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Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the *Drosophila* eye imaginal disc

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SUMMARY

During animal development, cell proliferation is controlled in many cases by regulation of the G_1 to S phase transition. Studies of mammalian tissue culture cells have shown that the G_1-specific cyclin, cyclin E, can be rate limiting for progression from G_1 to S phase. During *Drosophila* development, down-regulation of cyclin E is required for G_1 arrest in terminally differentiating embryonic epidermal cells. Whether cyclin E expression limits progression into S phase in proliferating, as opposed to differentiating, cells during development has not been investigated. Here we show that *Drosophila* cyclin E (DmcycE) protein is absent in G_1 phase cells but appears at the onset of S phase in proliferating cells of the larval optic lobe and eye imaginal disc. We have examined cells in the eye imaginal epithelium, where a clearly defined developmentally regulated G_1 to S phase transition occurs. Ectopic expression of *DmcycE* induces premature entry of most of these G_1 cells into S phase. Thus in these cells, control of *DmcycE* expression is required for regulated entry into S phase. Significantly, a band of eye imaginal disc cells in G_1 phase was not induced to enter S phase by ectopic expression of *DmcycE*. This provides evidence for additional regulatory mechanisms that operate during G_1 phase to limit cell proliferation during development. These results demonstrate that the role of cyclin E in regulating progression into S phase in mammalian tissue culture cells applies to some, but not all, cells during *Drosophila* development. Ectopic expression of *DmcycE* in the eye imaginal disc disrupts normal pattern formation, highlighting the importance of coordinating cell proliferation with developmental processes for correct patterning in the developing eye. These studies establish *DmcycE* as a target of regulatory mechanisms that coordinate cell proliferation with other developmental events.

Key words: cyclin E, G_1 phase, S phase, eye imaginal disc, *Drosophila*, pattern formation

INTRODUCTION

During metazoan development, regulation of cell proliferation by developmental mechanisms occurs at either the G_2 to M phase, metaphase to anaphase or at the G_1 to S phase transition. Developmental control of the G_2 to M phase transition and the metaphase to anaphase transition have been clearly demonstrated in differentiating cells (reviewed by Saint and Wigley, 1992). Regulation of the G_1 to S phase transition during development is less well characterised. This transition is regulated by members of the Cdk family of ser/thr protein kinases (reviewed by Pines and Hunter, 1991). The activity of Cdk protein kinases is controlled in part by their interaction with cyclins, many of which vary in abundance during the cell cycle (reviewed by Reed, 1992; Sherr, 1994). In mammalian cells the G_1 to S phase transition is regulated by the G_1 cyclins, cyclin D and cyclin E, which bind to and activate the Cdk4 and Cdk2 protein kinases, respectively (reviewed by Reed, 1992; Sherr, 1994). Of these two G_1 cyclins, cyclin E shows the most dramatic cell cycle variation in mRNA, protein and associated Cdk protein kinase activity, peaking in late G_1 phase just prior to S phase.

There is functional evidence in mammalian cells that cyclin E/Cdk2 is involved in G_1 regulation, since over expression shortens the G_1 phase and decreases the requirement for growth factors for the G_1 to S phase transition (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). Cyclin E/Cdk2 is also the target of growth inhibitory signals, such as contact inhibition and the negative growth factor TGFβ, that arrest cells in G_1 phase (reviewed by Elledge and Harper, 1994). This G1 arrest appears to be mediated by the p27 inhibitor that binds to and inhibits the activity of cyclinE/Cdk2 (Elledge and Harper, 1994).

Drosophila melanogaster offers a system in which to explore the regulation of the G_1 to S phase transition during animal development. The *Drosophila* homolog of human cyclin E, *DmcycE,* is required for the G_1 to S phase transition in cycle 17 embryonic cells (Knoblich et al., 1994). Developmental regulation of *Drosophila* cyclin E was first demonstrated with the observation that *DmcycE* encodes two proteins, with common C termini and unique N termini, that are expressed differentially during development (Richardson et al., 1993). The type II *DmcycE* mRNA is supplied maternally and is present during the first 13 parasynchronous, syncytial division cycles, whereas type I mRNA is zygotically expressed in all proliferating cells. When cells cease division in G$_1$ phase, *DmcycE* transcription is down-regulated (Richardson et al., 1993; Knoblich et al., 1994), suggesting that *DmcycE* is rate limiting for the G$_1$ to S phase transition during *Drosophila* embryogenesis.

In this report we show that DmcycE protein, like *DmcycE* mRNA, is present in S phase cells but absent in G$_1$ phase cells of the larval eye imaginal disc and optic lobe. Ectopic expression of *DmcycE* has previously been shown to drive terminally G1-arrested embryonic cells from G$_1$ phase into S phase (Knoblich et al., 1994), but a role for *DmcycE* in the regulation of the G$_1$ to S phase transition in proliferating cells during development has not been demonstrated. We have examined this by studying proliferating imaginal cells that have a developmentally regulated G$_1$ to S phase transition (Thomas et al., 1994; reviewed by Wolff and Ready, 1993). We report that ectopic expression of *DmcycE* is sufficient to force premature entry of some, but not all, G$_1$ phase cells in the eye imaginal disc into S phase. Ectopic expression of *DmcycE* causes a disruption in eye development, illustrating the importance of *DmcycE* transcriptional regulation in the coordination of cell proliferation with differentiation during development.

MATERIALS AND METHODS

Generation of DmcycE antisera, western analysis and antibody stainings

A GST-DmcycE fusion protein was generated by insertion of the 0.8 kb *Bgl*II fragment (1196-1973 bp corresponding to amino acids 152-409; Richardson et al., 1993) of *DmcycE* into pGEX-3X (Smith and Johnson, 1988). The entire *DmcycE* type I open reading frame with a *Nde*I site at the initiating ATG and a *Bam*HI site at the 3′ end was produced by the polymerase chain reaction (PCR) and cloned into the *Nde*I and *Bam*HI sites of the T7 expression vector, pRK171 (Rosenberg et al., 1987). PCR conditions were as described previously (Richardson et al., 1993). PCR primers were as follows.

5′ primer, 5′-CCCAT**ATG**AAGTTGGAACAGAAGC-3′
3′ primer 5′-CGGGATCCACTTAACGTAGACTGT-3′

The restriction sites are underlined and the initiating ATG is shown in bold.

To generate DmcycE antisera, SDS-polyacrylamide gel-purified DmcycE type I full-length protein was used to inoculate Balb-c mice. After two boosts, sera were harvested (DmcycE polyclonal sera) and spleen cells were isolated and used for the production of monoclonal antibodies.

