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Tissue-specific regulation of *cyclin E* transcription during *Drosophila melanogaster* embryogenesis

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SUMMARY

Cyclin E is an essential regulator of S phase entry. We have previously shown that transcriptional regulation of the gene that encodes *Drosophila* cyclin E, *DmcycE*, plays an important role in the control of the G_1 to S phase transition during development. We report here the first comprehensive analysis of the transcriptional regulation of a G_1 phase cell cycle regulatory gene during embryogenesis. Analysis of deficiencies, a genomic transformant and reporter gene constructs revealed that *DmcycE* transcription is controlled by a large and complex *cis*-regulatory region containing tissue- and stage-specific components. Separate regulatory elements for transcription in epidermal cells during cell cycles 14-16, central nervous system cells and peripheral nervous system cells were found. An additional *cis*-regulatory element drives transcription in thoracic epidermal cells that undergo a 17th cell cycle when other epidermal cells have arrested in G_1 phase prior to terminal differentiation. The complexity of *DmcycE* transcriptional regulation argues against a model in which *DmcycE* transcription is regulated simply and solely by G_1 to S phase transcription regulators such as RB, E2F and DP. Rather, our study demonstrates that tissue-specific transcriptional regulatory mechanisms are important components of the control of *cyclin E* transcription and thus of cell proliferation in metazoans.

Key words: *cyclin E*, Cell cycle, Transcriptional regulation, *Drosophila*, Development, S phase

INTRODUCTION

In recent years, knowledge of the regulatory molecules that control the eukaryotic cell cycle has grown dramatically. Two protein families, Cyclin-dependent serine/threonine kinases (Cdks) and cyclins, are central components of cell cycle regulation (reviewed in Pines and Hunter, 1991; Reed, 1992; Follette and O'Farrell, 1997). The G_1 cyclin, Cyclin E, was shown to be essential and rate limiting for progression into S phase in *Drosophila* embryonic and eye imaginal disc cells (Knoblich et al., 1994; Richardson et al., 1995) and also in mammalian cells (Ohtsubo, and Roberts, 1993; Resnitzky et al., 1994; Ohtsubo et al., 1995). We have previously shown that transcriptional regulation of the *Drosophila cyclin E* gene (*DmcycE*) is an important component in the control of cell proliferation during *Drosophila* development. The cycle 17 G_1 arrest that occurs in the embryonic epidermal cells prior to differentiation and the transient G_1 arrest of cells in the morphogenetic furrow of the developing eye disc both require downregulation of *DmcycE* (Knoblich et al., 1994; Richardson et al., 1995).

Multicellular development requires the coordination of cell division with morphogenic and developmental processes, suggesting that cell cycle progression must ultimately be controlled by developmental regulatory mechanisms. Given that different tissues exhibit widely variable cell cycle kinetics and that different regulators such as Dpp, Wingless, Notch and Prospero are responsible for proliferation or cell cycle arrest in different developmental contexts (Masucci et al., 1990; Skaer and Martinez-Arias, 1992; Horsfield et al., 1998; Go et al., 1998; Richter et al., 1998; Johnston and Edgar, 1998; Li and Vaessin, 2000; reviewed by Serrano and O'Farrell, 1997; Myster and Duronio, 2000), it will be important to determine the nature of the links between developmental regulators and cell cycle control if we are to understand the cell proliferation aspects of morphogenesis.

One mechanism by which developmental signals and cues could regulate cell proliferation is transcriptional control of cell cycle regulatory genes. *DmcycE* is a cell cycle regulatory gene that could be subject to this method of control, in order to link S phase with developmental processes during *Drosophila* development. The pattern of zygotic *DmcycE* transcription during embryonic development is complex and can be divided into three distinct modes, based on the type of modified cell cycle that is observed during embryogenesis.

Zygotic *DmcycE* transcription is first observed ubiquitously, at low levels, throughout the epidermis during mitotic cycles 14-16 (Richardson et al., 1993), although all cycles prior to cycle 17 can occur without zygotic transcription (Knoblich et al., 1994), presumably due to the presence of maternally

derived products. These embryonic cycles consist of a G_2 phase of variable length, mitosis and an S phase that initiates without an intervening G_1 phase (Edgar and O'Farrell, 1990). Cell division during these cycles occurs in a complex spatiotemporal pattern with groups of cells, termed mitotic domains, undergoing mitosis synchronously (Foe, 1989). The pattern of S phases, as monitored by bromodeoxyuridine (BrdU) incorporation, is complex, as a result of the preceding mitoses. During this period, S phases do not correlate with the ubiquitous epidermal *DmcycE* transcription (Richardson et al., 1993). A similar mode of regulation also appears to operate in the proliferating cells of the embryonic central and peripheral nervous systems (CNS and PNS) where cycles lack detectable G_1 phases (Hartenstein et al., 1987; Edgar and O'Farrell, 1990; Weigmann and Lehner, 1995). Transcription of *DmcycE* is then downregulated in most cells by stage 11, close to and perhaps at the time that cell division ceases after mitosis 16 (Richardson et al., 1993; Knoblich et al., 1994). Downregulation of *DmcycE* expression at this developmental stage is essential for the G_1 arrest observed in the epidermis prior to differentiation (Knoblich et al., 1994).

A second mode of *DmcycE* transcription is observed in a subset of epidermal cells in the lateral thoracic region (thoracic segments 1, 2 and 3) of the embryo. These cells, here termed the epidermal thoracic patches, complete a 17th mitotic cell cycle (Bate and Martinez-Arias, 1991; Knoblich et al., 1994) and are different to the remainder of the epidermal cells, which arrest in G_1 phase of cell cycle 17. In zygotic null *DmcycE* mutants, where maternal sources of Cyclin E become depleted after cycle 16, the epidermal thoracic patch cells arrest in G_1 phase and do not enter the 17th S phase (Knoblich et al., 1994), indicating that zygotic *DmcycE* transcription is essential for S phase progression in these cells. In addition, ectopic expression of *DmcycE* induces all dorsal epidermal cells, including the epidermal thoracic patch cells, to coordinately enter S phase, indicating that *DmcycE* transcription is limiting for entry of these cells into S phase (Knoblich et al., 1994). *DmcycE* transcription is downregulated coordinately in all epidermal cells, but is activated specifically in the cells of the thoracic patches in a pattern that precedes the 17th S phase (Knoblich et al., 1994). Thus the cycle 17 epidermal thoracic patch cells are the first cells to exhibit G_1, S, G_2 and M phases and are the first embryonic cells that are regulated at the G_1 to S phase transition by transcriptional regulation of *DmcycE*.

The third mode of *DmcycE* transcription is observed in endoreplicating tissues such as the gut, where the cell cycle consists of S phases with intervening gap phases but no mitoses (Smith and Orr-Weaver, 1991). *DmcycE* transcription in these tissues is spatially regulated and coincides with S phase (Knoblich et al., 1994). The cycling of *DmcycE* levels in endoreplicating cells is essential for the rounds of S phases observed in these cells (Follette et al., 1998; Weiss et al., 1998). These tissues are further examples of embryonic cells that have regulated G_1 to S phase transitions that are dependent on *DmcycE* transcription.

Developmental cues are implicated in the regulation of *DmcycE* transcription in *Drosophila*. For example, in *string (stg)* mutant embryos, *DmcycE* is transcribed in the correct spatiotemporal pattern and is downregulated at the time when cycle 17 G_1 phase arrest would normally occur, despite the fact that all cells remain in G_2 phase of cycle 14 (Knoblich et al., 1994). Thus in these cells, *DmcycE* transcription is independent of cell cycle progression and must be responding to developmental cues.

To investigate the relationship between embryonic development and *cyclin E* transcriptional control, we have dissected the *DmcycE* transcriptional regulatory region using a combination of *DmcycE* deficiencies, a genomic transformant and reporter gene constructs. In this first comprehensive analysis of developmental transcriptional regulation of a key G_1 phase cell cycle regulatory factor, we show that *DmcycE* has a large regulatory region containing multiple tissue-specific *cis*-acting sequences that act to coordinate the G_1 to S phase transition with embryonic development.

MATERIALS AND METHODS

Generation of constructs for germline transformation and generation of transgenic flies

The regions of genomic DNA used in all constructs are summarised in Fig. 1. The construct used to attempt genomic rescue consists of a 10.7 kb *Kpn*I genomic fragment covering the *DmcycE* type I transcript cloned into *P{CaSpeR-4}* to generate *P[w$^+$]TI*. Transgenic flies containing *P[w$^+$]TI* were obtained by *P*-element-mediated germline transformation of w^{1118} embryos and selection of w^+ transgenic flies. Three independent homozygous viable transformant lines were obtained and used in further analysis.

