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dpa, a member of the MCM family, is required for mitotic DNA replication but not endoreplication in *Drosophila*

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We have isolated the *Drosophila disc proliferation abnormal* (*dpa*) gene, a member of the MCM family of DNA replication factors. Members of this family of proteins are required for DNA replication in yeast. A *dpa* null mutant dies during pupal stages because imaginal tissues necessary for the formation of the adult fly fail to proliferate normally. Beginning in late embryogenesis BrdU labeling reveals DNA replication defects in mitotically proliferating cells. In contrast, *dpa* is dispensable for endoreplication, a specialized cell cycle consisting of consecutive rounds of S phases without intervening mitosis. Our studies suggest an essential role for *dpa* in mitotic DNA replication but not in endoreplication. Thus, *dpa* is not a general replication factor but may play a specialized regulatory role in DNA replication.

Keywords: DNA replication/*dpa*/*Drosophila*/MCM

Introduction

Our current understanding of DNA replication derives mainly from studies in *Escherichia coli*, bacteriophages, eukaryotic viruses and yeast. In the budding yeast *Saccharomyces cerevisiae*, a protein complex called origin recognition complex (ORC) has been shown to bind the A element of autonomously replicating sequences (ARSs) (Bell and Stillman, 1992). ORC appears to be bound to the ARS element throughout the cell cycle, as part of a multiprotein complex whose interaction with ARS changes during the cell cycle. There is more extensive protein interaction with ARS from the conclusion of mitosis until the next S phase (Diffley *et al.*, 1994). Interestingly, proteins encoded by *CDC46*, *MCM2* and *MCM3*, three members of a family of genes first identified for their cell division cycle (CDC) or minichromosome maintenance (MCM) defect, enter the nucleus following the end of mitosis and disappear from the nucleus after the initiation of S phase (Hennessy *et al.*, 1990; Yan *et al.*, 1993). These three genes are required for the initiation of DNA replication. Mutations in *MCM2* and *MCM3* result in full or partial loss of origin activity (Yan *et al.*, 1993). Together with the genetic interaction between *CDC46* and *ORC* (Li and Herskowitz, 1993), these observations indicate that the MCM family of proteins might modulate ORC activity in a cell cycle-dependent manner and have led to the proposal that the MCM gene products may be replication 'licensing factors' allowing one and only one round of DNA replication per cell cycle (Blow and Laskey, 1988; Heichman and Roberts, 1994).

On the basis of their mutant phenotypes and/or sequence similarity *MCM2 MCM3, CDC46*, the recently cloned *CDC54* (Whitbread and Dalton, 1995) and genes from *Schizosaccharomyces pombe*, *Xenopus*, mouse and man have been grouped into the MCM family. The *S.pombe cdc21*, *nda1* and *nda4* mutants show DNA replication defects (Coxon *et al.*, 1992; Miyake *et al.*, 1993). In addition, injection of antibodies raised against mammalian homologs inhibits DNA synthesis (Kimura *et al.*, 1994; Todorov *et al.*, 1994). Using *in vitro* licensing assays several groups could recently show the requirement of protein complexes containing *Xenopus* MCM3 in DNA replication licensing (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995) suggesting an important role for this gene family in DNA replication in higher eukaryotes.

DNA replication in *Drosophila* has been studied using a variety of approaches (Crevel and Cotterill, 1991; Smith and Orr-Weaver, 1991; Smith *et al.*, 1993) but only a few molecules required for DNA replication have been functionally characterized. They include DNA polymerase alpha, proliferating cell nuclear antigen (PCNA) and *plutonium*, a small ankyrin repeat containing protein which acts as an inhibitor of DNA replication during early embryogenesis (Yamaguchi *et al.*, 1991, 1992; Axton *et al.*, 1994; Henderson *et al.*, 1994). The study of DNA replication in *Drosophila* offers the advantage of two distinct cell cycles: mitotic cycles and cycles of DNA replication without mitosis (endocycles), the latter resulting in polyploid cells. During endocycles, heterochromatic regions of the genome become underrepresented, raising the possibility that the replication origins and regulatory molecules used are different from those used in mitotic cell cycles.

We report here that a gene identified in an enhancer trap screen (Bier *et al.*, 1989) is required for DNA replication and imaginal disc proliferation. The *disc proliferation abnormal* (*dpa*) gene product exhibits significant sequence similarity to the fission yeast *cdc21* gene product and other members of the MCM family of DNA replication factors. *dpa* is the first member of the cdc21 branch of the MCM family with characterized mutant phenotypes in multicellular organisms. A null mutation of this gene interferes with DNA replication in mitotically proliferating cells but not in endoreplicating cells.

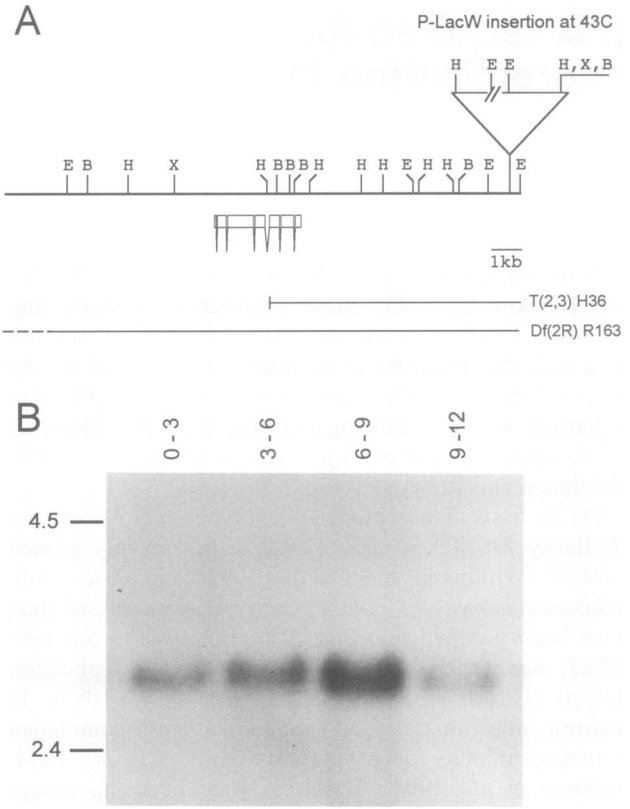

Fig. 1. Genomic organization of the *dpa* locus. **(A)** Restriction map and exon/intron organization of the *dpa* locus. The *dpa* transcript is represented by open bars, interrupted by six introns. The insertion site of the P element E5 2nd 23 ~7.5 kb 3′ to the end of the *dpa* transcription unit is shown. The translocation breakpoint of *T(2,3) H36* is indicated by the vertical bar at the left end of the solid line. The genomic DNA deleted in *Df(2R)R163* includes regions marked by the solid line and possibly those indicated by the dashed line. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. **(B)** Developmental Northern blot. Equal amounts of RNA isolated from Oregon R embryos were loaded and the Northern blot was probed with a full-length *dpa* cDNA. A single 2.8 kb transcript detected at 0–3 h of embryogenesis is probably maternally supplied. Zygotic expression reaches its peak level at 6–9 h. Reduced levels are detected between 9 and 12 h.