Western analysis of bacterially produced DmcycE or *Drosophila* protein extracts was performed using anti-DmcycE mouse polyclonal sera or monoclonal serum (no. 8B10). A biotinylated anti-mouse secondary antibody, followed by streptavidin-horseradish peroxidase (HRP) or a direct HRP linked anti-mouse secondary antibody were used for detection. The biotinylated anti-mouse antibody/streptavidin-HRP system detected two background bands in protein extracts from both early and late stages of embryogenesis (data not shown). These bands appear to be specific for western blots since late stage *DmcycE* deficiency embryos do not show staining with these sera (data not shown). Enhanced-chemi-luminescence (ECL kit, Amersham) was used for detection of the HRP conjugate.

Bacterially produced full-length DmcycE and GST-DmcycE fusion proteins were induced by addition of IPTG to bacterial cultures. Protein samples were prepared by centrifugation of cells, followed by sonication and boiling in sample buffer and were diluted 1000× prior to electrophoresis. *Drosophila* embryonic protein extracts were prepared as described by Lehner and O’Farrell (1989). Homozygous *DmcycE* deficiency embryos from *Df(2L)TE35D-1/CyO* flies, aged to ~6-16 hour AED (after egg deposition), were picked by their *snail* mutant phenotype (*snail* is also removed by the *TE35D-1* deficiency). A mixture of homozygous and heterozygous embryos from the same approx. 6-16 hour AED embryo collection from *Df(2L)TE35D-1/CyO* flies was used as a control. Heat-shocked *hsp70-DmcycE* protein extracts were prepared from a 0-16 hour AED embryo collection following a 30 minute heat shock at 37°C and 20 minutes recovery.

The distribution of DmcycE protein in *Drosophila* embryos or larval tissues was detected by incubation of fixed samples with the mouse polyclonal DmcycE antibody or the mouse monoclonal antibody (no. 8B10), followed by a biotinylated anti-mouse secondary antibody and a steptavidin-HRP detection system (Vectastain ABC kit, Vector labs. Inc.). Colour detection was achieved using diaminobenzidine (0.5 μg/ml) and H_2O_2 (0.045 μg/ml) and in most cases enhanced by the addition of NiCl (0.64 μg/ml). *DmcycE* deficiency embryos were obtained from *Df(2L)TE35D-3/CyO* P[*ry*$^+$*wg-LacZ*] flies. Wild-type embryos and larvae were Canton S.

Embryos were fixed in 4% paraformaldehyde for 20 minutes as described by Edgar and O’Farrell (1989). Larval disc-brain complexes were dissected and fixed in 4% paraformaldehyde for 30 minutes or as described by Van Vactor et al. (1991).

Construction of *DmcycE* transgenic flies and fly crosses

To obtain *DmcycE* under control of the *hsp70* heat-shock promoter, *DmcycE* type I cDNA (sequence position 415-2748; see Richardson et al., 1993) was cloned as an *Eco*RI fragment into pCaSpeR-hs (Pirotta, 1988). To obtain *DmcycE* under control of *GAL4(UAS)*, the same region from type I cDNA was cloned into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). Transgenic flies containing these constructs P[w$^+$ *hsp70-DmcycE*] or P[w$^+$ *GAL4*(*UAS)-DmcycE*] were obtained by P element-mediated germline transformation of *w*^{1118} embryos and selection of *w*$^+$ flies. A homozygous *hsp70-DmcycE* 3rd chromosome line was used for all heat-shock experiments. To examine the effect of ectopic expression of *DmcycE* in differentiating cells posterior to the MF, flies homozygous for P[w$^+$ *GAL4*(*UAS)-DmcycE*] on the 2nd chromosome and P[*ry*$^+$ *sevenless-GAL4*] on the 3rd chromosome (obtained from K. Basler), were generated.

Induction of *hsp70-DmcycE* expression, BrdU labelling, in situ hybridization and chromomycin A3 staining of larval tissues

Heat-shock induction of *DmcycE* in wandering third instar larvae was carried out by collecting larvae into an Eppendorf tube and incubating at 37°C for 30 minutes. The samples were subsequently returned to 25°C for 30-90 minutes before BrdU labelling, or for 60-180 minutes before fixation and chromomycin A3 staining. To analyse the effect of ectopic expression of *DmcycE* on eye development, staged larvae were heat shocked at 37°C for 60 minutes then returned to 25°C and allowed to develop into adults. Larvae from Canton S, or *hsp70-cyclin C* and *hsp70-string* fly strains (obtained from Dr P. O’Farrell) were used as controls. For BrdU labelling, eye-antennal discs or brain lobes were dissected and incubated with 60 μg/ml BrdU in Schneider’s tissue culture medium for 30 minutes at 25°C. Tissues were fixed as described above and BrdU-labelled cells were detected as described previously (Richardson et al., 1993). For chromomycin A3 staining, dissected eye discs were fixed as described above and incubated overnight in chromomycin A3 (Sigma) in 10% $MgSO_4$.

In situ hybridization to *DmcycE* mRNA in larval eye imaginal discs was carried out essentially as described for embryos (Richardson et al., 1993) with the following modifications. A digoxigenin-UTP-labelled *DmcycE* RNA probe was made by in vitro transcription from a linearised pBluescript plasmid containing the region from 415-2748 bp from *DmcycE* (Richardson et al., 1993). Larval eye imaginal discs were fixed in 4% paraformaldehyde, pH 7.5, for 20 minutes on ice, followed by treatment with 0.6% Triton X-100 in fixation buffer for 15 minutes. Discs were treated with 10 μg/ml proteinase K for 4 minutes and then post-fixed in 4% paraformaldehyde, 0.2% glutaraldehyde for 15 minutes. Hybridization was carried out at 55°C for 12 hours.

All samples were mounted on slides in 80% glycerol and photographed on a Ziess Axiophot microscope with Nomarski optics.

Preparation of adult eyes for electron microscope analysis and sectioning

Drosophila eyes were prepared for scanning electron microscopy by dehydration in ethanol and critical point drying, and then coated with palladium-carbon (as described by Kimmel et al., 1990). Sectioning of *Drosophila* adult eyes was carried out as described by Lockett et al. (1993). Photography was at 1000× magnification.

RESULTS

DmcycE is absent in G_1 phase cells in the larval optic lobe and eye imaginal disc

Down-regulation of *DmcycE* expression in the embryo is necessary for exit from cell proliferation prior to differentiation after the 16th mitosis (Knoblich et al., 1994). In other cases during *Drosophila* development, however, an extended developmentally regulated G_1 phase is followed by re-entry into the cell cycle. Examples of this occur in the lamina precursor cells of the larval optic lobe (Selleck et al., 1992) and in cells of the eye imaginal disc (Wolff and Ready, 1993; Thomas et al., 1994). We wished to determine whether these developmentally regulated G_1 phases correlate with the absence of DmcycE.