The *1.0TATAlacZ DmcycE* promoter-*lacZ* reporter construct (see Fig. 1) was made by cloning the 1.0 kb genomic *Hin*dIII/*Eco*RI fragment upstream of a minimal *hsp27* promoter and a nuclear targeted *lacZ* (*nlacZ*) reporter gene (from G. Riddihough). The assembled DNA was then cloned into the *P{CaSpeR-4}* transformation vector and used to transform the germline of flies using *P*-element-mediated transformation.

All remaining reporter gene constructs utilised the *DmcycE* promoter in place of the *hsp27* promoter. An *Nco*I site was created at the *DmcycE* ATG by site-directed mutagenesis. The resulting 2.9 kb *Nco*I fragment containing the *DmcycE* Type I 5′ UTR and proximal promoter sequences was ligated to the *Nco*I site corresponding to the ATG translation start site of *nlacZ* in a *pBluescriptII* (Stratagene) vector to create *pBST-2.9lacZ*. Constructs used in this study were derived from *pBST-2.9lacZ* by sequential addition of *DmcycE* genomic fragments. Most of the endogenous promoter constructs contained contiguous upstream *DmcycE* genomic sequences from the Type I *DmcycE* ATG, the *3.3+1.9lacZ* construct being the only non-contiguous endogenous promoter construct characterised in this study. Constructs were assembled in *pBluescriptII* (Stratagene), excised using *Kpn*I and *Not*I, cloned into the *P{CaSpeR-4}* transformation vector and transgenic flies generated as described above.

Whole-mount RNA in situ hybridisation, bromodeoxyuridine (BrdU) incorporation and immunohistochemistry

Whole-mount RNA in situ hybridisation to embryos was performed essentially as described by Tautz and Pfeifle (1989). For *DmcycE* RNA in situ hybridisation, *lacZ* expression from either a *Cy*O*wglacZ* or a *Cy*O*ftzlacZ* balancer chromosome was detected by antibody staining to allow unambiguous identification of the homozygous *DmcycE* mutant embryos. For BrdU labelling to detect S phases, embryos were labelled for 30 minutes with BrdU as previously described by Richardson et al. (1993). After labelling and fixation of *DmcycE* mutations, *lacZ* expression from either the *CyOwglacZ* or a *CyOftzlacZ* balancer chromosome was used to identify homozygous mutant embryos, as described above.

All embryos were mounted in 80% glycerol in 1 x PBS and visualised and photographed on a Zeiss Axiophot microscope using Nomarski optics.

RESULTS

Zygotic *DmcycE* transcription is controlled by a large *cis*-regulatory region

The *DmcycE* locus produces two transcripts, Type I and Type II, from different promoters (Richardson et al., 1993; L. Jones, M. Silson, W. Winnall, R. Saint and H. E. Richardson, unpublished). The Type I promoter drives embryonic zygotic transcription, while the Type II promoter is used to generate the maternally derived transcripts present in the embryo (Richardson et al., 1993; D. Crack, J. Secombe, A. Brumby, M. Coombe, R. Saint and H. E. Richardson, unpublished). Because of its role in embryonic zygotic *DmcycE* expression, the studies described here focus on the regulation of *DmcycE* Type I transcription. The extent of the *cis*-acting regulatory region controlling zygotic *DmcycE* (Type I) transcription was initially investigated by generating transgenic flies carrying a 10.7 kb *Kpn*I genomic DNA fragment that spanned the zygotic *DmcycE* transcript (*P[w*$^+$ *TI]*) and included approximately 4.8 kb upstream and 1.8 kb downstream of this transcript (Fig. 1). Two independent transformants homozygous for *P[w*$^+$ *TI]* failed to rescue to viability a *DmcycE* null allele (*DmcycE*^{AR95}) *trans*-heterozygous with either *Df(2L)TE35D-17* or *Df(2L)TE35D-19*, two deficiencies that uncover the *DmcycE* gene (data not shown; Knoblich et al., 1994).

To determine if the *P[w*$^+$ *TI]* transgene expressed the zygotic *DmcycE* transcript during embryogenesis, whole-mount in situ hybridisation was carried out on embryos homozygous for each of three independent lines of the transgene in a homozygous *DmcycE* deficiency background. In the absence of the transgene, no transcripts were detected in post-blastoderm embryos homozygous for either of the two *DmcycE* deficiencies *Df(2L)TE35D-19* or *Df(2L)TE35D-17* (Fig. 2C,H and results not shown). In embryos homozygous for a *DmcycE* deficiency and carrying the *P[w*$^+$ *TI]* transgene, *DmcycE* transcripts were detected in epidermal tissues during mitotic cycles 14-16 (data not shown) and in the proliferating CNS (Fig. 2B) in a pattern similar to that observed in *DmcycE*$^+$ sibling embryos, which express *DmcycE* in a wild-type pattern (Fig. 2A). The proliferating PNS cells of *P[w*$^+$ *TI]* transgenic, *DmcycE* deficient homozygous embryos also showed *DmcycE* expression in a pattern similar to *DmcycE*$^+$ siblings, with the exception of PNS neuroblasts in the maxillary and labial head segments, in which *DmcycE* transcripts were reduced or absent (Fig. 2B, compare with A). In addition, *DmcycE* transcripts from the transgene were not detectable in the epidermal thoracic patches (Fig. 2B,B′, compare with A,A′). The absence of *DmcycE* expression in the epidermal thoracic patches correlates with the absence of BrdU incorporation into these cells in *DmcycE* deficient embryos carrying the *P[w*$^+$ *TI]* transgene (Fig. 2E, compare with D). Finally, staining in the endoreplicating gut (Fig. 2F) was not detectable above the background observed using a *cyclin E* probe with *cyclin E* deficient embryos (Fig. 2G, compare with H). In *P[w*$^+$ *TI]* transgenic *DmcycE* deficient embryos, expression of *DmcycE* correlated with S phases in all tissues, as revealed by BrdU incorporation (Fig. 2E and data not shown). These data indicate that the 10.7 kb *Kpn*I genomic fragment could drive *DmcycE* expression at sufficiently high levels, in the tissues in which it is expressed, to induce entry into S phase.

These results demonstrate that the regions covering and closely flanking the zygotic Type I transcript in the *P[w*$^+$ *TI]* transgene contain *cis*-acting regulatory sequences that drive *DmcycE* transcription in the constitutive epidermal pattern during mitotic cycles 14-16, in the proliferating CNS and most of the proliferating PNS. However this pattern of *DmcycE* expression represents only part of the wild-type pattern seen during embryogenesis. The lack of *DmcycE* transcripts and S phases in the epidermal thoracic patches and a subset of the proliferating PNS cells shows that part or all of the *cis*-acting sequences regulating *DmcycE* transcription in these tissues are

