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The *Drosophila* cdc25 homolog *twine* is required for meiosis

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Summary

We have identified a second *cdc25* homolog in *Drosophila*. In contrast to *string* (the first homolog identified in *Drosophila*) this second homolog, *twine*, does not function in the mitotic cell cycle, but is specialized for meiosis. Expression of *twine* was observed exclusively in male and female gonads. *twine* transcripts are present in germ cells during meiosis, and appear only late during gametogenesis, well after the end of the mitotic germ cell divisions. The sterile *Drosophila* mutant, *mat(2)synHB5*, which had previously been isolated and mapped to the same genomic region as *twine* (35F), was found to carry a missense mutation in the *twine* gene. This missense mutation in *twine* abolished its ability to complement a mutation in *Schizosaccharomyces pombe cdc25*. Phenotypic analysis of *mat(2)synHB5* mutant flies revealed a complete block of meiosis in males and severe meiotic defects in females.

Key words: *cdc25*, *Drosophila*, phosphatase, meiosis, *string*, *twine*.

Introduction

The *cdc25* gene was initially identified in *Schizosaccharomyces pombe* where it acts as an important regulator of entry into mitosis (Russell and Nurse, 1986). The analysis of a *S. cerevisiae* homolog and of the *Drosophila* homolog encoded at the *string* locus emphasized the general importance (Russell et al.,1989; Edgar and O'Farrell, 1989), and studies in a variety of systems have revealed the biochemical basis of cdc25 function (for a recent review see Millar and Russell, 1992).

cdc25 function is required for entry into mitosis. Mutations in the *S. pombe cdc25* gene and in *string* lead to an arrest in the G2-phase of the cell cycle. Without cdc25 function, activation of the mitosis promoting activity of the $p34^{cdc2}$-kinase does not occur (Booher et al., 1989; Moreno et al., 1989). This activation requires the association of $p34^{cdc2}$ with cyclin B and the removal of inhibitory phosphate modifications from amino acid residues in the ATP binding site of the $p34^{cdc2}$-kinase (for a review see Nurse, 1990). Whereas in *S. pombe* this inhibitory phosphorylation occurs on Tyr-15 only, both Tyr-15 and the adjacent Thr-14 are phosphorylated in higher eukaryotes (Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991; Norbury et al., 1991). The biochemical characterization of cdc25 proteins from humans, *Drosophila* and *S. pombe* indicated that they function as phosphatases removing these inhibitory phosphate modifications (Dunphy and Kumagai, 1991, Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991). The dephosphorylation of Tyr-15 by the cdc25 phosphatase has been directly demonstrated and circumstantial evidence has indicated that the cdc25 phosphatase can also dephosphorylate Thr-14.

Sequence comparison revealed low, but significant similarities between phosphotyrosyl phosphatases and cdc25 phosphatases. The greatest similarity, however, was observed with a phosphatase from Vaccinia virus, VH1, which is capable of both tyrosyl and seryl dephosphorylation (Guan et al., 1991). The cdc25 phosphatase, therefore, appears to be a member of a novel class of phosphotyrosyl phosphatases capable of both seryl/threonyl and tyrosyl dephosphorylation.

Despite this apparently relaxed specificity, the cdc25 phosphatase appears to have an exceptionally high preference for the cyclin B-$p34^{cdc2}$ complex as a substrate. Recent findings with human cdc25 phosphatases provide a possible explanation for this behaviour. cdc25 phosphatase activity in vitro was found to be stimulated several fold by the addition of purified cyclin B (Galaktionov and Beach, 1991). Furthermore, transient association of the cdc25 phosphatase with the cyclin B-$p34^{cdc2}$ complex has been observed during M-phase in humans and *Xenopus* (Galaktionov and Beach, 1991; Jessus and Beach, 1992). The cdc25 phosphatase might therefore be maximally active only when associated with the cyclin B-$p34^{cdc2}$ complex.

In *Drosophila*, the expression of the *string* phosphatase is not only required for mitosis, but actually controls the timing of the embryonic cell divisions (Edgar and O'Farrell, 1990). Beginning at the cellular blastoderm stage, zygotic expression of *string* starts in a spatially defined and highly dynamic pattern which anticipates the pattern of the embryonic mitoses. Premature expression of *string* from a

heat inducible transgene results in a premature entry into mitosis. In contrast, overexpression of the $p34^{cdc2}$ kinase or the cyclin proteins does not affect the timing of mitoses (C.F.L., unpublished observations). These results, therefore, have indicated that the cells in the *Drosophila* embryo remain in the G2-phase until a pulse of *string* transcription produces cdc25 phosphatase activity which results in the activation of the cyclin B-$p34^{cdc2}$ kinase and entry into mitosis.

In addition to cyclin B and $p34^{cdc2}$, a variety of different cyclin proteins and cdc2-like kinases have been identified in *Drosophila* (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990a; Leopold and O'Farrell, 1991) and in other higher eukaryotes (Swenson et al., 1986; Elledge and Spottswood, 1991; Koff et al., 1991; Lew et al., 1991; Matsushime et al., 1991; Paris et al., 1991; Tsai et al., 1991; Xiong et al., 1991). Therefore, various additional cyclin-kinase complexes are likely to exist. In fact, such a complex has been clearly documented in the case of cyclin A and the cdk2 kinase in humans (Pines and Hunter, 1990; Tsai et al., 1991). In order to address whether multiple cdc25 homologs are involved in the regulation of such complexes, we have started a search for additional *Drosophila* cdc25 homologs. Here we report the existence of a second *Drosophila* cdc25 homolog. The same gene has been independently isolated with a different approach involving complementation of *S. pombe cdc25* and has been named *twine* (Jimenez et al., 1990; Alphey et al., 1992). Our analyses of *twine* expression and of the phenotype resulting from a mutation in *twine* indicates that this second cdc25 homolog is a meiotic regulator.

Materials and methods

Fly strains

The stock *mat(2)synHB5 cn bw/CyO,DTS-513* and other stocks with female sterile mutations on the second chromosome have been isolated by Schüpbach and Wieschaus (1989) and were kindly provided by T. Schüpbach, Princeton University, Princeton. The stock Df(2L)RN2/In(2LR)O carrying a defiency with the breakpoints 35E1.2;36A4.5 was kindly provided by M. Ashburner, University of Cambridge, Cambridge.

Enzymatic amplification experiments

For the identification of additional cdc25 homologs in *Drosophila* three degenerate primers corresponding to the most highly conserved regions in cdc25 phosphatases were synthesized:

primer 1: 5′-GGA GGATCC AT(ACT) GA(CT) TG(CT) (AC)GN T(AT)(CT) CCN TA(CT) GA-3′

primer 2: 5′-GGA TCTAGA (AG)TA NAT (CT)TC NGG (AG)TA NTG NA(AG) NGC NGG (AG)TA-3′

primer 3: 5′-GGA TCTAGA NGG NCC NC(GT) NTC NG(AT) N(CG)(AT) (AG)(AT)A (CT)TC (AG)CA-3′

In the putative amino acid sequence of the *twine* product (Fig. 1) the corresponding regions are found at the positions 241-248 (primer 1), 287-295 (primer 3) and 313-322 (primer 2). Primer 1 included a *Bam*HI restriction site at the 5′ end, and primer 2 and 3 a *Xba*I site.

