par-2, a gene required for blastomere asymmetry in Caenorhabditis elegans, encodes zinc-finger and ATP-binding motifs

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ABSTRACT  The par-2 gene of Caenorhabditis elegans functions in early embryogenesis to ensure an asymmetric first cleavage and the segregation of cytoplasmic factors. Both processes appear to be required to generate daughter blastomeres with distinct developmental potential. We isolated an allele of par-2 by using a screen for maternal-effect lethal mutations in a strain known for its high frequency of transposon insertion events. A transposable element was linked to this allele. Sequences flanking the site of transposon insertion were cloned and found to rescue the par-2 mutant phenotype. DNA in the par-2 region hybridized to a 2.3-kb germ-line-enriched mRNA. The cDNA corresponding to this germ-line-enriched message was cloned, sequenced, and used to identify the molecular lesions associated with three par-2 alleles. Sequence analysis of the par-2 cDNA revealed that the predicted protein contained two distinct motifs found in other known proteins: an ATP-binding site and a cysteine-rich motif which identifies the par-2 gene product as a member of a growing class of putative zinc-binding proteins.

Early embryogenesis in the nematode Caenorhabditis elegans is characterized by a series of asymmetric cleavages. The initial asymmetry of the early embryo becomes apparent during the first cell cycle, at which time cytoplasmic factors thought to be required for the specification of cell fate are segregated. The first cleavage produces a larger, anterior cell (AB) and a smaller, posterior cell (P1) which differ from each other in many respects including the orientation of subsequent cleavage planes, the timing of cell cycles, and the kinds of differentiated cell types they ultimately produce (1). For instance, germ-line-specific P granules are uniformly dispersed in one-cell embryos but become localized to the posterior cortex midway through the first cell cycle (2). At first cleavage, nearly all of the A granules are partitioned to the P1 blastomere. Furthermore, studies of gut development (3) have shown that factors required for specification of the intestine are present in P1, and not AB, at the two-cell stage. Blastomere fusion experiments have shown that these factors can be found in the cytoplasm of P1 (4).

The nature of the cytoplasmic mechanisms that generate asymmetry in the one-cell embryo are unknown, but microfilaments play a significant role in this process. Many of the manifestations of asymmetry in the early embryo can be disrupted by treatment of embryos with cytochalasin D (5). Treated one-cell embryos typically divide symmetrically and have mislocalized P granules; subsequent cleavage patterns are also abnormal. Presumably, disorganization of the microfilament system between fertilization and pronuclear fusion affects the segregation of factors that govern embryo polarity.

A class of genes has been identified that is required for the generation of asymmetry in the early embryo and whose mutant phenotype resembles the cytochalasin D-treated embryos described above (6). Mutations in any one of the five par genes (for partitioning-defective) result in embryos with defects in the position of the first cleavage furrow, altered orientation of the spindle at the second cleavage, synchronous early cleavages, defects in P-granule localization, and altered expression of intestinal cell differentiation markers. All mutations in these five genes result in strict maternal-effect lethality; therefore maternal expression of the par genes is required during oogenesis in order to produce viable embryos.

To gain further insight into the role of the par genes in cytoplasmic partitioning, molecular analysis of these genes is essential. As a step toward an understanding of the generation of asymmetry and cytoplasmic localization in the C. elegans embryo at the molecular level, we have isolated and sequenced the par-2 gene.

MATERIALS AND METHODS

Genetics. Nematode strains were maintained according to Brenner (7). The strain for identification of a transposon-induced allele of par-2 was constructed by crossing egl-23(n601) into the mutator strain TR679. Mutator strains in C. elegans undergo a high rate of transposition. The mutator activity in TR679, mut-2(r459), can be balanced by the chromosomal rearrangement mnt-1 (8). Homozygous egl-23(n601);mut-2(r459) animals were derived from the heterozygous strain egl-23(n601);+;mnt-1/mut-2(r459). These egl-23(n601);mut-2(r459) animals were the starting strain for the screen for maternal-effect lethal mutations. The screen was performed as described by Kemphues et al. (6).

