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The *three rows* Gene of *Drosophila melanogaster* Encodes a Novel Protein that Is Required for Chromosome Disjunction During Mitosis

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Zygotic expression of the *three rows* (*thr*) gene of *Drosophila melanogaster* is required for normal cell proliferation during embryogenesis. Mitotic defects in *thr* mutant embryos begin during mitosis 15, and all subsequent divisions are disrupted. Chromosome disjunction and consequently cytokinesis fail during these defective mitoses, although the initial mitotic processes (chromosome condensation, spindle assembly, metaphase plate formation, and cyclin degradation) are not affected. Despite the failure of chromosome disjunction and cytokinesis, later mitotic events (chromosome decondensation) and subsequent cell cycle progression continue. The *thr* gene has been isolated and shown to encode a 1209 amino acid protein that shares no extended sequence similarity with known proteins. *thr* mRNA is present as maternal mRNA that degrades at the time of cellularization. At this and all subsequent times during embryogenesis, zygotic expression correlates with mitotic proliferation. These observations, together with the observation that the zygotic phenotype of *thr* mutant embryos is influenced by the maternal genotype, suggest that the embryonic phenotype results from exhaustion of the maternal *thr* contribution and does not reflect a developmentally restricted requirement for *thr* function. Our results indicate that the novel *thr* product is required specifically for chromosome disjunction during all mitoses.

INTRODUCTION

Abundant stores of cellular components are stockpiled in the mature eggs of *Drosophila melanogaster*. After fertilization, these maternally derived products drive the cleavage cycles independently of zygotic gene expression (Gutzeit, 1980; Edgar *et al.*, 1986). The maternal contribution of most components required for proliferation appears to be sufficient for all of the embryonic cycles. Mutations in genes encoding such components therefore will not affect embryonic development. The absence of zygotic expression also will not affect larval development in these cases, because larval growth does not involve proliferation but is dependent on endo-replication and cell size increase only. However, pupal development is expected to be dependent on zygotic expression of these genes, because the imaginal cells proliferate during the larval stages. Accordingly, defects in genes required specifically for mitotic cell cycle progression are expected to result in late larval lethality (Shearn *et al.*, 1971; Szabad and Bryant, 1982; Gatti and Baker, 1989).

Defects in embryonic proliferation with consequent embryonic lethality, on the other hand, will result from mutations in genes encoding essential cell cycle functions if the maternal contribution is insufficient to provide for the entire set of embryonic cell cycles. This may be expected in the case of regulatory proteins, because the products of genes encoding regulatory functions are often unstable. The genetic analysis of two important cell cycle regulators (*string* and cyclin A) is consistent with this notion. Mutations in the *string* gene, which encodes a *Drosophila* homologue of the *Schizosacchar-*

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omyces pombe cdc25 gene, result in arrest of embryonic proliferation in the G2-phase before mitosis 14, the first embryonic division dependent on zygotic gene expression (Edgar and O'Farrell, 1989). The intricate transcriptional regulation of zygotic *string* expression controls the spatial and temporal program of the postblastoderm divisions (mitoses 14–16) in the embryo (Edgar and O'Farrell, 1990). Mutations in the gene encoding cyclin A, an unstable protein because of periodic mitotic degradation, result in an arrest of epidermal proliferation before mitosis 16 (Lehner and O'Farrell, 1989). To identify additional potential cell cycle regulators, we have analyzed mitotic proliferation in embryos homozygous for mutations that result in embryonic lethality. An extensive collection of such embryonic lethal mutations has been generated (Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984). In this report we describe the mutant phenotype and molecular characterization of *three rows* (*thr*), a gene whose zygotic expression is required for the embryonic mitoses.

MATERIALS AND METHODS

Drosophila Strains

thr^{1L}, thr^{1B}, and thr^{1IV} (Nüsslein-Volhard *et al.*, 1984) were provided by C. Nüsslein-Volhard. The dysgenic P-element allele, thr^{BH}, was provided by P. Gergen, Stonybrook, NY. The deficiency *Df(2R)Pcl11B*, *Df(2R)XM82*, and *Df(2R)PclW5* were provided by I. Duncan (Washington University, St. Louis, MO), G. Jürgens (University of Munich, Germany), and J. Kennison (NIH, Bethesda, MD), respectively. The rescue crosses described used the following stocks: *w*; ry^{506} *P*[$ry^{+}\Delta 2$-3](*99B*), *w*; *TM6b/TM3*, *w*; *CyO/Sco*, *w*; *cn* thr^{1B} *bw/SM6a*. Canton-S stocks were used for wild-type egg lays. For Southern analysis (see below) all *thr* mutants were crossed to the isochromosomal stocks *cn bw sp* or *b pr cn wx bw* (provided by R. Tearle, University of Adelaide, Australia).

Immunofluorescence Experiments

Embryos were collected and aged on apple juice agar plates at 25°C. For double labeling with affinity-purified rabbit anti-cyclin A antibodies and with mouse anti-cyclin B antisera, embryos were fixed and stained as described previously (Lehner and O'Farrell, 1990). Before labeling with anti β-tubulin antibodies (Amersham, Arlington Heights, IL), embryos were fixed as described by Karr and Alberts (1986). DNA was labeled with Hoechst 33258 (1 μg/μl) (Somerville, NJ). Pulse labeling with bromodeoxyuridine (BrdU), followed by immunolabeling with a monoclonal anti-BrdU antibody (Becton-Dickinson, Lincoln Park, NJ) was done as described by Edgar and O'Farrell (1990). Immunofluorescent labeling was analyzed with a Zeiss Axiophot (Thornwood, NY) microscope equipped for epifluorescence. Technical pan film (Kodak, Rochester, NY) was used for photography.

Molecular Characterization of Deficiencies and Chromosome Walking

Proximal (λ1C1) and distal (λN/N + 8) genomic clones from a region at 54F encompassing *grainyhead* were provided by S. Bray (Cambridge, U.K.). Terminal fragments from these were used to extend this walk. Clones were isolated from a Canton-S cosmid library (Tamkun, personal communication). The screening, isolation, and molecular analysis of these cosmid clones and all other molecular methods described were carried out using standard techniques described in Sambrook *et al.* (1989).

Southern blots of genomic DNA from adult flies were probed with DNA fragments from isolated cosmid clones. Fly DNA was prepared according to Jowett (1986), electrophoresed on 1.2% agarose gels, transferred to reinforced nitrocellulose (Schleicher and Schuell, Keene, NH), and hybridized with ^{32}P-labeled DNA fragments. Probes were prepared using a random primer labeling kit (Bresatec, Adelaide, Australia).

Genetic Transformation

The 11.0-kilobase (kb) *Not* I fragment shown in Figure 6 was subcloned into the w^{+} transformation vector pCaSpeR4 (Pirotta, personal communication). DNA was purified using QIAGEN-tip 100 columns (QIAGEN, Chatsworth, CA) and injected into embryos derived from a *w*; ry^{506} *P*[$ry^{+}\Delta 2$-3](*99B*) × *w*; *TM6b/TM3* mating. Transformant lines carrying the 11.0-kb *Not* I fragment were crossed to *thr* mutant lines to generate individuals carrying the transformed fragment in a *cn* thr^{321} *bw/cn* thr^{1B} *bw* background. Survival of such individuals indicated that the *thr* lethality was rescued by the presence of the 11.0-kb *Not* I fragment.

Isolation of cDNAs, Sequencing, and Sequence Analysis

Several cDNA clones were isolated from 0–4 h and 4–8 h embryonic cDNA libraries (Brown and Kafatos, 1988) using *Sal* I fragments derived from the genomic cosmid as probes. Fragments from one clone, UJA8, and from the 4.0-kb *Eco*RI genomic fragment were subcloned into pBluescript (Stratagene, La Jolla, CA) and used for sequence analysis. Double-stranded plasmid DNA was sequenced by the dideoxy method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical, Cleveland, OH). The entire UJA8 cDNA insert was sequenced on both strands. Genomic sequence overlapping the 5′ end of UJA8 (1.2 kb) was also determined in both directions. 3′ sequence analysis of a further nine distinct cDNA clones established the existence of alternative polyadenylation sites (see RESULTS).

Sequence comparisons were carried out using the computer programs of Staden (1980). Searches of GenBank and EMBL databases used the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs.