The primer combinations 1+2 and 1+3 were used in enzymatic amplification reactions (Saiki et al., 1985) using DNA isolated from a lambda zap ovary cDNA library. The isolation of the template DNA and the reaction conditions for enzymatic amplifica-

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                                                    TTCCAGCGTGCGCAGTGATTTCGATTCCCATGATGCCGAT   40

ATG GAA TAC CAG GCC AAA AGG CGA AAA TCA GCC GTG CAG GAG ACT    85
MET Glu Tyr Gln Ala Lys Arg Arg Lys Ser Ala Val Gln Glu Thr    15

CCG ATG CAA TGG ATG CTG AAG CGG CAT ATT CCT GCC AGC ACC ACC   130
Pro Met Gln Trp Met Leu Lys Arg His Ile Pro Ala Ser Thr Thr    30

GTT CTG TCG CCC ATC ACC GAA TTG TCG CAG AAT ATG AAT GGA GCC   175
Val Leu Ser Pro Ile Thr Glu Leu Ser Gln Asn Met Asn Gly Ala    45

CGC CTG GAT GGC ACT CCC AAG TCC ACC CAG AGA ATC CCA GCC AAC   220
Arg Leu Asp Gly Thr Pro Lys Ser Thr Gln Arg Ile Pro Ala Asn    60

AGG ACC CTG AAT AAC TTT AAT AGC CTG TCC TCG CGA ACA CTC GGC   265
Arg Thr Leu Asn Asn Phe Asn Ser Leu Ser Ser Arg Thr Leu Gly    75

AGT TTC AGT AGC TCC TGC TCG AGT TAC GAG TCG GGC AAC TCG CTG   310
Ser Phe Ser Ser Ser Cys Ser Ser Tyr Glu Ser Gly Asn Ser Leu    90

GAT GAT GAG TAC ATG GAC ATG TTC GAA ATG GAA TCA GCC GAG AAT   355
Asp Asp Glu Tyr Met Asp Met Phe Glu Met Glu Ser Ala Glu Asn   105

CAC AAT CTT GAG TTG CCC GAT GAC TTG GAA GTG CTT CTC AGC GGA   400
His Asn Leu Glu Leu Pro Asp Asp Leu Glu Val Leu Leu Ser Gly   120

CAG CTG AAA TCG GAG AGC AAT TTA GAG GAG ATG TCC AAC AAA AAA   445
Gln Leu Lys Ser Glu Ser Asn Leu Glu Glu Met Ser Asn Lys Lys   135

GGC TCC CTG CGT CGC TGC CTA AGC ATG TAT CCC AGT GAA CAG CCA   490
Gly Ser Leu Arg Arg Cys Leu Ser Met Tyr Pro Ser Glu Gln Pro   150

GAG GAA GCG GTT CAA GAA CCA GAT CAG GAG ACC AAC ATG CCC ATG   535
Glu Glu Ala Val Gln Glu Pro Asp Gln Glu Thr Asn Met Pro Met   165

AAG AAG ATG CAA CGG AAA ACT CTA TCA ATG AAC GAC GCC GAG ATC   580
Lys Lys Met Gln Arg Lys Thr Leu Ser Met Asn Asp Ala Glu Ile   180

ATG AGA GCA CTG GGA GAT GAA CCA GAG TTA ATC GGA GAT CTC AGC   625
Met Arg Ala Leu Gly Asp Glu Pro Glu Leu Ile Gly Asp Leu Ser   195

AAG CCA TGT ACC CTG CCC TGT TTG GCG ACA GGC ATT AGA CAT CGT   670
Lys Pro Cys Thr Leu Pro Cys Leu Ala Thr Gly Ile Arg His Arg   210

GAT CTA AAG ACC ATC TCC AGT GAC ACA TTG GCC AGA CTG ATT CAG   715
Asp Leu Lys Thr Ile Ser Ser Asp Thr Leu Ala Arg Leu Ile Gln   225

GGC GAG TTT GAT GAA CAA TTG GGA AGC CAG GGC GGA TAC GAG ATC   760
Gly Glu Phe Asp Glu Gln Leu Gly Ser Gln Gly Gly Tyr Glu Ile   240

ATA GAC TGC CGT TAC CCA TAC GAG TTC CTT GGC GGA CAC ATA CGG   805
Ile Asp Cys Arg Tyr Pro Tyr Glu Phe Leu Gly Gly His Ile Arg   255

GGA GCG AAG AAT TTG TAC ACA CGT GGG CAG ATA CAG GAG GCG TTT   850
Gly Ala Lys Asn Leu Tyr Thr Arg Gly Gln Ile Gln Glu Ala Phe   270

CCC ACG CTG ACC TCC AAT CAG GAA AAT CGA CGC ATC TAC GTC TTC   895
Pro Thr Leu Thr Ser Asn Gln Glu Asn Arg Arg Ile Tyr Val Phe   285

CAC TGT GAG TTC TCC TCG GAA CGG GGT CCC AAG CTA TTG CGC TAC   940
His Cys Glu Phe Ser Ser Glu Arg Gly Pro Lys Leu Leu Arg Tyr   300

CTG CGG AGC AAC GAC AGA AGT CAG CAT ACC CAC AAC TAT CCG GCA   985
Leu Arg Ser Asn Asp Arg Ser Gln His Thr His Asn Tyr Pro Ala   315

CTG GAC TAT CCT GAA CTT TAT ATC CTG CAC AAT GGA TAC AAG GAG 1030
Leu Asp Tyr Pro Glu Leu Tyr Ile Leu His Asn Gly Tyr Lys Glu   330

TTC TTC GGT CTA TAC TCG CAG CTA TGC CAG CCC AGC CAA TAT GTA 1075
Phe Phe Gly Leu Tyr Ser Gln Leu Cys Gln Pro Ser Gln Tyr Val   345

CCA ATG CTG GCA CCG GCG CAC AAT GAT GAG TTT CGA TAT TTT CGG 1120
Pro Met Leu Ala Pro Ala His Asn Asp Glu Phe Arg Tyr Phe Arg   360

GCC AAG ACC AAG TCC TGG CAA TGT GGC GAG GGC GGA GAC AGT GGA 1165
Ala Lys Thr Lys Ser Trp Gln Cys Gly Glu Gly Gly Asp Ser Gly   375

ATC GGC GGT GGA GGC TCT CGC GGC CTC CGG AAA TCA CGT TCA CGA 1210
Ile Gly Gly Gly Gly Ser Arg Gly Leu Arg Lys Ser Arg Ser Arg   390

CTG CTC TAC GCC GAG TGA CCATTTCAGGCTATAGATACAATTCTCGAATCGA  1262
Leu Leu Tyr Ala Glu                                         395

TCCACCTGCCATAAGCTCACAGAGATCTCAATGAGAAACGATGGCTGCCCAATTTTACT 1321

CATAGAATTATTTTGTAATATTTAAGGACGCACATTTTAAGGTTCATTTCTTGTTGCGT 1380

TGTTCAAATGTTGTGAAAGCACTTTATATTTATTTAGTTCTTGATTTTGTATTATGTTC 1439

CACGTTGTGTGGACACAGAGCACCCAAATGGGAACACCAAAGATGGGAAGAAGAACAGA 1498

AAATGGATATATGTTCTCTAGTCAGTGTATGTAATAGTTTCAAAATTAATTAAAGTTCA 1557

TTTTTGTGTTCGGAATTTAAAAGTTAACTTAAGAAAGTATTTCATATTTTATTTTAGTA 1616

TTTAAGCATAATAATCTAACCATTGGATTTAAGAACTTCAAAGGAAATTACGAAAATTG 1675

TCTATGTATGAATACAAACTATGCTGAAACTATTTATGTGGATTCCACCT(A)13     1738
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of a *twine* cDNA. The nucleotide sequence of a *twine* cDNA (1738bp) includes a long open reading frame encoding a protein of 395 amino acids. The predicted amino acid sequence is shown below the nucleotide sequence.

tion have been described previously (Lehner and O'Farrell, 1990b). The temperature cycle, however, was changed to 1 minute at 94°C, 2 minutes at 48°C, 3 minutes at 72°C, and the cycle was repeated 35 times. With both primer combinations, two main products in the expected size range were amplified. The two main reaction products obtained with the primer combination 1+2 were gel purified and used as a template in a reaction with the primer combination 1+3. This reamplification also yielded two products with the same size as those obtained with the same primer combination in primary enzymatic amplifications from total library DNA. The larger products were also obtained when a *string* cDNA was used as a template with both primer combinations. The smaller reaction product obtained with the primer combination 1+2 was gel purified and inserted into a Bluescript vector.