Embryos from candidate strains bearing maternal-effect lethal mutations were examined by Nomarski microscopy and assigned to a linkage group. One mutation identified in this screen, jb2, mapped to LGIII. Eggs from homozygous jb2 mothers exhibited synchronous and symmetric early cleavages. Since mutations in a known gene (par-2; see ref. 6) on LGIII result in a similar phenotype, complementation tests were performed between jb2 and par-2(it55s). jb2 failed to complement par-2(it55s).

Abbreviations: YAC, yeast artificial chromosome; SSCP, single-strand conformation polymorphism.
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The sequence reported in this paper has been deposited in the GenBank database (accession no. L26987).

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The jh2 mutation was backcrossed 10 times to homozygous daf-7(e1372ts), which was shown previously to map 1% to the left of par-2, to generate the strain of the genotype egl-23(m601); daf-7(e1372ts)/+; par(jh2). At 25°C, homozygous daf-7(e1372ts) animals arrest as dauer larvae and can therefore be used to balance the par-2(jh2) mutation.

The following strains were constructed for isolation of recombinants between par-2(jh2) and flanking markers: egl-23(m601); + dpy-1(e171); daf-7(e1372ts); par(jh2)+ and egl-23(m601); + dpy-1(e171); + par-2(jh2)+ and daf-7(e1372ts). These parent strains were maintained at 25°C, and homozygous Daf animals were picked to individual plates, allowed to recover at 16°C, and then examined for the production of progeny. Nonrecombinant animals are Daf and produce large numbers of dead eggs, whereas recombinant Daf non-Par animals produce many progeny. Recombinant genotypes are either daf-7+ dpy-1 daf-7 par-2+ or + dpy-1 daf-2 par-2+ daf-2. Strains homozygous for individual recombinant chromosomes were constructed. DNA was isolated from an expanded population of each strain and examined by Southern hybridization for the presence of a linked transposon.

The par-2 allele hw32 is an ethyl methanesulfonate-generated mutation generously provided by Jocelyn Shaw (University of Minnesota, St. Paul, MN). par-2(hw32) is an ethyl methanesulfonate-generated allele generously provided by Niansheng Cheng (Cornell University, Ithaca, NY). par-2(1w191) was isolated in a screen for mut-6-induced maternal-effect lethal mutations carried out in the lab of J. Priess (Fred Hutchinson Cancer Research Center, Seattle, WA). (C. Mello, B. Draper, K. Kemphues and J. Priess, personal communication).

Southern and Northern Analysis. For Southern analysis, genomic DNA was prepared by the proteinase K method (1). DNA was digested with restriction enzymes, separated by gel electrophoresis, and transferred to Nytran filters (Schleicher & Schuell) by standard techniques (9). For Northern analysis, RNA was isolated by the glass-bead method (10). Poly(A)+ RNA was purified on poly(U)-Sepharose and was subjected to electrophoresis through 0.4 M formaldehyde/1% agarose gels (9). After electrophoresis, gels were rinsed in distilled water and the separated RNAs were transferred to Nytran filters in 10× SSPE (1.8 M NaCl/100 mM sodium phosphate pH 7.8/10 mM EDTA). Radiolabeled DNA probes for both Northern and Southern analysis were prepared by the random-primer labeling method (11).

Cloning of 3.0-kb HindIII Fragment. A 3.0-kb HindIII-generated fragment containing the transposon TcI was identified that was present in the par-2(jh2) backcrossed strain and both parental strains but was absent from 20 Daf-7 non-Par recombinants in the interval to the left of par-2 and 70 Daf-2 non-Par recombinants in the interval to the right. Thus, this 3.0-kb HindIII fragment was tightly linked to par-2(jh2). This HindIII-generated fragment was isolated from the backcrossed egl-23(m601); daf-7(e1372ts)+ par-2(jh2)+ strain by gel purification of HindIII-digested genomic DNA of 2.5–4.0 kb and ligation of the purified DNA fraction to HindIII-cut pAM1499 DNA. The ligation was packaged in vitro (Gigapack, Stratagene) and plated on Escherichia coli C600 host cells. Plaques were transferred to Nytran filters and hybridized to radiolabeled pCE2001, a plasmid containing TcI sequences (12).