Results

Cloning of a gene at 43C with a S phase expression pattern

We initially identified *dpa* with a P element enhancer trap line which expressed lacZ in most or all cells of the embryonic central nervous system (CNS) and peripheral nervous system (PNS), and weaker staining in the developing gut and epidermis (Figure 4 and data not shown). This expression pattern is reminiscent of staining obtained with BrdU labeling of embryos (Bodmer *et al.*, 1989; Smith and Orr-Weaver, 1991; Duronio and O'Farrell, 1994). The P element insertion was mapped by *in situ* hybridization to the region 43C on the second chromosome (Figure 1A). Genomic DNA flanking the insertion site was cloned by plasmid rescue (see Materials and methods). The rescued genomic DNA was labeled with digoxigenin and used to detect RNA in whole-mount wild type embryos. One of the genomic fragments revealed an RNA distribution similar to the lacZ pattern in the enhancer trap line and was used to screen two independent cDNA libraries (see Materials and methods). We isolated one 2.8 kb cDNA clone from each library and found that each of the cDNA clones, when used as probes for *in situ* hybridization with wild type embryos, detected an expression pattern similar to the lacZ pattern of the P element enhancer trap line at 43C (data not shown). These probes hybridized to a single band of ~2.8 kb on a Northern blot of poly(A)$^+$ RNA from staged embryos (Figure 1B), suggesting that we isolated full length cDNA clones. The high levels of transcript in 0–3 h embryos indicate a maternally supplied message. Expression levels increased in 3–6 h embryos and reached maximum levels at 6–9 h. The transcript level was reduced by 9–12 h and only very low levels are detected after 12 h (Figure 1B and data not shown).

The gene at 43C encodes a protein with significant sequence similarity to fission yeast cdc21 and other members of the MCM family of DNA replication factors

Sequencing of one of the cDNA clones (see Materials and methods), revealed a large open reading frame that encodes a protein of 866 amino acids (96.6 kDa; Figure 2). The methionine codon at position 61 of the nucleotide sequence is preceded by stop codons in all three reading frames (Figure 2), as shown by sequencing both cDNA and genomic DNA, suggesting it is the translation start site.

The predicted protein sequence shows extensive sequence similarity to a family of proteins implicated in DNA replication (Figure 3), including the fission yeast cdc21 (45% identity), the budding yeast CDC54 (44% identity), CDC46 (34% identity), MCM2 (31% identity), MCM3 (31% identity) and the mammalian P1 protein (33% identity). Based on sequence similarity *dpa* can be assigned to the *cdc21/CDC54* branch of the MCM gene family.

The highest sequence similarity exhibited between members of this family of genes is found in the middle domain, whereas the amino- and carboxyl-terminal domains are far less similar (Figure 3). The N-terminal domain of the *dpa* gene product contains two interesting sequence motifs: a putative Zn finger and several cdc2 consensus phosphorylation sites. The Zn finger motif (Figure 2) which is conserved in cdc21, CDC54 and MCM2 (Yan *et al.*, 1991; Coxon *et al.*, 1992; Whitbread and Dalton, 1995) has been shown to be essential for MCM2 function (Yan *et al.*, 1991). dpa contains five potential phosphorylation sites for cdc2 protein kinase, (S/T)PXR, at its N-terminus (Nigg, 1991) and a sixth cdc2 phosphorylation consensus site, TPLK, following the putative Zn finger motif. cdc21, CDC54 and MCM3 also contain potential cdc2 phosphorylation sites, although the number and location of these sites differ in each case. Whether dpa is regulated by cdc2 phosphorylation, thereby providing a direct link between the cell cycle machinery and DNA replication, remains to be determined.

RNA expression pattern of dpa

Expression of *dpa* RNA was analyzed using *in situ* hybridization with a digoxigenin-labeled cDNA probe. The zygotic RNA expression closely resembles the pattern of DNA replication. Syncytial blastoderm embryos show strong staining (Figure 4A), indicating a maternally supplied transcript. Indeed, there is maternal expression in

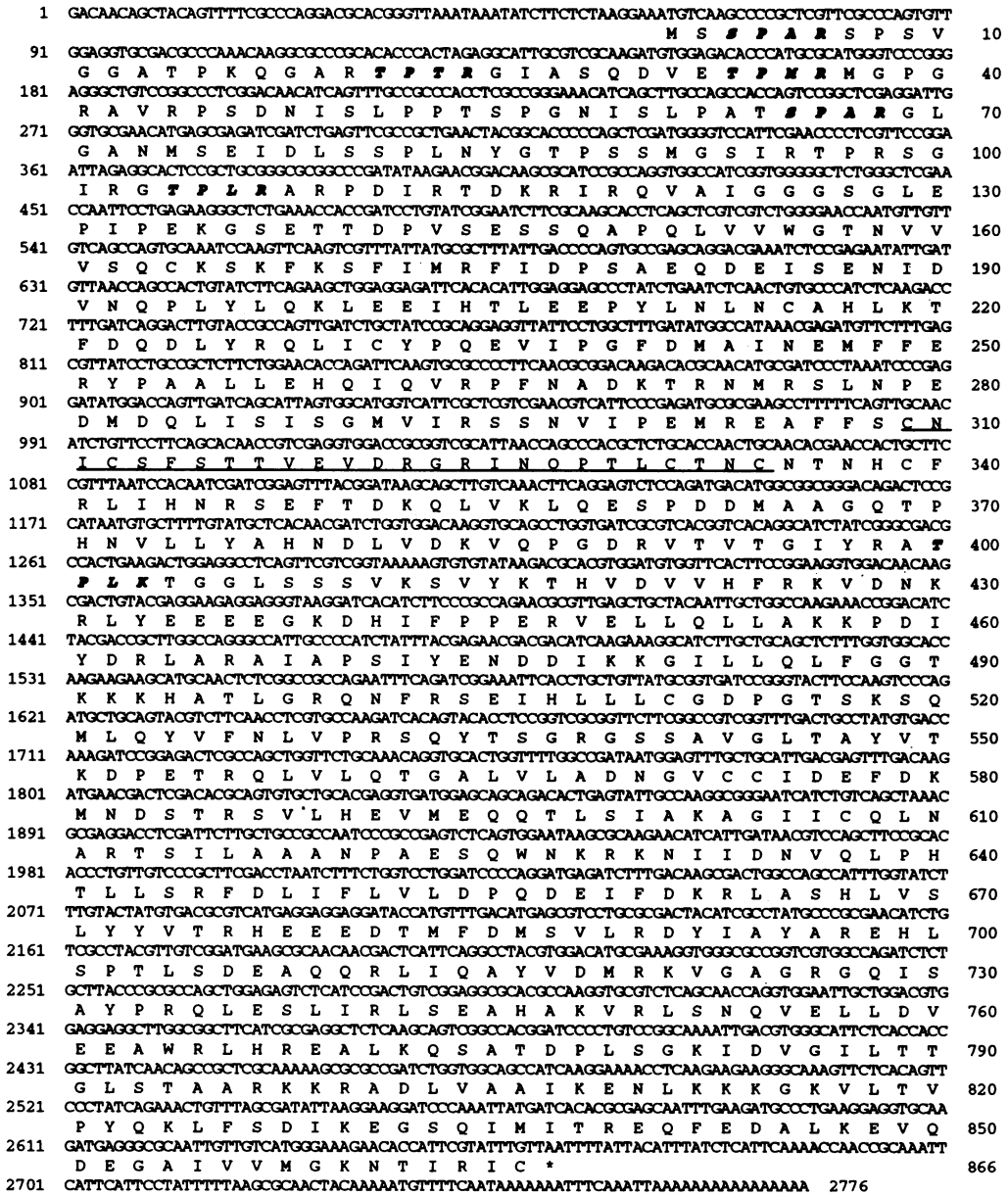

Fig. 2. Nucleotide sequence of the *dpa* cDNA and its conceptual translation. The predicted amino acid sequence of the longest open reading frame is displayed, using the single letter code. Nucleotides are numbered on the left, amino acids on the right. The putative Zn finger motif is underlined. The cdc2 consensus phosphorylation sites are bold and italicized.

