E2F mediates developmental and cell cycle regulation of ORC1 in *Drosophila*

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Throughout the cell cycle of *Saccharomyces cerevisiae*, the level of origin recognition complex (ORC) is constant and ORCs are bound constitutively to replication origins. Replication is regulated by the recruitment of additional factors such as CDC6. ORC components are widely conserved, and it generally has been assumed that they are also stable factors bound to origins throughout the cell cycle. In this report, we show that the level of the ORC1 subunit changes dramatically throughout *Drosophila* development. The accumulation of ORC1 is regulated by E2F-dependent transcription. In embryos, ORC1 accumulates preferentially in proliferating cells. In the eye imaginal disc, ORC1 accumulation is cell cycle regulated, with high levels in late G₁ and S phase. In the ovary, the sub-nuclear distribution of ORC1 shifts during a developmentally regulated switch from endoreplication of the entire genome to amplification of the chorion gene clusters. Furthermore, we find that overexpression of ORC1 alters the pattern of DNA synthesis in the eye disc and the ovary. Thus, replication origin activity appears to be governed in part by the level of ORC1 in *Drosophila*.

Keywords: cell cycle/*Drosophila*/E2F/origin recognition complex

Introduction

Study of the budding and fission yeasts has led to the following paradigm for the initiation of DNA synthesis in eukaryotes (Stillman, 1996). In *Saccharomyces cerevisiae*, origins of replication are bound throughout the cell cycle by a stable complex of six different proteins: the origin recognition complex (ORC) (Bell and Stillman, 1992; Diffley and Cocker, 1992). Early in G₁, the unstable regulatory factor CDC6 (CDC18 in *Schizosaccharomyces pombe*) is recruited to the origin-bound ORC (Cocker *et al.*, 1996). Addition of CDC6 is essential for the subsequent ordered addition of other factors, leading to the formation of a 'pre-replication complex' (pre-RC) which is poised to initiate DNA synthesis (Aparicio *et al.*, 1997; Donovan *et al.*, 1997; Tanaka *et al.*, 1997). Cell cycle regulators, including CDC7 and the cyclin-dependent kinases, then trigger initiation by mechanisms that are not yet fully understood. Following the onset of DNA synthesis, the RC is partially disassembled and a 'post-RC' consisting solely of the ORC remains bound to each origin. Thus, in budding yeast, initiation is regulated

neither by variations in the amount of ORC nor by the binding of ORC to origins. The abundance of ORCs does not appear to be regulated in *S.pombe* either (Muzi-Falconi and Kelly, 1995; Grallert and Nurse, 1996).

Most of our knowledge of ORC activity has come from studies of *S.cerevisiae* and *S.pombe*. Genes encoding ORC subunits have been identified in many other organisms (Gavin *et al.*, 1995; Gossen *et al.*, 1995), and it is generally believed that ORC function is conserved. ORCs have been purified from *Xenopus* and *Drosophila* (Gossen *et al.*, 1995; Rowles *et al.*, 1996). Although the properties of these complexes are not yet well defined, they appear to be essential for replication. Immunodepletion of ORCs blocks DNA replication in *Xenopus* egg extracts (Carpenter *et al.*, 1996; Rowles *et al.*, 1996) and mutations in *Drosophila* ORC2 cause a variety of DNA replication defects *in vivo* (Landis *et al.*, 1997). ORC2 is distributed uniformly among the cells of the early *Drosophila* embryo (Pak *et al.*, 1997). However, the regulation and distribution of other ORC constituents has not been analyzed in *Drosophila* or any other multicellular organism.

Previous work has shown that the level of ORC1 mRNA is regulated by E2F as cultured mammalian cells leave G₀ and enter S phase (Ohtani *et al.*, 1996), but not during subsequent G₁–S transitions (G.Leone and J.R.Nevins, personal communication). The E2F transcription factor is thought to play a role in regulating the transition from G₁ into S phase (reviewed by Dyson, 1998; Nevins, 1998). In response to proliferative signals in G₀ or G₁, E2F activity levels rise and the transcription of E2F-responsive genes is stimulated. These include genes that encode cell cycle regulators (e.g. *Cyclin E*, *Cyclin A*), enzymes required for the production of DNA precursors (e.g. *RNR*, *DHFR*) and replication factors (e.g. *ORC1*, *CDC6*, *MCMs*). Overexpression of E2F in serum-starved fibroblasts turns on the transcription of these target genes (DeGregori *et al.*, 1995) and drives the otherwise quiescent cells into S phase (Johnson *et al.*, 1993), demonstrating that elevated E2F levels can induce S phase entry.

