seen as distinct regions containing condensed chromatin. The three labeled areas exemplify cells 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 μ m.

sell and Nurse 1987a; Featherstone and Russell 1991). Deletion of this fission yeast gene causes cells to enter mitosis at half the size of wild-type cells. The *wee1*-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 p34^{*cdc2*} 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 *wee1*⁺ activity, although 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, its catalytic domain shows only 29% identity to that of *nim1*. This compares to a 59% identity between fission yeast *cdc2* and its functional *Drosophila* counterpart. On the other hand, *string*, the functional counterpart of *cdc25* (Edgar and O'Farrell 1989; Jimenez et al. 1990), shows just 35% identity to the fission yeast gene but only in a carboxy-terminal domain corresponding to ~39% of its length.

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 p34^{*cdc2*} 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*¹ fail to develop, as a consequence of totally aberrant mitotic events throughout the syncytial stages (Sunkel and Glover 1988). However, chromatin,

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)y* (Lin and Wolfner 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 *polo*¹ 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 *polo*¹ 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 *polo*¹ allele as having some residual function because maternal protein in larvae homozygous for the *polo*² 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 *polo*¹ 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 *polo*¹ 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 *polo*¹ 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 *polo*² 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 mutant third chromosomes from these stocks were tested for their ability to complement the *polo*¹ 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 *polo*². 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 (*l*²) 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 *polo*² also carried *l*². 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 *polo*², the *rec2* chromosome was subjected to hybrid dysgenesis to select for revertants of the *polo*² 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 77A3 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 λ EMBL4. 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 *Bam*HI fragment from phage E-1 (Fig. 2) was introduced into pW8 (Klemenz et al. 1987) and injected together with the helper element, pUChs 2-3, into embryos homozygous for *w¹¹⁸*. Lines were established in which the insertions were carried on either the X or second chromosomes. *w¹¹⁸* P[*w polo*] 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 polo*] *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 polo*]/*w¹¹⁸*; *ru st pol e ca/ru st pol 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 *Sal*I 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 *Sph*I–*Not*I 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* are replaced with 11 amino acids from the amino terminus of the T7 S10 protein. Protein preparations from bacterial cultures expressing these fusion proteins were fractionated by preparative SDS-PAGE. The protein specified by pUR9a2N was injected into a rabbit that produced a serum designated RB287. The protein specified by pAR9a2F was injected into mice that were used to raise several monoclonal antibodies as described in Harlow and Lane (1988). Polyclonal sera were absorbed against *E. coli* extracts enriched in β -galactosidase to remove antibodies against this component of the fusion protein. Immunostaining of embryos was performed with the polyclonal antiserum Rb287 and mAbs MA75 and MA81, all of which gave identical staining patterns.

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 *Nbo*I 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 μ Ci of [³⁵S]methionine (800–1200 Ci/mmmole, Amersham) in 20 μ l of rabbit reticulocyte lysate (Amersham) for 1 hr at 37°C. Before boiling for 3 min, 6.5 μ l of the reaction was prepared for SDS-PAGE by incubation with an equal volume of RNase (100 μ g/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 μ l 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 μ l) was added, and the mixture was incubated for 15 min at 4°C. IgG-sorb (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 μ l of SDS-gel-loading buffer. Following electrophoresis, the gel was fixed in methanol, and dried for autoradiography.

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