germline cells throughout oogenesis. The transcript accumulates in late oogenesis in the nurse cells before it is transferred to the oocyte (data not shown). The expression pattern in the embryo is dynamic during gastrulation and germband extension. Most tissues express the transcript at high levels but some areas have very little or no expression (Figure 4B). The epidermal expression remains high until late stage 11, when it is extinguished (Figure 4C). Expression is then found predominantly in the delaminated neuroblasts of the CNS and the sensory organ precursors of the PNS (Figure 4D and E). The dividing sensory organ precursors of the PNS continue to express the gene until stage 13/14 when expression ceases, concomitantly with the end of cell division. Thus, cells of the fully developed (mitotically quiescent) PNS do not express the transcript (not shown). Most or all cells in the ventral nerve cord and brain show high transcript levels from stages 10 through 12 whereas only a subset of CNS cells express the transcript from stages 13 onwards (Figure 4C–G). Expression in the brain continues until the end of embryogenesis (Figure 4F). The gene is also expressed in endoreplicating tissues, starting in the anterior and posterior midgut at stage 12 followed by hindgut expression at stage 13 and, shortly afterwards, expression in the malpighian tubules (Figure 4G). In late embryogenesis (stage 16/17) the gonads also express the transcript (Figure 4F). The pattern in the gut and malpighian tubules closely follows the pattern of the endocycles of DNA replication (Smith and Orr-Weaver, 1991).

We also detected expression of the *dpa* gene in cells that proliferate in third instar larvae to give rise to imaginal tissues. In the eye imaginal disc there is broad expression ahead of the morphogenetic furrow and in a tight band behind it, but not in the furrow itself (Figure 4H).

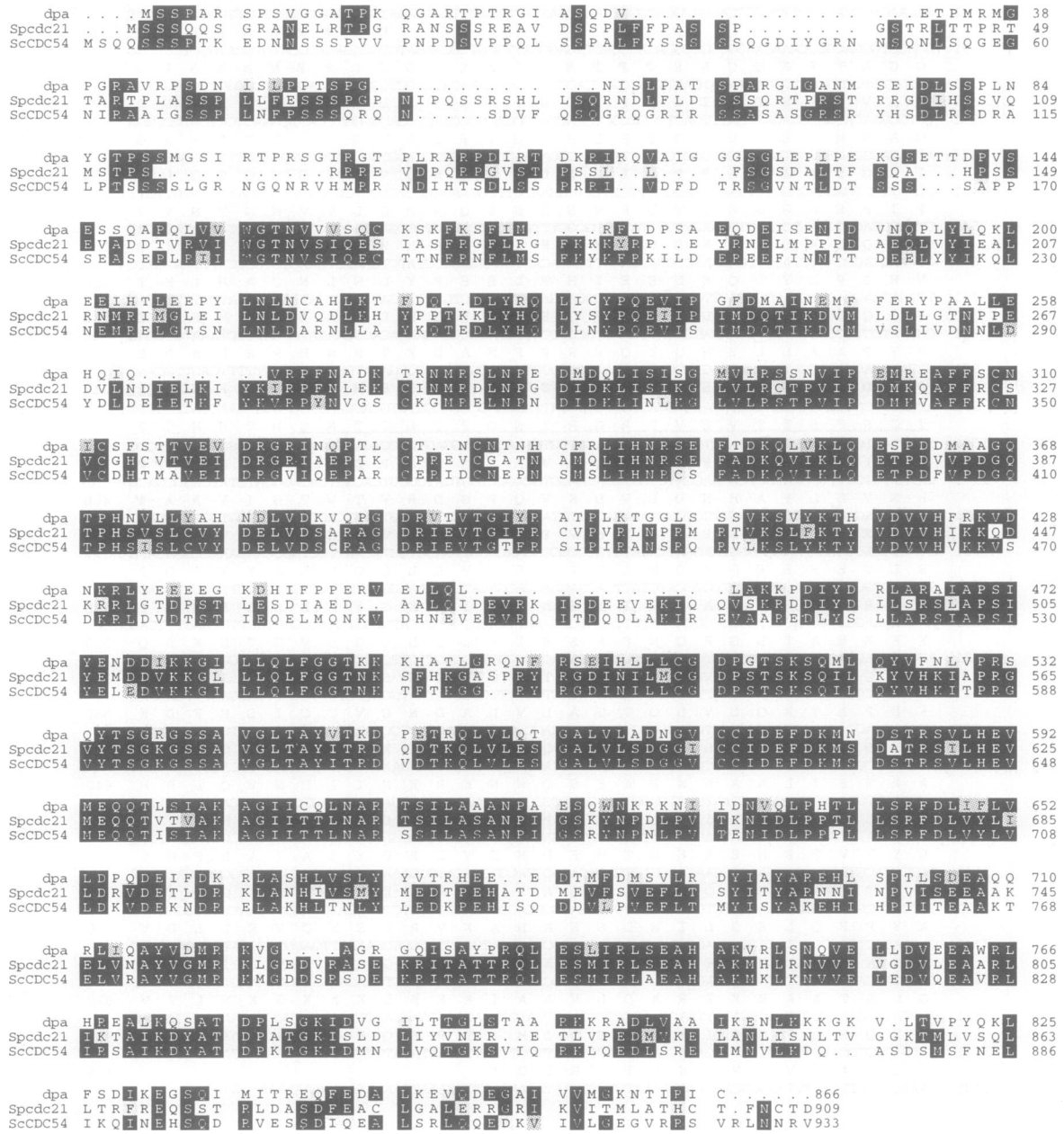

Fig. 3. Sequence similarity of the *dpa* gene product to members of the MCM family of DNA replication factors. The predicted dpa protein sequence is aligned to two related proteins: Spcdc21 from fission yeast (Coxon *et al.*, 1992) and budding yeast ScCDC54 (Whitbread and Dalton, 1995). Amino acid residues identical to the dpa sequence are boxed in black and similar amino acid residues are shaded in grey.

Expression thus coincides with the first and second mitotic waves of proliferating cells (Wolff and Ready, 1993). In the CNS of third instar larvae, expression is evident in the mitotically active optic lobes and in those neuroblasts that are producing ganglion mother cells (data not shown). In summary, the *dpa* transcript is expressed in all tissues undergoing DNA replication.

A null allele of dpa

The P element insertion at 43C is ~7.5 kb 3′ to the end of the *dpa* transcription unit (Figure 1A) and does not cause lethality. Imprecise excision of the P element (see Materials and methods) generated one line, R163, which did not complement the lethal phenotype of *Df(2R)ST1*, a deficiency uncovering the region 42B3-4 to 43E18. Further mapping using deficiencies and a panel of EMS-induced lethal mutations of the 43C region (Heitzler *et al.*, 1993) revealed that R163 does not complement the four different complementation groups defined by the lethal alleles *l(2)43Ca*^{1}, *l(2)43Cb*^{1}, *l(2)43Cc*^{1} and *l(2)43Da*^{2}. We therefore refer to this imprecise excision line henceforth as *Df(2R)R163*. *In situ* hybridization of embryos showed that no zygotic *dpa* transcript is present in *Df(2R)R163* homozygotes (data not shown).