The role of E2F is conserved between mammals and flies. Loss-of-function mutations in *Drosophila* E2F cause inefficient entry into S phase and proliferation defects (Royzman *et al.*, 1997; Duronio *et al.*, 1998). Conversely, overexpression of E2F in both embryos and imaginal discs drives otherwise quiescent cells into S phase (Asano *et al.*, 1996; Du *et al.*, 1996; Duronio *et al.*, 1996).

In this report, we investigate the regulation of ORC1 by E2F and the distribution of ORC1 during *Drosophila* development. Based on the experiments presented below, we draw three main conclusions. First, transcription of ORC1 is coupled to cell cycle progression via direct regulation by E2F. Secondly, the abundance of ORC1 is not constant, but varies during development and throughout the cell cycle. Thirdly, the level of ORC1 appears to limit

Fig. 1. Transcription of *ORC1* is E2F-dependent in embryos. The distributions of *ORC1* (top) and *RNR2* (bottom) mRNA as revealed by *in situ* hybridization in stage 13 wild-type (left) or *E2F⁻* (right) embryos. In wild-type embryos, both mRNAs are concentrated in the brain, the anterior midgut, the posterior midgut (arrowheads from left to right above), and the ventral nerve cord (arrowhead below). Much lower levels of both mRNAs are present in sibling *E2F⁻* embryos. Embryos are oriented anterior to the left, dorsal to the top throughout, except where noted otherwise.

origin utilization in at least two different cell types. Taken together, these observations suggest that *Drosophila* may regulate origin activity using additional mechanisms not found in the fission and budding yeasts.

Results

Transcription of ORC1 is regulated by E2F in embryonic and imaginal disc cells

Late in embryonic development, most cells enter an extended quiescent period, resuming DNA synthesis upon hatching. However, replication persists in three tissues (brain, ventral nerve cord, anterior- and posterior-midgut), and the mRNAs of E2F-regulated genes (such as *RNR2*) accumulate in these tissues (Duronio and O'Farrell, 1994). To determine whether transcription of *ORC1* is regulated by E2F, we examined the distribution of *ORC1* mRNA in wild-type and *E2F⁻* embryos by *in situ* hybridization. As shown in Figure 1, the distributions of *ORC1* and *RNR2* mRNAs are essentially the same in wild-type embryos at stage 13. Moreover, accumulation of either *RNR2* or *ORC1* mRNA is largely dependent on E2F function at this stage of development (Figure 1). Thus, transcription of *ORC1* is E2F-dependent in the embryo.

We next determined whether E2F regulates *ORC1* transcription in imaginal disc cells, which have canonical four-phase cell cycles. Since removing E2F function inhibits proliferation of the imaginal discs, we asked whether ectopic E2F synthesized under the control of the thermoinducible *hsp70* promoter could induce *ORC1* transcription. A brief heat pulse was administered to either control or *hsp70-E2F* transgenic larvae (Asano *et al.*, 1996), mRNA was prepared from imaginal discs at various time points, and the levels of various transcripts were measured by Northern analysis. As shown in Figure 2, accumulation of *ORC1* mRNA is induced following overexpression of E2F (~4-fold). By comparison, accumulation of three other E2F-regulated mRNAs—*PCNA*, *RNR1* and *RNR2*—is induced to a similar extent in these experiments (Figure 2). Thus, transcription of *ORC1* is also E2F-responsive in imaginal disc cells.

