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The Contribution of E2F-Regulated Transcription to *Drosophila PCNA* Gene Function

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Summary

E2F proteins control cell cycle progression by predominantly acting as either activators or repressors of transcription [1]. How the antagonizing activities of different E2Fs are integrated by *cis*-acting control regions into a final transcriptional output in an intact animal is not well understood. E2F function is required for normal development in many species [2–7], but it is not completely clear for which genes E2F-regulated transcription provides an essential biological function. To address these questions, we have characterized the control region of the *Drosophila PCNA* gene. A single E2F binding site within a 100-bp enhancer is necessary and sufficient to direct the correct spatiotemporal program of G1-S-regulated *PCNA* expression during development. This dynamic program requires both E2F-mediated transcriptional activation and repression, which, in *Drosophila*, are thought to be carried out by two distinct E2F proteins [2, 3, 8–11]. Our data suggest that functional antagonism between these different E2F proteins can occur in vivo by competition for the same binding site. An engineered *PCNA* gene with mutated E2F binding sites supports a low level of expression that can partially rescue the lethality of *PCNA* null mutants. Thus, E2F regulation of *PCNA* is dispensable for viability, but is nonetheless important for normal *Drosophila* development.

Results and Discussion

A 100-bp Enhancer Directs Patterned *PCNA* Expression during Development

Two sequences upstream of the *PCNA* transcription start site (Figure 1A) are capable of binding E2F in vitro [12, 13], and chromatin IP experiments have demonstrated that this region is occupied by both dE2F1 and dE2F2 in cultured SL2 cells [3]. The two E2F binding sites were previously reported to be necessary for *PCNA* expression in vivo, as determined by measuring the amount of β-Gal activity in whole-animal extracts from *PCNA-lacZ* transgenes [12]. However, this approach does not assess the contribution E2F binding sites make to endogenous patterns of gene expression that represent the complex spatiotemporal program of *PCNA* expression during development. To determine this, we engineered transgenic flies carrying *PCNA*-GFP fusion constructs (Figure 1B) and performed in situ hybridization of embryos carrying these transgenes with a GFP probe. The results indicate that a 100-bp sequence containing the two E2F binding sites reproduces the entire complex embryonic profile of cell cycle-correlated *PCNA* expression (see Figure S1, which is available in the Supplementary Material contained with this article online).

PCNA-GFP fusion protein (Figures 1D–1F) expression was also analyzed in several larval tissues by confocal microscopy. The final synchronous mitotic cycle prior to cell differentiation (called the "second mitotic wave") in third instar eye imaginal discs can be easily visualized as a stripe of cells that incorporate BrdU and that also express *PCNA* in an E2F-dependent fashion [3, 14, 15]. The accumulation of PCNA-GFP fusion protein from the reporter transgene faithfully reproduces this pattern of cell cycle control (Figure 1D). Similarly, Asano et al. have previously reported that E2F binding sites near the start of transcription are required for a virtually identical pattern of *ORC1* expression in eye discs [16]. In the optic lobe of the larval brain, PCNA-GFP expression correlates with the inner and outer proliferative zones, which are separated by a field of quiescent cells (Figure 1E) [17]. PCNA-GFP expression also accurately reports patterns of proliferation in the wing imaginal disc. Expression is absent in the zone of nonproliferating cells (ZNC) located at the juxtaposition of dorsal and ventral compartments in which E2F is thought to be inactive [16, 18], and it is present in the surrounding cells in a pattern consistent with known proliferation patterns in this part of the disc (Figure 1F). Thus, the 100-bp enhancer element containing two E2F binding sites accurately reproduces S phase-associated, E2F-dependent *PCNA* expression at many stages of *Drosophila* development. These transgenes should prove useful as tools to mark replicating cells in situ by using methods other than BrdU labeling (see, for example, [19]).