RNA Isolation and Analysis

RNA was extracted from staged embryos according to Chirgwin *et al.* (1979). Total RNA was fractionated on 0.66 M formaldehyde, 1.2% agarose gels (Davies *et al.*, 1986). RNA was immobilized by blotting onto nitrocellulose and hybridized with an antisense RNA probe synthesised by T7 RNA polymerase transcription of a 421-bp *Hin*dIII fragment (nucleotides −326–96, Figure 7) subcloned into a pBluescript-derived vector. Filters were washed at high stringency (0.1 × SSC, 65°C) and exposed to autoradiographic film.

Whole-mount in situ Hybridization

Whole-mount in situ hybridizations were performed with staged Canton-S and *Df(2L)PC4* embryos according to the protocol of Tautz and Pfeifle (1989) using digoxigenin probes (Boehringer Mannheim, Indianapolis, IN) prepared by random priming with 10 times the normal concentration of oligonucleotides (Oh and Edgar, unpublished modifications of Feinberg and Vogelstein, 1983). The UJA8 cDNA clone was labeled as a *thr*-specific probe.

RESULTS

three rows Mutations Cause a Failure of Chromosome Disjunction and Cytokinesis During Mitosis-15

Antibodies against cyclin A or cyclin B are powerful tools for the analysis of mitotic divisions in *Drosophila*

embryos. Cyclin A and cyclin B accumulate during interphase and are rapidly degraded during mitosis. Cells that are about to enter mitosis are intensely labeled, whereas cells that have just completed a mitotic division are not labeled by anti-cyclin antibodies (Lehner and O'Farrell, 1989, 1990).

An example of an immunofluorescent anti-cyclin A antibody labeling of a wild-type embryo is shown in Figure 1A. This embryo was fixed at the stage during which the cells of the dorsolateral epidermis are in the process of mitosis 15. The region of the dorsolateral epidermis outlined with the white box in Figure 1A is

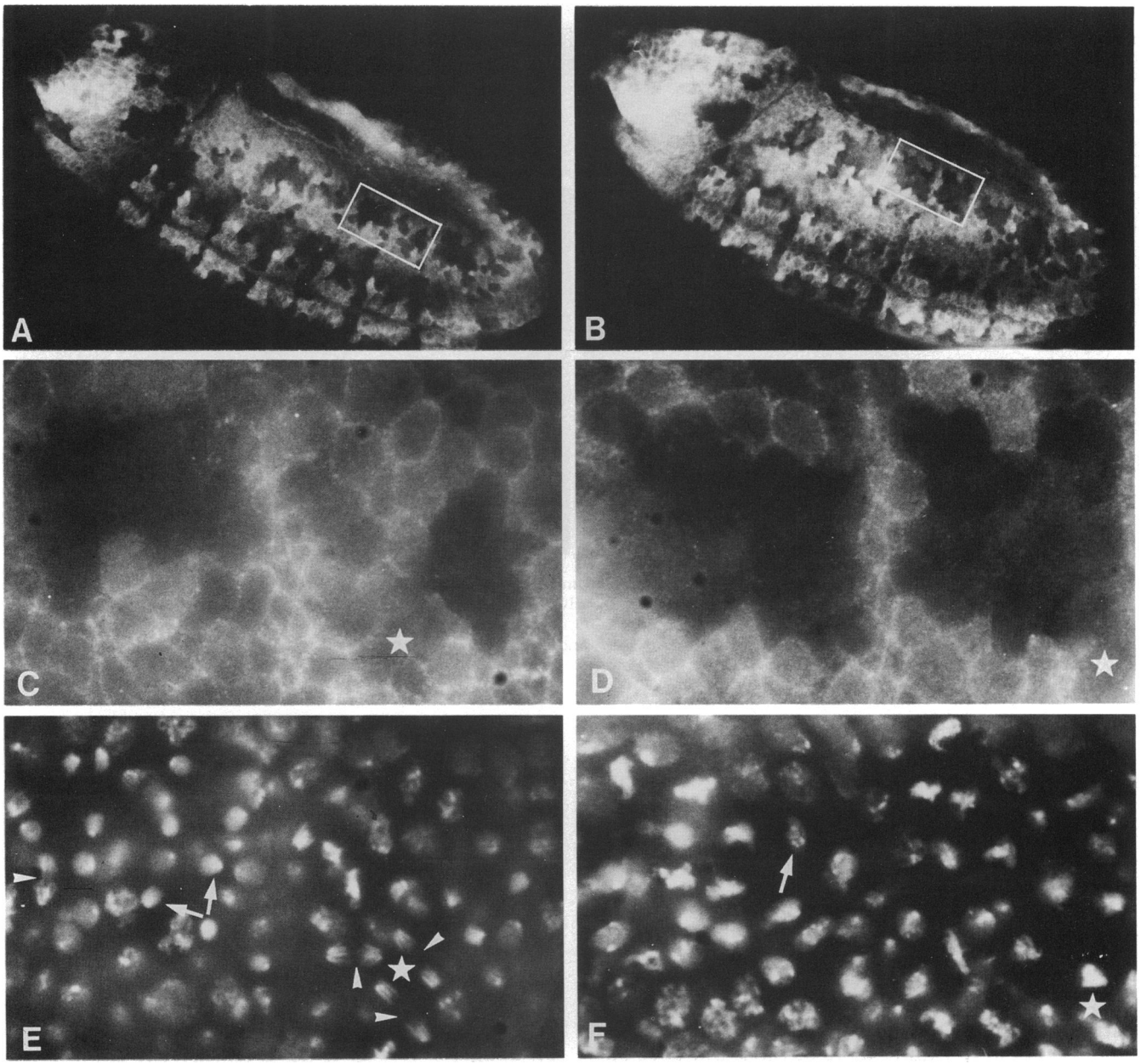

Figure 1. Mitotic defect in *thr* mutant embryos. Embryos were collected and aged to the stage during which the cells of the dorsolateral epidermis progress through mitosis 15. Progression through mitosis 15 was analyzed after double labeling with anti-cyclin A antibodies (A and B), anti-cyclin B antibodies (C and D), and with the DNA stain Hoechst 33258 (E and F). The region outlined in A and B is shown at higher magnification in C and E and D and F, respectively. In wild-type embryos (A, C, and E), anaphase figures (arrowheads) were readily detected in regions where anti-cyclin B labeling was very weak already (see star in C and E). In *thr* mutant embryos (B, D, and F), anaphase figures were never observed. Anaphase figures were also absent from cells with the weak anti-cyclin B labeling characteristic of anaphase (see star in D and F). During telophase, instead of the pairs of daughter nuclei that are present in wild-type (see for example arrows in E), a single nucleus was observed in *thr* mutants (see for example arrow in F).