Two approaches were used to confirm that the complementation group *l(2)43Ca*^{1} is mutant for *dpa*. First, we performed a Southern blot analysis on the translocation *T(2;3)H36* (Hilliker and Trusis-Coulter, 1987), which does not complement the lethal phenotype of *l(2)43Ca*^{1}, and found that the translocation *T(2;3)H36* had a breakpoint within the *dpa* transcription unit (Figure 1A and data not shown). This suggests that *T(2;3)H36* and *l(2)43Ca*^{1} are

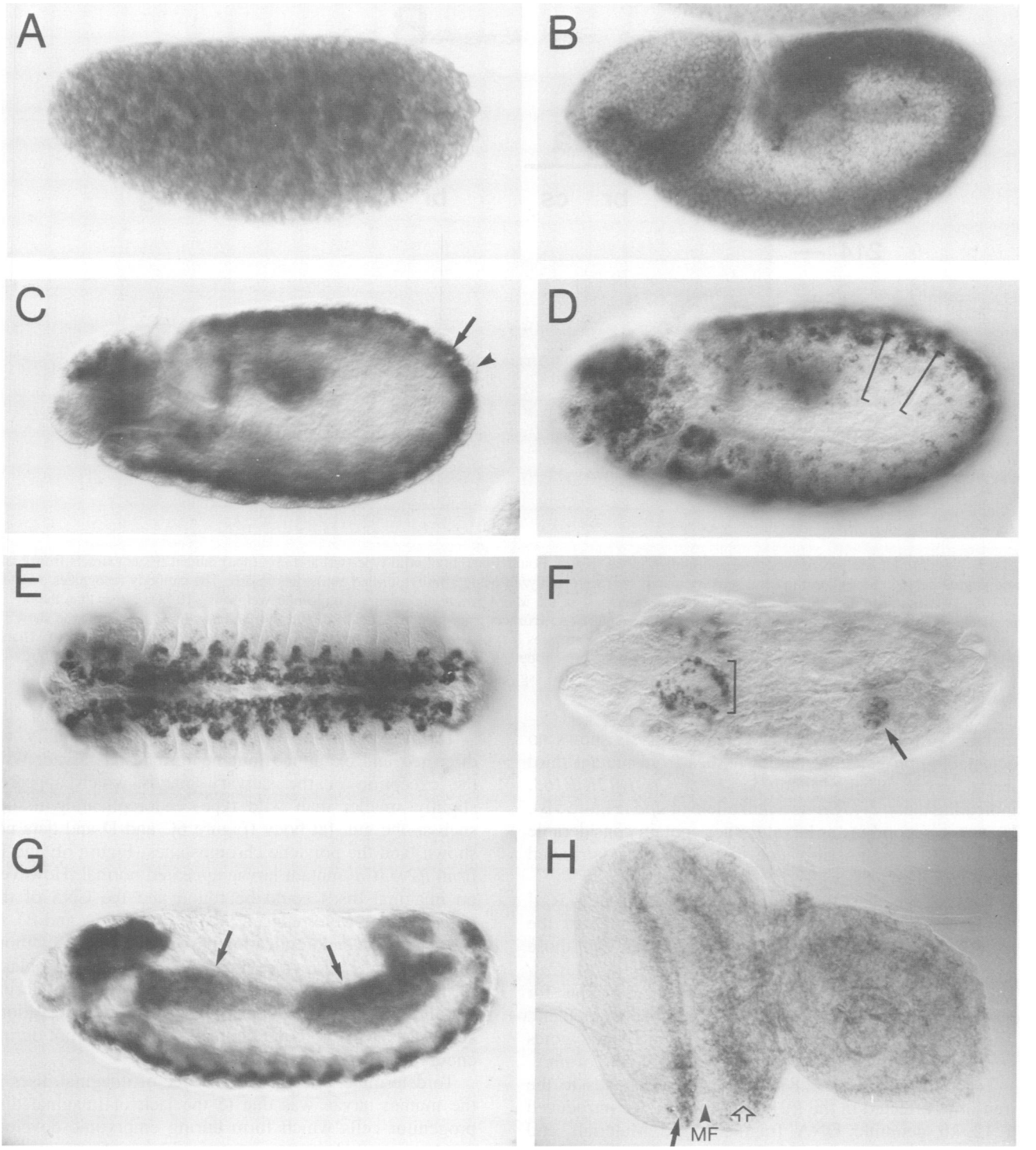

Fig. 4. Developmental profile of *dpa* expression. Expression pattern of *dpa* as revealed by *in situ* hybridization using a digoxigenin-labeled *dpa* cDNA probe on wild type embryos (A–G), oriented with the anterior to the left and ventral downward or towards the observer, and on an eye imaginal disc (H), oriented with the posterior to the left. (**A**) A stage 3 syncytial blastoderm embryo shows uniform distribution of maternally supplied *dpa* transcript. (**B**) Lateral view of a stage 7 embryo reveals broad epidermal *dpa* expression. (**C**) In a stage 11 embryo expression of *dpa* is predominant in the neuroblast layer (arrow), expression in epidermal cells (arrowhead) has ceased. (**D**) *dpa* transcript is expressed at high levels in the sensory organ precursors of the developing peripheral nervous system (brackets) in a stage 11 embryo. (**E**) Ventral view of a stage 12 embryo, showing strong expression in the CNS. (**F**) The *dpa* transcript persists in a subset of cells in the ventral nerve cord and the brain (bracket). This stage 17 embryo shows also *dpa* expression in the gonads (arrow). (**G**) A late stage 12 embryo revealing *dpa* expression in the endoreplicating tissues of the gut (arrows). (**H**) In the imaginal eye disc of a third instar larva, *dpa* is broadly expressed anterior to the morphogenetic furrow (open arrow) and a tight stripe (arrow) posterior to the morphogenetic furrow but not in the morphogenetic furrow (MF) itself. These two zones of *dpa* expression correspond to the first and second mitotic wave of proliferating cells.

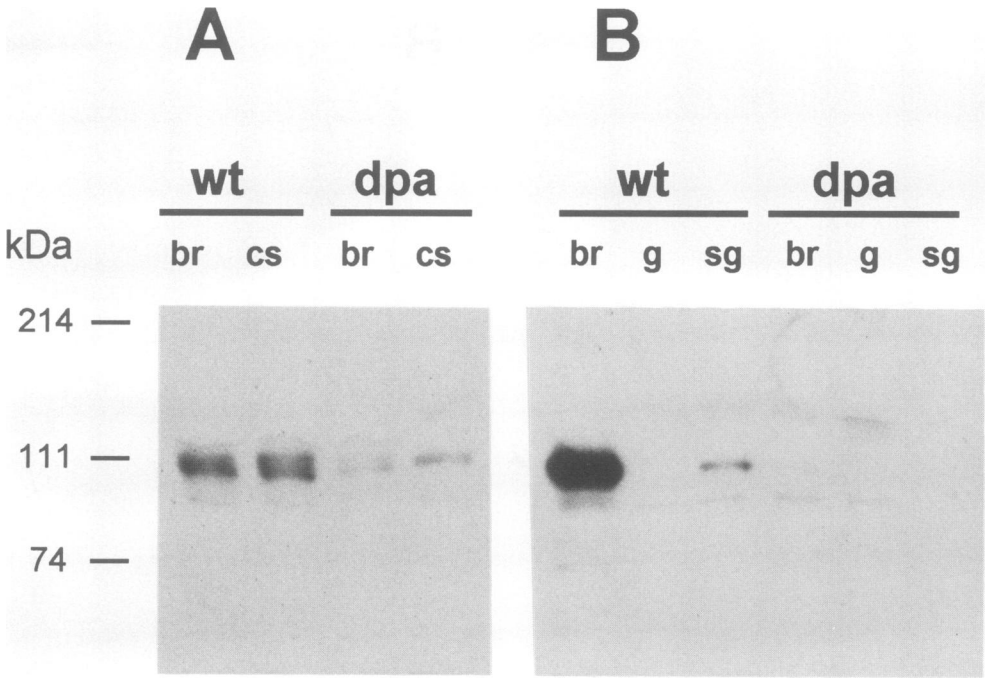

Fig. 5. Western blot analysis of wild type and mutant larval tissues. Equal amounts of wild type (wt) and *dpa* (dpa) mutant tissue extracts from brain and ventral nerve cord (br), gut (g) and salivary glands (sg) were analyzed using affinity-purified antibodies to dpa. The antibody recognizes a band of 105 kDa (similar to previous observations on other members of the MCM family, the apparent molecular weight is ~10 kDa larger than the predicted molecular weight for the primary translation product). (**A**) Extracts from second instar wild type (wt) and *dpa* (dpa) mutant larvae show reduced dpa protein levels in the CNS (br) and the remaining carcasses (cs) of *dpa* mutant animals. (**B**) Extracts from third instar wild type (wt) and *dpa* (dpa) mutant tissues. In contrast to the high levels of dpa protein detected in the brain and ventral nerve cord extracts (br) of wild type third instar larvae, only low amounts of dpa are present in salivary glands (sg) and no dpa protein is detected in gut extracts (g). dpa protein is undetectable in mutant tissues under these conditions.