For the analysis of mutations in the *twine* gene, the two primers corresponding to the regions flanking the coding sequence were synthesized:

primer 4: 5′-CGA GGATCC ATG GAA TAC CAG GCC AAA AGG-3′

primer 5: 5′-CGA TCTAGA GCC TGA AAT GGT CAC TC-3′

Primer 4 included a *Bam*HI restriction site, primer 5 a *Xba*I restriction site. Size comparison of the reaction products enzymatically amplified from genomic DNA or cDNA templates indicated that the coding sequence of the *twine* gene is not interrupted by introns. For the isolation of the *twine* coding sequence from *mat(2)synHB5* flies, 1 μg of genomic DNA isolated from homozygous males was used in an enzymatic amplification reaction using primers 4 and 5. The temperature cycle was adjusted to 1 minute at 94°C, 2 minutes at 53°C, 3 minutes at 72°C, and was repeated 35 times. The reaction products obtained in four independent reactions were gel purified and inserted into a Bluescript vector.

Isolation and characterization of cDNA clones and of the mutant allele

After sequence analysis of the fragment obtained by enzymatic amplification from the lambda zap ovary cDNA library, 10 cDNA clones were isolated from this library and plaque purified using the sequenced fragment as a probe after random primer labeling. After plasmid rescue from the lambda zap phages, the sizes of the cDNA inserts were determined, as well as the distance between the 5′ end of the insert and the region annealing to primer 3. For this purpose, the size of the products obtained by enzymatic amplification with primer 3 and either M13 primer or reverse primer (which flank the insert in the rescued plasmids) were determined after gel electrophoresis.

The largest insert was completely sequenced using double-stranded plasmid DNA as a template and a Sequenase kit (USB). In addition, the 5′ ends of two additional cDNA clones which, according to the PCR assay described above, extended far towards the 5′ end were sequenced. One of the clones started at position 35 of the nucleotide sequence shown in Fig. 1, the other at position 44.

For the analysis of the *mat(2)synHB5* mutation, one clone containing the *twine* coding sequence isolated by enzymatic amplification from *mat(2)synHB5* DNA was completely sequenced. To exclude the possibility that the identified mutation was introduced during enzymatic amplification, the result was confirmed by sequencing the corresponding region in three additional clones derived from independent PCR reactions.

Complementation experiments in S. pombe

The wild-type cDNA and the *twine* coding sequence isolated from *mat(2)synHB5* flies was inserted into the vector pSM1 (Russell, 1989). Transformation of *S. pombe* was done as described (Okazaki et al., 1990). The strain *cdc25-22 leu1-32 h*$^{-S}$ was transformed to leucine prototrophy at 25°C and colonies were then tested for the ability to divide at the restrictive temperature 36°C. Media used in genetic and physiological procedures have been described (Gutz et al., 1974; Nurse, 1975). These were Yeast extract (YE) containing 50 mg/ml each of adenine, uracil, leucine, lysine and histidine, Malt extract (ME), and EMM2 minimal, supplemented as required.

In situ hybridization experiments

Hybridizations to polytene chromosomes from salivary glands of wandering third instar larvae were carried out with a digoxigeninylated *twine* cDNA probe using reagents from the Genius kit (Boehringer).

In situ hybridization experiments with embryos were done according to Tautz and Pfeifle (1989) with minor modifications as described in Lehner and O'Farrell (1990b). In situ hybridization experiments with ovaries were done as described by Ephrussi et al. (1991). This method was also used for experiments with testis.

Cytological analyses of male and female meiosis

Testes and ovaries were dissected in Schneider's medium. Testes were fixed for 10 minutes in 4% formaldehyde/PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 4 mM KCl), stained with Hoechst 33258 (1 μg/μl in PBS) and mounted in 70% glycerol/PBS. Ovaries were fixed following the method of Puro (1991) with slight modifications. After dissection, ovaries were either directly fixed or incubated for 3 minutes in 75 mM KCl before fixation. Fixation was done during 1 hour in Carnoy's fixative (6:3:1 ethanol:chloroform:acetic acid). Subsequently, ovaries were incubated for 1 hour in ethanol and rehydrated by incubation in a series of reducing concentrations of ethanol in water. Ovaries were incubated in 1 N HCl for 10 minutes at room temperature and for 8 minutes at 60°C. After Feulgen staining, ovarioles were dissected and mounted in 70% glycerol in 15 mM $NaHSO_3$, 15 mM HCl.

Embryos from homozygous *mat(2)synHB5* females or wild-type females were collected for 10 minutes after several rounds of precollections. They were fixed and stained with Hoechst 33258 as described previously (Lehner and O'Farrell, 1989). Immunofluorescent labeling with a monoclonal antibody recognising the sperm tail (Karr, 1991) was done as described by Karr (1991).

Testes, ovarioles and embryos were analysed in a Zeiss Axiophot microscope using phase contrast, differential interference contrast and epifluorescence. Kodak technical pan film was used for photography.

Results

Identification of a second Drosophila *cdc25 homolog*

Degenerate primers corresponding to the most conserved regions in cdc25 phosphatases were used for enzymatic amplification from an ovary cDNA library template (for details see Materials and methods). One of the two resulting products was clearly different from *string*, but revealed strong sequence similarities to cdc25 phosphatases. Using this fragment as a probe, several cDNA clones were isolated from the ovary cDNA library. The DNA sequence derived from the clone with the largest cDNA insert is shown in Fig. 1. The open reading frame starting from the first methionine is 395 amino acids in length. Sequence comparison revealed that the corresponding gene was identical to the gene *twine* (L. Alphey and D. Glover, personal communication) which has been isolated independently in

a screen for *Drosophila* cDNAs complementing the temperature sensitive *cdc25* allele in *S. pombe* (Jimenez et al., 1990).

The alignment of the putative amino acid sequence of the *twine* product with other cdc25 homologs revealed extensive similarities in the C-terminal domain as shown in Fig. 2A. The most extensive conservation was found in the region with the HC motif, a signature sequence which contains residues essential for catalysis in all tyrosyl phosphatases (arrows in Fig. 2A). The N-terminal domain did not show similarities with other cdc25 homologs except for two motifs that were also present in the *string* protein (not shown). Comparison of sequence identities obtained after pairwise alignments clearly demonstrated that the two *Drosophila* sequences, *string* and *twine*, are most closely related to each other (43% identity, see Fig. 2B) and significantly less homologous to cdc25 of *S. pombe* or to the three human homologs (22-28% identity).

twine *expression is restricted to the female and male germ line*

The pattern of *twine* expression was analyzed by whole-mount in situ hybridization with a digoxygeninylated *twine* cDNA probe. *twine* expression was not observed in imaginal and larval tissues of third instar larvae. Labeling was also not detected in late embryonic stages (not shown). In contrast to *string*, which is expressed zygotically in dynamic patterns (Edgar and O'Farrell, 1989), *twine*, therefore, does not appear to be expressed zygotically in the embryo. In very young embryos, however, intense labeling was observed, uniformly distributed throughout the embryo (not shown), as has also been described in the case of *string*

A

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TWINE       GDEPE-LIGDLSKPCTLPCLATGIRHRDLKTISSDTLARLIQGEFDEQLG   233
STRING      RN...-....F..AYA..-.ME.-......S...E.V...LK...SDKVA   318
CDC25AHS    PRD---....F..GYLFHTV.-.-K.Q...Y..PEIM.SVLN.K.ANLIK   377
CDC25BHS    HR.---....Y..AFL.QTV-D.-K.Q...Y..PE.MVA.LT.K.SNIVD   420
CDC25CHS    SNQGH-....F..V.A..TV-S.-K.Q...YVNPE.V.A.LS.K.QGLIE   323
CDC25SP     TK.S.RF.SSHVEDLS...F--AVKEDS..R.TQE..LG.LD.K.KDIFD   431
              +   ++*++  +   ++       ++ +** ++ +++  ++ *+* +

TWINE       SQGGYEIIDCRYPYEFLGGHIRGAKNLYTRGQIQEAFPTL----------   273
STRING      .---.R..........E....E.......TE..LDE.L.VQQTELQQQQN   365
CDC25AHS    E---FV.........YE....K..V..HMEEEV-.D.L------LKKPIV   417
CDC25BHS    K---FV.V.......YE....KT.V..PLERDA-.S.L------LKSPIA   460
CDC25CHS    K---FYV........Y.....Q..L...SQEEL-FN.F------LKKPIV   363
CDC25SP     K---CI.....FE..Y.....ST.V..N.KQA.VD..-------LSKPLT   471
            +     ++***+ **+ **** +* **    +   +*
                      ↓     ↓
TWINE       TSNQENRRIYVFHCEFSSERGPKLLRYLRSNDRSQHTHNYPALDYPELYI   323
STRING      AESGHK.N.II............MS.F..NL..ERN.NA....H...I.L   415
CDC25AHS    PTD-GK.V.V............RMC..V.--E.DRLGNE..K.H.....V   464
CDC25BHS    PCSLDK.V.LI...........RMC.FI.--E.DRAVND..S.Y...M..   508
CDC25CHS    PLDTQK.I.I............RMC.C..--EEDRSLNQ....Y......   411
CDC25SP     -----H.VAL.....H.AH.A.H.ALHF.NT..RMNS.R..F.Y...V..   516
                 +*   +**** *++*+*++    *  +     + ** * ***+*+