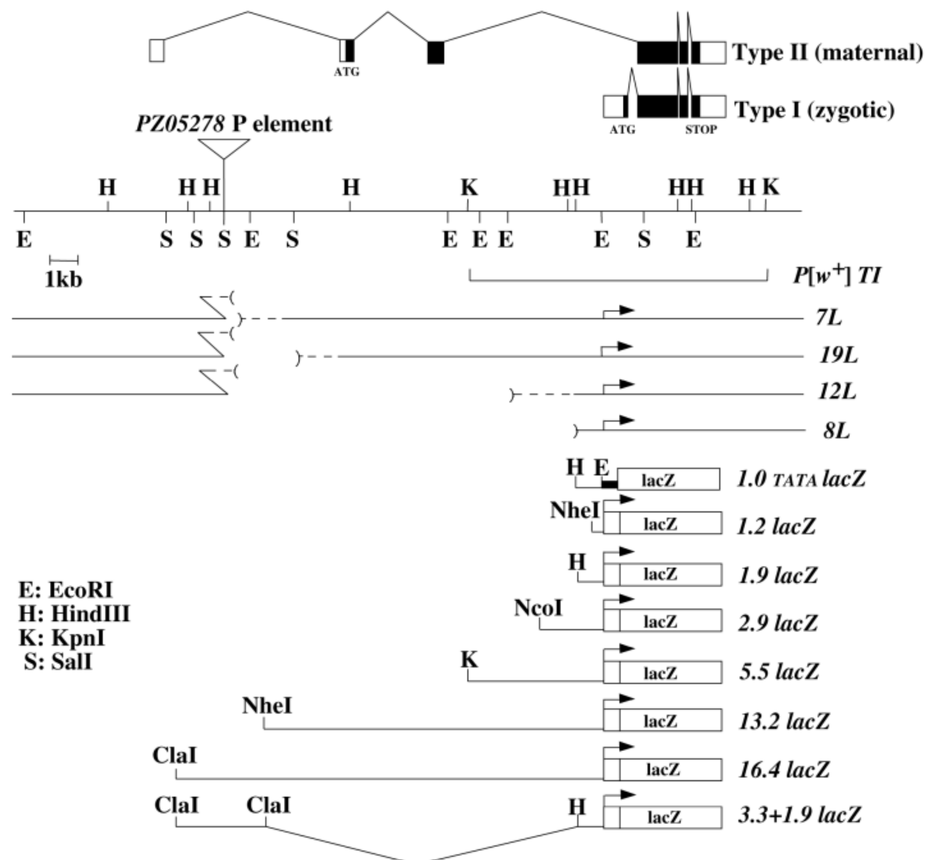

Fig. 1. Deficiencies and transformation constructs used in this study: a summary of the deletions and constructs used. The *TI* genomic fragment used in the rescue attempt, a series of P-element excision-derived deficiencies and the genomic fragments used to generate a series of reporter gene constructs are shown in relation to a genomic map of *DmcycE*. The broken lines indicate uncertainties in the position of deficiency breakpoints.

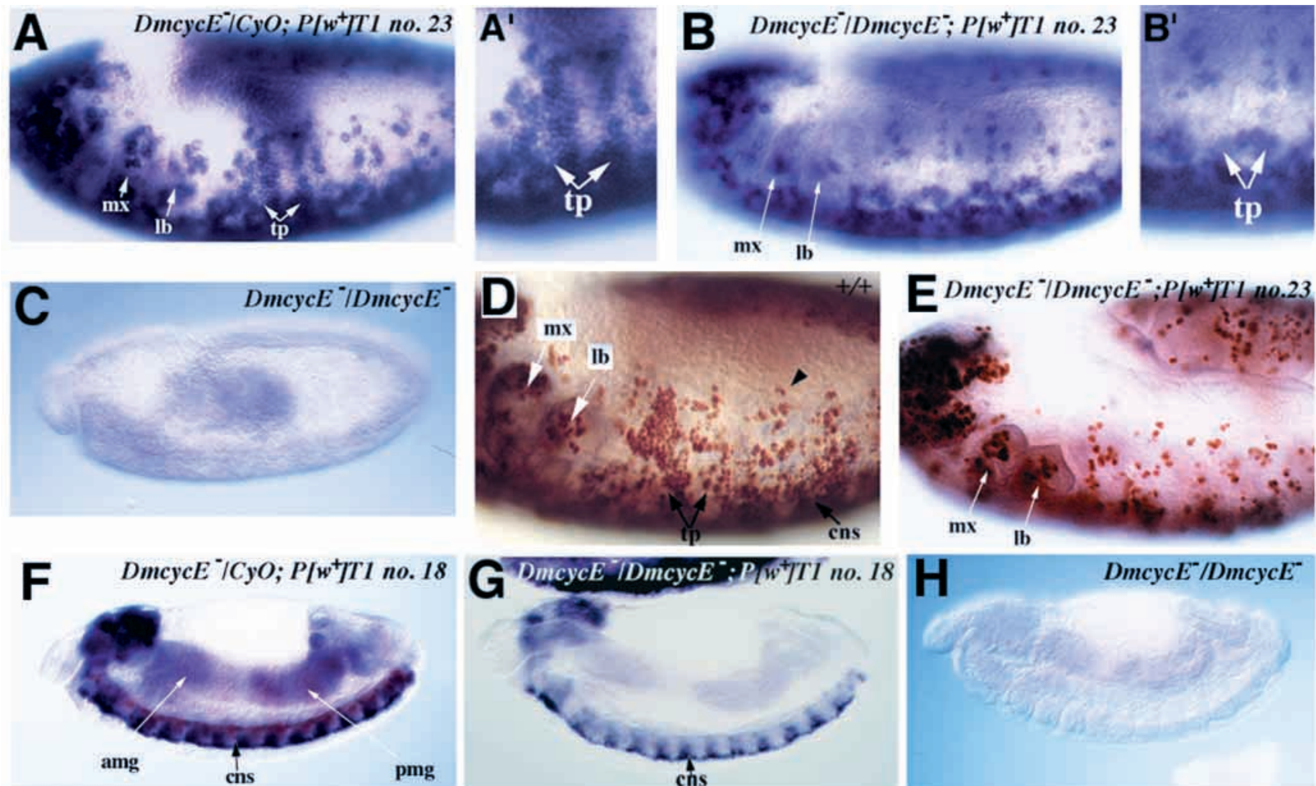

Fig. 2. The 10.7 kb *DmcycE* genomic transgene does not generate a complete pattern of *DmcycE* transcripts during embryogenesis. Whole-mount in situ hybridisation using a *DmcycE* probe (A-C,F-H), BrdU incorporation (D,E). (A) A stage-11 embryo heterozygous for a *DmcycE* deficiency and carrying the transgene, showing normal transcript levels in the CNS, PNS and epidermal thoracic patches. (A′) A higher magnification of thoracic patch expression in A, showing the epithelial nature of the cells expressing *DmcycE*. Out-of-focus CNS and PNS cells lie underneath these cells. (B) A stage-11 embryo homozygous for a *DmcycE* deficiency and carrying the *TI* transgene, showing transcripts in the CNS and PNS, but absence of transcripts in the epidermal thoracic patches and reduced levels of transcripts in the maxillary and labial neuroblasts. (B′) A higher magnification of the region that normally shows thoracic patch expression. Expression in CNS and PNS cells remains. (C) A stage-11 embryo homozygous for a *DmcycE* deficiency, showing absence of *cyclin E* transcripts. (D) A late stage-11/early stage-12 wild-type embryo showing incorporation of BrdU into cells of the thoracic patches and CNS and PNS cells. The arrowhead indicates an example of a PNS cell that has incorporated BrdU. (E) A late stage-11/early stage-12 embryo homozygous for a *DmcycE* deficiency and carrying the *TI* transgene, showing S phases in the CNS and PNS, but not in the epidermal thoracic patches. (F) A stage-13 embryo heterozygous for a *DmcycE* deficiency and carrying the transgene, showing normal transcription in the CNS and endoreplicating gut. The brown stain in this figure is the result of immunohistochemical staining of β-galactosidase derived from the balancer chromosome. (G) A stage-13 embryo homozygous for a *DmcycE* deficiency and carrying the *TI* transgene, showing transcripts in the CNS but no staining above background in the endoreplicating gut. (H) A stage-13 homozygous *DmcycE* deficiency control embryo. cns, central nervous system staining; mx, maxillary staining; lb, labial PNS staining; tp, thoracic patches.

located outside of the 10.7 kb *Kpn*I region contained in the *P[w⁺ TI]* transgene. The lack of these regulatory elements provides an explanation for the inability of this transgene to rescue a *DmcycE* null mutation. Significantly, these results indicate that regulation of *DmcycE* transcription during embryogenesis is driven by separable tissue-specific *cis*-acting regulatory regions.