In order to investigate DmcycE distribution in larval optic lobes and eye imaginal discs, mouse polyclonal and monoclonal antibodies were prepared to DmcycE protein. Western analysis with bacterially produced DmcycE proteins and protein extracts from *Drosophila* embryos (Fig. 1; and data not shown), showed that the DmcycE antisera are specific for DmcycE protein and recognise the region of DmcycE present in both the type I (zygotic) and type II (maternal) proteins. The specificity of the antibody is evident from the increase in abundance of zygotic DmcycE in heat-shocked *hsp70-DmcycE* (type I) embyros (Fig. 1A), and the absence of zygotic DmcycE as well as a dramatic reduction of maternal DmcycE in approx. 6-16 hour AED (after egg deposition) *DmcycE* deficiency embryos (Fig.1B; see Material and methods). To confirm the specificity of the antibody, DmcycE antibody stainings were carried out on wild-type and *DmcycE* deficiency embryos (Fig. 2). DmcycE antibody stainings of wild-type embryos revealed that DmcycE is a nuclear-localised protein and is present in mitotically proliferating and endoreplicating cells (Fig. 2A; data not shown). In *DmcycE* deficiency embryos, *DmcycE* mRNA is no longer detectable in somatic cells after cellularisation at G_2 of cycle 14 (Richardson et al., 1993). DmcycE antibody staining of *DmcycE* deficiency embryos undergoing S phases of cycles 15 and 16 (see Foe et al., 1993), showed only very low levels of protein (compare Fig. 2B with 2A). Slightly later in development, DmcycE protein could no longer be detected in any somatic tissues, yet was still present at high levels in the pole (presumptive germ) cells (Fig. 2C), where maternally supplied *DmcycE* mRNA persists (Richardson et al., 1993). These results demonstrate that the antisera is specific for DmcycE. Furthermore, the detection of maternally derived DmcycE protein during S phases of cycles 15 and 16 is consistent with the cycle 17 G_1-arrest phenotype of *DmcycE* deficiency embryos (Knoblich et al., 1994).

To examine whether the developmentally regulated G_1 phase in the larval optic lobe correlates with an absence of DmcycE, the protein distribution of DmcycE was compared with the pattern of S phases. Bromo-deoxyuridine (BrdU)

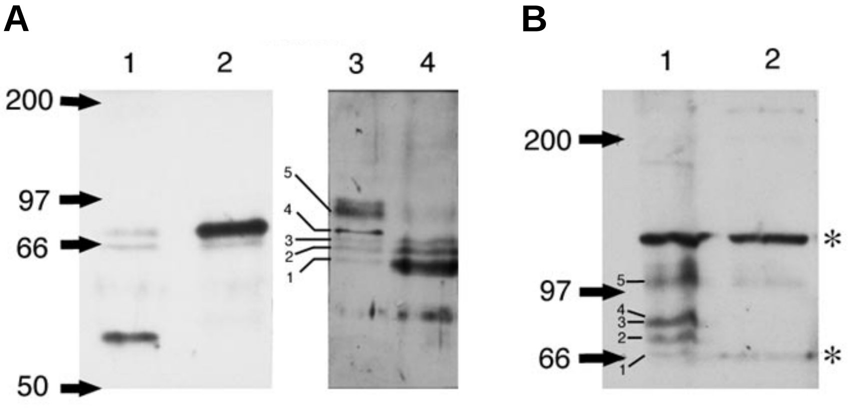

Fig. 1. Characterisation of DmcycE antisera. Western analysis of bacterially produced and *Drosophila* DmcycE protein using mouse polyclonal antiserum, raised to the full-length DmcycE type I protein. (A) Lane 1, bacterially produced GST-DmcycE fusion protein (58×10^3 M_r). Lane 2, bacterially produced full-length DmcycE type I protein (67×10^3 M_r). Lane 3, protein extract from a 0-16 hour (AED) collection of non-heat-shocked *hsp70-DmcycE Drosophila* embryos. Lane 4, protein extract from a 1-17 hour (AED) collection of heat-shocked *hsp70-DmcycE Drosophila* embryos. DmcycE protein bands were detected using the polyclonal sera followed by a HRP conjugated anti-mouse secondary antibody. DmcycE-specific bands are numbered. Note bands 1-3 are significantly increased in the heat-shocked *hsp70-DmcycE* sample and thus represent various forms of the type I (zygotic) protein. Approximately 2-fold more material was loaded onto lane 3 than lane 4 in order to visualise the zygotically produced DmcycE type I proteins. Bands 4 and 5 most likely represent maternally produced type II DmcycE protein and are under-represented in the older heat-shocked embryos (in lane 4). (B) Lane 1, protein extract from an approx. 6-16 hour collection (AED) of heterozygous *DmcycE* deficiency embryos (TE35D-1). Lane 2, protein extract from hand-picked homozygous *DmcycE* deficiency embryos from an approx. 6-16 hour collection from TE35D-1 heterozygous parents. DmcycE protein bands were detected using the polyclonal sera followed by a biotinylated anti-mouse secondary antibody and a streptavidin-HRP detection system. DmcycE-specific bands are numbered. Note that the zygotic DmcycE bands 1-3 are not present in *DmcycE* deficiency embryos. There is also a reduction in maternal DmcycE bands in the *DmcycE* deficiency embryo sample, possibly due to the skewed collection of older embryos. The bands at 150×10^3 M_r and at 65×10^3 M_r (marked with asterisks) are background bands due to reaction with the secondary or tertiary reagents (see Material and methods) and show that equal amounts of protein are present in both tracks.