TWINE       LHNGYKEFFGLYSQLCQPSQYVPMLAPAHNDEFRYFRAKTKSWQCGEGGD   373
STRING      .........ESHVE..E.HA.RT..D..Y.EAY.H....S...N-----G   460
CDC25AHS    .KG......MKCQSY.E.PS.R..HHEDFKEDLKK..T.SRT.-------   507
CDC25BHS    .KG......PQHPNF.E.QD.R..NHE.FK..LKT..L.TR..-------   551
CDC25CHS    .KG..RD..PE.ME..E.QS.C..HHQD.KT.LLRC.SQS.VQ-------   454
CDC25SP     L.G...S.YENHKNR.D.IN..P.NDRS.VMTCTKAMNNF.R-------N   559
            *+++*++*+      *+*  * +*   +   +    + + +

TWINE       SGIGGGGSRGLRKSRSRLLYAE      395
STRING      D.L..ATG.-.K......ML        479
CDC25AHS    -----A.EKSK.EMY...KKL        523
CDC25BHS    -----A.E.SR.ELC...QDQ        567
CDC25CHS    -----E.E.Q..EQIAL.VKDMSP     473
CDC25SP     ATFMRTK.YTFWPKCVSFPRR        580
                + +  +     +
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B

	TWINE	STRING	CDC25AHS	CDC25BHS	CDC25CHS	CDC25SP
TWINE	100	43	26	25	28	23
STRING		100	27	27	26	22
CDC25AHS			100	41	32	20
CDC25BHS				100	33	20
CDC25CHS					100	21
CDC25SP						100

Fig. 2. Amino acid sequence comparison of cdc25 homologs.(A) The amino acid sequences of the conserved C-terminal domains of the *twine* protein and the *string* protein from *Drosophila* (Edgar and O'Farrell, 1989), the three cdc25 homologs isolated from humans (CDC25AHS, CDC25BHS and CDC25CHS) (Sadhu et al., 1990; Galaktionov and Beach, 1991) and the cdc25 protein from *S. pombe* (CDC25SP; Russell and Nurse, 1986) are aligned. Gaps introduced for optimal alignment are indicated by dashes. Amino acids that are identical to the *twine* sequence are represented by dots. Positions with identical amino acids in all the cdc25 homologs are marked with asterisks in the bottom line, and positions where most of the cdc25 homologs are similar are indicated by a +. Arrows point to residues that are essential for catalysis in the *string* protein (Gautier et al., 1991) and are part of the HC motif, a signature sequence present in all phosphotyrosyl phosphatases (Millar and Russell, 1992). (B) The amino acid sequences of cdc25 homologs were aligned in pairs and the extent of identity was determined and is listed (% identity).

(Edgar and O'Farrell, 1989). Since zygotic transcription has not yet started in these early embryos, this labeling must reflect maternally derived *twine* mRNA (see below). *twine* mRNA was detected during all of the early, syncytial stages. After the last syncytial division (mitosis 13), concomittant with cellularization, signals were fading rapidly and were detected predominantly in the yolky interior. After gastrulation, only background levels were observed.

The expression of the maternal *twine* mRNA was analyzed by in situ hybridization experiments with ovaries (Fig. 3; for a description of oogenesis see Mahowald and Kambysellis, 1980). Strong labeling was observed in the nurse cells of stage 10 oocytes (Fig. 3B). In stage 11 egg chambers (Fig. 3C), labeling was seen not only in the nurse cells, but also in the oocyte, indicating that *twine* mRNA is translocated from the nurse cells into the oocyte during nurse cell contraction. During the early stages of oogenesis, signals above background were not seen prior to stage 7 and only weak signals were observed during stage 8 and 9 (Fig. 3A). Thus, *twine* expression does not occur in the somatic follicle cells while they proliferate mitotically during stages 2-5. In contrast to *twine*, (and in contrast to the results of Alphey et al., 1992), *string* was found to be expressed also during earlier stages of oogenesis (Fig. 3D). In these early stages, however, *string* expression occurred predominantly in the somatic follicle cells (Fig. 3D, arrow). After the end of follicle cell proliferation (stage 6), *string* mRNA was no longer found in the follicle cells, and in later stages (Fig. 3E,F), *string* expression was observed in germ line cells where it closely paralleled the expression of *twine*.

The expression pattern of the *twine* cdc25 homolog during oogenesis is consistent with a role either in meiosis or in early embryonic mitoses which are known to rely on maternally provided components. In order to distinguish between these two possibilities, it was of interest to analyze the expression of *twine* in male gonads, where only meiosis and no concurrent production of stores for the early embryo occurs (for a description of spermatogenesis see Lindsley and Tokuyasu, 1980; Gönczy et al., 1992). The results of these experiments are shown in Fig. 4. *twine* expression was not detected at the apical tip of the testis where the stem cells divide mitotically to form the cysts containing 16 syncytial spermatocytes (Fig. 4B). However, during the premeiotic growth phase of the cysts, labeling was clearly present. As illustrated in Fig. 4C, labeling was not only present in the premeiotic, 16-cell cysts (arrowhead), but also after meiosis I in 32-cell cysts (arrow). No labeling was seen after meiosis II, during the process of spermatid differentiation (star).

In situ hybridization experiments with the *string* probe yielded complementary results. *String* expression was detected at the apical tip where the mitotic divisions of the germ line cells lead to the formation of the cysts. During the meiotic stages, signals with the *string* probe were not above background (C.F.L., unpublished observation, see also Alphey et al., 1992).

Identification of a mutation in twine

The results of the in situ hybridization experiments suggested that the *twine* product serves a role during meiosis in both sexes Accordingly, we anticipated that mutations in *twine* would result in female and male sterility. Interestingly, the female sterile mutation, *mat(2)synHB5*, isolated by Schüpbach and Wieschaus (1989) had been mapped to the same genomic region as the *twine* gene (35F; according to in situ hybridizations to polytene chromosomes, not shown). The analysis of homozygous *mat(2)synHB5* males revealed that this mutation also resulted in complete male sterility (see below). Male and female sterility were also observed in flies with the *mat(2)synHB5* chromosome over the deficiency Df(2L)RN2, which deletes the *twine* gene. Thus, the *mat(2)synHB5* mutation had the characteristics expected for a *twine* allele, and was characterized further. By in situ hybridization experiments, *twine* mRNA was detected at wild-type levels in early embryos derived from mothers homozygous for *mat(2)synHB5* (not shown). This observation suggested that *mat(2)synHB5* was either a mutation in the coding sequence or not a *twine* allele at all. This was analyzed by sequencing the coding region of the *twine* gene isolated from *mat(2)synHB5* flies by enzymatic amplification (see Material and methods). As shown in Fig. 5, the sequence revealed the presence of one missense mutation. The codon for a proline, which is conserved in all the known cdc25 homologs and is located C-terminally in the HC motif containing the active site, was found to be changed into a leucine codon.

In order to demonstrate that the observed mutation would impair the function of the *twine* product, we constructed plasmids that allowed complementation experiments in *S. pombe* (not shown). The construct with the wild-type *twine* cDNA transformed into an *S. pombe* strain with a temperature sensitive mutation in *cdc25* was able to restore growth at the restrictive temperature. In contrast, the construct with the mutant *twine* allele did not allow growth at the restrictive temperature. These results indicate that the wild-type *twine* product is a functional cdc25 homolog and that the mutation present in *mat(2)synHB5* does impair its function.