Identification of *cis*-acting sequences required for *DmcycE* transcription in the epidermal thoracic patches and the PNS

Three large *P*-element-mediated deletions $DmcycE^{8L}$, $DmcycE^{12L}$ and $DmcycE^{19L}$ (see summary in Fig. 1), which remove putative *cis*-acting regulatory sequences important for zygotic *DmcycE* transcription during embryogenesis, have been generated from a lethal *P*-element insertion allele of *DmcycE* (*l(2)05278*) that appears to specifically disrupt endoreplication S phases (L. Jones, M. Silson, W. Winnall, R. Saint and H. E. Richardson, unpublished). Whole-mount in situ hybridisation analysis of $DmcycE^{8L}$ homozygous mutant embryos (the largest *P*-element-induced deletion; Fig. 1) revealed the presence of *DmcycE* transcripts in the epidermis and the proliferating CNS in a pattern similar to wild type, although the overall level of transcripts detected was reduced when compared with $DmcycE^{+}$ sibling embryos (Fig. 3B, compare with A). However, transcripts were not detected in the proliferating PNS, nor in the epidermal thoracic patches (Fig. 3B). A similar result was obtained with homozygous embryos of the second largest deficiency, $DmcycE^{12L}$, except that transcripts in the epidermis and CNS were at wild-type levels (Fig. 3D, compare with C). *DmcycE* transcripts were also present in one or two PNS cells per parasegment in this deficiency, but were not detected in the thoracic patches (Fig. 3C,D and results not shown). BrdU incorporation revealed that the distribution of S phases mirrored

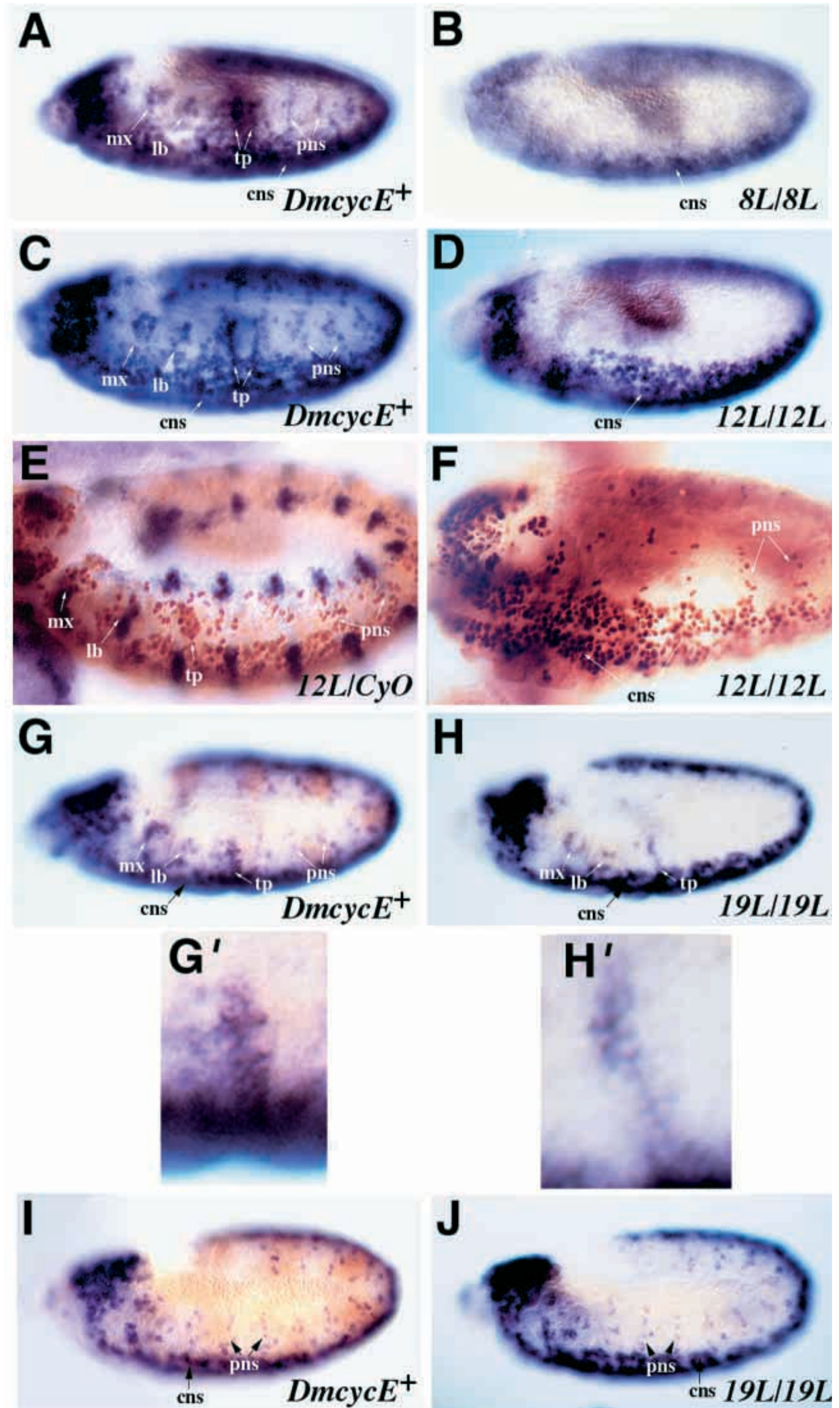

Fig. 3. Phenotypic analysis of the *8L*, *12L* and *19L* deficiencies during embryogenesis. Whole-mount in situ hybridisation using a *DmcycE* probe (A-D,G,G′,H,H′,I,J) and BrdU incorporation (E,F). Homozygous embryos were detected by the absence of *ftzlacZ* expression, in the case of B,D,H,J, and by the absence of *wglacZ* staining in F. (A-F) Stage-11 embryos. (A) *DmcycE*$^{+}$ sibling of the *DmcycE*^{8L} embryo in B, showing the wild-type pattern of transcripts in the CNS, PNS and epidermal thoracic patches. (B) Homozygous *DmcycE*^{8L} embryo showing very reduced levels of transcripts in the CNS and absence of transcripts in the PNS and epidermal thoracic patches. (C) *DmcycE*$^{+}$ sibling of the *DmcycE*^{12L} embryo in D showing the wild-type pattern of transcripts in the CNS, PNS and epidermal thoracic patches. (D) Homozygous *DmcycE*^{12L} embryo showing transcripts in the CNS, but very reduced PNS expression and no epidermal thoracic patches transcripts. (E) BrdU incorporation in a heterozygous *DmcycE*^{12L} embryo showing the wild-type pattern of S phases. *wglacZ* expression is detected by the blue staining. (F) BrdU incorporation in a homozygous *DmcycE*^{12L} embryo showing S phases in the CNS, but fewer S phases in the PNS and no S phases in the epidermal thoracic patches. (G-H′) Late stage-11/early stage-12 embryos. (G) *DmcycE*$^{+}$ sibling of the *DmcycE*^{19L} embryo in H, showing the wild-type pattern of *cyclin* E expression. The brown stain is the result of immunohistochemical staining of β-galactosidase derived from the balancer chromosome. (G′) Higher magnification of thoracic patch expression in G, showing the epithelial nature of these cells. (H) Homozygous *DmcycE*^{19L} embryo showing transcripts in the CNS and PNS, but fewer cells in the epidermal thoracic patches expressing *DmcycE* than in G, most obviously in the first thoracic segment. (H′) Higher magnification of thoracic patch expression in H showing the epithelial nature of these cells. (I,J) Early stage-12 embryos. (I) *DmcycE*$^{+}$ sibling of the *DmcycE*^{19L} embryo in J, showing the wild-type pattern of *cyclin* E expression. The brown stain is the result of immunohistochemical staining of β-galactosidase derived from the balancer chromosome. (J) Homozygous *DmcycE*^{19L} embryos showing transcripts in the CNS and PNS. cns, central nervous system staining; pns, peripheral nervous system staining; mx, maxillary staining; lb, labial PNS staining; tp, thoracic patches.

the distribution of *DmcycE* transcripts in homozygous *DmcycE*^{12L} embryos (Fig. 3E,F).

Characterisation of embryos homozygous for *DmcycE*^{19L}, one of the smallest of the deletions (Fig. 1), revealed the presence of *DmcycE* transcripts in the CNS and PNS in a pattern similar to wild-type embryos, although the level of *DmcycE* transcripts in the PNS cells initially appeared to be less than in heterozygous sibling embryos (Fig. 3H,J, compare with G,I; data not shown). Interestingly, the *DmcycE*^{19L} deletion partially disrupted *DmcycE* expression in the epidermal thoracic patches, as only a subset of the thoracic patch cells of the first thoracic segment expressed *DmcycE* (Fig. 3G,G′, compare with H,H′), suggesting that these patches of expression may constitute a complex domain of *DmcycE* regulation.