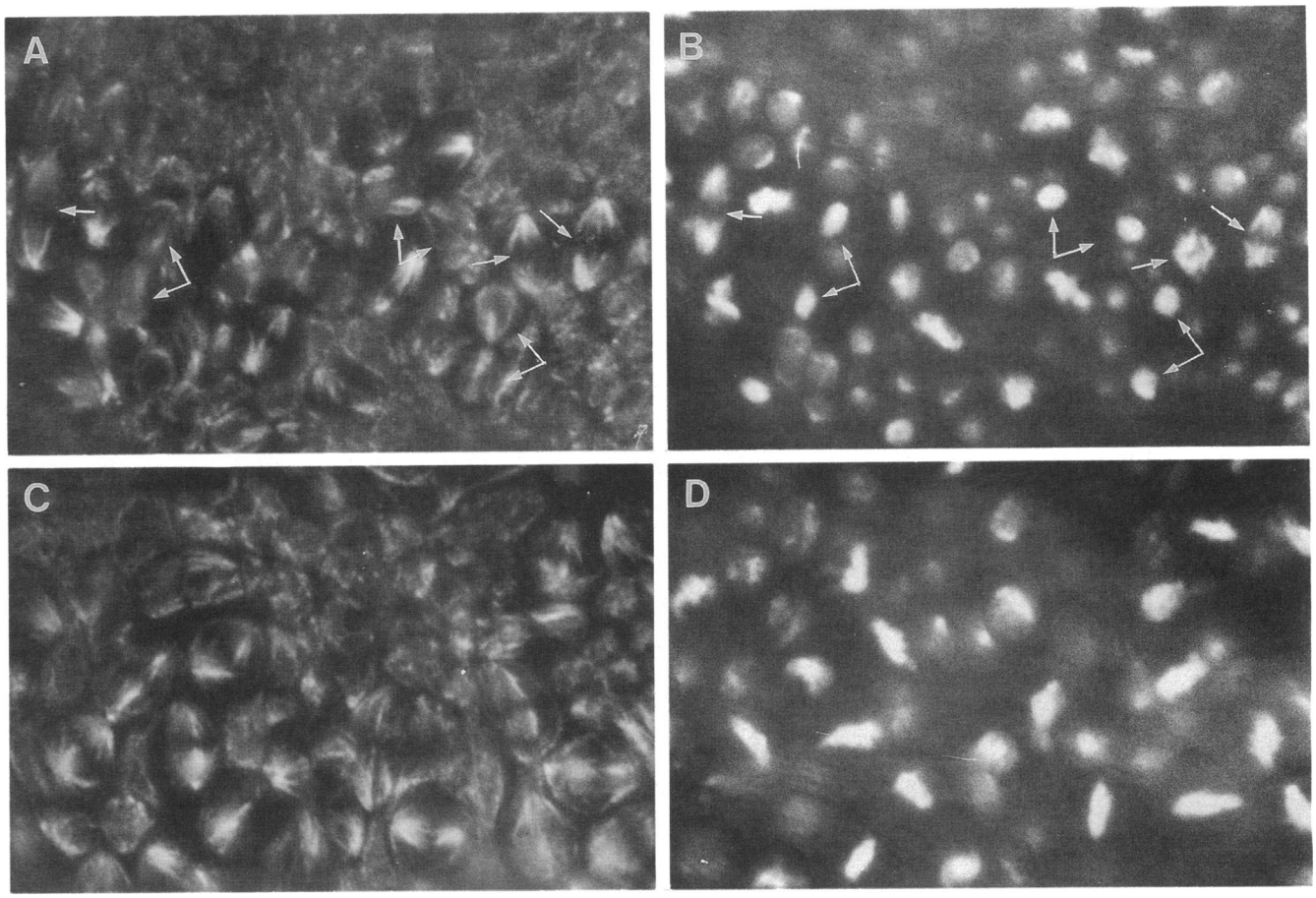

Figure 2. M-phase 15 in *thr* mutant embryos. Embryos were collected and aged to the stage during which the cells of the dorsolateral epidermis progress through mitosis 15. Progression through this division was analyzed by double labeling with anti-β-tubulin antibodies (A and C) and the DNA stain Hoechst 33258 (B and D). Corresponding regions from the dorsolateral epidermis are shown. Arrows indicate anaphase and telophase figures that were readily detected in wild-type embryos (A and B) but not in *thr* mutant embryos (C and D).

shown at higher magnification after double labeling with anti-cyclin B antibodies (Figure 1C) and a DNA stain (Figure 1E). A great number of mitotic figures, including anaphase and telophase figures (see arrowheads and arrows, respectively, in Figure 1E), can readily be observed in areas where anti-cyclin B labelling is absent or weak.

An embryo that is homozygous for the *thr* mutant allele thr^{1B} is shown in Figure 1, B, D, and F. Anti-cyclin A antibody labeling (Figure 1B) revealed that the embryo is at approximately the same developmental stage as the wild-type embryo described above (Figure 1, A, C, and E). A close comparison, however, indicates that the mutant embryo is slightly more advanced in development than the wild-type embryo. The areas in the dorsolateral epidermis that are no longer labeled with anti-cyclin antibodies (Figure 1, C, and D) are somewhat larger in the mutant embryo than in the wild-type embryo. More cells are expected, therefore, to have completed mitosis 15. However, the DNA labeling demonstrates that the completion of mitosis 15 is abnormal in mutant *thr* embryos. In contrast to wild-type, anaphase figures were not found in mutant embryos (Figure 1F). Anaphase cells in wild-type embryos can be identified after double labeling with anti-cyclin antibodies. They are not labeled with anti-cyclin A antibodies but are still very weakly labeled with anti-cyclin B antibodies (see star in Figure 1, C and E). However, in mutant embryos, regions that displayed the characteristic absence of anti-cyclin A and weak anti-cyclin B labeling of anaphase cells contained no anaphase figures (see star in Figure 1, D and F). In addition, normal telophase figures were also absent in mutant embryos. Instead of the characteristic pairs of daughter nuclei with decondensing chromatin, which were observed in wild-type embryos (see arrows in Figure 1E), chromatin decondensation was observed only in single nuclei in mutants (see arrow in Figure 1F) and not in pairs. The results of these double labeling experiments with anti-cyclin antibodies and a DNA stain indicate that chromosome separation is defective in *thr* mutant embryos.

Labeling with anti-tubulin antibodies provides an alternative way to visualise mitotic cells in the *Drosophila* embryo. Figure 2 presents regions from the dorsolateral epidermis from wild-type and *thr* mutant embryos fixed at the stage of mitosis 15 and double labeled with anti-β-tubulin antibodies and a DNA stain. The results of these labelings fully confirm the finding that chromosome disjunction is defective in *thr* mutant embryos. Although anaphase and telophase figures were readily observed in wild-type embryos (Figure 2, A and B, arrows), *thr* mutant embryos appeared devoid of normal anaphase and telophase figures (Figure 2, C and D).

The analysis of subsequent development in *thr* mutant embryos indicated that *thr* function is specifically required for chromosome disjunction, because other processes were not affected and continued normally. After mitosis 15, cells returned to a normal interphase organisation despite the fact that chromosome separation and consequently cytokinesis had not occurred during M-phase 15. Pulse-labeling with BrdU, which is incorporated into DNA during S phase, and visualization with a monoclonal anti-BrdU antibody (Edgar and O'Farrell, 1990) indicated that entry into the next S phase (S phase 16) occurred normally in *thr* mutant embryos (Figure 3).

Cell cycle progression continued in *thr* mutant embryos after S phase 16. M-phase 16 occurred at the correct developmental stage in *thr* mutant embryos (Figure 4), but double labelling with anti-β-tubulin antibodies and a DNA stain failed to reveal normal anaphase and telophase figures (Figure 4, C and D). Figures 3 and 4 also show that the cycle 16 *thr* mutant nuclei are fewer in number and larger in size than wild-type cycle 16 nuclei because of the failure of disjunction at mitosis 15. After mitosis 16, cell cycle progression continued in the peripheral nervous system (PNS) and CNS following the normal developmental program of embryonic proliferation. In these tissues, the increase in cell size resulting from the polyploidisation during the progression through multiple cell cycles lacking chromosome separation and cytokinesis was even more apparent than that observed after M-phase 16 (Stratmann and Lehner, unpublished observations). Despite these abnormalities, major developmental events such as germband retraction, a complex morphogenetic movement after M-phase 16, proceeded normally in *thr* mutant embryos (D'Andrea, Stratmann, Lehner, John, and Saint, unpublished observations). Cell death occurred at very late stages with a higher frequency in mutant compared to wild-type embryos presumably as a consequence of the polyploidisation and cell size increase. The same observation has been made previously in another mutant in which a defect in cytokinesis leads to polyploidisation and cell size increase (Hime and Saint, 1992).

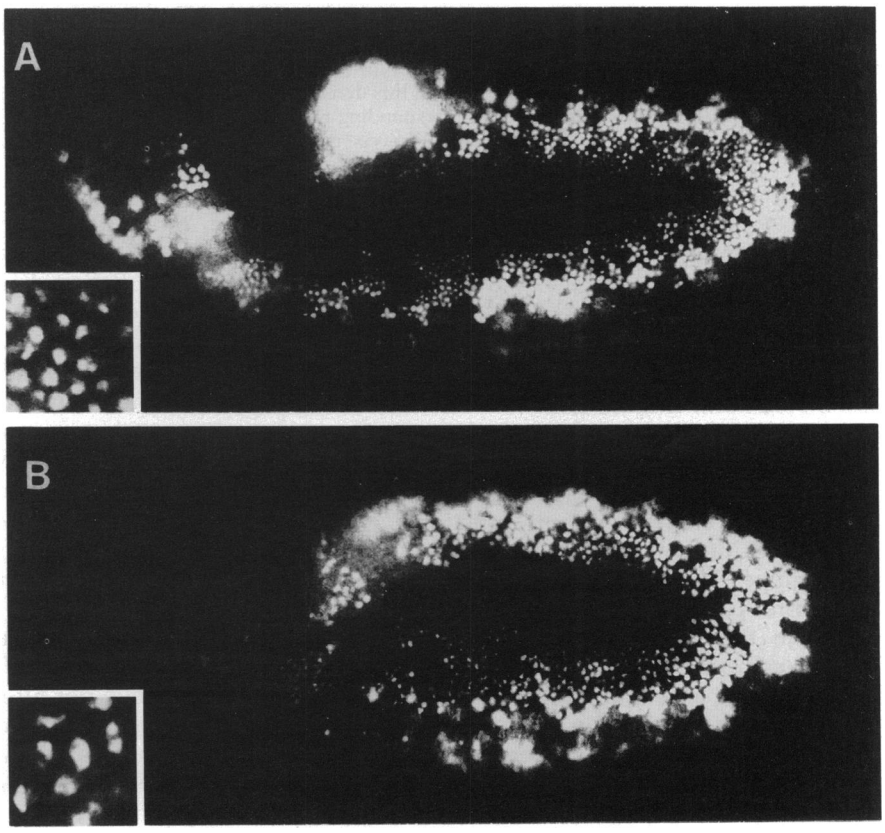

Figure 3. S-phase 16 in *thr* mutant embryos. Wild-type (A) and *thr* mutant embryos (B) were pulse-labeled with BrdU during the stage of S phase 16. Subsequently, embryos were fixed and stained with a monoclonal antibody against BrdU. S phase 16 occurs in *thr* mutant embryos despite the failure of chromosome disjunction and cytokinesis during the previous mitosis. These mitotic defects result in the characteristic reduction of cell density (insets) allowing the identification of mutant embryos.