E2F/RBF Control of *PCNA* Expression Is Mediated through the 100-bp Enhancer

To test whether our *PCNA*-GFP constructs respond to genetic manipulations of E2F activity similarly to the endogenous gene, we examined GFP expression in different *dE2F1* or *RBF* mutant backgrounds [2, 8, 9, 20]. Mutation of *dE2F1* caused a loss of *PCNA*-GFP expression relative to phenotypically wild-type sibling controls at a stage at which endogenous *PCNA* expression is also lost in *dE2F1* mutants (Figures 2A and 2B). As with the endogenous gene, *PCNA*-GFP expression is not terminated on schedule in embryos lacking maternal and zygotic *RBF* function (Figures 2C and 2D), and this inappropriate expression persists in epidermal cells that are normally quiescent and do not express *PCNA* (Figures 2E and 2F). Simultaneous overexpression of dE2F1 and dDP via heat shock hyperactivates E2F and mimics loss of *RBF* function in embryos [21]. This treatment also resulted in ectopic *PCNA*-GFP expression in cells that

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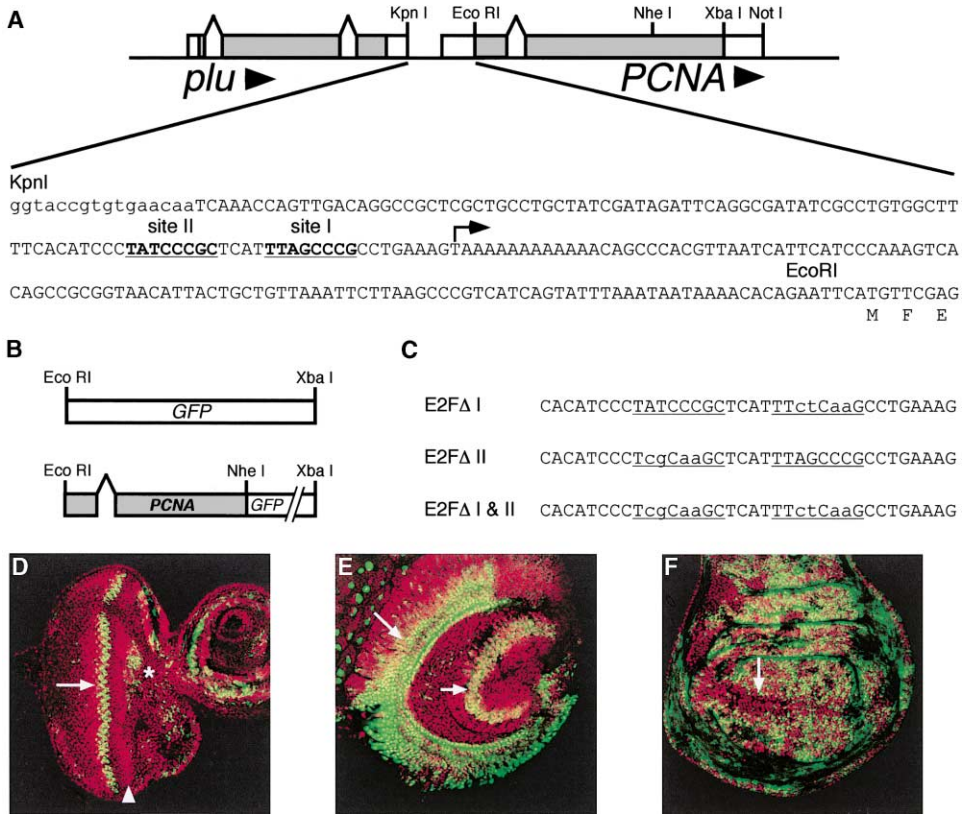

Figure 1. The *PCNA* Upstream Control Region Confers Correct Expression to a Heterologous Gene during Development

(A) A schematic of the *plutonium* (*plu*) and *PCNA* loci and nucleotide sequence of the *PCNA* promoter. Boxes represent exons, with the shaded portion indicating the protein coding regions (see the Supplementary Material for additional details). The nucleotide sequence of the control region and promoter is shown below the diagram. Lower case nucleotides indicate vector sequence, and upper case nucleotides indicate the endogenous *PCNA* sequence. The two E2F binding sites (numbered according to [12]) located just upstream of the *PCNA* transcription start site (bent arrow) are underlined.