These analyses define the location of separable *DmcycE* regulatory elements. Elements essential for *DmcycE* transcription in the PNS lie between the 3′ breakpoints of the *DmcycE*^{19L} and *DmcycE*^{12L} deletions (see Fig. 1), although other PNS enhancers required for initial maximal expression lie outside of this region. This region also contains elements required for a subset of the expression pattern in the epidermal

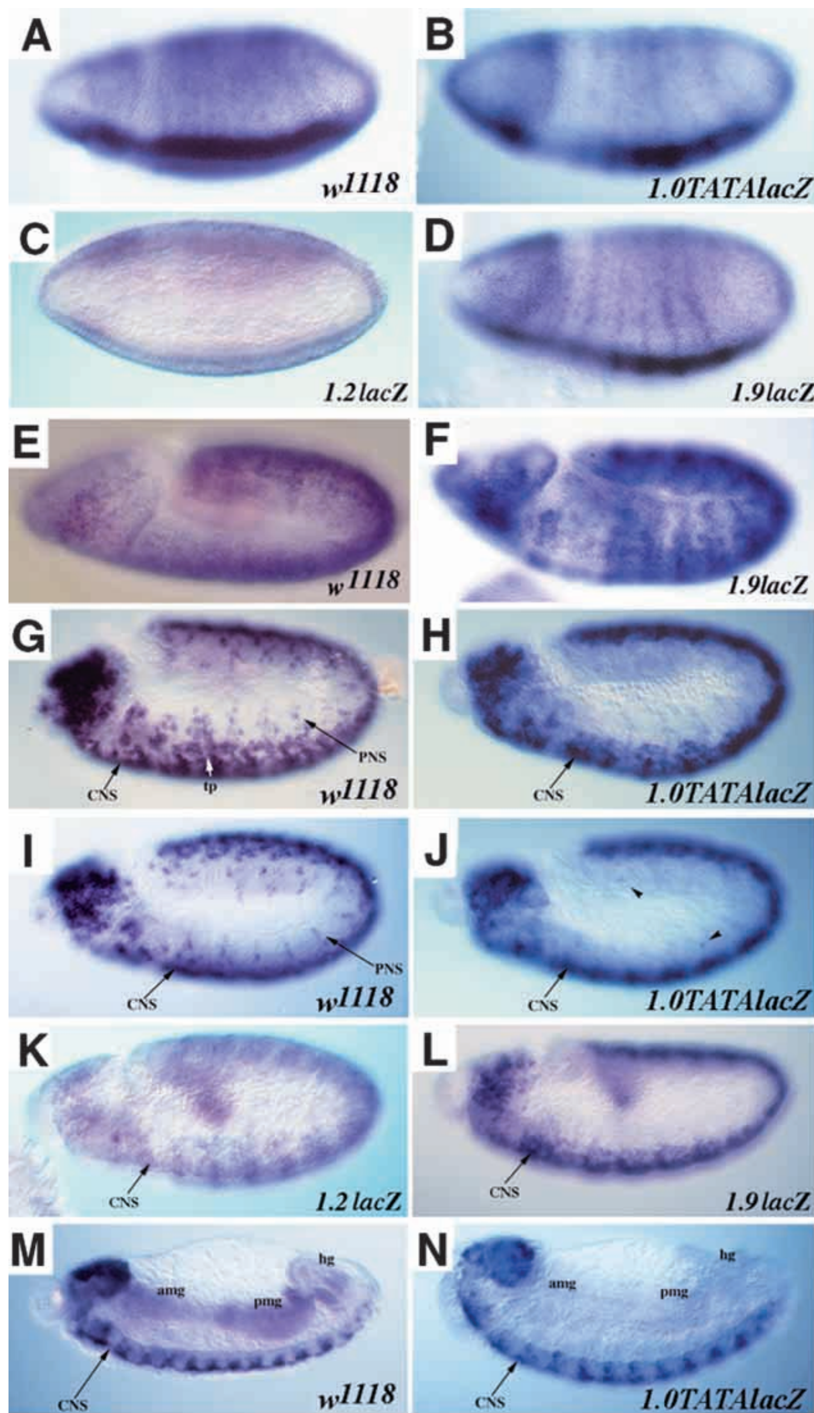

Fig. 4. *DmcycE cis*-acting elements that regulate expression in the CNS and epidermis during embryogenesis. Whole-mount in situ hybridisation using a *DmcycE* or *lacZ* reporter gene probe as indicated. (A-D) Stage 5-6 embryos. (A) w^{1118} embryo, *DmcycE* probe; (B) *1.0TATAlacZ* embryo, *lacZ* probe; (C) *1.2lacZ* embryo, *lacZ* probe; (D) *1.9lacZ* embryo, *lacZ* probe. (E,F) Late stage-9 embryos. (E) w^{1118} embryo, *DmcycE* probe, showing general epidermal expression of *DmcycE*. (F) 1.9*lacZ* embryo, *lacZ* probe, showing general epidermal expression of the *lacZ* reporter gene. (G,H) Stage-11 embryos. (G) w^{1118} embryo, *DmcycE* probe; (H) *1.0TATAlacZ* embryo, *lacZ* probe. Note the lack of PNS and epidermal thoracic patch transcripts. (I-L) Late stage-11 embryos. (I) w^{1118} embryo, *DmcycE* probe. Note that epidermal thoracic patch staining has gone by this stage; (J) *1.0TATAlacZ* embryo, *lacZ* probe showing a few faintly stained PNS cells indicated by arrowheads; (K) *1.2lacZ* embryo, *lacZ* probe; (L) *1.9lacZ* embryo, *lacZ* probe. (M,N) Stage-13 embryos. (M) w^{1118} embryo, *DmcycE* probe; (N) *1.0TATAlacZ* embryo, *lacZ* probe. Note the absence of transcripts in the endoreplicative gut. CNS, central nervous system; PNS, peripheral nervous system; tp, thoracic patch; amg, anterior midgut; pmg, posterior midgut; hg, hindgut.

thoracic patches. Regulatory elements that are required for the complete pattern of *DmcycE* transcription in the epidermal thoracic patches are located between the 3′ breakpoints of the $DmcycE^{19L}$ deletion and a group of small *P*-element-mediated deletions represented by $DmcycE^{7L}$ in Fig. 1. Embryos that are homozygous for these small deletions have a wild-type *DmcycE* transcription pattern in the CNS, PNS and the epidermal thoracic patches (results not shown). The locations of regulatory elements defined by these deficiencies are consistent with the locations determined by the analysis of the *P[w⁺ TI]* transgene.

Reporter gene analysis further defines *DmcycE* tissue-specific *cis*-acting regulatory elements

To confirm and extend the results obtained from the previous analyses, a series of *DmcycE* promoter-*lacZ* reporter gene constructs (summarised in Fig. 1) were generated and introduced into flies using *P*-element-mediated germline transformation. All but one of the *lacZ* reporter constructs contained the immediate endogenous zygotic promoter sequences and the entire *DmcycE* 5′ untranslated region (UTR) fused to the ATG of an open reading frame encoding a nuclear targeted β-galactosidase. The endogenous *DmcycE* promoter was favoured over a heterologous minimal TATA promoter because zygotic *DmcycE* transcription is under the control of a TATA-less promoter (L. Jones, M. Silson, W. Winnall, R. Saint and H. E. Richardson, unpublished). The only reporter gene construct in which a heterologous promoter was used contained the promoter proximal 1.0 kb *Hin*dIII/*Eco*RI genomic fragment joined to the *hsp27* TATA box and *lacZ* sequences (the *1.0TATAlacZ* construct, Fig. 1). With one exception, embryos homozygous for at least three independent insertions of each transgene were characterised using whole-mount in situ hybridisation to determine the pattern of *lacZ* reporter gene transcription. The exception was the *16.4lacZ* construct, where only one line was characterised. The activity of this transformant was assumed to be unaffected by local chromosome position effects, as the *lacZ* expression patterns produced were consistent with those of other constructs.