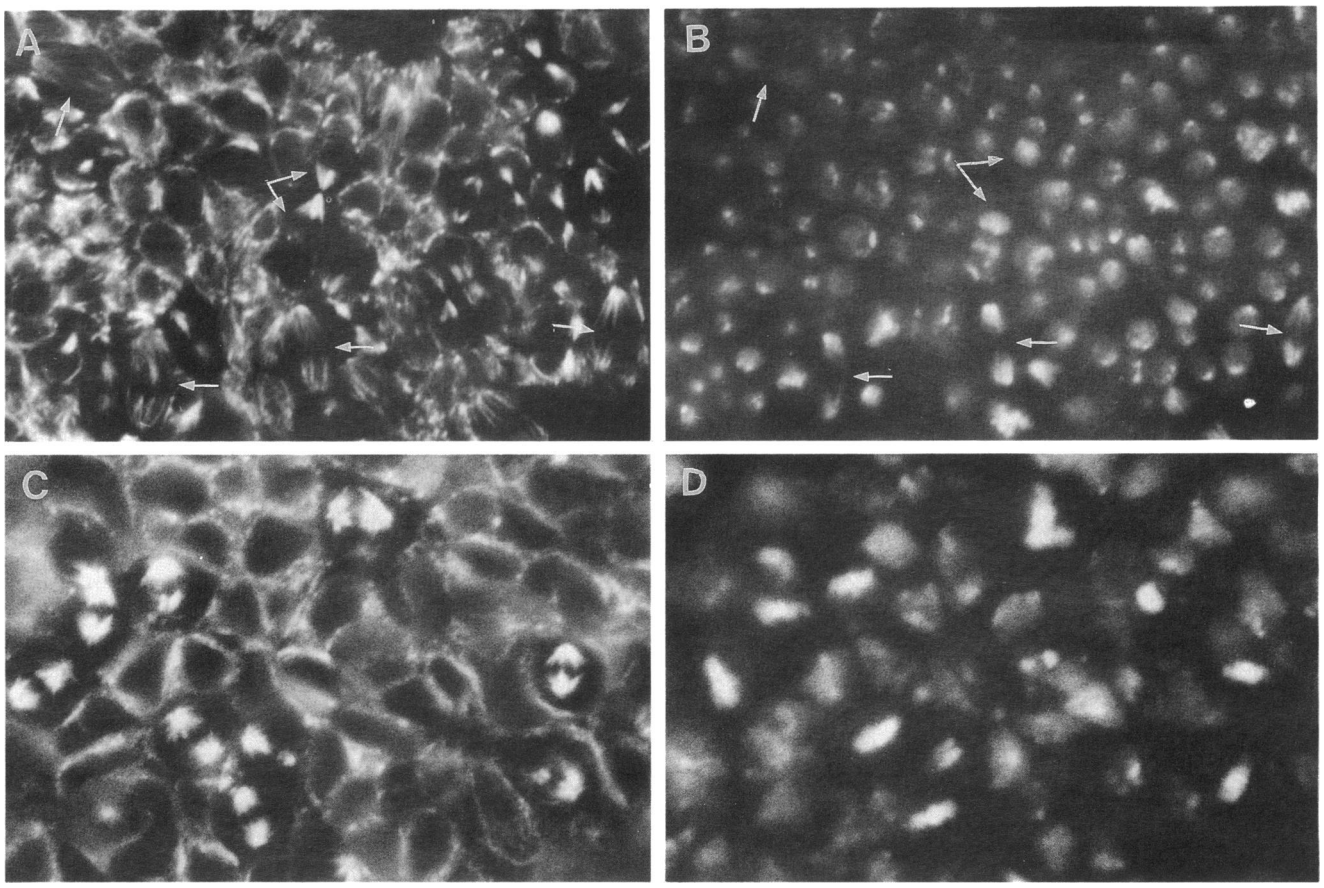

Figure 4. M-phase 16 in *thr* mutant embryos. Embryos were collected and aged to the stage during which the cells of the dorsolateral epidermis progress through mitosis 16. Progression through this division was analyzed by double labeling with anti-β-tubulin antibodies (A and C) and the DNA stain Hoechst 33258 (B and D). Corresponding regions from the dorsolateral epidermis are shown. Arrows indicate anaphase and telophase figures that were readily detected in wild-type embryos (A and B) but not in *thr* mutant embryos (C and D).

Failure of Chromosome Disjunction in three rows Mutants Does Not Result from Absence of Mitotic Spindles

All the previous observations indicate that *thr* function is specifically required for chromosome separation. Figures 2 and 4 also indicate that mitotic spindles are still assembled in *thr* mutant embryos. The careful comparison of mitotic cells from wild-type (Figure 5, A–E) and *thr* mutant embryos (Figure 5, F–L) during M-phase 15 at high magnification suggests that the mitotic spindle in *thr* mutant embryos is fully functional at least until metaphase. Metaphase spindles were indistinguishable in wild-type (Figure 5A) and *thr* mutant embryos (Figure 5F). Moreover, the alignment of condensed chromosomes in the metaphase plate appeared completely normal (compare Figure 5′, A and F). The mitotic spindle in *thr* mutant embryos, therefore, is not defective in promoting the congression of the mitotic chromosomes into the metaphase plate. Later in mitosis, the spindle elongation of anaphase B was clearly visible in wild-type embryos (Figure 5, C and D). However, only rudimentary signs of spindle elongation were detected in *thr* mutant embryos (Figure 5, G–I). These extended spindles in mutants were always much more irregular than the wild-type counterparts and were not accompanied by separation of chromosomes (Figure 5, G′–I′). Only in rare cases was separation of chromosomes observed to a limited extent (Figure 5, K′ and L′).

The rudimentary spindle elongation that was observed in *thr* mutant embryos during mitosis 15 was no longer observed during mitosis 16 (Figure 5M), suggesting that some residual, maternally contributed *thr* function might still have been present during mitosis 15, allowing the rudimentary spindle extension. Another interesting difference between mitoses 15 and 16 was noticed. During mitosis 16, but not mitosis 15, multipolar metaphase spindles were often observed (arrow in Figure 5M). This result suggests that the centriole cycle is not affected in *thr* mutants, because the centrioles appear to be duplicated despite the failure of chromosome disjunction and cytokinesis during mitosis 15. The resulting four centrioles appear to nucleate bipolar spindles in most cells, but occasionally tripolar or tetrapolar spindles were nucleated.