Binding of E2F to the ORC1 promoter positively regulates transcription in vivo

To determine whether the regulation described above is mediated by the direct action of E2F, we first isolated the

Fig. 2. Transcription of *ORC1* is regulated by E2F in imaginal discs. Northern blots of samples prepared from eye-antennal imaginal discs dissected from either wild-type larvae, which do not overproduce E2F (-), or from *hsp70-E2F* transgenic larvae which do (+). Samples were prepared either from unshocked larvae (t=0) or from larvae at 1.5 or 3 h after a 30 min heat shock (indicated above). The left panel shows the time course of *ORC1* mRNA accumulation. The right panel shows the relative level of three different E2F-regulated mRNAs 3 h after heat shock. (Similar kinetics were observed for all four E2F-regulated mRNAs.) The unregulated *rp49* mRNA serves as a loading control. In the experiment shown, *RNR1*, *RNR2* and *PCNA* mRNAs were induced by factors of 4, 4.5 and 3.5, respectively.

ORC1 promoter. Within the 400 nt upstream of the major transcriptional start site, four candidate E2F binding sites with similarity to the canonical site in the adenovirus *E2* promoter (TTTCGCGC) were identified by inspection, two at approximately -340 nt and two overlapping sites at -13 nt (Figure 3A). Characterization of other E2F-responsive promoters has shown that binding sites close to the transcriptional start site frequently play a predominant role in regulation (Fry *et al.*, 1997), and thus we focused on the overlapping sites at -13. To test whether E2F indeed can bind to these sites, we prepared two DNA fragments, one bearing nt -27 to +12 of the *ORC1* promoter and an otherwise identical mutant fragment bearing substitutions within the E2F binding sites. These DNAs were used as competitors in gel mobility-shift experiments in which the binding of E2F in *Drosophila* S2 cell extracts to overlapping E2F binding sites in the *Polα* promoter was assayed (Ohtani and Nevins, 1994). As shown in Figure 3B, the wild-type *ORC1* fragment, but not the mutant fragment, competes for binding to E2F. In similar experiments, radiolabeled wild-type *ORC1* promoter fragment binds to the E2F in these extracts (data not shown). Thus, *Drosophila* E2F binds to the *ORC1* promoter just upstream of the start site of transcription.

Fig. 3. Binding of E2F to the *ORC1* promoter activates transcription *in vivo*. (A) Sequence of the proximal *ORC1* promoter. The major transcriptional start site is shown with an arrow. The dashed lines indicate potential E2F binding sites, and the solid lines indicate the E2F binding sites mutated in the experiments described below. (B) E2F–DNA complexes detected in a gel mobility-shift experiment. In lanes 1, 3 and 5, complexes between the E2F in a nuclear extract prepared from S2 cells and a radiolabeled probe bearing the *Polα* E2F binding sites are visible near the top of the photograph, and unbound probe is visible near the bottom. Reactions also contained unlabeled competitors as follows: lane 1 (none), 2 (wild-type *ORC1*), 3 (mutant *ORC1*), 4 (wild-type adenovirus E2), 5 (mutant adenovirus E2). (C and D) Activity of the wild-type (C) and mutant (D) *ORC1* promoters in eye-antennal imaginal discs. Activity is revealed by the accumulation of Ftz–GFP–Myc-tag fusion protein. The numbers in (C) refer to four different regions of the eye disc, as described in the text. The morphogenetic furrow, marked in Figures 5 and 6 with arrowheads, corresponds to region 2. Seven independent lines of each transgene showed essentially identical expression patterns. (E) Activity of the wild-type *ORC1* promoter in part of a wing imaginal disc, visualized as described above. The ZNC, which is diagrammed schematically in (F), falls in the middle of the presumptive wing blade. The arrowheads in (E) and (F) point to a stripe of cells within the anterior compartment of the ZNC that are arrested in G₁. Note that the *ORC1* promoter is also inactive along the anteroposterior boundary. Discs are oriented posterior to the left throughout.

To test the role of these E2F sites *in vivo*, we prepared transcriptional reporter genes in which either the wild-type *ORC1* promoter or a mutant derivative bearing substitutions within the proximal E2F binding sites (described above) drives the expression of a cDNA encoding an unstable Ftz–GFP–Myc tag fusion protein (Figure 3C and D). As described below, the half-life of this fusion protein evidently is short with respect to the length of the cell cycle; thus, its accumulation monitors newly synthesized protein and permits examination of cell cycle-regulated transcription *in vivo*. The wild-type and mutant *ORC1* promoter reporters were reintroduced into flies by P element-mediated transformation, and the distribution of fusion protein was examined in transgenic flies.