(B) The two *PCNA*-GFP fusion constructs employed. One is a simple replacement of the *PCNA* coding region with the coding region of GFP, and the other is a protein fusion at the NheI site of *PCNA*. The pattern of GFP transcription is identical for each.

(C) Mutations that abrogate E2F binding are indicated by lower case nucleotides in each E2F binding site (underlined). These sequences are identical to the mutations engineered by Yamaguchi et al. [12] and were obtained by PCR of plasmids kindly provided by Masa Yamaguchi.

(D–F) *PCNA*-GFP reporter expression in larval tissues. Brains and imaginal discs dissected from *PCNA*-GFP third instar larvae were fixed and stained with propidium iodide to detect nuclei (red). GFP expression (green) was detected by illumination of the samples at 488 nm with a Zeiss 410 confocal microscope. (D) An eye imaginal disc with anterior oriented toward the right. The region of asynchronous cell proliferation anterior to the MF is indicated by an asterisk, and S phase of the second mitotic wave is indicated with an arrow. Note that PCNA-GFP expression does not occur in G1 cells within the MF (arrowhead). (E) Larval brain lobe. The long and short arrows indicate the outer and inner proliferative zones of the optic lobe, respectively. (F) A wing imaginal disc. The arrow indicates the anterior portion of the ZNC.

normally do not express the reporter (Figures 2G and 2H). Thus, our *PCNA*-GFP reporter constructs respond to genetic manipulation of embryonic E2F function in essentially the same way as the endogenous gene.

A Single E2F Site Mediates both Repression and Activation of *PCNA* In Vivo

Various GFP transgene reporters were engineered to test the contribution of each of the two E2F binding sites to *PCNA* expression in vivo (Figure 1C) [12]. Simultaneous mutation of site I and site II significantly reduced expression of the GFP reporter in embryos and in eye discs (Figures 3A, 3E, and 3F). Embryonic expression was reduced at all stages, including prior to germ band retraction (not shown) when zygotic mutation of *dE2F1* or *dDP* has little if any effect on endogenous *PCNA* expression [2, 8, 20]. This result suggests that maternal dE2F1/dDP drives *PCNA* expression during early embryogenesis. Mutation of just the E2F binding site nearest the start of transcription (called site I; Figures 1A and 1C) had no effect on *PCNA*-GFP expression in embryos or eye discs (Figures 3B, 3C, and 3G). Interestingly, a construct with a mutant site II, but retaining E2F binding site I, did not drive patterned GFP expression in either embryos or eye discs, but lacked detectable expression, as with the double site mutant construct (Figures 3D and 3H). These data indicate that site I and site II, which reside only 4 bp apart (Figure 1A), are not redundant for control of *PCNA* expression. Indeed, site II binds dE2F1 better than site I in gel shift experiments, and reporter constructs with a mutated site I are as active as wild-type in cell culture CAT reporter assays [12]. Moreover, this result indicates that a single E2F binding site can mediate both the activation and repression necessary to generate patterned *PCNA* expression.