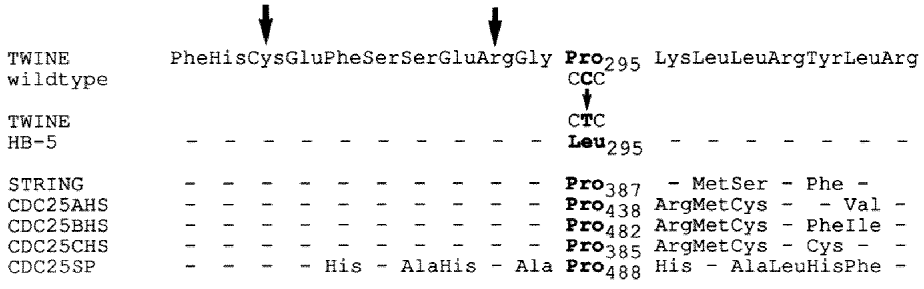

Fig. 5. Analysis of the *twine* gene in *mat(2)synHB5*. The sequence of the coding region of the *twine* gene isolated from *mat(2)synHB5* flies was determined and revealed a missense mutation in the codon for the proline at position 295. This proline is conserved in all the known cdc25 homologs and is immediately downstream from residues thought to be involved in catalysis (arrows; Gautier et al., 1991)

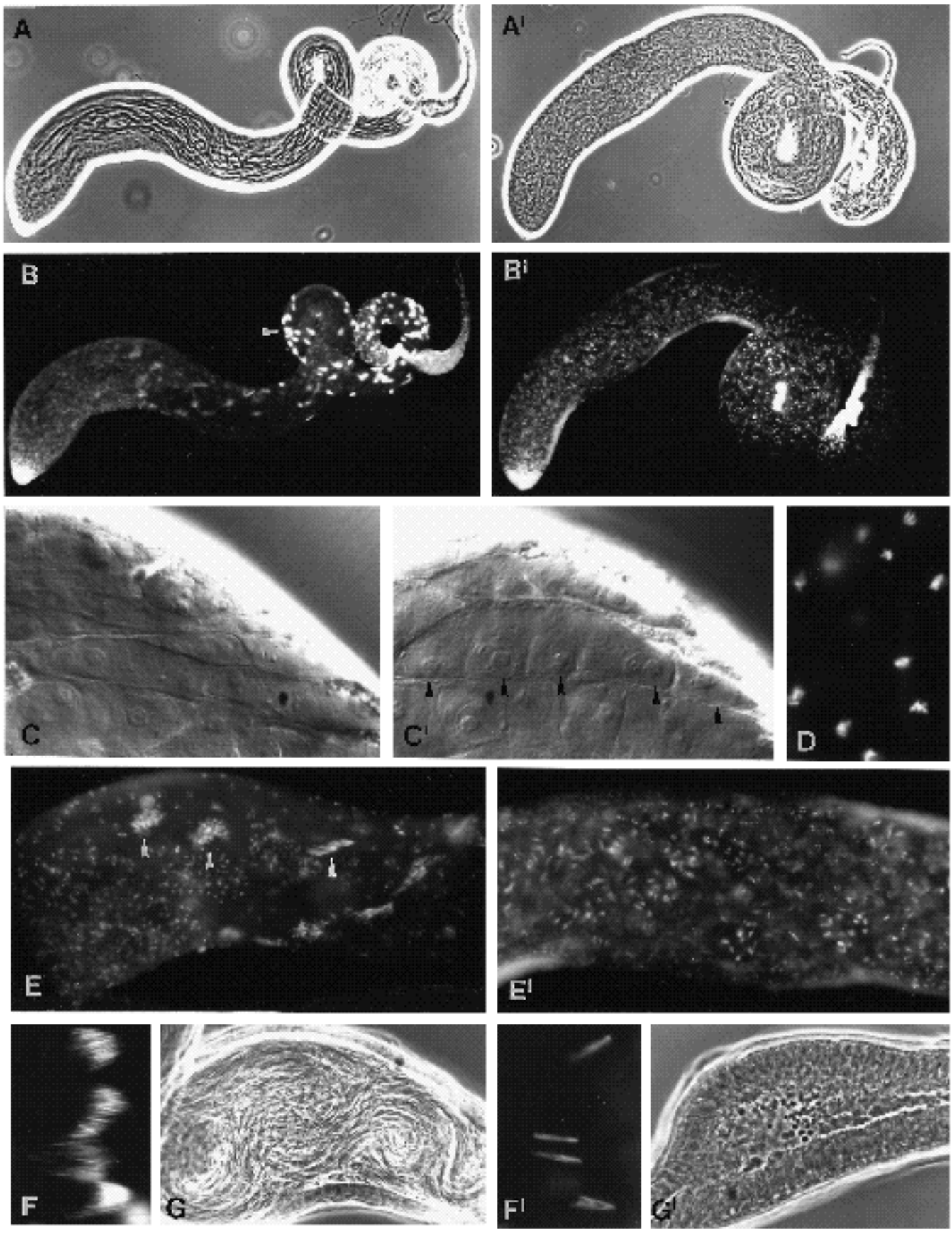
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Fig. 6. Phenotypic analysis of spermatogenesis in homozygous *mat(2)syn HB5* males. Testes from wild-type (A-F) and homozygous *mat(2)synHB5* males (A′-F′) were fixed and stained with Hoechst. (A,A′) Phase contrast micrographs revealing similar morphology in whole-mount preparations of wild-type and mutant testes. (B,B′) Hoechst staining revealing intensely labeled sperm head clusters only in the distal region of the wild-type testis (white arrowhead). (C,C′) Differential interference contrast micrographs of the apical tips indicating that the formation of the cysts is not affected in the mutants. Black arrowheads point to nuclei in a single cyst in the mutant. (D) High magnification view of prometaphase figures from a wild-type cyst in meiosis I with condensed chromosomes stained with Hoechst. No meiotic figures were seen in the mutants. (E,E′) Postmeiotic clustering of the nuclei in cysts (white arrowheads) is only seen in the wild type. (F,F′) Sperm heads are significantly larger in the mutant and not thightly clustered as in the wild type. (G,G′) Seminal vesicles contain motile sperm in the wild type and no sperm in the mutant.

We cannot exclude, however, that the *mat(2)synHB5* product has some residual activity.

Absence of meiosis in mat(2)syn HB5 *males*

The phenotypic consequences of the *mat(2)synHB5* mutation were studied cytologically. Fig. 6 illustrates the results obtained with testes. Testes developed in homozygous *mat(2)synHB5* males (Fig. 6A′,B′) and displayed a similar morphology as testes from wild-type males (Fig. 6A,B). Inspection of the apical tip at high magnification (Fig. 6C and C′) clearly demonstrated that premeiotic cysts with normal appearance were formed in the mutant testis. The arrowheads in Fig. 6C′ point to several clearly visible nuclei in a cyst of a mutant. However, whereas in wild-type testis meiotic figures with condensed chromosomes during either meiosis I (Fig. 6D) or meiosis II (not shown) were readily observed after staining with a DNA stain, meiotic figures were never observed in mutant testes. Accordingly, the cysts with densely packed clusters of either 32 or 64 nuclei produced by meiosis I or II, respectively, were only detected in the wild type (Fig. 6E, arrowheads) and not in the mutants (Fig. 6E′). Despite the absence of meiosis, the processes of sperm differentiation including elongation of sperm tails and compaction of the nuclear DNA into sperm heads occurred in the mutant. Nevertheless, spermatids were clearly abnormal, and the mutant sperm heads were always considerably larger and never thightly bundled as in the wild type (compare Fig. 6F and F′). Moreover, in contrast to wild type, no motile sperm was present in the seminal vesicle of mutant males (Fig. 6G and G′). In conclusion, these observations demonstrate that meiotic divisions do not occur in the mutant males.

Meiotic defects in mat(2)synHB5 *females*

As in the males, the premeiotic mitoses in the germ line were also not affected by the *mat(2)synHB5* mutation in the female (Fig. 7). Completely normal egg chambers containing nurse cells and oocyte were seen in the mutant up to the onset of the meiotic nuclear envelope break down in stage 13 of oogenesis (see below). A single nucleus with the DNA characteristically compacted in the karyosome was present in all the oocytes (arrowheads in Fig. 7).