DNA Sequencing. cDNA libraries analyzed during the course of this work were generously provided by Stuart Kim (Massachusetts Institute of Technology, Cambridge, MA), Irene Schauer (University of Colorado, Boulder, CO), Chris Martin (Columbia University, New York, NY), Bob Barstead (University of Missouri, St. Louis, MO), and Julie Ahring (University of Wisconsin, Madison, WI). Restriction fragments isolated from the cDNA clones were inserted into the plasmid vector pBluescript SK or KS (Stratagene).

Clones were sequenced by the chain-termination technique using Sequenase enzyme (United States Biochemical). All sequencing was performed on double-stranded template DNA; both strands of all cDNA subclones were sequenced.

Preparation of Yeast Artificial Chromosome (YAC) DNA for Injection. The Y11F11/rol-6 YAC was generated by first inserting an EcoRI fragment containing the dominant rol-6 gene into the plasmid pSL42-2 (a YIp5 derivative from S. Carl Falco, DuPont), which contains the yeast LYS2 gene. The resulting plasmid, pL2R6, was linearized at the unique SafI restriction site and transformed into the yeast strain bearing the Y11F11 YAC. The desired recombination product was recovered by selecting for lysine prototrophy. One liter of yeast cells harboring the Y11F11/rol-6 YAC were grown at 30°C under selective conditions. Cells were harvested, lysed, and subjected to centrifugation through two sucrose gradients (see ref. 13 for details) to obtain fractions enriched in YAC DNA. par-2(it5s) animals were injected as described (14).

Polymerase Chain Reaction (PCR), Single-Strand Conformation Polymorphism (SSCP) Analysis, and Sequencing. PCR assays were performed on individual animals as described (15). Reaction conditions differed from those described in that the final reaction mixture contained 1 mM MgCl2, 0.2 μM primers, and 0.1 mM dNTPs. Reactions were cycled 35 times: 94°C for 1 min, 57°C for 2 min, 72°C for 2 min. Primers DL15 (5′-CAACATGCTGGTCGAGAA-3′) and DL16 (5′-GAAAGGGTTGACATGC-3′), which hybridize to sequences in the fifth exon of the par-2 cDNA, were used for all reactions. For SSCP analysis (16), 7 μCi (259 kBq) of (α-32P)dATP was added to each reaction mixture. Two microliers of each reaction was added to 18 μl of 95% formamide/1 mM NaOH/2 mM EDTA and was boiled for 10 min; then 2.5 μl was loaded onto a polyacrylamide gel (6% (wt/vol) acrylamide/5% (vol/vol) glycerol/1× TBE (89 mM Tris/89 mM boric acid/2 mM EDTA); running buffer, 0.5× TBE) and subjected to electrophoresis at 52 V for 5 hr. Ten par-2 alleles were tested for a SSCP with DL15 and DL16. Only PCR DNA from par-2(hw32) showed evidence of a polymorphism. The par-2(1w191) insertion/duplication was detected by PCR with DL15 and DL16 in single worms. The sequences of the PCR products of both the par-2(hw32) and par-2(1w191) mutant alleles were determined by the chain-termination technique. PCRs were scaled up to 100 μl and the products were purified by using Genelean glass beads (Bio 101) according to the manufacturer's protocol. Approximately 1 μg of DNA was added to 0.2 μmol of primer, boiled for 3 min, and then sequenced according to the Sequenase protocol (United States Biochemical). PCR products from at least two individual worms were sequenced separately for each allele.

RESULTS

par-2 Mutant Phenotype. The par-2 mutant phenotype has been described previously (6). Briefly, embryos from homozygous par-2 mothers exhibit a symmetric first cleavage. The two resulting daughter cells then divide at the same time and in the same orientation and produce a four-cell embryo with blastomeres of equal size. Mutant par-2 embryos arrest as balls of cells without undergoing morphogenesis, although cellular differentiation of most cell types has been observed (6). Temperature-shift experiments have shown that the temperature-sensitive period of the par-2 gene product begins 12 hr before fertilization and ends before the first cleavage (17).