As shown in Figure 3C and D, activity of the *ORC1*

promoter is dependent on the integrity of the E2F binding sites at –13 nt. In flies bearing the wild-type promoter construct, fusion protein is detectable in cells throughout most regions of the imaginal discs, with some notable exceptions that are described below. However, in flies bearing the mutant promoter construct, essentially no fusion protein is detectable in any of the imaginal discs. These observations suggest that E2F acts directly by binding to the *ORC1* promoter and stimulating transcription. Furthermore, the spatiotemporal pattern of *ORC1* promoter activation within two specialized groups of cells in the eye and wing imaginal discs supports the idea that E2F couples transcription of *ORC1* to cell cycle progression, as described below.

During the third larval instar, a developmentally regu-

Fig. 4. Modulation of ORC1 abundance in embryos. (A) Accumulation of ORC1 revealed by Western analysis. Samples were prepared from embryos of various ages (in hours after egg deposition at the top). (B–E) Distribution of ORC1 at various stages of development in wild-type embryos: (B) syncytial blastoderm, (C) the onset of gastrulation, (D) germ band extension, (E) germ band retraction, embryonic stage 13. Note that the protein is localized to nuclei throughout development. The arrowheads at the top of (E) highlight accumulation in the brain, anterior midgut, posterior midgut (from left to right), and the arrowhead at the bottom highlights accumulation in the ventral nerve cord. (F) Distribution of ORC1 in a sibling *E2F⁻* embryo at stage 13. Note that protein is detectable only in the brain at a low level (left arrowhead), and in the germ line precursor cells (right arrowhead). We assume that persistence of maternal mRNA or protein accounts for the presence of appreciable ORC1 in the germ line precursors, which are born when the embryo contains very high levels of both [i.e. earlier than the embryo shown in (B)].

lated cell cycle transition takes place as a wave of differentiation sweeps across the eye imaginal disc (Wolff and Ready, 1993). The wave front is marked by the morphogenetic furrow. During differentiation, four regions can be identified as follows (see Figure 3C). (1) Anterior to the morphogenetic furrow (including the antennal disc), undifferentiated cells cycle asynchronously. (2) As they enter the furrow, cells arrest in an extended G_1 phase. (3) Immediately posterior to the furrow, some cells are recruited into ommatidial pre-clusters and begin neural differentiation while others synchronously enter S phase. (4) Posterior to this synchronous wave of S phase, most cells cease cycling and terminally differentiate.

The pattern of *ORC1* promoter activity in the eye imaginal disc suggests that it is turned on late in G_1 , near the G_1 –S boundary. In particular, the *ORC1* promoter is activated in a random pattern among the asynchronous cells in the anterior region of the disc: turned off as cells enter the morphogenetic furrow and G_1 , turned on in cells as they emerge from the furrow late in G_1 phase, and then turned off in the quiescent cells in the posterior region of the eye (Figure 3C).

Another developmentally programmed cell cycle arrest has recently been described in the wing imaginal disc (Johnston and Edgar, 1998). At the dorsoventral boundary of the disc, *Notch* and *wingless* signaling establishes a zone of non-proliferating cells (ZNC) in which no S phase is detectable. Cells throughout the posterior ZNC and in the center of the anterior ZNC arrest late in G_1 , at a point when expression of *Cyclin E* can drive them into S; flanking cells in the anterior ZNC arrest in G_2 (Figure 3F). Among the cells of the ZNC, we find that the *ORC1* promoter is active only in G_1 -arrested cells and not in those arrested in G_2 (Figure 3E). These observations

support the idea that activation of E2F in G_1 stimulates transcription of *ORC1* in a variety of cell types.

Accumulation of ORC1 protein is coupled to proliferation and cell cycle progression

In *Drosophila*, many genes have been shown to be transcriptionally regulated by E2F during the G_1 –S transition. These include *Cyclin E*, *RNR*, *Pol α* , *PCNA*, *MCM2* and *MCM3* (Duronio and O'Farrell, 1994, 1995). However, only in the case of *Cyclin E* has it been shown that protein levels are cell cycle regulated (Richardson *et al.*, 1995), presumably at least in part as a consequence of E2F action. In the other cases, either the protein distribution has not been reported or the protein level is constant throughout the cell cycle. Therefore, to determine whether the level of ORC1 is modulated as a result of E2F-dependent regulation, we prepared antibodies that specifically recognize the protein and examined its distribution in embryos and imaginal discs.