Surprisingly, in contrast to the mutant males, entry into the first meiotic division occurred at the correct stage of oogenesis in mutant females (Fig. 8). Whereas early stage-13 oocytes still contained a single nucleus with compacted DNA (Fig. 8A) in a clearly recognisable nuclear envelope

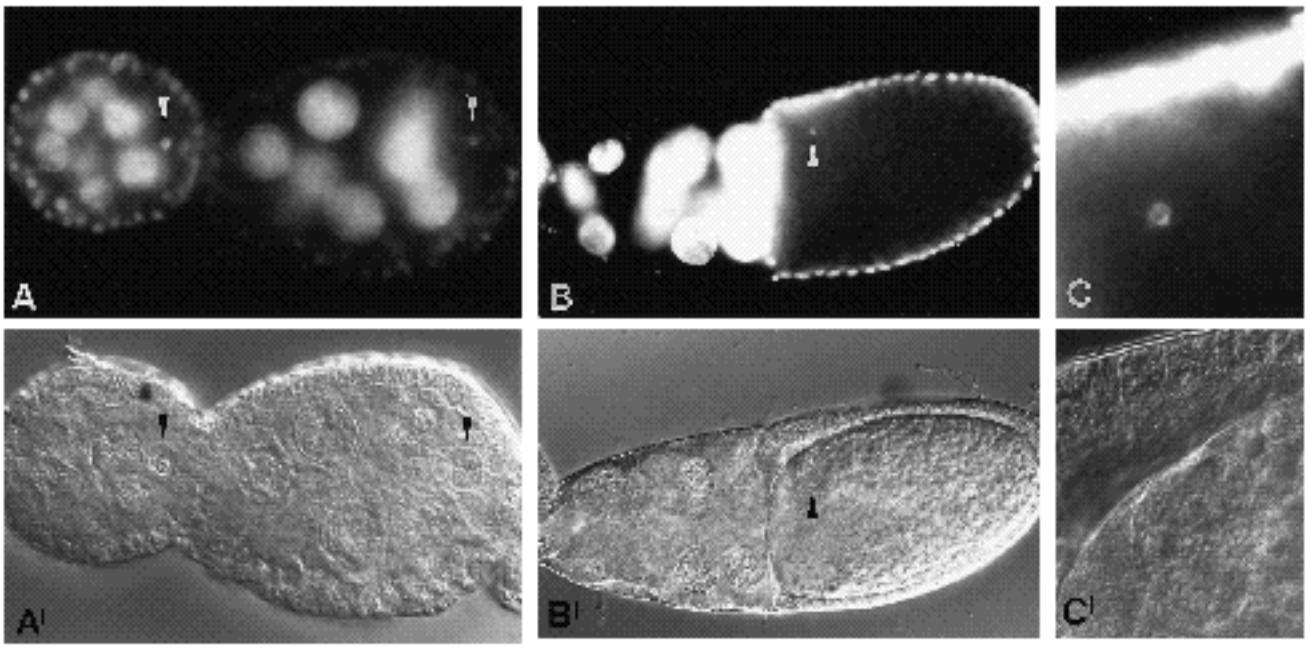

Fig. 7. Early oogenesis in homozygous *mat(2)synHB5* females. Ovaries from mutant females were fixed, Feulgen stained and viewed using epifluorescence (A-C) or differential interference contrast (A′-C′); (A,A′) stage 5 (left) and stage-7 (right) egg chamber; (B,B′) stage-11 egg chamber; (C,C′) high magnification view of the region with the oocyte nucleus of the stage-11 egg chamber shown in (B,B′). Nurse cells and a single oocyte nucleus (arrowheads) are present and of wild-type appearence.

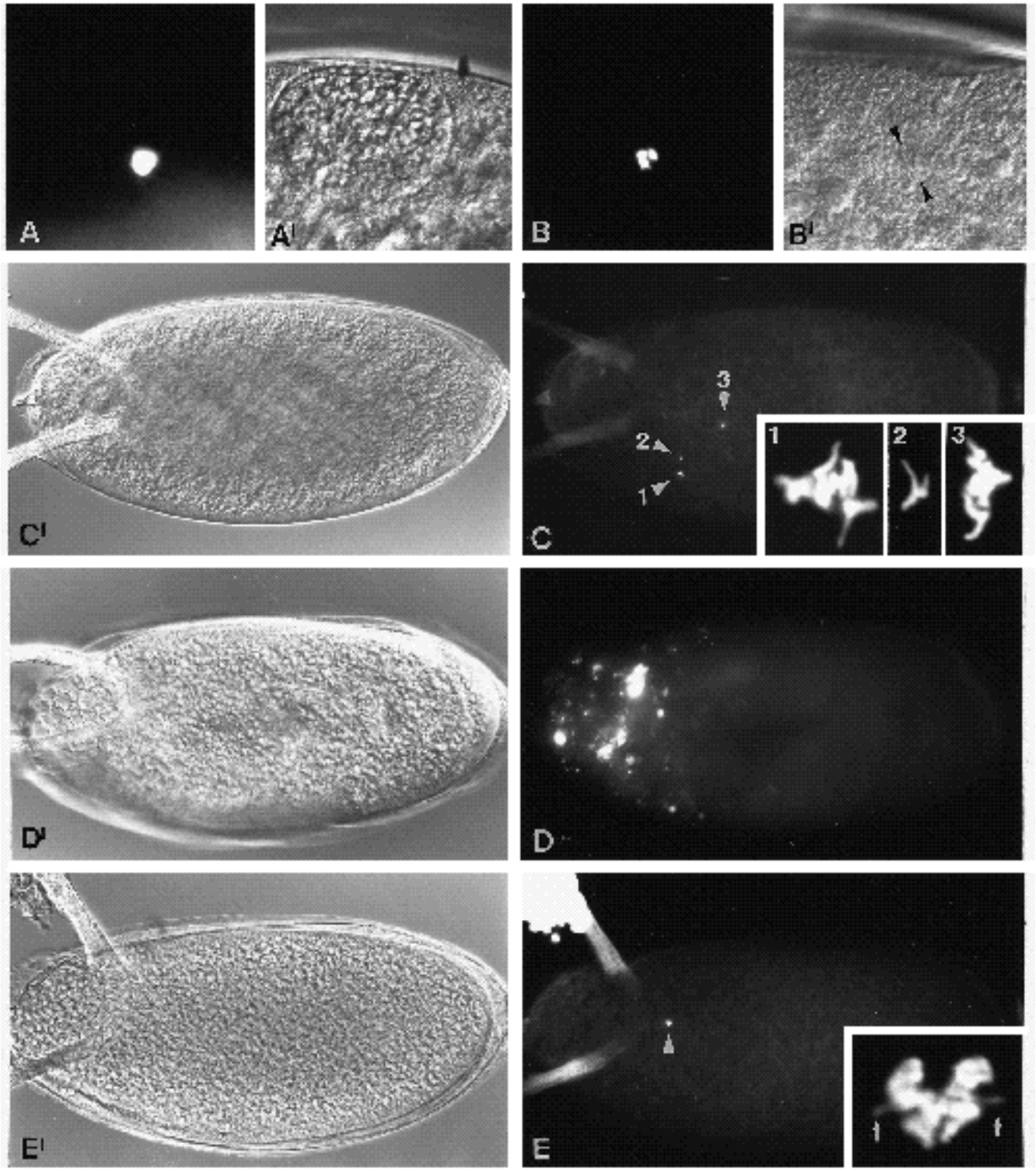

Fig. 8. Meiotic defects during late stages of oogenesis in homozygous *mat(s)synHB5* females. Ovaries were fixed, Feulgen stained and viewed using epifluorescence (A-E) or differential interference contrast (A′-E′). (A,A′) A normal oocyte nucleus with nuclear envelope and karyosome is observed in an early stage-13 oocyte from a mutant female. (B,B′) Entry into the first meiotic division in a stage-13 oocyte from a mutant female. Separation of bivalents and dissassembly of nuclear envelope are clearly detectable. Fibrillar structures, most likely representing spindle microtubules, are associated with the chromosomes and are marked with arrowheads. (C,C′) Mature stage-14 oocyte from a mutant female. Three clusters with condensed chromosomes are observed and shown at higher magnification in the inserts. (D,D′) Mature stage-14 oocyte from a mutant female with abnormally dispersed chromatin. (E,E′) Mature stage-14 oocyte from a wild-type female with a characteristic meiotic figure (inset). The white arrows in the inset point to the fourth chromosome univalents.