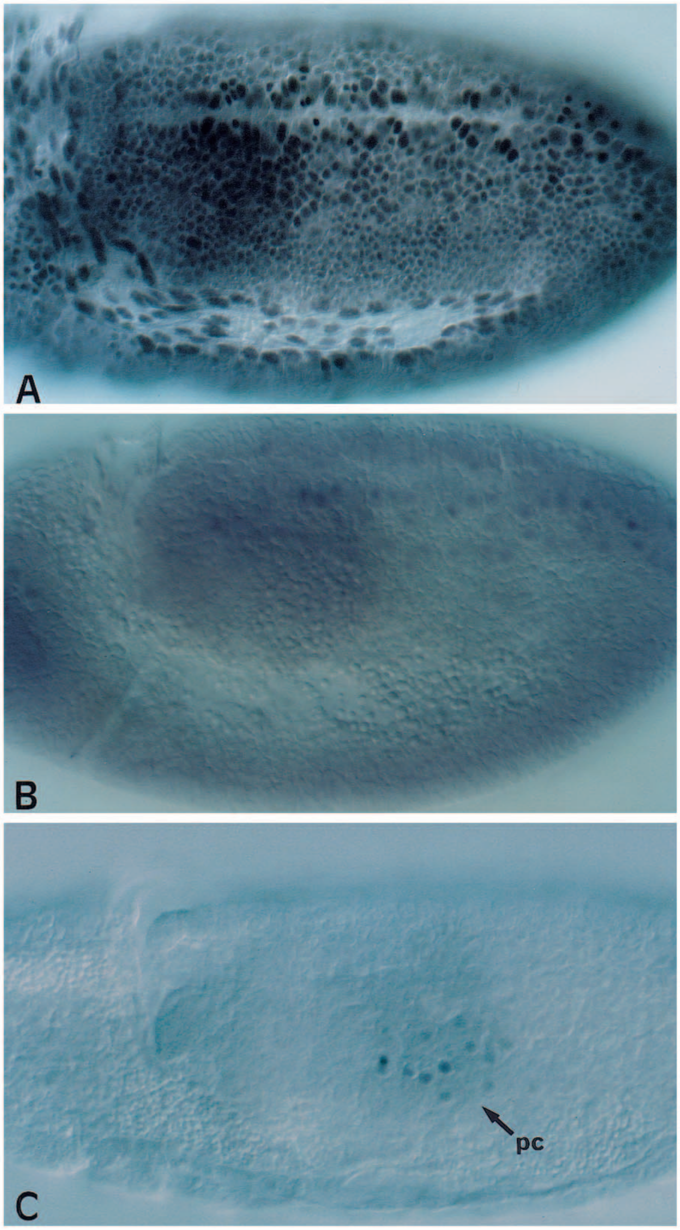

Fig. 2. DmcycE protein in *DmcycE* deficiency embryos. (A) A wild-type embryo undergoing S phase 15 (approx. 4 hour AED) showing DmcycE protein in all cells. (B) A *DmcycE* deficiency embryo at the same stage as the embryo in A showing only low levels of DmcycE in the epidermal cells and slightly greater levels of DmcycE in the neuroblasts. (C) A *DmcycE* deficiency embryo at a later stage (approx. 5 hour AED) near the completion of cycle 16, showing no detectable staining with the DmcycE antibody in somatic tissues. The only cells that contain DmcycE are the pole cells (identified from their characteristic position and morphology) where maternal *DmcycE* mRNA is also known to persist (Richardson et al., 1993). Embryos are orientated anterior to the left and ventral side down. pc, pole cells.

labelling (Fig. 3A) was used to show the two major zones of proliferation in the larval optic lobe, the outer proliferating centre (opc) and the inner proliferating centre (ipc). Between these two zones and immediately posterior to the lamina furrow, a band of cells known as the lamina precursor cells undergo a synchronous S phase. Prior to the lamina furrow, these cells are in G_1 phase and progression into S phase is a developmentally regulated event (Selleck et al., 1992). DmcycE protein distribution in the larval optic lobes is similar to the pattern of S phases, being present in the opc (out of the plane of focus in Fig. 3B), the ipc and in a band corresponding to the S phase lamina precursor cells. Notably, DmcycE is absent in the G_1 phase lamina precursor cells. Curiously, DmcycE is present in the lamina in a region where only a subset of cells are in S phase (Fig. 3) indicating that in these cells, DmcycE is not sufficient for entry into S phase (see Discussion).

The second example of a developmentally programmed G_1 to S phase transition occurs in the larval eye imaginal disc. Differentiation of the single-layer epithelium of the eye imaginal disc occurs from posterior to anterior in a wave associated with a prominent indentation known as the morphogenetic furrow (MF). Following logarithmic growth that occurs during much of larval development, cells in a band anterior to the MF remain in G_1 phase for an extended period (Wolff and Ready, 1993; Thomas et al., 1994; see Fig. 4A,B). A subset of these G_1 phase cells are induced by patterning mechanisms to terminally differentiate into ommatidial precluster cells, while the other cells synchronously enter S phase. DmcycE protein

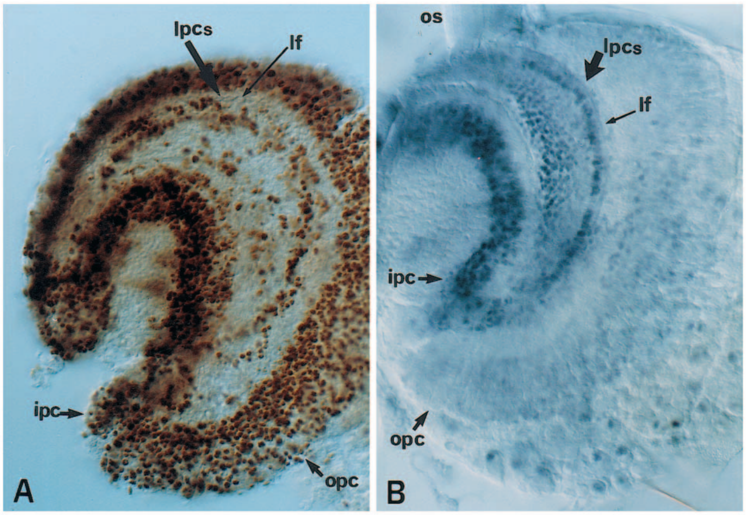

Fig. 3. Cyclin E protein distribution in the larval optic lobes compared with S phases. (A) S phases, as revealed by BrdU incorporation (30 minute pulse), showing 2 regions of proliferation, the outer proliferating centre (opc) and inner proliferating centre (ipc). Between these regions the lamina precursor cells (lpcs) enter S phase immediately posterior to the lamina furrow (lf). (B) DmcycE protein distribution, showing a similar pattern to the pattern of S phases except for cells within the lamina (the region posterior to the lamina furrow), where all cells contain DmcycE but only a few are in S phase. The opc cells contain DmcycE, but are out of the plane of focus in this photograph. Anterior is to the right. ipc, inner proliferating centre; opc, outer proliferating centre; lpcs, lamina precursor cells; lf, lamina furrow; os, optic stalk.