Cloning of Sequences Linked to par-2. We identified an allele of par-2 in a transposon mutagenesis screen designed to identify maternal-effect lethal mutations (see Materials and Methods). C. elegans strains have been found that have
increased transposition rates of the transposon Tcl (8) and that consequently have an increased rate of spontaneous mutation. The par-2 allele jb2 was isolated from such a strain, and was backcrossed 10 times to the wild-type strain C. elegans var. Bristol (N2). By recombination analysis, we analyzed this strain for the presence of Tcl elements associated with the mutation. We identified a Tcl-containing 3.0-kb HindIII DNA fragment that was located in the interval between 0.05% to the left and 0.1% to the right of par-2 (see Materials and Methods for details). We cloned the 3.0-kb HindIII-generated fragment containing Tcl from a size-selected library made from the backcrossed strain by probing with radiolabeled Tcl and analyzing positive clones by restriction analysis. DNA containing the Tcl was excised and the flanking DNA was used to probe existing cosmids and YAC libraries. In collaboration with John Sulston, Alan Coulson, and coworkers, who have physically mapped 95% of the worm genome (18), we identified one cosmids, F58B6, and one YAC, Y11F11, that contain DNA flanking the Tcl insertion in par-2(jb2).

Rescue of the par-2 Mutant Phenotype by Microinjection.

We used the 80-kb Y11F11 YAC in microinjection experiments designed to rescue the par-2 mutant phenotype. To identify successful injection of YAC DNA, we first inserted the dominant rol-6 gene (14) into one arm of the YAC by recombination in yeast. DNA from this YAC, Y11F11/rol-6, was partially purified from yeast chromosomes and was injected at a concentration of 100 ng/µl into par-2(it5ts) hermaphrodites raised at the permissive temperature (16°C). Injected animals were placed at the permissive temperature. When the F1 progeny of the injected animals reached the L3-L4 larval stage, we shifted them to restrictive temperature (25°C). After the F1 progeny matured and self-fertilized, we examined the plates for the presence of F2 larvae instead of dead eggs. One out of 42 injected animals produced F2 larvae at the restrictive temperature; many of these F2 progeny were rollers. In subsequent generations, only the Rol animals produced progeny at 25°C. This transformed line continued to grow at 25°C for several months. In contrast, when uninjectected par-2(it5ts) animals or par-2(it5ts) animals injected with rol-6 alone are shifted to 25°C, 96% of the eggs produced are dead and the small number of eggs that hatch are not rollers. And F2 DNA isolated from the transformed line confirmed the presence of YAC DNA in the strain (data not shown). The cosmids F58B6 failed to rescue the par-2 mutant phenotype. Subsequent analysis showed that the cosmids was deleted for portions of the par-2 gene (data not shown).

Identification of an Allele-Specific par-2 Polymorphism. The microinjection results described above indicate that the par-2 gene is present on the Y11F11 YAC but that transformation rescue would not permit us to delimit further the sequences encoding par-2. As an alternative approach, we searched for allele-specific DNA polymorphisms by using small subclones of Y11F11 to probe DNA from par-2 mutant alleles. Using a 1.0-kb Pst I-Xba I fragment from the cosmids F58B6, we detected a restriction fragment length polymorphism in the strain bearing the par-2 allele it46. DNA isolated from the heterozygous strain carrying a 2.3-kb fragment of a 4.3-kb hybridizing Cla I fragment in addition to the wild-type 2.8-kb Cla I fragment (Fig. 1, lanes 7 and 8). The polymorphic fragment resulted from fusion of two adjacent Cla I fragments by deletion of a Cla I site and surrounding sequences in the it46 mutant allele. Examination of wild-type and mutant DNA digested with EcoRI (Fig. 1, lanes 3 and 4) showed that a total of 4.5 kb was deleted. The parental strains from which it46 was isolated did not exhibit this polymorphism (data not shown). Restriction mapping in this region indicated that the site of the it46 deletion was 12 kb from the site of Tcl insertion in the jb2 allele.