mutants of *dpa*. Second, Western blot analysis shows no detectable *dpa* gene product in tissues from mutant third instar larvae of the genotype: *l(2)43Ca^1/Df(2R)R163*, indicating that *l(2)43Ca^1* is a null allele of *dpa* (Figure 5B). However, second instar larval tissues contain considerable amounts of dpa, possibly due to maternally contributed supplies (Figure 5A).

To test whether the EMS-induced lethal *l(2)43Ca^1* behaves genetically as a null allele, we compared the phenotypes of homozygous *l(2)43Ca^1* animals with those of animals hemizygous for *l(2)43Ca^1* [*l(2)43Ca^1/Df(2R)R163*]. Animals homozygous or hemizygous for *l(2)43Ca^1* die during the larval/pupal stage of development. The lack of enhancement of the phenotype in hemizygotes is further evidence that *l(2)43Ca^1* is a null allele of *dpa*.

To confirm that the 2.8 kb cDNA corresponds to the transcript affected in the *l(2)43Ca^1* mutant, we introduced a 12 kb genomic DNA fragment (see Materials and methods) encompassing the entire *dpa* transcription unit into flies using P element-mediated germline transformation. This construct rescued the *l(2)43Ca^1* mutant to full viability and fertility. Together, these data demonstrate that *l(2)43Ca^1* is a *dpa* mutant.

dpa mutant larvae fail to develop imaginal discs

Homozygous *l(2)43Ca^1* mutant animals as well as animals hemizygous for *l(2)43Ca^1*, [*l(2)43Ca^1/Df(2R)R163*], developed into third instar larvae of normal size but pupariation was slightly delayed (1–2 days) compared with control larvae. *dpa* mutant animals die during pupal development. The tissues of the mutant pupae disintegrate, leaving empty pupal cases.

To investigate the cause of the pupal lethality we dissected and examined mutant third instar larvae. With the exception of the salivary glands which appeared slightly smaller than wild type, endoreplicating tissues such as the gut, fat body (Figure 6C and D and data not shown) and the polytene chromosomes (Figure 6E and F) from *l(2)43Ca^1* mutant larvae appeared normal. However, no imaginal discs could be found and the CNS of the mutant larvae were reduced in size (Figure 6A and B). In contrast to the endoreplicating tissue which grows without cell division, cells of the imaginal discs and neuroblasts of the larval CNS undergo many cycles of mitosis during larval development (Cohen, 1993). These observations suggest that *dpa* is required during mitotic but not during endoreplicating cell cycles.

To determine whether the absence of imaginal discs in the mutant larvae was due to the lack of imaginal disc progenitor cells which form during embryonic development, we stained mutant embryos with two disc primordia-specific probes, a digoxigenin-labeled *escargot* probe for *in situ* hybridization (Hayashi *et al.*, 1993) and antibodies specific for the snail protein (Mauhin *et al.*, 1993). The disc primordia appeared normal in *dpa* mutant embryos (data not shown), even though no imaginal discs were found in mutant third instar larvae. This suggests that in the *dpa* mutant the imaginal primordia do not proliferate normally during larval development.

It has been proposed that members of the MCM family might function as licensing factors allowing only one round of DNA replication per cell cycle. To determine whether the absence of imaginal discs in *dpa* mutants is due to mitotically proliferating cells undergoing additional