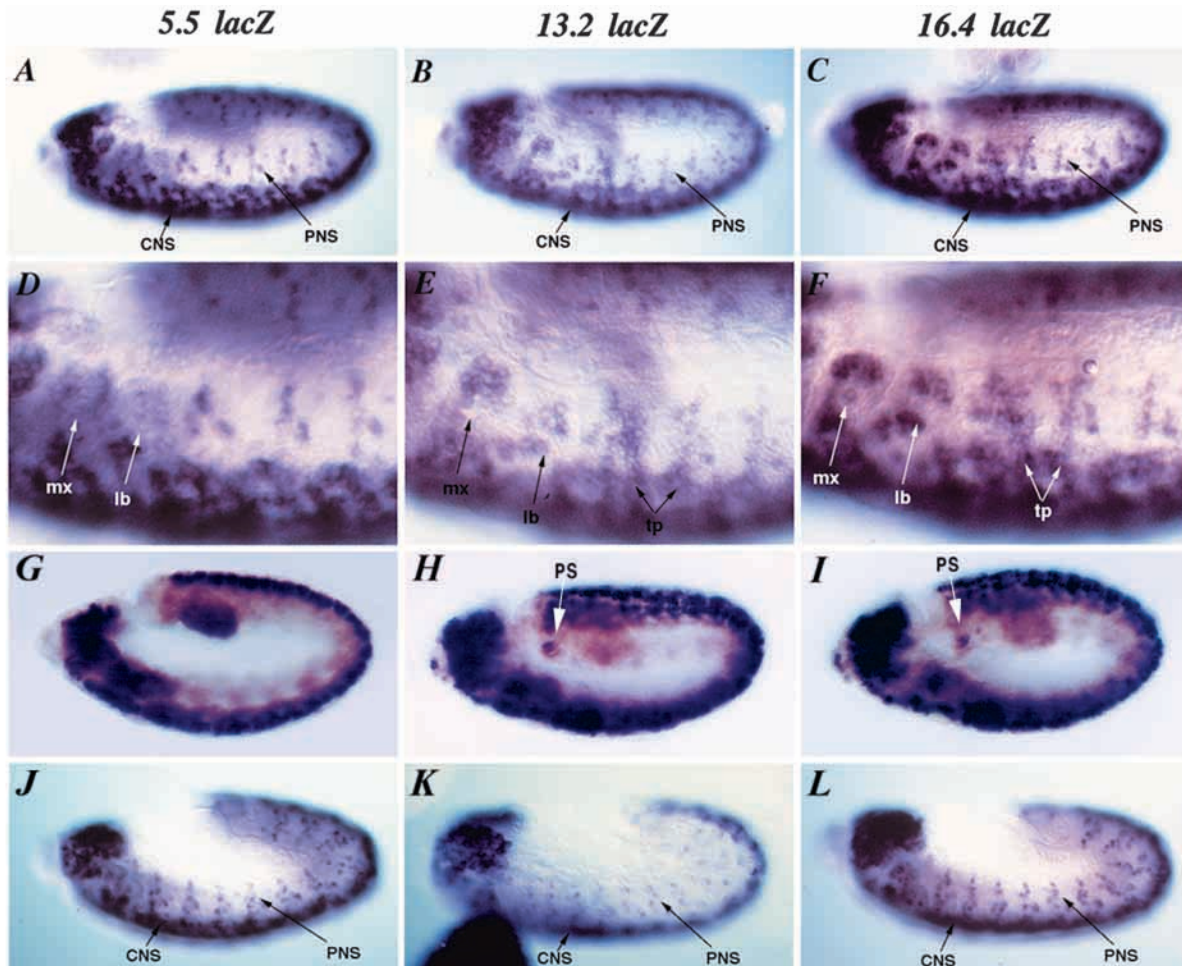

Fig. 5. *DmcycE cis*-acting elements that regulate expression in the PNS and epidermal thoracic patches. Whole-mount in situ hybridisation using a *lacZ* probe in stage-11 embryos (A-F). (A) *5.5lacZ* embryo showing expression in the CNS and PNS cells; (B) *13.2lacZ* embryo showing expression in the CNS and PNS cells; (C) *16.4lacZ* embryo showing expression in the CNS and PNS cells; (D) higher magnification of the *5.5lacZ* embryo shown in A. Expression is absent in the neuroblasts of the maxillary and labial segments as well as in the epidermal thoracic patches. (E) Higher magnification of the *13.2lacZ* embryo shown in B, exhibiting expression in the CNS and PNS cells, including the neuroblasts of the maxillary and labial segments and expression in epidermal thoracic patch cells. (F) Higher magnification of the *16.4lacZ* embryo shown in C, exhibiting expression in the CNS and PNS cells, including the neuroblasts of the maxillary and labial segments and expression in epidermal thoracic patch cells. (G-I) Late stage-11 embryos. (G) 5.5*lacZ* embryo showing absence of expression in the posterior spiracle primordium; (H) 13.2*lacZ* embryo showing expression in the posterior spiracle primordium; (I) 16.4*lacZ* embryo showing expression in the posterior spiracle primordium. (J-L) Stage-12 embryos. (J) *5.5lacZ* embryo showing expression in the CNS and PNS cells; (K) *13.2lacZ* embryo showing expression in the CNS and PNS cells; (L) *16.4lacZ* embryo showing expression in the CNS and PNS cells. See Figs 2, 4 for abbreviations.

CNS and early epidermal expression

The *1.0TATAlacZ* heterologous promoter construct and the *1.9lacZ* endogenous promoter construct extend the same distance 5′ from the start of zygotic *DmcycE* transcription (Fig. 1). The patterns of *lacZ* transcripts derived from these two constructs are essentially identical (Fig. 4 and data not shown), indicating that there was no obvious difference between the endogenous *DmcycE* and heterologous *hsp27-TATA* promoters.

Expression of the *lacZ* reporter gene from the *1.0TATAlacZ* and *1.9lacZ* endogenous promoter constructs was detected in the early epidermis (Fig. 4A,B,D-F) and also in the proliferating CNS (Fig. 4G-J,L-N) in a pattern similar to that normally seen for *DmcycE*. This indicates that *cis*-acting sequences required for driving *DmcycE* transcription in the epidermis during cycles 14-16 and in the CNS cells are located within this 1.0 kb fragment. Significantly, epidermal reporter gene expression was downregulated by the end of stage 11 (Fig. 4J,L, compare with G), suggesting that the sequences driving expression of the reporter constructs include the regulatory elements responsible for the downregulation of wild-type *DmcycE*.

In contrast to the normal pattern of epidermal and CNS expression from the *1.0TATAlacZ* and *1.9lacZ* constructs, there was a dramatic absence of *lacZ* transcripts in most of the proliferating PNS cells. Very low levels of *lacZ* expression were detected in one or two PNS cells per segment (Fig. 4G-J,L). This result is consistent with analysis of the $DmcycE^{12L}$ regulatory region deletion (see above) and indicates that *DmcycE* transcription in the CNS and PNS cells is regulated by different *cis*-acting sequences. In addition, *lacZ* transcripts

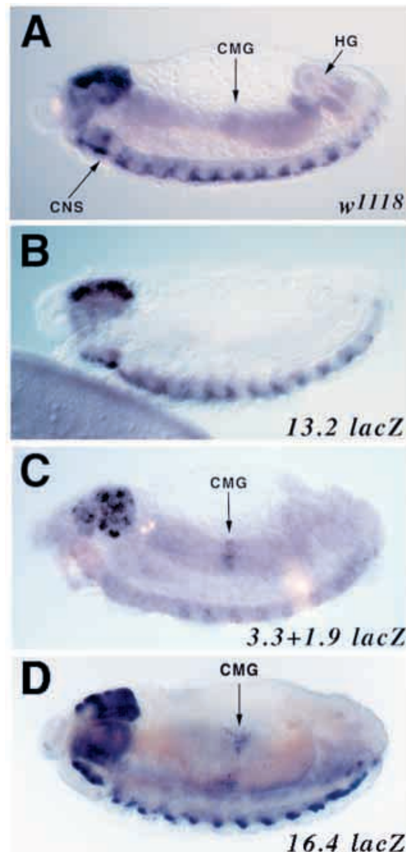

Fig. 6. *DmcycE cis*-acting elements that regulate expression in the endoreplicative gut. Whole-mount in situ hybridisation using a *DmcycE* or *lacZ* reporter gene probe as indicated, showing reporter gene expression in an endoreplication domain in the central midgut driven by sequences more than 12kb 5′ of the transcription start site. All embryos are shown at stage 13. (A) w^{1118} embryo, *DmcycE* probe; (B) *13.2lacZ* embryo, *lacZ* probe; (C) *3.3+1.9lacZ* embryo, *lacZ* probe; (D) 16.4*lacZ* embryo, *lacZ* probe. CMG, central midgut; HG, hindgut; CNS, central nervous system.

were not detected in the epidermal thoracic patches nor in endoreplicating tissues (Fig. 4G,H,M,N), suggesting that essential regulatory sequences controlling *DmcycE* transcription in these tissues are located outside of the 1.0 kb *Hin*dIII/*Eco*RI genomic fragment, again consistent with the phenotypic analysis of deficiencies and the genomic fragment described above (summarised in Fig. 7). Consistent with all necessary epidermal and CNS elements being localised to the 1.0 kb *Hin*dIII/*Eco*RI fragment, all larger constructs containing this region exhibited expression of *lacZ* in the epidermis during mitotic cycles 14-16 and in the proliferating CNS cells (data not shown).

Embryos homozygous for the *1.2lacZ* construct, which contains approximately the last 400 bp of the 1.0 kb *Hin*dIII/*Eco*RI fragment, expressed *lacZ* at a very low level in both the epidermis and the CNS (Fig. 4C,K). This suggests that the enhancers necessary for this pattern of *DmcycE* transcription are located in this 400 bp fragment. However, as the level of *lacZ* transcripts is markedly reduced relative to the *1.0TATAlacZ* and *1.9lacZ* constructs, regulatory elements that increase the level of transcription in this pattern must be located in the remaining 600 bp region of the 1.0 kb *Hin*dIII/*Eco*RI fragment.