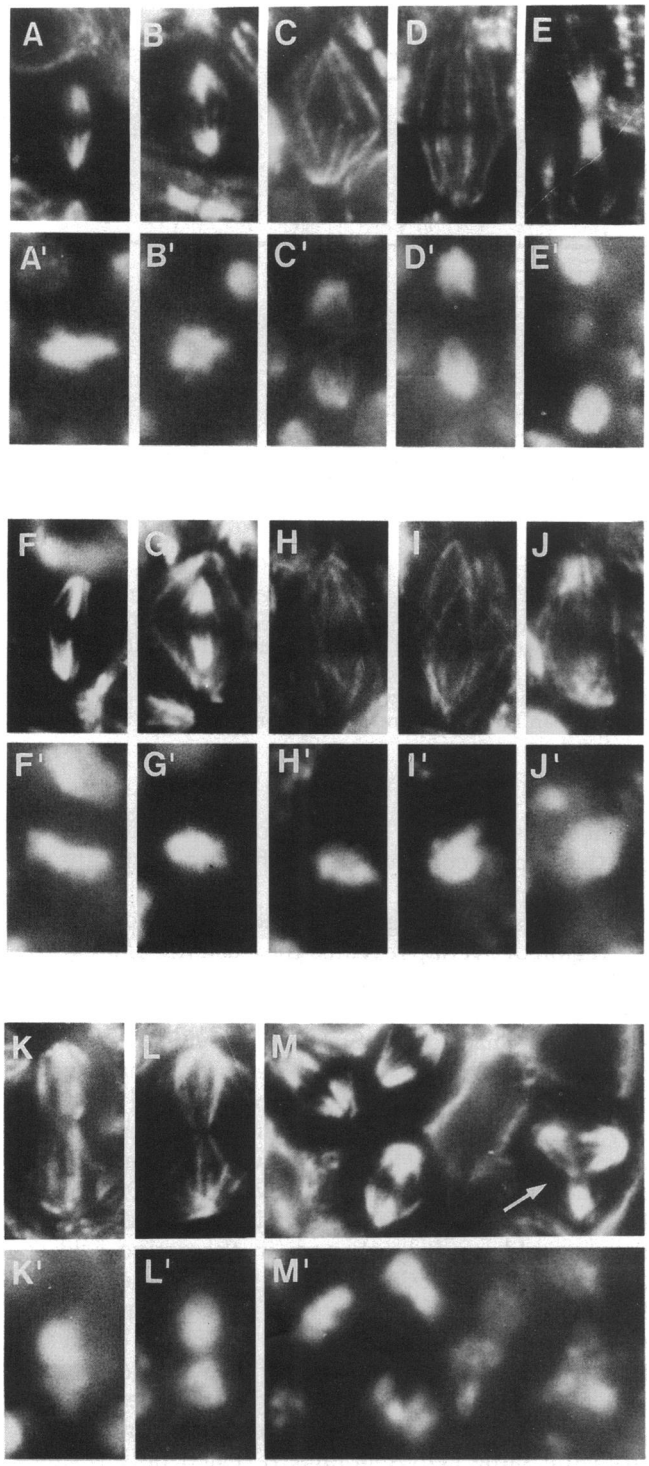

Figure 5. Mitotic spindles in *thr* mutant embryos. Wild-type embryos (A–E) or *thr* mutant embryos (F–M) were fixed during mitosis 15 (A–K) or mitosis 16 (M) and double labeled with anti-β tubulin antibodies (A–M) and with the DNA stain Hoechst 33258 (A'–M'). Epidermal cells during the completion of mitosis are shown. (A and F) Metaphase, (B and G) early anaphase, (C and H) anaphase, (D and I) late anaphase, and (E, J–L) telophase. Chromosome disjunction does not occur in *thr* mutants except in rare cases where some chromatin separation is apparent (K and L). Cells with multipolar spindles (arrow in M) are present in *thr* mutant embryos during mitosis 16.

The results described above were also obtained in embryos that were homozygous for the allele thr^{1L} or for *Df(2R)XM82*, which deletes the *thr* locus (Stratmann and Lehner, unpublished observations). Therefore, this phenotype reflects complete loss of *thr* function, and the alleles thr^{1B} and thr^{1L} are likely to be amorphic.

Mapping and Cloning of the three rows Gene

The *thr* mutation was previously mapped genetically to position 2–86 (Nüsslein-Volhard *et al.*, 1984). Genetic and cytological mapping located *thr* to the interval 54F–55A between the proximal breakpoints of *Df(2R)Pcl11B* and *Df(2R)PclW5* (Tearle, personal communication; Saint, unpublished data). This placed *thr* between the *grainyhead* (*grh*) locus (Bray and Kafatos, 1991) and the protein phosphatase Y gene (Dombradi *et al.*, 1989) (D'Andrea and Saint, unpublished observations).

The region containing *thr* was isolated in a chromosome walk that was initiated from the 3' end of the *grh* gene. Cosmid 3.3a was isolated with DNA probes from *grh* (Figure 6). Comparison of the signal intensity when terminal restriction fragments of this cosmid were used to probe genomic DNA isolated from flies heterozygous for *Df(2R)Pcl11B* or from flies carrying an isochromosomal 2nd chromosome demonstrated that the proximal breakpoint of *Df(2R)Pcl11B* lay within this cosmid (Figure 6) (D'Andrea, John, and Saint, unpublished observations). In addition, Southern analysis with the terminal 6.5-kb *Sal* I fragment of this cosmid revealed the presence of a complex polymorphism in the thr^{BH} allele, an allele derived from a hybrid dysgenic cross (Lindsley and Zimm, 1992; Gergen, personal communication). The restriction mapping data of the thr^{BH} mutant chromosome is consistent with two insertion events, one of an apparently intact P-element and a second of unknown type (Figure 6). To characterize the nature of these polymorphisms in more detail, *Eco*RI fragments from thr^{BH}/*cn bw sp* genomic DNA were cloned into a λgt10 vector. An *Xba* I-*Sal* I fragment spanning the polymorphic fragments was used to probe for recombinants containing these fragments. A 0.6-kb internal *Sal* I fragment was identified as a fragment covering the site of the P element insertion and was subcloned for sequence analysis. This defined the insertion site of the P element (Figures 6 and 7). The eight nucleotides adjacent to the insertion site show five matches with the consensus derived for P element integration sites by O'Hare and Rubin (1983). The exact nature of the second lesion in the adjacent *Eco*RI fragment was not determined, although Southern hybridization results are consistent with a non-P-element insertion generating the novel *Eco*RI fragment of 3.7 kb. Genetic analysis revealed that the thr^{BH} allele was not revertable in the presence of P transposase. We have not determined whether this nonreversion was because of a defect in the P-element insertion or because of an effect of the second insertion on expression of the *thr* gene.

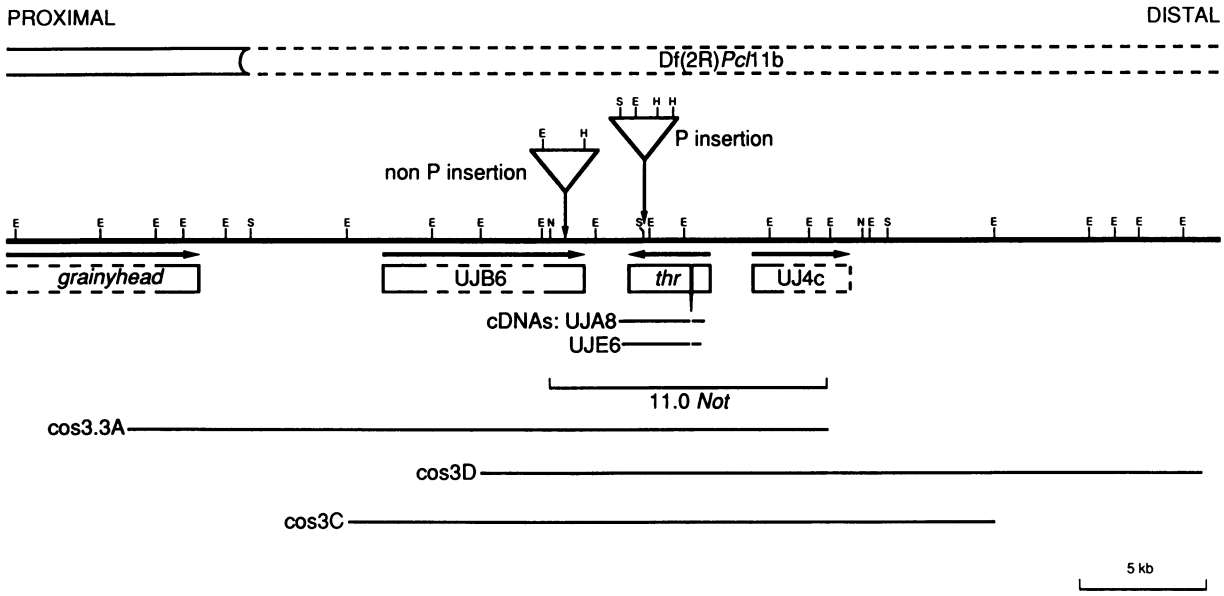

Figure 6. Restriction map of the *grh-thr* region. The region deleted in chromosomal deficiency *Df(2R)Pcl 11B* is marked by dashes. Two insertion polymorphisms in the thr^{BH} allele relative to the *iso-1* isochromosomal line from which the cosmid library was derived (Tamkun, personal communication), one of which was shown to be P element derived, are shown. The position and orientation of the *grh* and *thr* genes are indicated, as well as two cDNA clones UJB6 and UJ4c from transcription units flanking *thr*. The longest *thr* cDNA clones UJA8 and UJE6 are represented. The region spanned by three cosmid clones is shown, as well as that of an 11-kb fragment, derived from cosmid 3.3A, which was found to be capable of rescuing the *thr* mutant phenotype.