(Fig. 8A′), entry into the first meiotic division as evidenced by chromosome appearance (Fig. 8B) and nuclear envelope breakdown (Fig. 8B′) was observed in stage-13 oocytes. Inspection at high magnification also revealed fibrillar structures in the region of the condensing chromosomes presumably indicating the formation of a meiotic spindle (see arrowheads in Fig. 8B′).

Analysis of mature stage-14 oocytes, however, clearly revealed abnormalities in the mutants. In the wild type, mature stage-14 oocytes are naturally arrested in metaphase of meiosis I. Normally, meiosis is completed only after egg activation, which occurs during egg laying. Egg activation and consequentially resumption of meiosis can be induced experimentally by incubation in hypotonic medium (Mahowald et al., 1983; Theurkauf and Hawley, 1992). An early anaphase figure of the first meiotic division which was typically observed, if mature stage-14 oocytes were fixed after a 3 minute incubation in hypotonic medium, is shown in the inset in Fig. 8E. The white arrows presumably mark the small fourth chromosome univalents which are known to be segregated even before the onset of anaphase (Puro, 1991, Theurkauf and Hawley, 1992). The other, not yet completely segregated chromosomes are found in more central positions. Such normal anaphase figures were essentially never observed in mutant stage-14 oocytes. Instead, abnormalities of varying severity were detected. Fig. 8C illustrates a mild phenotype and Fig. 8D a more severe phenotype. In the mutant oocyte shown in Fig. 8C, three discrete clusters of condensed chromosomes were detected (see arrowheads 1-3 and corresponding insets in Fig. 8C). Many mutant oocytes had even more dispersed clusters of chromatin of varying size which sometimes appeared condensed (not shown) and sometimes decondensed as in Fig. 8D.

Despite defective meiosis, mutant eggs could be fertilized with sperm as revealed by labeling with an antibody recognizing the sperm tail (not shown). As in the case of the mature stage-14 oocytes, freshly laid eggs of mutant mothers displayed various degrees of abnormalities (Fig. 9A-C). Often two (Fig. 9A,B) or more (Fig. 9C) clusters of intensely labeled chromatin of either decondensed (Fig. 9A) or condensed appearance (Fig. 9B, inset) were recognized. Normal eggs with three polar bodies (arrows and upper inset in Fig. 9D) and two pronuclei (arrowhead and lower inset in Fig. 9D) as seen in freshly laid eggs from wild-type mothers were never detected among the progeny of mutant mothers.

All these observations clearly indicate that meiosis is

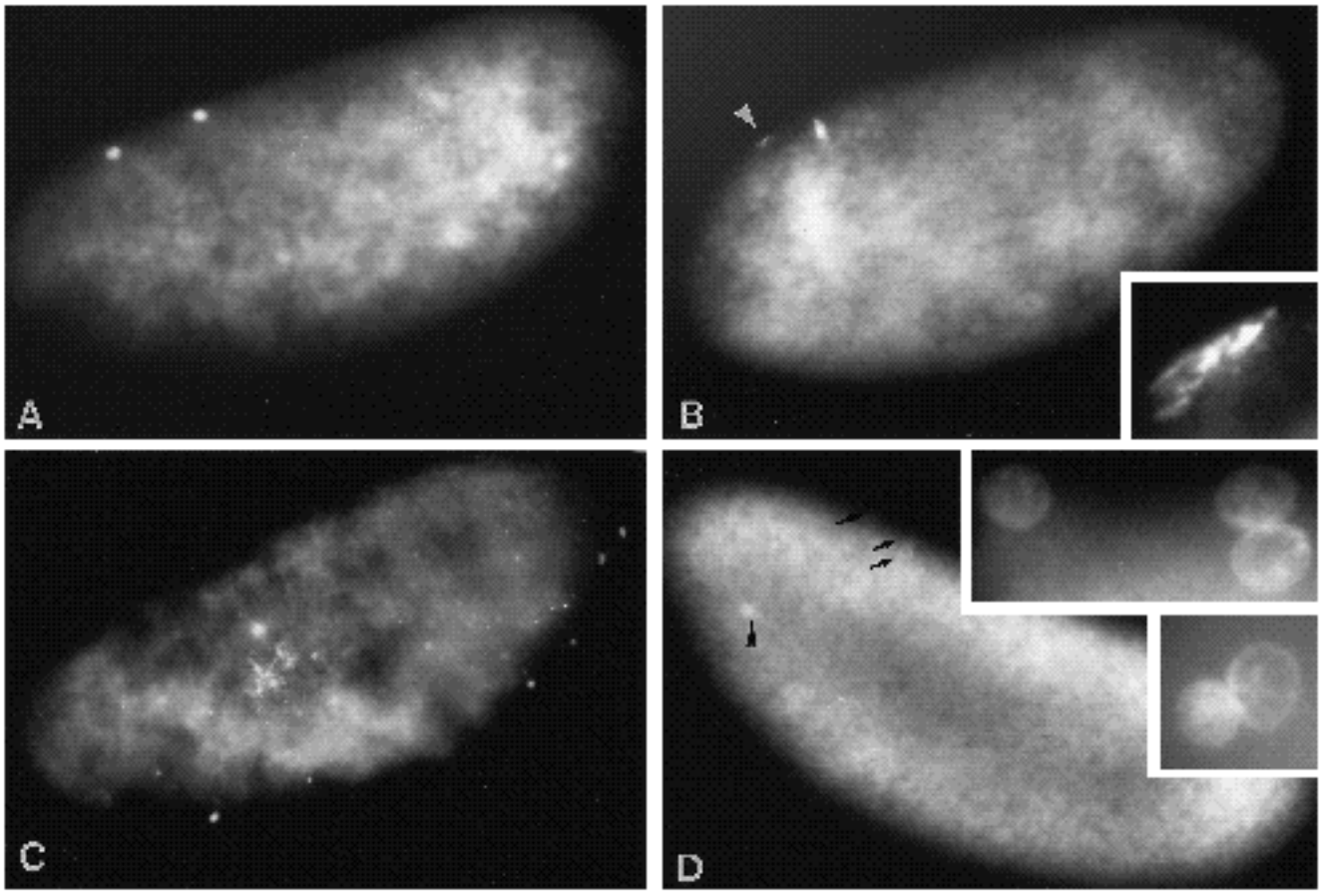

Fig. 9. The stages of completion of meiosis in freshly laid eggs. Eggs were collected for 10 minutes from either homozygous *mat(2)synHB5* females (A-C) or from wild-type females (D) and then fixed and stained with Hoechst. A variable number of intensely staining nuclei with either decondensed or condensed chromatin (inset in B) were present in eggs of mutant females. In the egg derived from a wild-type female (D), the normal completion of meiosis and fertilization is indicated by the presence of two pronuclei (arrowhead and lower insert) and three polar bodies (arrows and upper insert).

defective in the mutant females. The defects, however, start only after entry into meiosis. Our observations suggest that the arrest at metaphase of meiosis I which normally occurs in mature stage-14 oocytes does not occur in the mutant oocytes. Instead, chromosomes become dispersed, eventually thoughout the oocyte. According to the signal intensities observed after DNA labeling, this chromatin dispersal must involve DNA replication. The observed phenotype is most easily explained by assuming that the mutant oocyte continues to progress through aberrant cell cycles during which chromosomes are replicated and segregated irregularily.

Discussion

The *Drosophila* cdc25 phosphatase encoded at the *string* locus is required for entry into mitosis (Edgar and O'Farrell, 1989), and the regulated expression of *string* directs the intricate patterns of the embryonic cell divisions (Edgar and O'Farrell, 1990). We have identified a second *Drosophila* cdc25 homolog. Expression of this second homolog, *twine*, was only detected in the germ line starting at relatively late stages of gametogenesis, well after the end of the mitotic germ cell divisions. In both male and female germ cells, *twine* transcripts are present during the stages of meiosis, and our genetic analyses indicate that *twine* is required for meiosis.