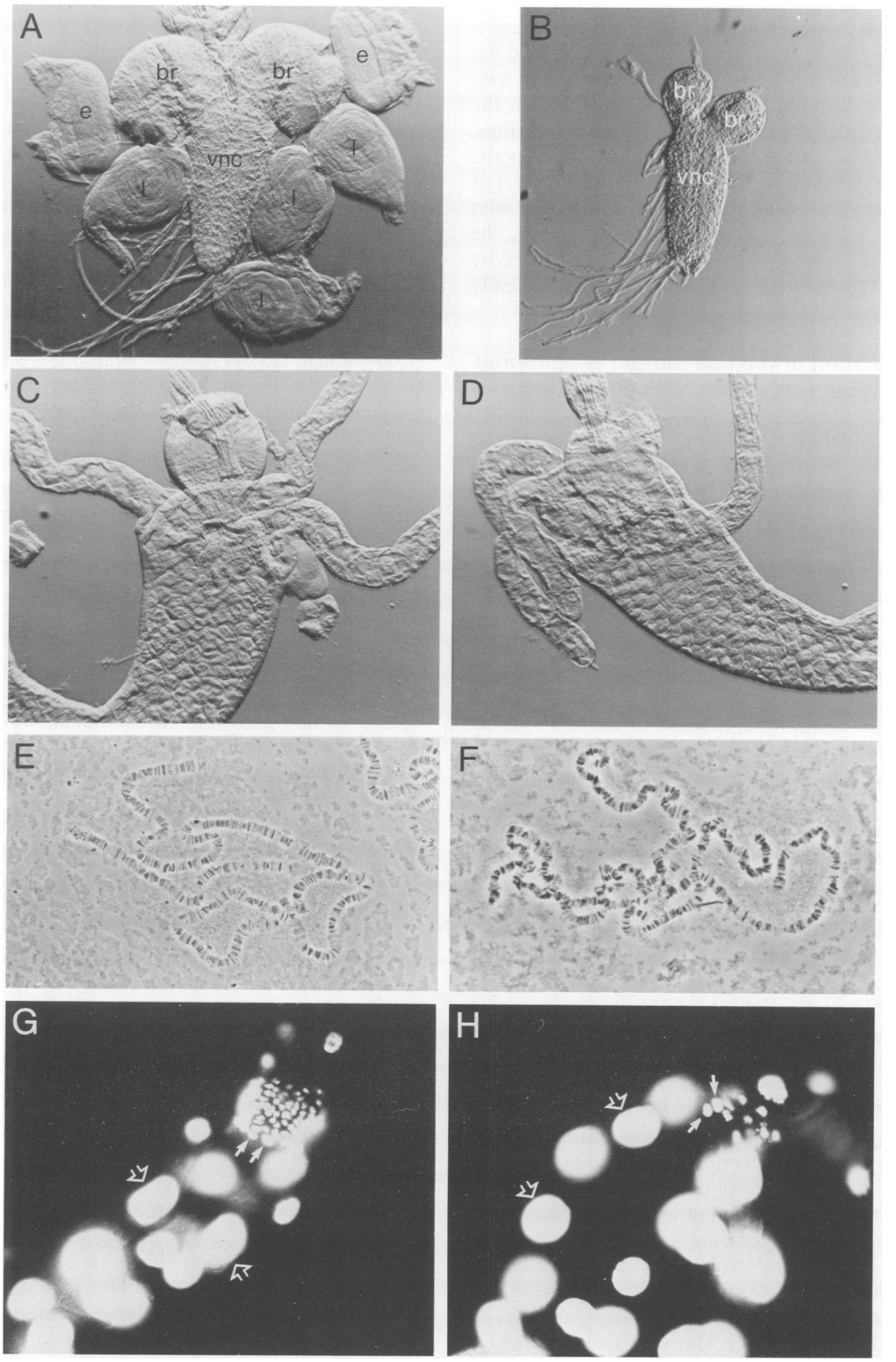

Fig. 6. *dpa* function is required for the proliferation of imaginal tissues. Wild type (A, C, E and G) and *dpa* mutant (B, D, F and H) tissues of third instar larvae. Corresponding wild type and mutant tissues are shown at the same magnification. (**A**) Wild type third instar ventral nerve cord (vnc) and brain lobes (br) with the attached eye (e) and leg (l) imaginal discs. (**B**) The ventral nerve cord and brain of a *dpa* mutant third instar larvae are smaller than those of a wild type larva and no imaginal discs are found. (**C**) Part of the foregut dissected from a third instar wild type larva. (**D**) The size and morphology of the foregut are unaffected in the *dpa* mutant. (**E**) Wild type banding pattern of salivary gland polytene chromosomes. (**F**) Loss of *dpa* function does not affect the size and banding pattern of salivary gland polytene chromosomes. (**G**) Polytene (open arrows) and imaginal cells (arrows) in wild type salivary glands were stained with the DNA stain Hoechst 33258. (**H**) *dpa* mutant salivary glands have fewer imaginal cells. The size and staining intensity of polytene and remaining imaginal cells seem unchanged.

rounds of DNA replication without intervening cytokinesis, resulting in large polyploid nuclei, we stained the salivary glands and the CNS of third instar larvae with the nuclear dye Hoechst 33258. In the *dpa* mutant, the diploid cells of the CNS and cells of the imaginal rings, which are adjacent to the large polyploid nuclei in the salivary glands, are reduced in number but have nuclei of normal size and intensity of staining compared with wild type (Figure 6G and H and data not shown). Thus, the lack of imaginal discs in the *dpa* mutant is not caused by multiple rounds of endoreplication of normally mitotically proliferating tissues.

BrdU incorporation in the dpa mutant is abnormal

Since the *dpa* gene product shows significant sequence similarity to genes involved in DNA replication, we asked whether *dpa* is involved in DNA replication by examining the incorporation of the thymidine analogue BrdU into mutant animals at various times during development. In the wild type, progressively more and more cells in the CNS become mitotically quiescent towards the end of embryogenesis until only a few cells in the brain can be labeled with BrdU (Prokop and Technau, 1991). BrdU labeling of mutant embryos (see Materials and methods) revealed no abnormality before stage 15 (not shown). However, when BrdU was microinjected into stage 16 embryos and the BrdU pattern in the larval CNS was examined, more cells were labeled in the *dpa* mutant (Figure 7A and B). The additional cell labeling could be due either to ectopic S phases or delay in completion of prior S phases. When *dpa* mutants were labeled with BrdU later in embryogenesis or during early larval stages, ectopic BrdU incorporation was observed in progressively fewer cells (compare Figure 7B and D). The latter observation is more consistent with prolonged S phases and not ectopic initiation of S phases.

In freshly hatched wild type larvae, BrdU labeling reveals five pairs of neuroblasts in the larval brain, which rapidly divide in a stem cell-like division pattern to give rise to clusters of ganglion mother cells and neurons surrounding the neuroblast (Figure 7C; Truman and Bate, 1988). In *dpa* mutant larvae BrdU is incorporated abnormally into cells scattered all over the brain and ventral nerve cord (Figure 7D). Despite the ectopic BrdU labeling, mutant larvae showed fewer ganglion mother cells surrounding the putative neuroblasts, revealed either by BrdU incorporation or by staining with antibodies against prospero (Figure 7D and data not shown), indicating that the cell cycle is either prolonged or blocked after the first division(s). Thus *dpa* function is required for the regulation of DNA replication in neuroblasts.

In vitro BrdU labeling (see Materials and methods) of third instar mutant larvae revealed that only a small number of CNS cells still incorporated BrdU whereas massive labeling was seen in the CNS of wild type larvae (Figure 7G and H). In mutant larvae the BrdU incorporation was mostly in surface-associated cells and cells of the mid-line. Prokop and Technau (1994a) have examined BrdU incorporation into surface-associated glial cells in third instar larvae. These glial cells do not divide during larval development and are therefore endoreplicating. It is unclear whether the BrdU-positive cells in the CNS of the mutant are mitotically replicating or endoreplicating. The lack of BrdU incorporation into most neuroblasts suggests that *dpa* function is required for DNA replication in mitotically proliferating cells.

In contrast to the strong mutant phenotype evident during first instar larval development, BrdU incorporation into CNS of second instar larvae in the *dpa* mutant was sometimes indistinguishable from wild type (Figure 7E and F). During second instar larval development many neuroblasts which had become mitotically quiescent during late embryogenesis commence proliferation (Truman and Bate, 1988). We believe that ectopic BrdU incorporation in first instar larvae represents prolonged S phases of cells which through multiple cell divisions have diluted maternal *dpa* product. However, those neuroblasts which have been mitotically quiescent since late embryogenesis may have retained sufficient maternal dpa protein, as suggested by Western blot analysis (Figure 5A), to initiate DNA replication during the second instar larval stage.

BrdU incorporation into endoreplicating tissue is not affected by dpa

Cells of the larval tissues, unlike the imaginal disc cells, commence a specialized cell cycle consisting only of alternating gap phases and phases of DNA synthesis, referred to as endocycle or endoreplication (Smith and Orr-Weaver, 1991). We found that BrdU incorporation into endoreplicating tissue of the gut, fat body and salivary gland in first and third instar mutant larvae showed no significant difference from that in wild type larvae (Figure 7I–J and data not shown), suggesting that *dpa* is not affecting DNA replication in endocycles.

Interestingly, Western blot analysis of wild type third instar tissue extracts showed that the endoreplicating tissues of the gut and salivary glands contain drastically less *dpa* gene product than the mitotically proliferating cells of the brain and imaginal discs (Figure 5 and data not shown).

Discussion

We have isolated a gene, *dpa*, which shows strong sequence similarity to fission yeast genes *cdc21*, *nda1* and *nda4*, budding yeast genes *CDC54, MCM2, MCM3* and *CDC46* as well as to the mammalian DNA polymerase alpha-associated P1 protein. These similarities suggest that *dpa* is involved in an early step of DNA replication in *Drosophila.* Loss of *dpa* function prevents imaginal progenitor cells from giving rise to imaginal discs (mitotic tissue) without apparent effects on endoreplicating tissues. Defects in the incorporation of the thymidine analog BrdU into mutant larvae indicate that *dpa* is required for mitotic DNA replication but is dispensable for DNA replication during larval endocycles.

dpa is an essential gene

The null *dpa* mutant $l(2)43Ca^{1}$ fails to develop imaginal discs and dies during pupal development. Abnormal S phases in the CNS are evident during late embryogenesis. It seems likely that mitosis during early embryonic development in the mutant embryo is sustained by the large maternal contribution of *dpa* transcript (Figures 1B, 4A and 5A), similar to what has been found for *Dmcdc2* (Stern *et al.*, 1993). Indeed, microinjections of antibodies