Mutation of *dE2F2* results in slightly elevated levels

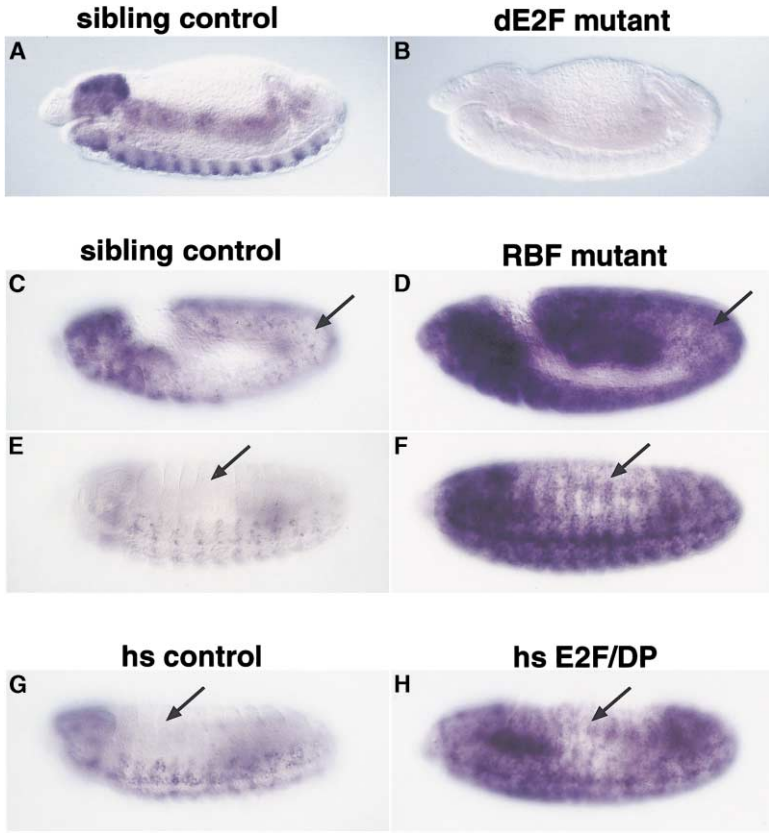

Figure 2. The *PCNA* Upstream Control Region Is Regulated by E2F/RBF

(A–H) All panels show whole-mount in situ hybridization of *PCNA*-GFP embryos (anterior oriented toward the left) using digoxigenin-labeled antisense RNA probes for GFP. (A), (C), and (E) each show embryos that are siblings (i.e., from the same collection and histochemical reaction) of the embryos shown in panels B, D, and F, respectively.

(A) Stage-13 phenotypically wild-type embryo.

(B) Stage-13 dE2F1^{7172} homozygous mutant embryo that lacks reporter gene expression.

(C) Stage 12 phenotypically wild-type embryo. The arrow indicates G1-arrested epidermal cells that lack *PCNA*-GFP reporter gene expression.

(D) Stage-12 maternally and zygotically *RBF* mutant embryo prepared as in [9]. The arrow indicates ectopic expression of the reporter in *RBF* mutant epidermal cells.

(E) Ventral/lateral view of a phenotypically wild-type stage-13 embryo. Arrow as in (C).

(F) Similar view of a stage-13 *RBF* mutant embryo. Arrow as in (D).

(G) Control embryo derived from a *yw*^{67} × *PCNA*-GFP cross subjected to a 30-min 37°C heat shock. Arrow as in (E).

(H) An embryo derived from a hsp70-dE2F1/dDP × *PCNA*-GFP cross subjected to a 30-min 37°C heat shock. The arrow indicates ectopic expression of the reporter in epidermal cells after simultaneous overexpression of E2F and DP.

of endogenous *PCNA* transcript in eye discs [3]. To determine the binding sites through which dE2F2 acts, the activity of each *PCNA* reporter construct was observed in *dE2F2* mutant eye discs by in situ hybridization. The expression of *PCNA*-GFP and *PCNA*-Δ site I-GFP in *dE2F2* mutant eye discs was very similar to wild-type (Figures 3I and 3K, respectively), suggesting that dE2F1 is sufficient to provide both activating and repressing activities necessary to generate the pattern of *PCNA* expression. *PCNA*-Δ site II-GFP was expressed