DNA probes from this region were used to isolate cDNA clones from early embryonic libraries (Brown and Kafatos, 1988). Southern blotting and restriction enzyme cleavage mapping allowed the transcribed regions to be positioned accurately on the molecular map of this region (Figure 6). Three transcription units were identified (Figure 6). One transcription unit, defined by cDNA clone UJA8, was shown to span the P-element insertion site in thr^{BH}. DNA sequence analysis of the mutant allele revealed that the UJA8 coding region was interrupted by insertion of the P element (Figure 6).

An 11-kb genomic DNA fragment, which covered this transcript but excluded parts of the neighbouring transcripts (Figure 6), was introduced into the *Drosophila* germline by P element-mediated transformation. Two independent transformants, crossed into a homozygous *thr* mutant background, rescued the *thr* mutant phenotype (D'Andrea, John, and Saint, unpublished observations), confirming that this transcription unit corresponds to the *thr* gene.

Structure of the thr Gene and Its Product

Despite repeated attempts at screening cDNA libraries for full length cDNA clones, no clone that contained the entire protein coding region was isolated. Consequently, the 5′ sequence of *thr* was obtained from genomic fragments derived from cosmid clones (Figure 7). The assembled cDNA and genomic sequences (Figure 7) revealed a large open reading frame (ORF) encoding a predicted 1209 amino acid polypeptide. The ORF thus defined was shown to be contiguous within the mRNA by RNase protection analysis (John and Saint, unpublished observations).

Comparison of the genomic and cDNA sequences in the region of overlap revealed the existence of an intron of 58 base pairs (bp) between positions 779 and 780 of the ORF (Figure 7). This intron contains consensus splice sequences (Shapiro and Senapathy, 1987) at the junctions with the ORF. Although restriction mapping of cDNA and genomic DNA fragments failed to detect any other introns, the existence of other similarly sized introns cannot be excluded. Six nucleotide substitution polymorphisms were found between the cDNA clone UJA8 and genomic DNA-derived sequences. Three of these would result in changes in the amino acid sequence of the encoded product, two of the changes being conservative (Figure 7). These polymorphisms may be the product of error prone replication by reverse transcriptase in construction of the cDNA library. However, the sequence of an additional seven independent cDNA clones with different 5′ termini, derived from the same cDNA library, revealed only a single nucleotide difference (nucleotide 2328, Figure 7) in a 2.4-kb region of overlap with the longest clone, UJA8. These data and the silent or conservative nature of five of the six changes make it more likely that the polymorphisms observed between the cDNA and genomic DNA sequences reflect true sequence differences between the different strains used to generate the cDNA and genomic libraries.

Figure 7. Nucleotide sequence and derived amino acid sequence of *thr*. The nucleotide sequence of the *Drosophila thr* gene has a long ORF encoding a theoretical protein of 1209 amino acids. The derived amino acid sequence is shown in one letter code below the nucleotide sequence. The 5′ termini of the two longest cDNA clones UJA8 and UJE6 are shown. The extent of nucleotide sequence derived from subcloned fragments of cosmid 3.3a are arrowed. A 58-bp intron revealed by sequencing of genomic fragments and six nucleotide substitution polymorphisms relative to the cDNA sequence are indicated. Differences in the genomic nucleotide sequence are shown above the cDNA sequence. Amino acid substitutions that result are shown below the UJA8 amino acid sequence. A further nucleotide substitution polymorphism and consequent amino acid sequence difference detected in cDNA clone UJA9 is indicated. Nucleotide sequence derived from the dysgenic allele thr^{BH} extends from the arrowhead marked "BH Genomic" to the position indicated as "P ele" where the cDNA sequence is interrupted by a consensus P element right arm (O'Hare and Rubin, 1983). The nucleotide at which poly A tails commence in cDNA clones UJA8 and UJE6 is arrowed in the 3′ untranslated sequence. Putative polyadenylation signals are underlined. A region of amino acid sequence with similarity to part of the product of the *nuc2* gene of *S. pombe* is boxed. Four potential PEST sequence with PESTFIND scores (Rogers *et al.*, 1986) of 9.4, 14.9, 2.7, and 2.5 lie between residues 395–409, 1076–1099, 1142–1163, and 1188–1204 respectively.

Sequence analysis also indicated the existence of alternate polyadenylation sites in *thr* transcripts. Of 10 distinct cDNA clones obtained from a 4- to 8-h library, eight (including UJE6, Figure 7) had a 111 bp 3′ untranslated region, whereas UJA8 (Figure 7) and one other independent clone had a 356 bp 3′ untranslated region. Consensus polyadenylation signals (Proudfoot, 1991) were found 51 and 21 bp, respectively, upstream of the poly A tails (Figure 7).

The only large ORF derived from the sequence analysis encodes a highly basic (basic/acidic 182/124) serine-rich (9.8%) protein of predicted molecular weight 137 849 Da. A hydropathy profile (Kyte and Doolittle, 1982) suggested that *thr* encodes a protein with no extended stretches of hydrophobic residues (John and Saint, unpublished observations). Given the highly basic nature of the encoded product, particularly at the C-terminal end, the occurrence of three potential nuclear targeting sequences (Figure 7) of the bipartite type (Dingwall and Laskey, 1991) is not surprising. The putative *thr*-encoded protein also contains four regions 3 at the C-terminal end (see legend to Figure 7) that give high scores with the algorithm for identifying PEST sequences (Rogers *et al.*, 1986). PEST sequences are sequences rich in proline, glutamic acid, aspartic acid, serine, and threonine, flanked by basic amino acids that are present in many unstable proteins.

Comparisons with sequence databases using the program FASTA (Pearson and Lipman, 1988) revealed no extended sequence similarity to any known protein. By contrast, the program BLAST (Altschul *et al.*, 1990), which optimizes matches between short motifs, indicated that a sequence at residues 535–559 of the encoded product exhibited 44% identity (smallest Poisson probability 0.015) with residues 220–244 of the product of the *nuc*2 gene of *Schizosaccharomyces pombe* (Figure 8). *nuc*2 encodes a 665–amino acid residue protein with an essential role in mitosis (Hirano *et al.*, 1988). The possible significance of this similarity is discussed below.

Developmental Expression of the thr Gene

Hybridization of a *thr* cDNA probe to a developmental Northern blot of total RNA detected high levels of *thr* transcripts in embryos ≤4 h postfertilization (Figure 9). Two species of transcript of size 4.4 and 4.7 kb were observed. The basis of the difference between the two transcripts has not been elucidated, although the alternative 3′ polyadenylation sites contribute, at least in part,

Figure 8. A region of amino acid sequence similarity between the *thr*-encoded product and the *S. pombe nuc2* product. Identical residues are boxed and conservative substitutions using the following grouping: A, L, V, I, M; K, R; D, E; S, T; N, Q; Y, F are circled. The derived *nuc*2 sequence is from Hirano *et al.* (1988).

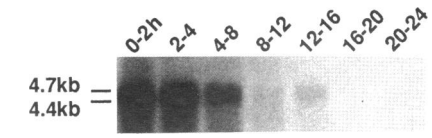

Figure 9. Detection of *thr* transcripts during *Drosophila* development. Northern blot of poly(A)$^+$RNA isolated from staged embryos probed with a 421 nucleotide antisense RNA probe. Two hybridizing bands corresponding to 4.4- and 4.7-kb transcripts were apparent on the original autoradiogram but are obscured in the long exposure shown here to illustrate the levels of *thr* mRNA during embryogenesis.

to this difference. Beyond 4 h, transcript levels were reduced but remained detectable throughout embryogenesis. RNase protection experiments also detected *thr* transcripts in third instar larvae, adult males and females and *Drosophila* Schneider line 2 cultured cells (John and Saint, unpublished observations), consistent with an association between *thr* expression and cell proliferation in all tissues. Adult female transcripts presumably include maternal *thr* transcripts generated during oogenesis (see below).