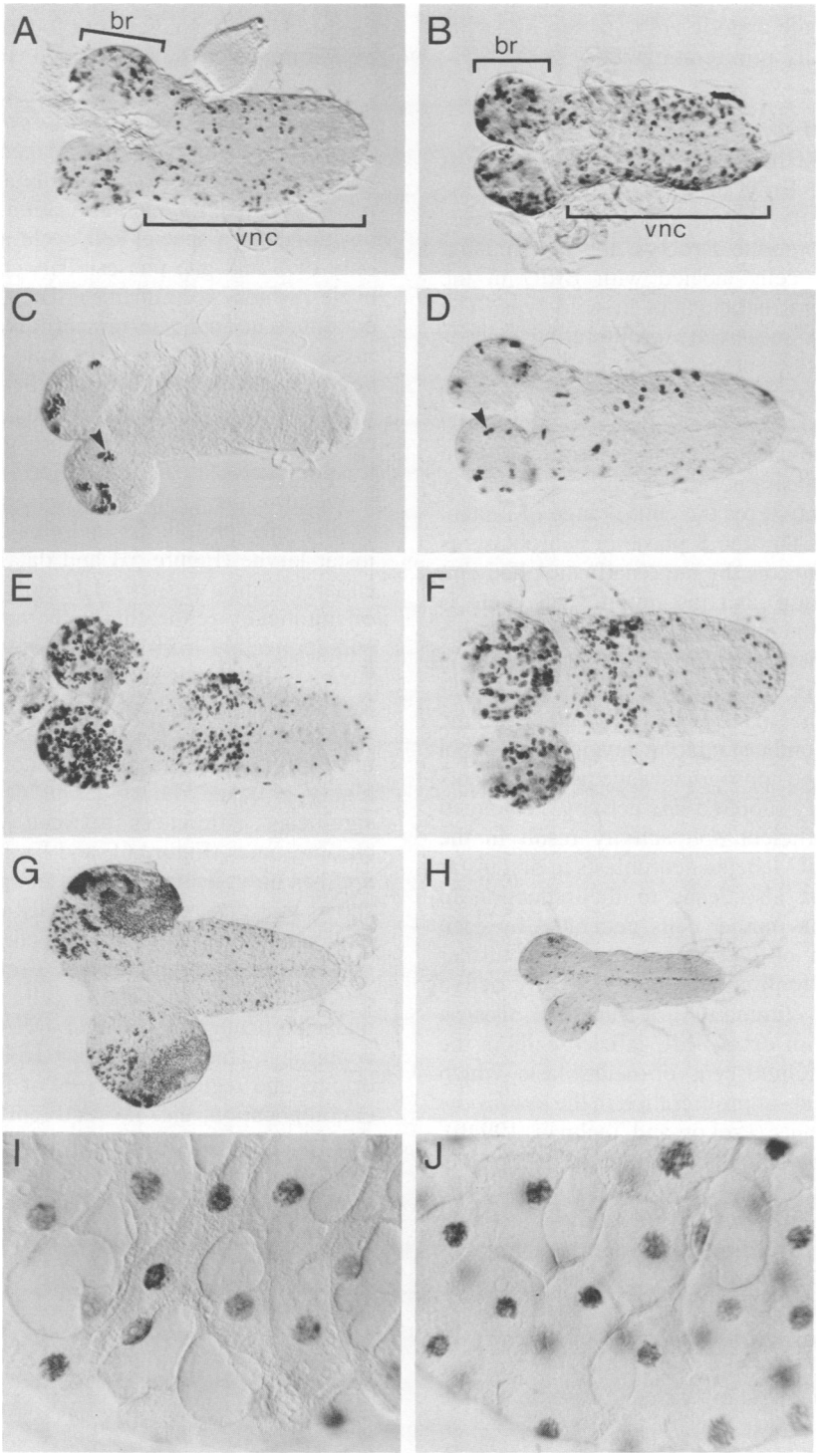

Fig. 7. Loss of *dpa* function causes abnormal S phase pattern. Wild type (A, C, E, G and I) and *dpa* (B, D, F, H and J) mutant animals of the genotype *l(2)43Ca*^{1}*/Df(2R)R163* were labeled with BrdU (see Materials and methods) to analyze the *dpa* function. (**A**) Wild type embryo injected with BrdU at stage 16 shows labeling in the brain (br) and ventral nerve cord (vnc). (**B**) More cells are labeled with BrdU in a *dpa* mutant embryo injected at stage 16. (**C**) Wild type first instar larvae incorporate BrdU into the brain, but not the ventral nerve cord, in a characteristic and invariant pattern. Arrowhead marks a cluster of ganglion mother cells produced by a neuroblast. (**D**) *dpa* mutant first instar larvae show extensive BrdU labeling in the vnc and the brain (br). Only one or two cells are associated in a cluster (e.g. in the cluster marked by the arrowhead, corresponding to the cluster marked in C), suggesting reduced proliferation. (**E**) Wild type second instar larvae showed extensive BrdU incorporation into the brain and ventral nerve cord. (**F**) BrdU labeling of *dpa* mutant second instar larvae resembles the wild type pattern. (**G**) BrdU-positive cells in a third instar CNS. (**H**) Only a few cells label with BrdU in *dpa* mutant third instar CNS. (**I**) BrdU incorporation in polytene nuclei of third instar wild type salivary glands. (**J**) Loss of *dpa* function does not affect BrdU labeling of third instar salivary gland nuclei.

against dpa into early embryos results in chromosome bridges (T.T.Su *et al.*, in preparation). This is consistent with a role of *dpa* in DNA replication, as blocking DNA replication in early embryos with aphidicolin also results in chromosome bridges (Raff and Glover, 1988). Thus, the *dpa* function, initially supplied maternally and sub-

sequently produced in the zygotic embryo, serves a vital function in all mitotically proliferating cells.

Mutant phenotype of dpa

During embryonic stage 16 a far greater number of cells in the CNS incorporate BrdU in *dpa* mutants than in the wild type animals. As development progresses, progressively fewer cells incorporate BrdU in the *dpa* mutant, resulting in far fewer cells labeled with BrdU in the CNS of third instar *dpa* mutant than in wild type. The requirement for *dpa* in mitotically proliferating cells is evident from the reduced number of progeny cells produced by a neuroblast and by the absence of third instar imaginal discs in *dpa* mutants. Paradoxically, BrdU incorporation into neuroblasts that resume proliferation in second instar larvae appears normal in *dpa* mutants.

One possible explanation for the entire range of mutant phenotypes observed is that the S phase in neuroblasts is prolonged in *dpa* mutants, as the maternally supplied *dpa* product becomes limiting, and the mitotic cell cycle is eventually blocked in the absence of *dpa* activity. In this model, the developmental program of cell proliferation is not directly affected in *dpa* mutants; neuroblasts that normally proliferate either in late embryogenesis or resume proliferation during second instar larval development begin BrdU incorporation at the appropriate stage in *dpa* mutants by relying on maternally supplied *dpa* activity. Prolonged S phases due to insufficient *dpa* activity result in the appearance of extra BrdU-labeled neuroblasts in first instar larvae; insufficient dpa also leads to the reduction in the number of ganglion mother cells generated by each neuroblast. Exhaustion of *dpa* activity prevents further DNA replication and proliferation of mitotically active cells, leading to the elimination of imaginal tissues. Although the presence of extra BrdU-labeled cells in the first instar larvae is reminiscent of neuroblasts which undergo additional rounds of proliferation in the *mushroom body defect (mud)* mutants (Prokop and Technau, 1994b), unlike *mud* mutants, *dpa* mutants do not show overgrowth of the brain tissues but instead have smaller brains. The range of *dpa* phenotypes appears more easily compatible with a prolonged S phase rather than an increase in mitosis of these neuroblasts. Treisman *et al.* (1995) have found a similar range of phenotypes for a *Drosophila MCM2* mutant. Another explanation for the complex phenotypes seen in the *dpa* mutant is that other members of the MCM gene family might compensate for some of the *dpa* function.