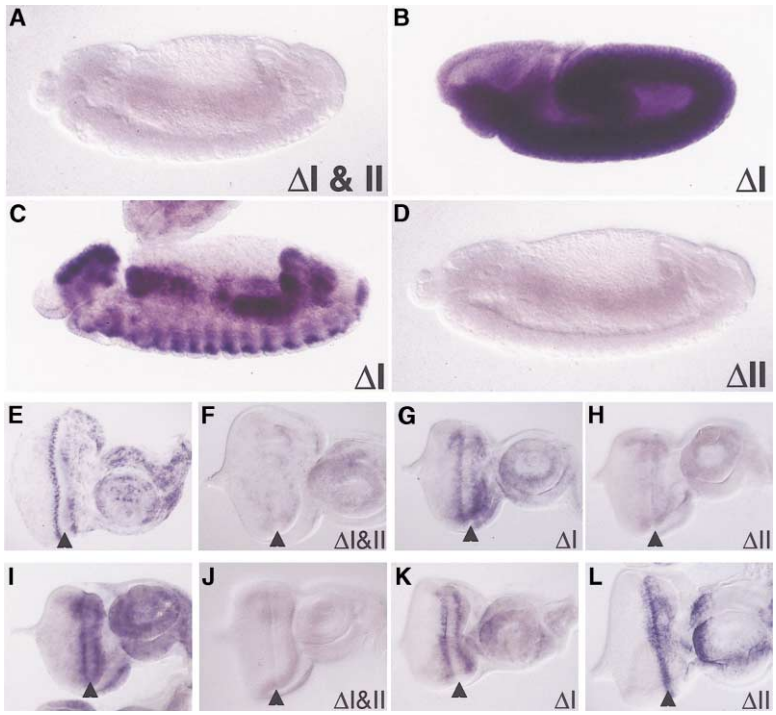

Figure 3. A Single E2F Binding Site Can Mediate Normal *PCNA* Expression

All panels show whole-mount in situ hybridization with a GFP probe.

(A) Stage-13 Δ site I&II *PCNA*-GFP embryo.

(B) Stage-9 germ band-extended E2F Δ site I *PCNA*-GFP embryo.

(C) Stage-13 germ band-retracted E2F Δ site I *PCNA*-GFP. Note that the pattern of GFP expression after mutation of E2F binding site I is normal (compare [B] and [C] to Figures S1B and S1D, respectively).

(D) Stage-13 E2F Δ site II *PCNA*-GFP embryo. For each of these experiments, three independent transgenic insertions were evaluated, and all gave similar results.

(E–H) Third instar eye/attenal discs carrying the indicated transgenes in a wild-type background.

(I–L) Third instar eye/attenal discs in a *dE2F2*^{329}/*dE2F2*$^{1-188}$ mutant background hand selected for genotype by using a Kr-GFP-expressing balancer chromosome. (E) and (I) contain *PCNA*-GFP. Arrowheads indicate the location of the morphogenetic furrow.

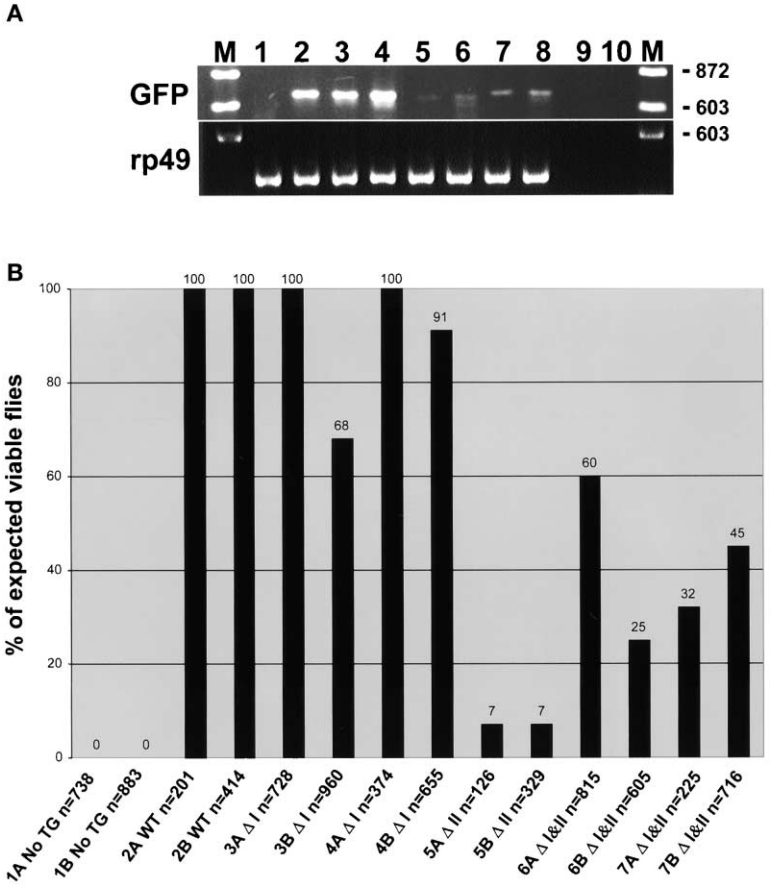

Figure 4. Complementation of *PCNA* Null Alleles with *PCNA* Transgene Constructs that Express at a Low Level