The notion that the control of meiotic and mitotic divisions is mechanistically similar is old and based on a large body of evidence. In *Xenopus* oocytes, for example, entry into the meiotic divisions can be induced by injection of the active cyclin B-p34^{cdc2} kinase complex. The same result is also observed after injection of *string* mRNA (Gautier et al., 1991). Moreover, in *S. pombe*, *cdc25* is not only involved in mitosis but also in meiosis (Grallert and Sipiczki, 1989). The existence of a distinct, meiotic cdc25 homolog in *Drosophila* presumably reflects a need for additonal levels of controls required for meiosis in the context of gametogenesis in higher organisms.

twine *function during the early embryonic mitoses?*

The *twine* mRNA that accumulates during oogenesis persists in the oocyte beyond meiosis and is detectable in the early embryo until cellularization. Therefore, *twine* might not have an exclusively meiotic function, but could also act during the early embryonic mitoses. However, during the early embryonic stages, not only *twine* mRNA but also maternally derived *string* mRNA is present (Edgar and O'Farrell, 1989). The function of either *twine* or *string* during these stages has not yet been analyzed. It remains possible that the *twine* protein is inactivated after meiosis, and that the *string* protein is the only cdc25 phosphatase active during the early embryonic mitoses. At least in later mitotic divisions, when *twine* is no longer expressed, *string* function is sufficient for entry into mitosis. Whereas *string* is expressed in apparently all mitotically dividing cells in the embryo (Edgar and O'Farrell, 1989), in the larvae (J. Knoblich and C.F.L., unpublished observations) and in the adult (e.g. during the follicle cell proliferation, see Fig. 3), *twine* expression, with the sole exception of the early embryonic cycles, is not observed during mitotic cell cycles. Therefore, we propose that *twine* is specialized for meiosis and that it is not active in the early embryo.

Identification of a mutant twine *allele*

The observation that *twine* is not only expressed during oogenesis but also during spermatogenesis where, in contrast to oogenesis, no stockpiling of components for early embryonic development occurs, strongly suggested that *twine* might function during meiosis. A mutation in *twine* was therefore expected to result in both female and male sterility. Our analysis of mutant fly lines, which had been isolated in a screen for female sterile mutations on the second chromosome by Schüpbach and Wieschaus (1989) and which had been mapped to the same genomic region as *twine*, revealed that one line, *mat(2)synHB5*, was not only female sterile but also male sterile. Sequence analysis of the *twine* gene from *mat(2)synHB5* flies revealed one missense mutation in a position that is conserved in all the known cdc25 homologs. Moreover, this missense mutation in *twine* is in the immediate neighbourhood of amino acid residues that are known to be part of the active site in phosphotyrosyl phosphatases (Gautier et al., 1991; Guan et al., 1991; Millar et al., 1991). The results of our complementation experiments demonstrate that the function of *twine* which allows complementation of a mutant cdc25 allele in *S. pombe* is impaired by this missense mutation.

twine *function in male meiosis*

Meiosis in males homozygous for *mat(2)synHB5* is completely blocked. Meiotic figures with condensed chromosomes were never observed in mutant testes indicating that *twine* is required for entry into meiosis. Other aspects of spermatogenesis are not affected. The development and growth of premeiotic cysts appears completely normal. Moreover, postmeiotic differentiation processes also continue despite the absence of meiosis. Sperm tails elongate and the formation of sperm heads is attempted in mutant testes, although the compaction of the premeiotic, presumably 4N nuclei into the typical rod shape does not occur to the same extent as in the case of the postmeiotic 1N nuclei in wild-type testes.

twine expression starts during the growth phase of cysts many hours before the onset of the meiotic divisions. *string* expression on the other hand is not observed during the meiotic stages. If this does not simply reflect an inability to detect transient, low level expression, it indicates that entry into meiosis is controlled by a mechanism different from that controlling entry into mitosis in the cellularized embryo, where the transcriptional control of *string* expression is thought to determine the time of entry into mitosis (for a review see Lehner, 1991). During the embryonic cell division cycles, *string* expression is sufficient to force G2-cells into mitosis, and starts immediately (about 25 minutes) before mitosis in an intricate pattern which accurately anticipates the pattern of the subsequent division (Edgar and O'Farrell, 1990).

twine *function in female meiosis*

As in testes from homozygous *mat(2)synHB5* males, mitotic divisions of germ line cells were completely normal

in mutant females and defects were only observed during meiosis. However, whereas entry into meiosis appears to be completely blocked in mutant males, it is still accomplished in mutant females. Nuclear envelope breakdown and chromosome condensation occur at the correct stage in mutant oocytes. But, in contrast to wild-type oocytes, mutant oocytes do not arrest at metaphase of the first meiotic division. Instead, chromosomes are replicated and dispersed into irregular nuclei of variable size. Such irregular nuclei were never observed in mutant testes. The extent of this chromatin dispersal in mature mutant oocytes varies considerably. This variability most likely reflects the fact that mature stage-14 oocytes can be retained for several hours before fertilization and egg deposition. We assume that the severity of the phenotype increases with increasing retention time, which is affected by feeding of the adult females. Our observation that the proportion of eggs with few nuclei is higher in collections from well fed females than in collections from starved females is consistent with this assumption (C.F.L., unpublished observation).

Our analysis of *string* expression suggests an attractive explanation for the phenotypic differences observed in males and females. Whereas we have been unable to detect *string* transcripts during the meiotic stages of spermatogenesis, *string* transcripts are clearly present in oocytes undergoing meiosis. Normal levels of *string* transcripts were also detected in mutant oocytes (data not shown). It is therefore possible that the *string* activity allows entry into meiosis in mutant oocytes. The results of experiments in *S. pombe* clearly indicate that *string* and *twine* have similar activities since both can complement cdc25 alleles (Edgar and O'Farrell, 1989; Jimenez et al., 1990; this paper). Moreover, the presence of *string* activity in mutant oocytes might not only allow entry into meiosis but also further cell cycle progression. Our observations, however, showed that the oocyte nucleus does not proceed through regular mitotic cycles but is fragmented rapidly in the mutant oocytes. While an ordered cell cycle progression might be hampered for a variety of reasons, we would like to point out that the centrosome which might well be required for an ordered mitotic cycle is missing in late oocytes and is contributed only during fertilization by the sperm (Mahowald and Kambysellis, 1980).

According to our interpretation, *twine* would act formally analogous to CSF (cytostatic factor), an activity that causes the arrest at metaphase II during *Xenopus* egg maturation (Masui and Markert, 1971; for recent references on CSF see Hunt, 1992). Mechanistically, CSF acts by stabilizing the activity of MPF (maturation promoting factor), a factor which according to recent results represents the active complex of cyclin B and the $p34^{cdc2}$ kinase. In *Drosophila* oocytes, MPF activity might be stabilized by *twine* activity and cause the arrest at metaphase of meiosis I until *twine* is inactivated after egg activation. Testing this idea will require a biochemical analysis of *string* and *twine* activity during female meiosis. Fortunately, recent progress in the development of methods allowing mass isolation and in vitro activation of mature oocytes should render such investigations feasible. In addition, such studies might also reveal which of the various cyclin-cdc2 (or cdc2-like) kinases is a target of *twine* activity.

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Fig. 3. Expression of *twine* and *string* during oogenesis. The results of in situ hybridizations with a *twine* probe (A-C) or a *string* probe (D-F) are shown. A,D: stages 1-8; B,E: stage 10; C,F: stage 11. An egg chamber at stage 5, during which maximal levels of string transcripts are observed in the follicle cells is indicated by an arrow. The graded signals observed in stage 11 oocytes (C,F) presumably reflect differential permeability of the vitelline membrane.

Fig. 4. Expression of *twine* during spermatogenesis A whole-mount preparation of a testis after in situ hybridization with a *twine* probe is shown in A. The apical tip is shown at higher magnification in B and the region were meiosis occurs in C. *twine* expression is not found in the proliferation center at the apical tip, but starts in cysts during the growth phase. It is detected in premeiotic cysts (arrowhead) and in cysts after meiosis I (arrow), but not during the postmeiotic stages (star).