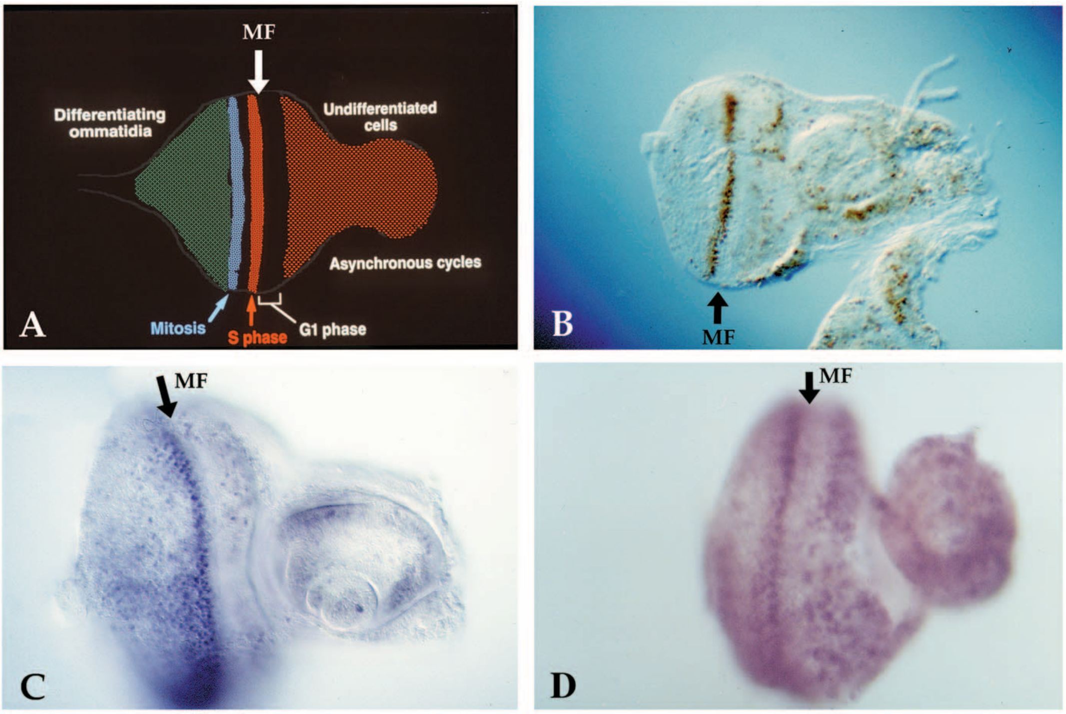

Fig. 4. DmcycE distribution in the eye reveals transcriptional control. (A) Schematic of proliferating and differentiating cells in the developing eye-antennal imaginal disc. After asynchronous divisions, cells arrest in G_1 just anterior to the morphogenetic furrow (MF). Posterior to the MF, some cells undergo differentiation and form the photoreceptor preclusters while the surrounding cells undergo a synchronous S phase followed in some cases by mitosis. (B) S phases revealed by bromodeoxyuridine (BrdU) labelling of third instar larval eye imaginal discs. Note the absence of S phases anterior to the MF. BrdU-labelled cells in the most posterior region of the disc correspond to subretinal cells (Wolff and Ready, 1993). (C) DmcycE protein distribution in the developing eye disc. (D) *DmcycE* mRNA distribution in the developing eye disc, as revealed by in situ hybridization with a digoxigenin-labelled probe. *DmcycE* mRNA and protein are present in a similar pattern to the pattern of S phase cells and are absent from the G_1 cells anterior to the MF. (B) 200× magnification; (C,D) 400× magnification. Larval eye imaginal discs are orientated with anterior to the right. Arrows indicate the morphogenetic furrow (MF).

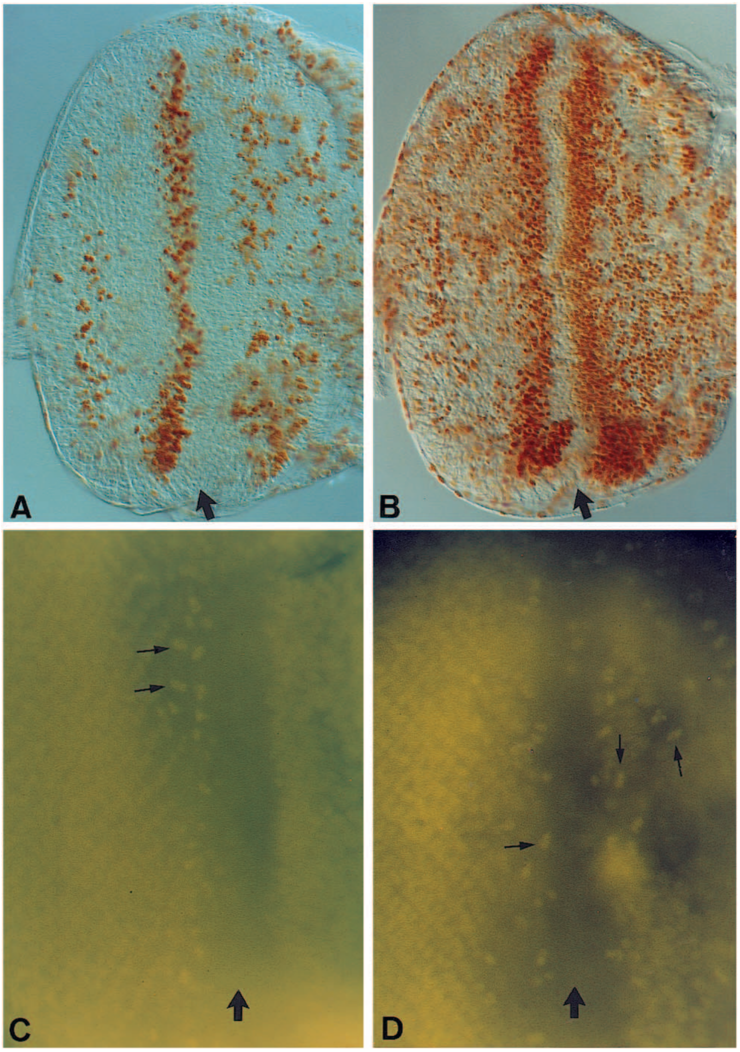

Fig. 5. Heat-shock-induced ectopic expression of cyclin E induces G_1 phase-arrested eye imaginal disc cells into S phase and through a complete cell cycle. The pattern of S phases, as revealed by BrdU incorporation, in a heat-shocked eye disc from a control larval (A), or a heat-shocked eye disc from a *hsp70-DmcycE* larva (B). Third instar larvae were heat-shocked and allowed to recover for 60 minutes before BrdU labelling. 400× magnification. (C,D) Heat-shocked larvae were allowed to recover for 120 minutes before fixing and staining with chromomycin A3. (C) A heat-shocked control larval eye disc. (D) A heat-shocked *hsp70-DmcycE* larval eye disc. The large arrow indicates the morphogenetic furrow. In C and D small arrows point to examples of mitotic cells as revealed by the presence of condensed DNA. 1000× magnification. Anterior is to the right.