To characterize the spatial and temporal distribution of *thr* mRNA during embryonic development, digoxigenin-labeled cDNA, derived from clone UJA8, was hybridized in situ to fixed embryos (Figure 10). Throughout the syncytial cleavage divisions before cellularization, embryos exhibited high levels of *thr* transcripts (Figure 10A) (John and Saint, unpublished observations). In these syncytial embryos, the *thr* transcripts appeared to be concentrated around the nuclei. A similar distribution has been observed for transcripts of cyclin B (Lehner and O'Farrell, 1990) and has been shown to be dependent on microtubule integrity (Raff *et al.*, 1990) and sequences in the 3′ nontranslated region (Dalby and Glover, 1992). After cellularization, levels of *thr* transcripts were seen to remain high and evenly distributed, except for a higher concentration in the region of the pole cells (Figure 10B). During germ band extension, transcript levels were high except in the amnioserosa (Figure 10C) but declined during cycles 15 and 16 (Figure 10D) to be undetectable in most tissues before germ band retraction. Significantly, there was no indication of any stage-specific regulation of *thr* transcript levels during cycle 15. At the germ band extended stage and the early stages of retraction, *thr* expression was clearly observed in cells of the central and peripheral nervous systems (Figure 10, E and F). After germ band retraction, *thr* transcripts were confined to a subset of cells within the ventral nerve cord and in the proliferative centres of the brain lobes (Figure 10, G and H). These are the only proliferating tissues at this stage of development. Expression was not detected in cells undergoing endo-replication cycles, cycles of DNA replication that proceed in the absence of mitosis to generate polytene chromosomes (Smith and Orr-Weaver, 1991).

To follow the fate of the maternal contribution during embryonic development, embryos derived from parents

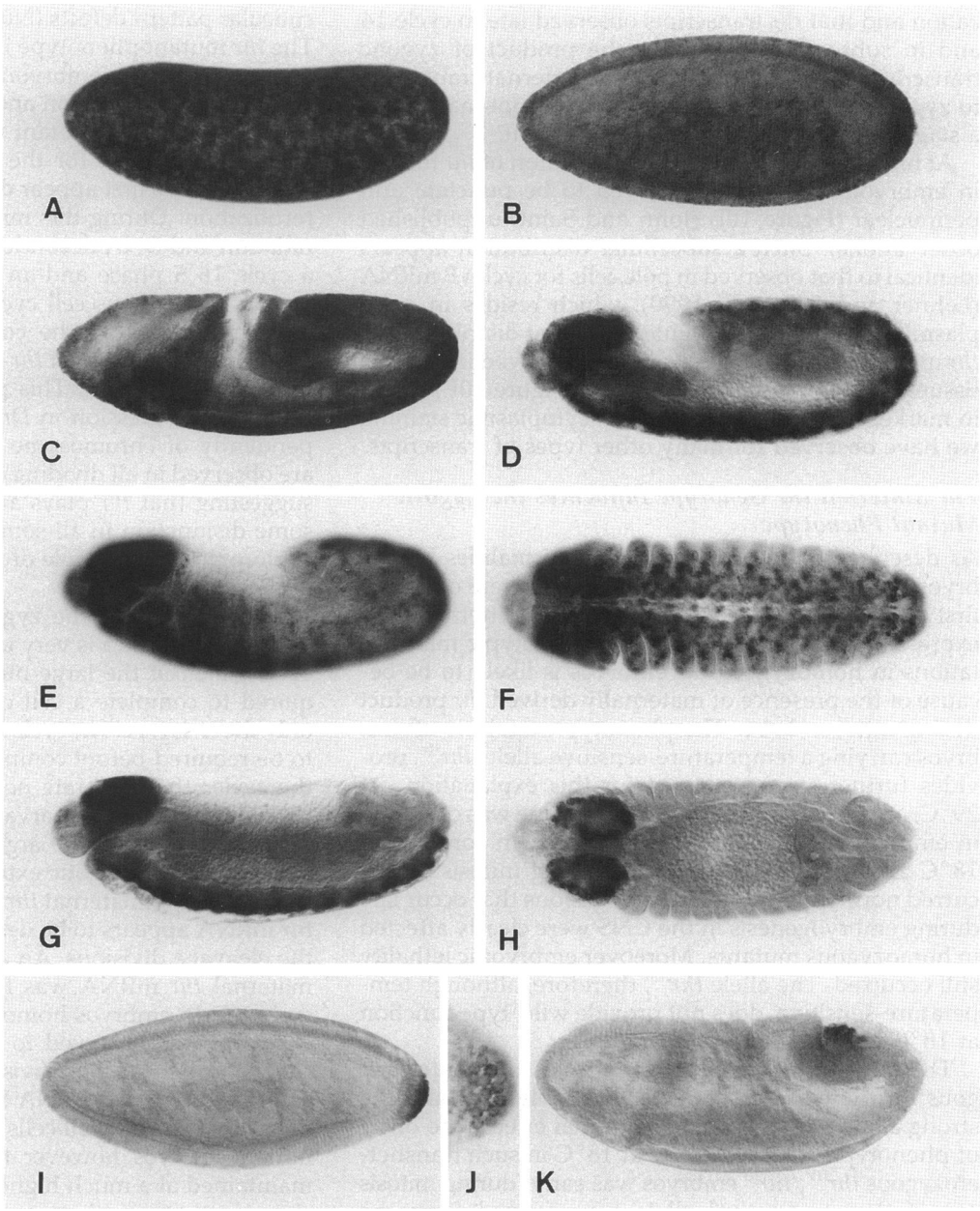

Figure 10. Expression of the *Drosophila thr* gene during embryonic development in wild-type and *thr*-deficient embryos. Whole mount embryos were hybridized with the UJA8 cDNA labeled by incorporation of digoxigenin-11-dUTP. Hybridization was detected using an alkaline phosphatase coupled secondary antibody detection system. All embryos are oriented with anterior to the left and ventral side down unless otherwise indicated. (A) Cleavage stage embryo exhibiting perinuclear distribution of transcript. (B) An embryo at cellular blastoderm with relatively uniform message distribution. (C) Germ band extended embryo with uniform staining in all but the amnioserosa. (D) Germ band extended embryo with reduced staining in all but cells of the CNS. (E) Embryo in early stage of germ band retraction showing expression in cells of the CNS and PNS. (F) Ventral view of an almost complete germ band retracted embryo showing transcripts in segmentally reiterated cells of the CNS and PNS. (G) Fully germ band retracted embryo with staining in ventral nerve cord and brain lobe (CNS). (H) Dorsal view of fully germ band retracted embryo with transcripts confined to cells in the proliferating margins of the brain lobes. (I–K) Progeny of *Df(2R)PC4/CyO* parents. *Df(2R)PC4* removes *thr* in its entirety. One-quarter of the embryos at cellularization and later stages were lacking *thr* transcripts. (I) An embryo during cellularization showing lack of transcripts in all cells except pole (presumptive germ) cells. (J) Cellular blastoderm at higher magnification showing punctate staining in pole cells. (K) Early germ band extended embryo with transcripts restricted to the pole cells.

heterozygous for the deficiency *Df(2L)PC4*, which removes *thr*, were examined by in situ hybridization. During the syncytial divisions, all embryos exhibited uniformly high levels of transcript, as observed in the progeny of wild-type parents (results not shown). During cellularization and early germ band extension, a majority of the embryos again resembled wild-type. At these stages and later in development, however, approximately one-quarter of the progeny exhibited dramatically lower levels of *thr* transcripts in all cells except the pole cells (Figure 10, I–K). These embryos presumably correspond to the homozygous deficiency embryos. Double staining with the DNA stain Hoechst 33258 showed that the unstained fully germ band extended embryos exhibited the mitotic defect characteristic of homozygous *thr* embryos, as expected (John and Saint, unpublished observations). We conclude that maternal *thr* mRNA is rapidly degraded at the time of cellulari-

zation and that *thr* transcripts observed late in cycle 14 and in subsequent cycles are the product of zygotic transcription. The transition from maternal transcripts to zygotic transcripts during cycle 14 is not marked by a stage in which *thr* transcripts are absent.

At higher magnification, the distribution of *thr* mRNA in embryonic cells was observed to be punctate and perinuclear (Figure 10J) (John and Saint, unpublished observations). Such a subcellular distribution appears identical to that observed in pole cells for cyclin B mRNA (Lehner and O'Farrell, 1990), which resides in cytoplasmic granules. The irregular, granular distribution of *thr* mRNA within germ cells is also observed in somatic tissues (see staining in cells of PNS, Figure 10E) and is in marked contrast to the uniform cytoplasmic staining we have observed for many other types of transcripts.