Northern Analysis. To test whether the it46-associated deletion might cover the par-2 gene, we asked whether there was an mRNA transcribed from the region deleted in it46 with an expression pattern consistent with our expectations for the par-2 gene. We predicted that transcription from a strictly maternal-effect lethal gene such as par-2 should be highest in the female germ line. We compared mRNA isolated from a mutant strain with an exclusively female germ line to mRNA isolated from a mutant strain with a dramatically reduced germ line. The fem-2(2b42sts) mutation transforms XX animals, which are normally hermaphrodites, into fertile females; i.e., at the restrictive temperature these animals do not make sperm (19). The glp-l(e214lts1) mutation affects germ-line proliferation so that only 4-10 germ cells are formed at restrictive temperature. The somatic gonad is essentially wild type in these animals (20). We used DNA corresponding to the deleted region in the it46 allele to probe Northern blots containing poly(A) RNA from fem-2(2b42sts) and glp-l(e214lts1) animals grown at the restrictive temperature. The probe hybridized to a 2.3-kb RNA from fem-2 "female" animals (Fig. 2, lane 1). The 2.3-kb RNA was not present in RNA isolated from glp-1 animals (Fig. 2, lane 2).

Isolation of a par-2 cDNA. We used DNA corresponding to the deleted region in the it46 allele to probe cDNA libraries. The largest cDNA we identified was 2.2 kb. When this cDNA was used to probe a Northern blot, it hybridized to a single germ-line-enriched 2.3-kb mRNA, suggesting that this cDNA corresponded to the same mRNA as that identified by the genomic fragment. Primer extension analysis and PCR amplification of a cDNA pool synthesized from RNA by using an oligonucleotide homologous to SSL2 [a 22-nucleotide transspliced leader sequence (21)] and an oligonucleotide homologous to the sequenced cDNA suggested that the putative par-2 message was trans-spliced (data not shown).

We hybridized a 5'-specific cDNA probe and a 3'-specific cDNA probe to restriction enzyme digests of the cosmids, YAC, and genomic DNA so that we could orient the cDNA relative to the allele-specific polymorphisms we described earlier. These data are summarized in Fig. 3A. The par-2 gene spans >20 kb of genomic DNA. The it46 deletion, as de-

![Fig. 1. Identification of an allele-specific restriction fragment length polymorphism.](image-url)
Fig. 2. Northern blot of RNA isolated from fem-2 and glp-1 animals. Three to four micrograms of poly(A)+ RNA was run in a formaldehyde/1% agarose gel and blotted to a Nytran filter. The filter was hybridized to radiolabeled DNA isolated from the region of the par-2(1666) deletion and to a radiolabeled anc-54 (myosin heavy-chain gene) to control for amount of RNA loaded in each lane. The filter was washed with 1× SSPE/0.25% SDS at 65°C for 1 hr. Lane 1, RNA from fem-2(b2434ts) animals grown at the restrictive temperature; lane 2, RNA from glp-1(e2141ts) animals grown at the restrictive temperature. The anc-54 probe hybridized to a 6.0-kb message; the genomic DNA from the region of the par-2(1666) deletion hybridized to a 2.3-kb message.

scribed above, encompasses the Cts I site that is also present in the 5′ end of the cDNA and thus deletes coding sequences contained in this germ-line-enriched cDNA clone. The Tci I insertion site in the jb2 allele is located 3′ to sequences in this cDNA.

Identification of Additional Allele-Specific Polymorphisms.

To verify that this cDNA clone represents the par-2 mRNA, we identified two additional lesions in this transcribed region that are associated with par-2 alleles. A polymorphism was found in the par-2 allele lw32 by SSCP analysis, and a polymorphism was found in the par-2 allele, zu191, by analysis of PCR-amplified DNA from single worms. The location of both these polymorphisms is shown in Fig. 3A. Sequence analysis has revealed that the lw32 lesion is a C → T transition at nucleotide 711 of the cDNA resulting in the formation of a TGA stop codon. The truncated protein is 233 amino acids long and is missing a putative ATP-binding site (see below). The zu191 polymorphism is a 19-nucleotide insertion/duplication shown in Fig. 3B. This insertion into the gene causes a stop codon at the point of insertion, truncating the open reading frame to 197 amino acids.

Sequence Analysis of the par-2 cDNA. The par-2 cDNA encodes a single large open reading frame of 628 amino acids. When the putative protein product derived from the par-2 cDNA was compared with known protein sequences in the GenBank, Swiss-Prot, and PIR databases (October, 1993), two distinct motifs were observed. The first motif was a cysteine-rich region belonging to a recently recognized, rapidly growing class of proteins. The second motif is present in proteins derived from viruses, yeast, flies, mice, frogs, and humans (22–25), and a peptide containing this motif has been shown to bind zinc (22). The Par-2 version of this motif is shown in Fig. 4A, compared with the domain present in other members of the family. As in other family members, the putative zinc-binding motif of Par-2 is found in the amino-terminal end of the protein. While many of the proteins in this family harbor nuclear-localization signals, no such signal has been identified in the predicted Par-2 protein.