Antisera were prepared by immunizing animals with glutathione *S*-transferase (GST) fusion proteins bearing three different portions of ORC1. Several lines of evidence suggest that the antibodies specifically recognize ORC1 both on Western blots and in fixed tissue. First, antisera raised against all three antigens in both rabbits and rats recognize a 120 kDa protein on Western blots (Figure 4A) that corresponds to ORC1 by several criteria (Materials and methods). Secondly, when used to detect ORC1 by standard immunohistochemical methods, many of these sera exhibit similar patterns in embryo and imaginal disc preparations. Thirdly, using transgenic flies in which transcription of an *ORC1* cDNA is driven by the *hsp70* promoter, ubiquitous induction of ORC1 is readily detectable both by Western blots and *in situ* (Figures 6, 7 and 9).

We initially examined the distribution of ORC1 during embryonic development. The first 13 nuclear division cycles that occur in a syncytium are parasynchronous. However, upon formation of the cellular blastoderm and the onset of gastrulation, this synchrony breaks down. Subsequent cell divisions are synchronous within cohorts of adjacent cells, but cell cycles within adjacent 'mitotic domains' are out of register (Foe *et al.*, 1993).

The distribution of ORC1 at various stages of embryonic development is shown in Figure 4. During the first 13 synchronous cell cycles, maternally synthesized ORC1 is uniformly distributed among the embryonic nuclei (Figure 4B). However, coincident with the formation of the cellular blastoderm and the onset of gastrulation, the ORC1 distribution changes dramatically, such that different nuclei contain very different levels of protein. For example, mesodermal precursors along the ventral midline, which comprise one of the mitotic domains, accumulate relatively high levels of protein at the onset of gastrulation (Figure 4C). During germ band extension, ORC1 levels are highest among the mitotically active neuroblasts and in domains of epidermal precursor cells (Figure 4D). Later, in stage 13 embryos, when most cells in the embryo are cell cycle arrested, ORC1 accumulates preferentially in cells of the nervous system and midgut that continue to cycle (Figure 4E). In summary, the level of ORC1 in the *Drosophila* embryo is not constant as is the case in *S.cerevisiae*. Instead, the protein is developmentally regulated such that high levels of protein are found in proliferating cells.

Two lines of evidence suggest that E2F-dependent transcriptional regulation is responsible (at least in part) for this differential accumulation of ORC1. First, in stage 13 *E2F*⁻ embryos, essentially no ORC1 is detectable (Figure 4F). (Analysis of E2F-dependence in earlier embryonic stages is confounded by the maternal supply of E2F.) Secondly, the pattern of ORC1 accumulation is mirrored by the patterns of *ORC1* promoter activity and *ORC1* mRNA accumulation during embryonic development (Figure 1; data not shown). Therefore, we conclude that E2F-dependent transcriptional regulation, at least in part, couples ORC1 accumulation to proliferation.

We next examined the distribution of ORC1 in eye-antennal imaginal discs, where a developmentally regulated cell cycle transition takes place as the morphogenetic furrow sweeps across the disc. As shown in Figure 5A, the level of ORC1 changes dramatically during this G₁-S transition. The level of protein initially is low among the G₁-arrested cells in the furrow. As cells emerge from the furrow late in G₁, the level of ORC1 rises. Following the completion of S phase, ORC1 levels fall, returning to the basal level seen in the furrow. Two additional observations suggest that these changes in ORC1 levels are not peculiar to cells in and around the morphogenetic furrow. First, cells with high and low levels of ORC1 are randomly interspersed in the anterior region of the eye disc and the antennal disc where cells cycle asynchronously (Figure 5A). Secondly, within the ZNC of the wing imaginal disc, cells arrested in G₁ accumulate high levels of ORC1, whereas G₂-arrested cells do not (data not shown).

To determine more precisely when ORC1 levels rise and fall with respect to the beginning and end of S phase, the following double-labeling experiment was performed. Cells in S phase were identified by pulse-labeling dissected

Fig. 5. Cell cycle regulation of ORC1 in imaginal discs.