(as revealed by anti-DmcycE antibody stainings; Fig. 4C) and *DmcycE* mRNA (as revealed by in situ hybridization; Fig. 4D), are present in a subset of the asynchronously proliferating cells. DmcycE protein and mRNA are also present in a band of cells immediately posterior to the MF, corresponding to S phase cells (Fig. 4B) but, significantly, are not detected in the band of G_1 phase cells within and anterior to the MF (Fig. 4C,D).

Ectopic expression of *DmcycE* drives G_1 phase cells in the larval eye imaginal disc through a complete cell cycle

To determine whether down-regulation of *DmcycE* anterior to the MF in the eye imaginal disc is important in establishing the G_1 phase, we ectopically expressed *DmcycE* in these cells by heat-shock induction of an *hsp70-DmcycE* transgene and monitored S phases by BrdU labelling.

Ectopic expression of *DmcycE* from the *hsp70-DmcycE* transgene resulted in a dramatic increase in the number of BrdU-labelled cells in the eye-antennal disc 60-90 minutes after heat shock (Fig. 5B), compared with the control (Fig. 5A). *hsp70-DmcycE* expression in the eye imaginal disc triggers entry of the majority of the G_1 phase cells anterior to the MF into S phase. In addition, the band of S phase cells posterior to the furrow is wider and contains more labelled cells than the control. Thus it appears that the differentiating precluster cells are driven into S phase. Interestingly, a narrow band of cells in the MF is not triggered to enter S phase (see Discussion). In addition to the dramatic effects adjacent to the MF, there is a general increase in the number of BrdU-labelled cells throughout the disc, both in the region of undifferentiated asynchronously dividing cells and in the terminally differentiating region of the disc. Thus G_1 phase cells anterior to the MF, many differentiating cells posterior to the MF, and many proliferating cells in the undifferentiated region of the eye imaginal disc are induced by DmcycE to enter S phase prematurely.

To determine whether the additional S phase cells are induced to proceed through a complete cell cycle, heat-shocked *hsp70-DmcycE* larvae were allowed to recover for 120 minutes or 180 minutes before dissection and staining with chromomycin A3 to visualise mitotic cells (Fig. 5C,D). As expected, control discs showed a band of mitotic cells posterior to the MF, and no mitoses were observed immediately anterior to the furrow (Fig. 5C; and see Fig. 4A). After 120 minutes recovery, heat-shocked *hsp70-DmcycE* discs showed an additional band of mitotic cells anterior to the MF (Fig. 5D), corresponding to the additional band of S phase cells seen after heat shock. More mitotic cells were observed immediately posterior to the MF (Fig. 5D; and data not shown). Not all of the cells anterior to the MF were in mitosis at one time, possibly due to asynchronous entry into, and the short duration of, mitosis. We conclude that *hsp70-DmcycE* expression in the eye imaginal disc induces at least some cells to complete an ectopic cell cycle.

Ectopic expression of *DmcycE* alters the normal pattern of development of the eye imaginal disc

To examine the consequence of the ectopic S phase on subsequent development of the eye imaginal disc, heat-shocked *hsp70-DmcycE* larvae were allowed to develop into adults, and their eyes examined using scanning electron microscopy (Fig. 6). Eye imaginal discs from controls, heat-shocked Canton S, heat-shocked *hsp70-DmcycC* (containing the *hsp70* promoter fused to a cDNA encoding the candidate G_1 cyclin, *Drosophila* cyclin C; Leopold and O’Farrell, 1991) and heat-shocked *hsp70-string* (containing the *hsp70* promoter fused to the mitotic inducer, string/cdc25 phosphatase cDNA; Edgar and O’Farrell, 1989) did not show any abnormalities (Fig. 6A; and results not shown). However, ectopic expression of *DmcycE* results in abnormal development of the adult eye (Fig. 6B-D). Scanning electron micrographs revealed roughening in a band of ommatidia running in a dorsal-ventral axis across the eye in the heat-shocked *hsp70-DmcycE* individuals (Fig. 6B), indicating irregular formation of ommatidia. Indeed, higher magnification of the eyes from heat-shocked *hsp70-DmcycE* individuals revealed irregularity in the size and position of ommatidia (Fig. 6C,D) and increased numbers of bristles associated with each ommatidia (see Discussion).

To examine the consequence of ectopically expressing *DmcycE* in differentiating cells immediately posterior to the MF, transgenic flies were generated in which *DmcycE* was expressed in the *sevenless* pattern (Basler et al., 1989) using the GAL4 system (see Materials and methods). Expression of *DmcycE* in the *sevenless* pattern resulted in disorganisation throughout the eye (Fig. 6E,F). These results indicate that at least part of the eye disorganisation observed using the *hsp70-DmcycE* transgenic flies, is due to the effect of ectopic expression of *DmcycE* on differentiating cells posterior to the MF.

To investigate the nature of eye disorganisation at a cellular level, the photoreceptor cell arrangement was examined in sections of eyes from heat-shocked control and heat-shocked *hsp70-DmcycE* flies (Fig. 7). As expected from the band of roughening observed across the eye, a band of disorganised ommatidia surrounded by relatively undisturbed ommatidia was observed in sections of the heat-shocked *hsp70-DmcycE* samples (Fig. 7B). The disorganised region contained ommatidia with altered complements of photoreceptor cells (Fig. 7B,C). In addition, patches of apparently undifferentiated cells and large vacuoles were observed in eye imaginal discs from heat-shocked *hsp70-DmcycE* flies (Fig. 7B,C). Thus, ectopic *DmcycE* expression leads to disorganisation of the eye by altering the number of photoreceptor cells per ommatidium and the development of the surrounding cells.