(A) Two-step RT-PCR was performed with equal amounts of total RNA prepared from 8- to 12-hr-old embryos with primers specific for GFP (top) or rp49 (bottom). M indicates lanes with DNA markers. Lane 1: yw^{67} control, lane 2: *PCNA*-GFP, lanes 3 and 4: two independent lines of *PCNA*-Δ site I-GFP, lanes 5 and 6: two independent lines of *PCNA*-Δ site II-GFP, lanes 7 and 8: two independent lines of *PCNA*-Δ site I&II-GFP, lane 9: RT-PCR with no input cDNA template, lane 10: RT-PCR with no input RNA. A total of 10% of the final PCR reaction was loaded in lanes 2–4, and 20% was loaded for all other lanes.

(B) Each bar along the *x* axis represents a cross between female flies heterozygous for a null allele of *PCNA* (i.e., *mus209*) and male flies heterozygous for the M173 deficiency, which deletes *PCNA*, either without (1, "No TG") or with (2–7) the indicated *PCNA* transgene. The *y* axis indicates the percentage of viable flies expected if all homozygous *PCNA* mutant flies actually eclosed (1/3 of the total, since balancer homozygotes are inviable). The indicated percentage values are based on the total number of adult flies that were scored (n), which were derived from many individual crosses. Experiments 2 and 5 represent data for a single wild-type and a single Δ site II transgene, respectively. Experiments 3 and 4 and 6 and 7 represent data for two independent Δ site I and Δ site I&II transgenes, respectively. Labels "A" and "B" refer to females of genotype $mus209^{D\text{-}292}$/SM1 and $mus209^{775}$/SM5, respectively [27]. Male genotypes were as follows. 1: Df(2R)M173/CyO, 2: Df(2R)M173/CyO; P{*PCNA*}, 3: Df(2R)M173/CyO; P{Δ site I-*PCNA*}-8, 4: Df(2R)M173/CyO; P{Δ site I-*PCNA*}-60, 5: Df(2R)M173/CyO; P{Δ site II-*PCNA*}-8, 6: Df(2R)M173/CyO; P{Δ site I&II-*PCNA*}-8, 7: Df(2R)M173/CyO; P{Δ site I&II-*PCNA*}-12. Crosses 3A, 4A, and 4B were not significantly different than wild-type, while all others were ($p < 0.0001$ using Fisher's Exact Test).

within the morphogenetic furrow of *dE2F2* mutant eye discs (Figure 3L), which is in contrast to the lack of expression of this reporter in wild-type eye discs (Figure 3H). This ectopic expression appears to require dE2F1, because *PCNA*-Δ site I&II-GFP was not expressed in *dE2F2* mutant eye discs (Figure 3J). Thus, dE2F1 and dE2F2 could compete for site I when site II is absent, suggesting that activating and repressing influences on the *PCNA* gene *can* act through the same E2F binding site. However, the relevance of this observation to endogenous *PCNA* regulation is unclear, since E2F binding site II alone is sufficient to drive the spatiotemporal pattern of *PCNA* expression. Perhaps *dE2F2* modulates the overall level of output of *PCNA* transcription by binding to site I, rather than whether the gene is fully repressed or not.