PNS expression

The *5.5lacZ* construct is the smallest endogenous promoter construct that gave a pattern of *lacZ* expression in PNS cells similar to that observed for wild-type *DmcycE* (Fig. 5A,D,J). Interestingly, the pattern of PNS *lacZ* transcripts was not complete as it lacked expression in PNS neuroblasts of the maxillary and labial segments (Fig. 5D). This result is consistent with the pattern of zygotic *DmcycE* transcription observed from the 10.7 kb *Kpn*I *P[w⁺ TI]* genomic rescue construct, which extends to the same 5′ *Kpn*I restriction enzyme site used in the *5.5lacZ* construct. The next smallest endogenous promoter construct, the *2.9lacZ*, fails to express *lacZ* in the PNS in all but 1 or 2 cells per segment (data not shown), so the regulatory sequences responsible for driving *DmcycE* transcription in proliferating thoracic and abdominal PNS cells must be located in a 2.6 kb *Kpn*I/*Nco*I genomic fragment (summarised in Fig. 7).

Embryos homozygous for either the *13.2lacZ* or the *16.4lacZ* endogenous promoter constructs showed *lacZ* transcripts in all of the proliferating PNS cells (Fig. 5B,C,E,F,K,L). This pattern was indistinguishable from the wild-type *DmcycE* PNS expression pattern, since *lacZ* transcripts were now present in the maxillary and labial segment PNS neuroblasts (Fig. 5B,C,E,F). The detection of *lacZ* PNS transcripts in the maxillary and labial head segments from the *13.2lacZ* and *16.4lacZ* constructs and the absence of this pattern from the *5.5lacZ* construct indicate that enhancer sequences necessary for *DmcycE* transcription in the maxillary and labial segment PNS cells are located in the 7.7 kb *Nhe*I/*Kpn*I genomic fragment (summarised in Fig. 7).

Expression in cycle 17 thoracic epidermal cells

lacZ transcripts in the epidermal thoracic patches were observed in embryos homozygous for the *13.2lacZ* and *16.4lacZ* promoter constructs (Fig. 5B,C,E,F). The pattern is similar to wild-type *DmcycE* transcription in the thoracic patches in the epidermis of the first and second thoracic segments. *DmcycE* expression can also be observed in a small patch in the ventral epidermis of the third thoracic segment, but this expression occurs in a much smaller group of cells and more transiently than in the first and second thoracic patches. As a result it was very difficult to observe and was not analysed in detail. As *lacZ* transcripts in the epidermal thoracic patches were not observed from embryos homozygous for the *5.5lacZ* construct (Fig. 5A,D), sequences required for driving *DmcycE* transcription in the epidermal thoracic patches must be located within the 7.7 kb *Nhe*I/*Kpn*I fragment (see Fig. 7). The location of this *cis*-acting regulatory element is consistent with the analysis of *DmcycE* transcription in embryos homozygous for the $DmcycE^{12L}$ regulatory region deletion, which removes this 7.7 kb *Nhe*I/*Kpn*I genomic fragment and lacks *DmcycE* transcription in the epidermal thoracic patches (see above).

Expression in the posterior spiracle primordia

Stage-11 and -12 embryos express *Rnr2* in the posterior spiracle primordia prior to S phase entry at this stage (Duronio and O’Farrell, 1995). *DmcycE* is also expressed in these cells (data not shown). *LacZ* transcripts in the posterior spiracle

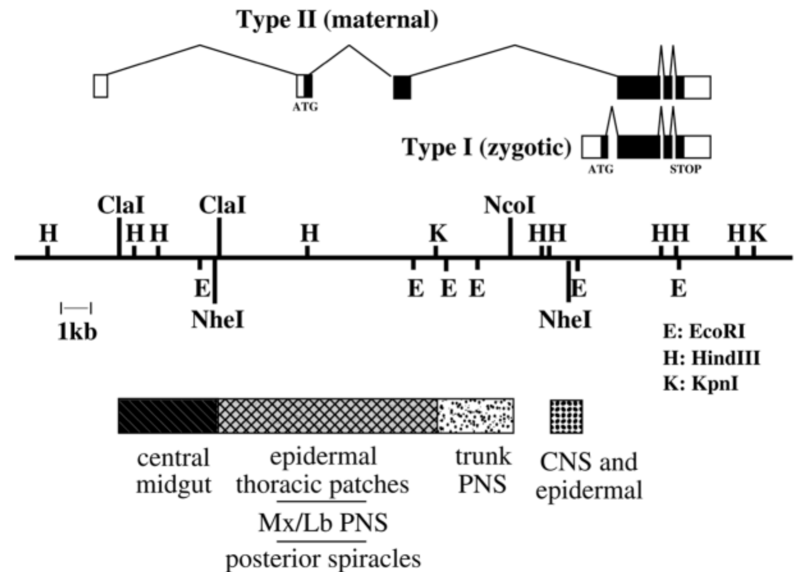

Fig. 7. The *DmcycE* genomic region and identified regulatory elements. Diagrammatic representation of the *DmcycE* genomic region, summarising the locations of tissue-specific *cis*-acting regulatory sequences, defined in this study, in relation to the *DmcycE* transcription unit. Note that this is not a complete map of the enzymes indicated. Only the *Nco*I *Nhe*I *Cla*I and *Kpn*I sites used to generate the constructs are indicated.

primordia were observed in embryos carrying the 13.2*lacZ* and *16.4lacZ* constructs (Fig. 5H,I), but not in embryos carrying the 5.5*lacZ* construct (Fig. 5G). This indicates that the regulatory region responsible for *DmcycE* expression in the posterior spiracle primordia is located within the 7.7 kb *Nhe*I/*Kpn*I fragment, the same region that contains the regulatory sequences required for expression in the epidermal thoracic patches.

Expression in endoreplicating tissues

Analysis of the series of *DmcycE* promoter-*lacZ* reporter constructs, up to and including the *13.2lacZ* construct, failed to identify the location of any regulatory elements responsible for driving *DmcycE* transcription in the endoreplicating tissues (Figs 4N, 6B; data not shown), indicating that such elements are located outside of the 13.2kb *Nhe*I/*Nco*I region covered in this analysis. This conclusion is consistent with our observations that the 10.7 kb *Kpn*I *P[w+ TI]* rescue construct exhibits no detectable *DmcycE* expression in endoreplicating cells (see Fig. 2G).

Interestingly, analysis of two *DmcycE* promoter-*lacZ* reporter constructs has identified a 3.3 kb *Cla*I genomic fragment that contains regulatory elements important for transcription in a subset of the endoreplicating midgut. Embryos homozygous for either the *16.4lacZ* construct or the *3.3+1.9lac*Z (see Fig. 1), show a low level of *lacZ* expression in cells of the embryonic central midgut (Fig. 6C,D), which is not present in embryos containing the *13.2lacZ* construct (Fig. 6B). However, regulatory elements that are required for *DmcycE* transcription in the anterior and posterior midgut, hindgut, salivary glands and Malpighian tubules remain to be identified.

DISCUSSION

We have previously shown that regulated *DmcycE* transcription is required to maintain the normal pattern of S phases during *Drosophila* development (Knoblich et al., 1994; Richardson et al., 1995; Secombe et al., 1998). However, the mechanism by which developmental signals and patterning cues are integrated to coordinate *DmcycE* transcription could occur in many ways. For example, *DmcycE* could be regulated in a relatively simple manner, with multiple developmental signals inducing the expression of a common *trans*-acting factor that acts on a small number of *DmcycE* regulatory elements. Alternatively, developmental regulation of *DmcycE* transcription may be more direct, with a complex set of *DmcycE cis*-regulatory elements being responsible for integrating developmental signals and patterning cues.