DISCUSSION

During metazoan development, cell proliferation must be coordinated with developmental processes. The G_1 phase is an important control point where decisions are made to continue cell proliferation or to differentiate (Pardee, 1989). A simple example of developmental decisions made during G_1 is observed in the budding yeast, *Saccharomyces cerevisiae*, where controls act to ensure that cells are arrested in G_1 before they decide to mate or sporulate (reviewed by Reed, 1992). In budding yeast, arrest in G_1 in response to these environmental signals requires the inactivation and down-regulation of the G_1 cyclins (Reed, 1992). Over-expression or stablization of G_1 cyclins can prevent these developmental G_1 arrests. In metazoans the decision to proliferate or differentiate may also

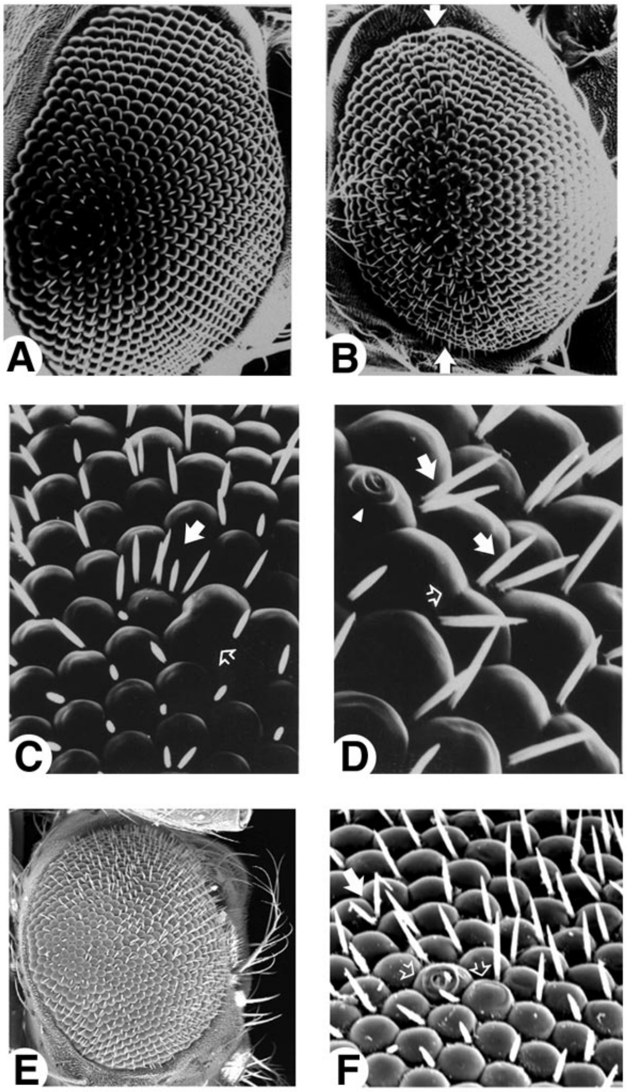

Fig. 6. Scanning electron micrographs of adult eyes after ectopic expression of *DmcycE* reveals that eye development is disrupted. The effect of ectopic expression of *DmcycE* in the eye imaginal disc development was analysed in *hsp70-DmcycE* transgenic flies (B-D) or by expression in the *sevenless* pattern in homozygous *GAL4(UAS)-DmcycE*; *sev-GAL4* flies (E,F). (A-D) Control and *hsp70-DmcycE* third instar larvae were heat shocked and allowed to develop. (A) An eye from a heat-shocked control fly showing the organised array of ommatidia with a bristle at every alternate vertex. (B-D) Eyes from heat-shocked *hsp70-DmcycE* showing disorganised and irregular ommatidia and bristle multiplications. In B, arrows indicate the band of roughness. In C and D, arrows indicate examples of bristle multiplications, open arrows indicate fused ommatidia and (in D) the arrowhead indicates a lens blister. (A,B) 200× magnification; (C)1000× magnification and (D) 2000× magnification. (E) An eye from a homozygous *GAL4(UAS)-DmcycE*; *sev-GAL4* fly at 150× magnification showing generalised roughness throughout the eye. (F) The same eye at 750× magnification showing duplicated bristles (arrow) and lens blisters (open arrows).

be controlled primarily by the regulation of the G_1 cyclins. Here we present evidence that transcriptional regulation of cyclin E is important in the regulation of the G_1 to S phase transition in response to developmental cues.

Cyclin E protein is absent in G_1 phase cells

The expression of *DmcycE* mRNA during embryonic development correlates with cell proliferation and is absent in terminally differentiating tissues which are known to be in G_1 phase (Richardson et al., 1993; Knoblich et al., 1994). Here we show that during development of the larval optic lobe and eye imaginal disc, DmcycE protein distribution correlates with cell proliferation. In particular, DmcycE is not detectable in the larval optic lobe lamina precursor cells, which undergo an extended developmentally controlled G_1 phase as they move into the lamina furrow (Selleck et al., 1992). However, once these cells move out of the furrow and are induced to enter S phase by innervation of the optic nerves (Selleck et al., 1992), DmcycE is present at high levels. DmcycE is also absent during the extended G_1 phase of cells in the region of the MF in the eye imaginal disc. In both these cases the down-regulation of *DmcycE* may be important in limiting cell proliferation.

Curiously, DmcycE is present in the larval optic lobe lamina, where most cells appear to have ceased proliferation. The cell cycle phase of these cells is not known, so they may either be arrested in G_1 or G_2 phase. If arrested in G_1 phase, cell cycle progression may be blocked by the presence of cell cycle inhibitory proteins, such as homologs of p21 and p27, that act to inhibit cyclin E/Cdk activity (reviewed by Elledge and Harper, 1994). If arrested in G_2 phase, the presence of DmcycE may have no effect. Alternatively it remains possible that DmcycE plays a non-cell cycle role in this tissue.

Induction of *DmcycE* expression is sufficient for the G_1 to S phase transition

Heat-shock induction of *DmcycE* in third instar larvae results in cells in two regions of the G_1 band of the eye imaginal disc aberrantly entering S phase. The first of these regions is immediately anterior to the MF and contains undifferentiated G_1 phase cells. The second is immediately posterior to the furrow where some of the cells normally enter S phase, while the others have initiated differentiation to form ommatidial preclusters. Thus, expression of *DmcycE* is sufficient to drive both undifferentiated and differentiating G_1 phase cells into S phase. There is also an increase in S phase cells in the anterior region of the eye, where cells are undergoing asynchronous cycles. As these cells are in a variety of cell cycle phases, it is likely that these additional S phase cells arise by premature induction of G_1 phase cells into S phase by the ectopic expression of *DmcycE*. Thus, control of the length of the G_1 phase of asynchronously dividing *Drosophila* imaginal cells, like mammalian tissue culture cells, appears to require regulated expression of cyclin E.

Interestingly, a band of cells in the G_1-arrested region is not induced to enter S phase by ectopic expression of *DmcycE*. It is possible that the inability of DmcycE to induce these cells to enter S phase may be due to the expression in this region of a cyclin E/Cdk inhibitor. A possible candidate for such an inhibitor that is expressed in this region is *decapentaplegic* (Masucci et al., 1990), a homolog of the mammalian negative growth factor TGFβ, which acts by inducing the p27 inhibitor leading to the inhibition of cyclin E/Cdk2 activity (reviewed by Elledge and Harper, 1994). The possibility that *decapentaplegic* is involved in the observed refractiveness of these cells to *DmcycE* is under investigation.