The requirement for MCM family members in DNA replication, their interaction with ORC and their transient appearance in the nucleus during the cell cycle led to the proposal that these proteins function as licensing factors to ensure that only one round of DNA replication occurs per S phase (Heichman and Roberts, 1994). Such a licensing factor could function positively to activate DNA replication or negatively to prevent re-replication of DNA during the same S phase. While we have not directly shown that *dpa* acts as a licensing factor, the mutant phenotype is consistent with *dpa* acting as a positive regulator of DNA replication. It is not compatible with models in which the licensing factor functions as a negative regulator to prevent extra DNA replication without cytokinesis, because we have not found ectopic polyploid cells in *dpa* mutant tissues examined.

dpa is dispensable for endoreplication

In contrast to the yeast MCM gene products, *dpa* is not generally required for the regulation of DNA replication: it is dispensible for endoreplication. During late embryonic development a special cell cycle emerges which consists only of S and gap phases. This endoreplication or endocycle, which accompanies the growth of larval cells, continues normally in mutant larvae up to the last larval instar. Although we cannot rule out the persistence of very low levels of maternally derived *dpa* activity even in late third instar larvae, residual zygotic *dpa* activity is clearly insufficient to support mitotic proliferation in imaginal cells.

Western blot analysis shows only very small amounts of the dpa protein in endoreplicating tissues of third instar larvae (Figure 5B and data not shown). Since the endoreplicating tissues analyzed contain a small number of mitotically replicating cells we do not know whether endoreplicating cells are devoid of dpa protein. This suggests that dpa is either dispensable or required at lower levels in endoreplication than in mitotic DNA replication. Our analysis of salivary gland chromosomes which undergo multiple rounds of endoreplication and become highly polytene during larval development showed no significant differences between wild type and mutant chromosomes (Figure 6E and F). It thus seems likely that *dpa* has no essential function in endocycles. Our analysis shows that dpa is not a general factor required for DNA replication but serves a specialized function in DNA replication during mitotic cell cycles.

Although endoreplicating cells become polyploid they under-replicate parts of their DNA even under wild type conditions. This might suggest a different use of replication origins and regulatory factors of DNA replication during endoreplication, e.g. no requirement for licensing might be expected. The differential involvement of *dpa* in these two modes of DNA replication might help to elucidate the differences in how these modes are initiated or regulated.

Materials and methods

Drosophila stocks and genetics

All fly stocks were kept and crosses performed according to standard procedures. The following stocks were used in this study: enhancer trap E5 2nd 23 (Bier *et al.*, 1989), *l(2)43Ca^{1} bw^{D}*, *l(2)43Cb^{1}cn bw sp*, *l(2)43Cc^{1} bw^{D}*, *l(2)43Da^{2} bw^{D}*, (Heitzler *et al.*, 1993), *Df(2R)ST1* (Ashburner *et al.*, 1981) and *T(2;3)H36* (Hilliker and Trusis-Coulter, 1987). To determine the genotype of larvae, the mutants were balanced over *In(2LR)O, Cy, P[y^{+}]* unless otherwise stated.

To generate imprecise excisions, the homozygous viable PlacW enhancer trap line at 43C was mobilized using the P transposase source Δ2-3. Of the 200 excisions, one was recessive lethal when crossed to *Df(2R)ST1* (deficient for 42B3-4 to 43E18). Further mapping this excision using the lethal alleles at 43C-D (Heitzler *et al.*, 1993) showed that it uncovered four lethal complementation groups, *l(2)43Ca^{1}*, *Cb^{1}*, *Cc^{1}* and *Da^{2}* and is therefore designated as *Df(2R)R163*.

Cloning and sequencing dpa

The *dpa* gene was isolated using the plasmid rescue technique (Pirotta, 1986). Using the part of the rescued DNA that gives the expected pattern of *in situ* hybridization on whole-mount embryos, we screened embryonic cDNA libraries of 9–12 h (a gift from K.Zinn) and 3–12 h (Poole *et al.*,

1985) and isolated one cDNA clone from each library, both identical in size. Approximately 300 bp at the 5′ and 3′ end of each cDNA were sequenced and revealed identical sequences. The cDNA clone isolated from the 3–12 h library was then sequenced on both strands using the Sequenase II system (USB). To investigate the exon/intron organization, we isolated genomic lambda clones from an Oregon R library (Lambda DASH, Stratagene). The 1.4 and 4.8 kb *Hin*dIII fragments hybridizing to the cDNA probe (Figure 1A) were subcloned into Bluescript (Stratagene) and sequenced, revealing the exon/intron structure (Figure 1A).

Transformation rescue

A 12 kb *Eco*RI fragment of genomic DNA containing the entire *dpa* transcription unit was cloned into the transformation vector pCaSpeR4 (Pirotta, 1988). Several transformant lines were established and crossed to $l(2)43Ca^1$ to assay for rescue of the recessive lethality.

Analysis of RNA expression

Northern blot analysis was performed as described in Vaessin *et al.* (1987) using the ^{32}P-labeled cDNA isolated from the 3–12 h library as a probe. *In situ* hybridization to whole-mount embryos, imaginal discs and ovaries were performed following the method of Tautz and Pfeifle (1989), using digoxigenin-labeled DNA and RNA probes (Boehringer Mannheim).

Determination of the lethal phase of the dpa mutant

The lethal phase of the *dpa* amorphic allele $l(2)43Ca^1$ was determined using a $yellow^+$-marked second chromosome balancer. Homozygous larvae were selected and aged at 25 or at 18°C. In homozygous (*l(2)43Ca1*) and hemizygous mutant larvae ($l(2)43Ca^1/Df(2R)R163$) pupariation was slightly delayed. Both genotypes are pupal lethal.

BrdU labeling

BrdU labeling of embryos was performed according to Bodmer *et al.* (1989). Microinjection of BrdU into stage 16–18 embryos was performed as described in Prokop and Technau (1991) with the exception that larvae were dissected shortly after hatching and immunostained for BrdU incorporation. Labeling of first instar larvae followed the protocol of Truman and Bate (1988) with the following modification. Feeding of mutant and wild type larvae was monitored by adding a small amount of food dye to the BrdU-containing food which stains the intestines of feeding larvae. BrdU-labeled larvae were dissected in PBS and then fixed in 10% formaldehyde solution in PBS for 10 min instead of Carnoy's fixative. BrdU incorporation into third instar larvae was essentially as described in Truman and Bate (1988), except that 30 μg/ml BrdU was used and tissues were fixed for 20 min with 10% formaldehyde in PBS.

Western blot analysis

Larval tissues were dissected by hand and homogenized in 20 mM Tris pH 7.5, 100 mM NaCl and 0.5 mM PMSF, mixed with 2× SDS–PAGE loading buffer and boiled for 5 min. Equal amounts of protein were separated on SDS–PAGE and blotted onto Immobilon P nylon membranes (Millipore) using a semi-dry blotter (LKB). The membranes were blocked with PBT containing 5% non-fat dry milk and incubated with affinity purified antibodies raised in rabbits against amino acids 132–866 of the *dpa* gene product in a 1:500 dilution (T.T.Su *et al.*, in preparation). After washing the membranes were incubated with donkey anti-rabbit conjugated to horseradish peroxidase, washed and developed using ECL reagents (Amersham, UK).

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