The Maternal thr Genotype Influences the Zygotic Mutant Phenotype

As described above, phenotypic abnormalities in embryos homozygous for strong *thr* alleles were observed first at mitosis 15. On the basis of the patterns of *thr* expression, this delayed onset of phenotypic manifestations in homozygous *thr* embryos is likely to be because of the presence of maternally derived *thr* product during mitoses 1–14. The phenotypic analysis of embryos carrying a temperature-sensitive allele, thr^{1lv}, provides further strong support for this explanation. At 29°C, an essentially amorphic phenotype was observed in embryos homozygous for this allele. In contrast, at 18°C all divisions up to and including mitosis 16 occurred normally. However, the divisions that occur late during embryogenesis in the CNS were clearly affected in homozygous mutants. Moreover embryonic lethality still occurred. The allele thr^{1lv}, therefore, although temperature-sensitive, does not provide wild-type function at 18°C.

The phenotypic analysis of embryos transheterozygous for the temperature-sensitive allele thr^{1V} and the strong allele thr^{1B} revealed a maternal effect. The onset of phenotypic manifestations at 18°C in such transheterozygous thr^{1V}/thr^{1B} embryos was early (during mitosis 16), if the strong thr^{1B} allele was derived from the mother, and late (during the late divisions in the CNS), if the weak thr^{1lv} allele was derived from the mother (Stratmann and Lehner, unpublished observations). As the only differences between these two types of progeny were the maternal versus paternal differences in genotype, the difference in phenotype can only be accounted for by a maternal effect of the *thr* gene. Thus decreased maternal contribution provided by the mothers that are heterozygous for the strong allele must have exhausted earlier than the maternal contribution provided by the mothers with the weak allele.

DISCUSSION

The *thr* gene of *Drosophila* was identified in a screen for zygotic embryonic lethal mutations that result in cuticular pattern defects (Nüsslein-Volhard *et al.*, 1984). The *thr* mutant phenotype is shown here to result from disruption of the embryonic cell cycles. Specifically, chromosome disjunction and, consequently, cytokinesis are affected in the mutant embryonic mitoses. In embryos homozygous for the strongest *thr* alleles, these mitotic defects first appear during the 15th mitosis after fertilization. During this mitosis, however, cyclin degradation and DNA decondensation occurs. In addition, a cycle 16 S phase and an abnormal cycle 16 mitosis are observed. Thus cell cycle events appear not to be affected, leading to the conclusion that the primary consequence of a loss of *thr* function is a failure of chromosome disjunction. This phenotype also shows that cell cycle progression in *Drosophila* can proceed independently of chromosome disjunction. *thr* transcripts are observed in all dividing cells during embryogenesis, suggesting that *thr* plays an essential role in chromosome disjunction in all somatic mitoses. We are yet to explore the possible role of *thr* in germline mitoses and in meiosis.

Manifestation of the zygotic *thr* mutant phenotype as early as cycle 15 is very unusual for a *Drosophila* cell cycle gene. Of the large number of genes that are required to complete a cell cycle, zygotic expression of only the *string*, *pebble*, cyclin A, and *fizzy* genes is known to be required before completion of the first 16 cycles, the cycles that generate nearly all but the neural and imaginal tissues of the larva. The genetic and molecular evidence reported here argues strongly that this early requirement for zygotic expression results from the instability of the maternal *thr* contribution. The maternal *thr* mRNA appears to be destabilized upon cessation of the cleavage divisions. An apparently constant pool of maternal *thr* mRNA was found in all cleavage stage embryos. In embryos homozygous for a *thr* deficiency, this pool was observed to rapidly decrease after cessation of the cleavage divisions, so that at the time of cellularization, *thr* mRNA levels were reduced to undetectable levels in all cells except pole (germ) cells. In normal embryos, however, the levels of *thr* mRNA were maintained at a much higher level during these stages, showing that zygotic transcription was supplementing the rapidly degrading maternal mRNA. The disappearance of maternal *thr* transcripts at this time is consistent with the temporal onset of the mutant phenotype. In embryos homozygous for amorphic alleles, sufficient *thr* product is presumably translated from the maternal *thr* mRNA before its degradation for a normal 14th mitosis to occur and perhaps for a rudimentary mitosis 15. Such a persistence of the maternal *thr* protein is also suggested by the observation that the maternal *thr* genotype affects the time at which the mutant phenotype becomes evident. The dependence of the zygotic phenotype on the maternal genotype, the absence of any stage-specific regulation of *thr* transcripts during cycles 14 and 15, and the expression of *thr* in all proliferating embryonic cells argues against the possibility that the

thr embryonic phenotype results from a developmentally restricted requirement for *thr* function. Rather, our results are consistent with the idea that *thr* function is provided maternally and that in homozygous mutant embryos the maternal contribution becomes insufficient before mitosis 15, at which stage the mutant phenotype manifests itself because of a failure to produce functional zygotic *thr* products.

The apparent stability of the maternal *thr* mRNA during the cleavage divisions, compared with the instability during cellularization suggests that, upon completion of the cleavage divisions, RNA degradative pathways, which are specific for certain transcripts, are activated. The change in stability is dramatically evident for maternal *string* transcripts (Edgar and O'Farrell, 1989) and for maternal *Drosophila* cyclin E transcripts (Richardson *et al.*, 1993). The basis for the decreased stability of these maternal transcripts after the cleavage divisions is yet to be determined for any of these genes.

The reason for the failure of chromosome disjunction in *thr* mutants has not been determined. It appears not to result from failure to form the mitotic spindle. Antibodies against β-tubulin revealed the presence of normal mitotic spindles in *thr* mutants. The *thr* sequence does not suggest a biochemical role for the *thr* product. *thr* transcripts have an ORF that encodes a 1209–amino acid protein with no extended sequence similarity with any known protein. *thr* is thus unlikely to be directly responsible for chromosome movement, having no homology to any known motor protein. The only potential clue to the function of the *thr* protein provided by the sequence is a very small but apparently significant similarity to sequences found in the *nuc2* protein of *S. pombe*. *nuc2* encodes a protein that is localized to the nuclear scaffold (Hirano *et al.*, 1988) and, like *thr*, is required for chromosome disjunction. The region of similarity may correspond to a functional domain because it lies within a small region of the *nuc2* protein defined in vitro as a DNA binding domain (Hirano *et al.*, 1990). It should be noted, however, that *thr* appears not to be a true homologue of the *nuc2* gene. The protein encoded by *nuc2* exhibits another domain that consists of repeats of the Tetratricopeptide snap helix (Sikorski *et al.*, 1990), a motif that is not present in the *thr* product. Conversely, *bimA*, the putative *nuc2* homologue of *Aspergillus nidulans* (O'Donnell *et al.*, 1991) encodes a product that lacks sequences resembling the region of similarity between *thr* and *nuc2*. Finally, in contrast to *thr*, *nuc2* mutants do not initiate a further round of replication after their failure to separate sister chromatids but appear to undergo a true cell cycle arrest in a metaphase-like stage (Hirano *et al.*, 1988). Thus the significance, if any, of this limited but apparently significant homology remains to be demonstrated.

Several other possible roles for the *thr* product remain. It could form part of the kinetochore or be involved in the generation of proper kinetochore structure required for the normal attachment or activity of motor proteins. Alternatively, it could be involved in an intracellular signaling mechanism that triggers separation of the chromosomes during mitosis. Finally, it could be required for the physical detachment of the sister chromatids that allows them to be separated. In this regard, topoisomerase has been implicated in the detachment of sister chromatids. Topoisomerase inhibitors can phenocopy the *thr* mutant phenotype, but only if low doses are used (Buchenau *et al.*, 1993). This study showed, however, that high doses of topoisomerase inhibitors affect chromosome condensation, a process that is not affected in *thr* mutants. It seems unlikely, therefore, that the inhibitors target a function that directly involves the *thr* protein.

The early embryonic phenotype observed in *thr* mutants may be informative with regard to *thr* function. Previous work suggested that the maternal contribution of essential mitotic cell cycle function is sufficient for all embryogenesis (Shearn *et al.*, 1971; Szabad and Bryant, 1982; Gatti and Baker, 1989). Mutations in genes providing such essential mitotic functions are therefore not associated with embryonic lethality but result in lethality much later at the larval/pupal boundary. The early *thr* phenotype that results from a higher level of instability of the maternal *thr* contribution, suggests that *thr* may play a regulatory rather than a structural role during mitosis. This would be consistent with the fact that the only other characterized cell cycle genes that have been shown to exhibit embryonic lethality, *string* and cyclin A (Edgar and O'Farrell, 1989; Lehner and O'Farrell, 1989), are known cell cycle regulatory genes. A test of this suggestion awaits a detailed biochemical characterization of the novel *thr* product and of its essential role in chromosome disjunction during mitosis.

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