The predicted Par-2 also possesses a putative ATP-binding site of the myosin class (type A; see ref. 28). Fig. 4B shows the type A ATP-binding site consensus sequence, the Par-2 putative ATP-binding site, and the sequence of selected proteins containing this motif for comparison. Par-2, however, lacks other hallmarks of myosin proteins such as actin-binding domains or an a-helical coiled-coil structure.

**DISCUSSION**

We report the isolation and sequence analysis of the maternal-effect lethal gene par-2. par-2 plays an essential role in partitioning of cytoplasmic factors; the isolation of the gene is a step toward understanding this process at the molecular level. We present evidence that DNA sequences adjacent to and including the site of a transposon-associated mutation in par-2 rescue the mutant hz-2 phenotype. These data allow us to identify a cDNA clone that is likely to be derived from the par-2 mRNA. The transcription unit represented by this cDNA lies on an 80-kb YAC that rescues the par-2 mutant phenotype; the transcript is germ-line enriched, as one would expect for the par-2 transcript, and there are lesions in exons of this transcript associated with three par-2 alleles. We feel that these data provide compelling evidence that we have isolated the par-2 gene. The Tci I insertion site in par-2(b2) does not appear to be in coding sequences and thus may disrupt a 3′ regulatory region. Alternatively, insertion of the transposon may not cause the mutant phenotype and merely may be linked to par-2. Sequencing of this mutant allele will verify whether other sequence alterations are present.

The presence of a possible zinc-binding motif in the predicted Par-2 protein is intriguing. Many proteins containing similar motifs have been found to bind specifically to nucleic acids via a structure termed the zinc finger (30). Presently, it is unclear whether Par-2 and other proteins with the conserved cysteine-rich motif bind to nucleic acids. Evidence on individual members of this class has led to predictions of DNA binding (23, 25), RNA binding (24, 31), and protein-protein interactions (30, 32). If Par-2 functions as a DNA-binding protein, it may activate downstream genes responsible for establishing or maintaining asymmetry in the early
A zinc-binding motif

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B ATP-binding motif

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<th>C. elegans myosin</th>
<th>Yeast MYO1</th>
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Fig. 4. (A) Zinc-binding motif. The consensus sequence for the zinc-binding region is shown along with this motif from Par-2 and from several other members of this class. The references for the sequences listed here are as follows: xenf7 (26); ring1 (22); neu (27); RAD18 (25); consensus sequence (27). (B) ATP-binding motif. The consensus sequence for the type A ATP-binding site is shown along with several members of this class of proteins and the putative par-2 ATP-binding site. C. elegans myosin and bovine ATPase B sequences are from ref. 28. Yeast MYO1 sequence is from ref. 29.

embryo. If Par-2 binds to RNA, it could participate in generating asymmetry by translocating RNA molecules or by regulating the activity of specific RNAs, as is seen in the negative regulation of hunchback mRNA by the zinc-finger-containing protein encoded by the nanos gene (33). If Par-2 is not involved in nucleic acid binding, it may instead interact with other proteins such as components of the microfilament structure, the transport machinery, or partitioned determinants.

The predicted Par-2 protein also contains a putative ATP-binding site of the myosin class. The presence of this motif in Par-2 is consistent with an active, energy-consuming role in cytoplasmic sorting. However, no requirement can be ascribed to either the ATP-binding site or the cysteine-rich domain until specific mutations in these motifs are shown to affect function.

Studies of early C. elegans embryogenesis cited above imply the existence of cytoskeletal architecture dedicated to the process of moving or preventing movement of molecules involved in the process of determining cell fate. Because all five par genes affect the distribution of cytoplasmic components in gene-specific ways (6, 34), it is likely that they function in a common process required for the segregation of cytoplasmic determinants. Additional molecular analysis of par-2 and the other par genes should elucidate their precise role in this process.

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