E2F Regulation Is Important, but Not Essential, for *PCNA* Gene Function

RT-PCR was used to directly measure whether loss of E2F binding sites could support some expression below the level detectable by cytological methods (Figure 4A). Whereas GFP transcripts were undetectable in wild-type embryos (lane 1), they were detected at similar levels in both *PCNA*-GFP and *PCNA*-Δ site I-GFP lines (lanes 2–4). GFP transcripts were also reproducibly detected in Δ site II (lanes 5 and 6) and Δ site I&II (lanes 7 and 8) lines compared to controls (lanes 1, 9, 10). In the Δ site I and Δ site I&II lines, the amount of GFP mRNA detected was lower than with *PCNA*-GFP or *PCNA*-Δ site I-GFP lines, although there was enough line to line variability within a single construct that we could not make conclusive quantitative comparisons between constructs. Nevertheless, these data indicate that the *PCNA* enhancer can support transcription in the absence of functional E2F binding sites. Similarly, simultaneous loss of *dE2F1* and *dE2F2* reduces, but does not eliminate, endogenous *PCNA* expression in the eye disc [3].

Chronic derepression in the absence of E2F proteins might provide sufficient expression to permit cell cycle progression. Thus, the essential function of the *PCNA* gene could possibly be provided in the absence of E2F-regulated transcription. To test this, we constructed *PCNA* minigenes that lacked one or both of the E2F binding sites (by fusing the site mutants used above to the *PCNA* coding region instead of GFP) and asked if these transgenes could complement the lethality of *PCNA* null mutants. As expected, both a wild-type control transgene and two independent Δ site I-*PCNA* transgenes fully complemented the lethality of *PCNA* null

mutant animals (Figure 4B). The two independent Δ site I&II-*PCNA* and one Δ site II-*PCNA* transgenes tested in this assay also complemented *PCNA* lethality, although less efficiently than wild-type or Δ site I transgenes (Figure 4B). The Δ site II-*PCNA* rescue was the least efficient (7% of expected for each of two *PCNA* alleles), although, since only a single line was tested, we cannot exclude an inhibitory effect from insertion position. Nevertheless, it is possible that E2F2-mediated inhibition through site I further inhibits expression from this transgene relative to the Δ site I&II-*PCNA* construct. In sum, while E2F regulation is not absolutely essential for *PCNA* gene function, it appears to provide a level of *PCNA* expression necessary for normal *Drosophila* development.

Conclusions

Loss of *dE2F1* function is lethal, and replication in cells that lack *dE2F1* is impaired relative to that in wild-type cells [2, 8, 20, 22, 23]. Because *dE2F1* is also required for the expression of genes encoding essential replication factors, *dE2F1*-mediated activation appears to be necessary for normal cell cycle progression. Nevertheless, sufficient *PCNA* function is provided in the absence of E2F regulation to support development. This expression may result from the absence of E2F/RBF-dependent repression and/or the presence of other factors (e.g., DREF [24]) that bind within the 100-bp enhancer and contribute to *PCNA* activation. *S. cerevisiae* cells can proliferate in the absence of cell cycle-regulated activation of transcription of replication factors [25]. Similarly, DNA synthesis in the second mitotic wave appears rather normal in eye imaginal discs lacking both *dE2F1* and *dE2F2* [3]. Here, direct control of cyclin E transcription by the Ci transcription factor in response to Hh signaling appears to control the onset of S phase [26].

If sufficient biosynthesis of essential replication factors like PCNA can be achieved in the absence of E2F, then why is E2F essential? Our data indicate that the absence of E2F-regulated *PCNA* expression is not tolerated very well. Thus, the optimal level of *PCNA* gene function requires E2F input. Since *PCNA* is not expected to provide a cell cycle regulatory role, per se, we suspect that the lack of cell cycle regulation and the consequent low level of ubiquitous expression is not the problem (see the Supplementary Material available with this article online). Rather, the lack of high-level expression achieved during S phase may attenuate DNA synthesis and inhibit normal development [15, 20]. This may be true for many E2F target genes, such that the coordinated loss of entire E2F-directed programs of gene expression is much more detrimental than the loss of E2F regulation of a single gene like *PCNA*.

Supplementary Material

Supplementary Material including data indicating that the 100-bp enhancer containing two E2F binding sites directs normal embryonic *PCNA* expression and data showing that PCNA protein can be detected in unreplicating cells that do not express PCNA mRNA is available at http://images.cellpress.com/supmat/supmatin.htm.

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