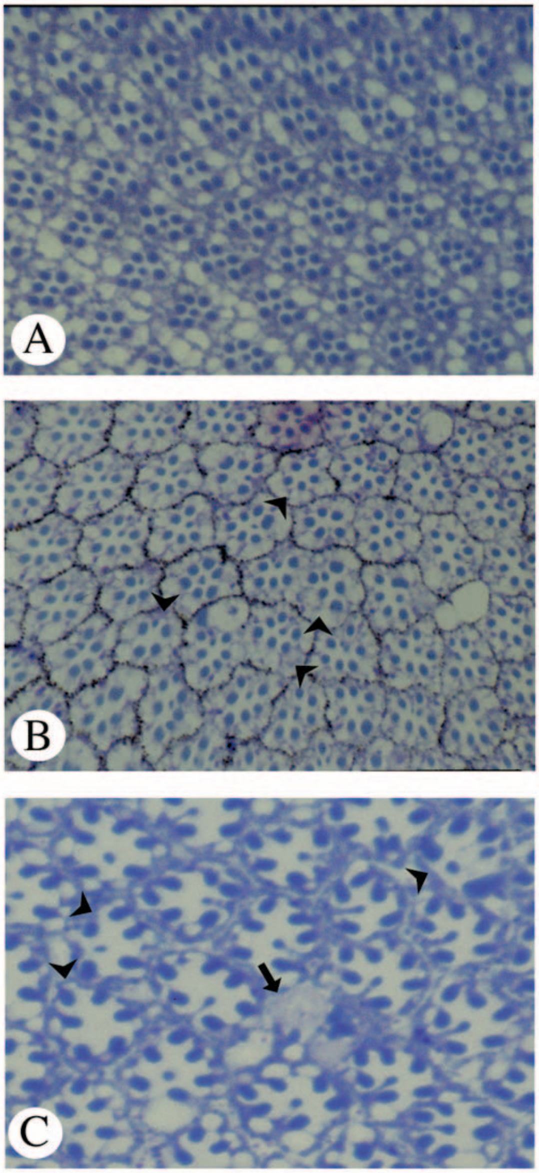

Fig. 7. Sections of adult eyes after ectopic expression of *DmcycE* reveals that pattern formation in the eye is disrupted. Control and *hsp70-DmcycE* third instar larvae were heat-shocked and allowed to develop. (A) A heat-shocked control eye showing the organised array of ommatidia. Each ommatidium contains a ring of pigment cells surrounding six large photoreceptor cells and one smaller photoreceptor cell. (B,C) Heat-shocked *hsp70-DmcycE* adult eyes. In B a region containing disorganised ommatidia is surrounded by normal ommatidia on the left and the right hand sides. The pigment cells have stained more intensely in B than in A and C. The arrowheads indicate examples of abnormal ommatidia with altered complements of photoreceptor cells and (in C) the arrow indicates a patch of apparently undifferentiated cells. Note the large vacuoles in B. C is shown at a 2.3× higher magnification relative to A and B.

Down-regulation of cyclin E expression is important for correct eye development

The induction of an inappropriate cell cycle in the eye imaginal disc by *hsp70-DmcycE* results in abnormal development of the adult eye. The specific eye defects include altered complements of photoreceptor cells per ommatidium as well as patches of undifferentiated cells and bristle multiplications. Considering the number of additional cells that are driven into S phase by ectopic expression of *hsp70-DmcycE*, it is surprising that eye disorganisation is not more severe. By utilising the *sevenless* enhancer we showed that expression of *DmcycE* specifically in differentiating cells posterior to the MF also results in eye disorganisation. Thus, the eye disorganisation observed after ectopic expression of *hsp70-DmcycE* is, at least partially, due to the expression of *DmcycE* in the differentiating cells posterior to the MF. The effect of the additional cells, generated by ectopic expression of *hsp70-DmcycE*, on patterning in other regions of the developing eye remains to be determined. These additional cells may be eliminated by the apoptotic mechanism that normally functions in the eye at the final phase of pattern formation (Wolff and Ready, 1993).

The effect of ectopic expression of *DmcycE* on eye development may be related to that observed in a *roughex* mutation where cells fail to enter an extended G_1 phase anterior to the MF (Thomas et al., 1994). The *roughex* mutation results in more extreme errors in pattern formation and eye roughening (Thomas et al., 1994) than ectopic expression of *DmcycE*, possibly because the *roughex* mutation completely eliminates the G_1 phase so that all cells are proliferating when differentiation is induced. The *roughex* mutation leads to an advance in expression of the G_2 cyclins, cyclin A and cyclin B, anterior to the MF and preliminary results suggest that ectopic expression of cyclin E also occurs in this region (B.J. Thomas, personal communication).

Why does ectopic expression of cyclin E in differentiating photoreceptor cells cause eye disorganisation? One explanation is that the generation of extra cells alters the nature of cell-cell contacts that are known to be important in pattern formation in the eye (Wolff and Ready, 1993). In addition, expression of *DmcycE* in differentiating ommatidial preclusters posterior to the MF at the time at which their cell fate is being determined, may lead to their duplication and a subsequent increase in the number of photoreceptor cells per ommatidia. Indeed this is observed, although not all ommatidia in heat-shocked *hsp70-DmcycE* individuals exhibited this increase in photoreceptor cell numbers. Another possiblity is that induction of *DmcycE* and re-entry of differentiating cells into the cell cycle inhibits their differentiation or prevents cell death. Indeed patches of apparently undifferentiated cells were often observed. Alternatively, induction of differentiating cells into the cell cycle may trigger apoptosis as has been observed to occur in other systems (reviewed by Harrington et al., 1994). This possibility may explain the occurrence of ommatidia with a decreased complement of photoreceptor cells as well as the large vacuoles and general disorganisation of the heat-shocked *hsp70-DmcycE* eyes. The reason for the bristle multiplications is unknown, since bristle cell determination does not occur until pupal development (reviewed by Wolff and Ready, 1993). However, bristle duplications are often observed in eye patterning mutants (eg. Saint et al., 1988) and may be a general feature of eye disorganisation.

In conclusion, these results illustrate the importance of G_1 phase control for correct pattern formation during eye development. They also identify *DmcycE* as a target of developmental mechanisms that control G_1 to S phase progression in proliferating eye imaginal cells.

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