The data presented in this study supports the second hypothesis. Our analyses revealed the presence of multiple independent tissue-specific regulatory elements acting to control *DmcycE* transcription during embryogenesis. These include (1) at least two different elements required for expression in different cells of the peripheral nervous system, (2) an element required for expression in the epidermal cells and central nervous system and (3) an element required for expression in patches of thoracic epidermal cells that undergo a 17th G_1 phase-regulated cycle, in the PNS of the labial and maxillary segments and in the posterior spiracle primordia. These elements were identified by the presence or absence of tissue-specific expression in a genomic transformant, in animals carrying small regulatory region deletions generated by *P*-element excisions and in genomic fragment-reporter gene constructs. The results obtained with each of these different approaches were consistent with the others in terms of the location of the different elements. One notable group of elements that were not fully defined in this study are those responsible for *DmcycE* expression in endoreplicating tissues. Only one element was identified that was responsible for driving *DmcycE* transcription in the central midgut. Presumably elements responsible for regulating the remaining patterns of *DmcycE* in embryonic endoreplicating tissues lie further 5′ or 3′ of the genomic regions examined in this study. Our failure to define more than the one endoreplicative regulatory sequence is surprising. It is possible that other regulatory sequences lie outside the region studied, but it is also possible that identification of these domains may be hampered by the negative autoregulatory nature of *DmcycE* expression in

these endoreplication domains (Duronio and O'Farrell, 1995; Sauer et al., 1995). If the elements carrying the endoreplication regulatory sequences also carry the autorepression sequences, expression may be inhibited by the endogenous *DmcycE*. It should also be noted that additional complexity is likely to exist in the regulation of *DmcycE* transcription. For example, *DmcycE* was expressed in a subset of the epidermal thoracic patch cells in *DmcycE*^{19L} deficient embryos, indicating that these patches may themselves be complex domains of *DmcycE* transcriptional regulation.

Although our analyses have identified separable tissue-specific *cis*-acting elements in the *DmcycE* regulatory region, we have yet to elucidate the factors that operate on these elements to drive *DmcycE* transcription. Some clues to the *trans*-acting factors that could be regulating *DmcycE* transcription come from studies of cell cycle control mechanisms in mammalian cells, where *cyclin E* transcriptional regulation also plays an important role in control of the G_1 to S phase transition (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). A well-established model of G_1 phase transcriptional regulation in mammalian cells postulates a cascade of events initiated by extracellular growth factor signalling that leads to activation of the Cyclin D/Cdk4 complex, which in turn phosphorylates the tumor suppressor Retinoblastoma (Rb), disassociating it from the S phase-specific transcription factors E2F and DP and allowing transcription of S phase-specific genes, such as *cyclin E* (reviewed in Dyson, 1998).

In *Drosophila*, where we have an opportunity to examine G_1 phase-regulatory events within a developmental context, *dE2F* and *dDP* are required for embryonic *DmcycE* transcription in endoreplicating tissues, but are dispensable for *DmcycE* transcription in the CNS divisions that lack G_1 and G_2 phases (Duronio and O'Farrell, 1995; Royzman et al., 1997; Duronio et al., 1998). It is not known whether *dE2F* and *dDP* are required for *DmcycE* expression in the early embryonic PNS and epidermal divisions, because maternal products of these genes may mask any requirement. Nonetheless, the fact that these transcription factors are dispensable for *DmcycE* transcription in the CNS cells argues against the universality of the mammalian model of *cyclin E* regulation by E2F. In addition, the mammalian model predicts that E2F-dependent transcriptional regulation of *DmcycE* in endoreplicating tissues should be mediated through an E2F/DP responsive element. In the experiments described here, we have shown that this model is an oversimplification of *DmcycE* regulation in *Drosophila*, as only one element in the 16 kb analysed was found to drive *DmcycE* expression in a subset of endoreplicating tissues. Thus a minimum of two regulatory elements are required for driving *DmcycE* expression in endoreplicating tissues. The fact that expression of *DmcycE* in these tissues has been shown to be dependent on the E2F/DP complex appears at first sight to be paradoxical. The paradox would be resolved if expression requires activation by both E2F/DP and tissue-specific activators. An absolute requirement for a developmental signal cannot however exist, as ectopic expression of E2F and DP together induces *DmcycE* expression in all G_1-arrested epidermal cells (Duronio et al., 1996). It remains possible that the high levels of E2F and DP expressed following heat shock induction of the respective transgenes overrides the tissue-specific regulatory component.

Downregulation of *DmcycE* transcription during cycle 16 is essential for cycle 17 G_1 arrest in epidermal cells prior to differentiation (Knoblich et al., 1994). Significantly, the regulatory element that drives constitutive *DmcycE* expression in the epidermal cells during cycles 14-16 showed transcriptional downregulation, characteristic of wild-type *DmcycE* expression. If this downregulation requires active repression of *DmcycE* transcription, then the regulatory sequences necessary for this repression must also be located in the 1.0 kb regulatory element defined here. Alternatively, the downregulation could be a consequence of the loss of activation of *DmcycE* transcription in the epidermis. Interestingly, a study by Du and Dyson (1999), revealed that *DmcycE* is ectopically expressed in a subset of normally G_1-arrested epidermal cells in embryos that are deficient for *RBF* (the *Drosophila Rb* homolog). However, *DmcycE* transcription is initially downregulated normally and a G_1 cycle 17 arrest is established in the absence of RBF. These data suggest that *DmcycE* is actively repressed in G_1-arrested epidermal cells and that RBF is required to maintain this repression. Although *dE2F* or *dDP* do not result in ectopic *DmcycE* transcription in epidermal cells (Duronio and O'Farrell, 1995; Royzman et al., 1997; Duronio et al., 1998) a RBF/E2F/DP complex may still mediate this repression if maternal E2F and DP are not depleted in the respective mutant embryos at this stage. A transcriptional repression mechanism of *DmcycE* at this stage may also be mediated by the second *Drosophila* E2F, E2F2, which has been shown to act as a transcriptional repressor of S phase genes in tissue culture cells (Sawado et al., 1998). Alternatively, E2F and/or DP may be needed to activate ectopic *DmcycE* expression in the absence of RBF. The initial mechanism acting to downregulate *DmcycE* transcription remains to be determined. Further dissection of this regulatory element may identify separate activation and repression sequences.

The regulators of *DmcycE* transcription in other developmental contexts may be more difficult to identify, as many candidate genes may be involved. In some cases it is possible to suggest the involvement of particular regulatory genes. For example, the discrete expression of *DmcycE* in the epidermal thoracic patches, which undergo a G_1-regulated 17th cell cycle, is much stronger in the first thoracic segment and is absent in the abdominal segments. The products of the homeotic genes of the Bithorax and Antennapedia Complexes, which are key components of anterior/posterior patterning (Heuer and Kaufman, 1992; Castelli-Gair and Akam, 1995) are therefore candidate upstream regulators of *DmcycE* transcription in this tissue.

Complex transcriptional regulation of cell cycle regulatory genes: a common theme?

Our understanding of cell cycle regulation has primarily derived from the single cell yeast, from cultured cells and from oocytes or very early embryos in which the complex patterning of embryogenesis has not yet begun. In contrast, cell cycles in the embryos of a multicellular organism respond to a variety of developmental cues that give different tissue types and different cell cycle kinetics. The significance of the relationship between embryo patterning and cell cycle control is evident from the pioneering work of Edgar and O'Farrell (1989, 1990), in which regulation of the cycles that occur during gastrulation

and germ band extension in *Drosophila* embryos was shown to be mediated by the transcriptional regulation of the *string* (*stg*) mitotic activator gene. This transcriptional regulation is mediated by the patterning genes active during these stages.

The *stg* mitotic inducer gene exhibits a remarkably complex transcriptional regulatory region that responds to a variety of patterning genes to control cell cycle progression during early embryogenesis (Edgar et al., 1994; Lehman et al., 1998). The analysis of *DmcycE* regulation presented here offers an interesting parallel with *stg* in that both genes exhibit unexpected complexity in transcriptional regulation. It is striking that both *DmcycE* and *stg*, the first cell cycle regulators to have their transcriptional regulation examined in a developmental context, contain tissue-specific regulatory elements. This indicates that transcriptional regulation is mediated differently in distinct developmental contexts, such as during the complex events of gastrulation when cycles 14-16 occur or during the 17th cycle in cells of the epidermal thoracic patches.

Why is *DmcycE* transcriptional regulation so complex? One clue comes from the observation that the G_1 regulators *cyclin D*, *cyclin E*, *Rb* and *E2F/DP* are highly conserved between insects and mammals, but have no specific orthologs in yeast (Reed, 1996). Perhaps these genes represent a specialised mechanism that evolved to deal with G_1 phase regulation in multicellular organisms. Complex transcriptional regulation of cell cycle genes such as *DmcycE* may be part of such a mechanism, which permitted the universal eukaryotic cell cycle regulatory genes to be brought under the influence of the more recently evolved transcriptional regulatory mechanisms operating in different tissues and at different developmental stages.

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