(A) The distribution of ORC1 in a wild-type eye-antennal imaginal disc. The morphogenetic furrow is marked with an arrowhead. (B and C) High magnification views of a portion of *dpp-LacZ* eye discs double-labeled to reveal the distribution of cytoplasmic LacZ in blue and either nuclear ORC1 in brown (B) or incorporated nuclear BrdU in brown (C). Expression of LacZ is under the control of *decapentaplegic* (*dpp*) regulatory elements and begins as cells enter the morphogenetic furrow. Note that the treatment required to detect incorporated BrdU alters the nuclear morphology. Note also that the imaginal discs are not flat and some of the cells containing ORC1 and incorporated BrdU are out of the focal plane.

discs with BrdU, and incorporated BrdU was detected by immunohistochemical methods. As this protocol destroys the ORC1 antigen, we measured the time lag between the accumulation of ORC1 or the onset of S phase and the onset of LacZ accumulation in *dpp-lacZ* discs (Blackman *et al.*, 1991). In these discs, expression of *LacZ* under the control of *dpp* regulatory elements begins in the morphogenetic furrow, prior to the G₁-S transition.

As shown in Figure 5B and C, LacZ is first detectable within the morphogenetic furrow. With respect to this landmark, high levels of ORC1 accumulate in cells 3–4 rows to the posterior and S phase begins in cells 5–6 rows to the posterior. Since a new row of cells emerges from the furrow every 1.5 h, this suggests that ORC1 accumulates ~1.5–3 h before the onset of S phase. Incorporated BrdU is detectable in cells ~9–10 rows behind the LacZ landmark; but ORC1 levels are high throughout this region and persist in cells even more

distant from the landmark (rows 11–12 to the posterior). This suggests that ORC1 levels fall only after the completion of S phase. In summary, the data presented in Figure 5 show that the level of ORC1 is cell cycle regulated, with peak accumulation during late G₁ and throughout S phase.

Ectopic ORC1 alters the pattern of DNA synthesis in vivo

Given the coupling of ORC1 abundance to cell cycle progression described above, we wished to determine whether the abundance of ORC1 regulates DNA synthesis. No such modulation has been described for ORCs in *S.cerevisiae*, where neither the level of the ORC nor its binding to origin DNA varies throughout the cell cycle (Diffley and Cocker, 1992). To test this idea, we constitutively overexpressed ORC1 by preparing transgenic flies in which transcription of an *ORC1* cDNA is driven by the *hsp70* promoter. In all the experiments described below, flies were not heat shocked, and therefore the excess ORC1 is derived from basal transcription of the *hsp70-ORC1* (*HS-ORC1*) transgene.

Flies carrying one copy of the *HS-ORC1* transgene exhibit no marked phenotypes. However, flies with various different combinations of either three or four copies of the transgene exhibit a wide array of defects including: rough eyes; lethality during embryonic, larval and pupal stages; male sterility; and female sterility. Overall, these phenotypes are clearly dependent on the dose of the transgene and not the site of transgene insertion; in total, 34 different combinations of transgenes have been analyzed. The severity of each phenotype is somewhat variable among different combinations of transgenes, presumably due to slight differences in the level of ORC1 expression.

As described below, we have examined the pattern of DNA synthesis in two of the tissues that are affected by overexpression of ORC1. In each case, we observe nuclei (or sub-nuclear zones) that normally have low levels of ORC1 and do not synthesize DNA driven to incorporate BrdU by ectopic ORC1. One interpretation of these observations is that the level of endogenous ORC1 limits S phase entry, at least in these cell types.

DNA synthesis in the morphogenetic furrow of HS-ORC1 eye imaginal discs

Eyes from wild-type and 4X *HS-ORC1* flies are shown in Figure 6A and B. The orderly array of ommatidia and bristles evident in the wild-type eye is disrupted in the *HS-ORC1* eye. Western analysis of third instar eye-antennal imaginal discs reveals that *HS-ORC1* discs contain ~4-fold more ORC1 than isogenic wild-type discs (Figure 6C). The ectopic ORC1 appears to be uniformly distributed throughout cells of the disc; because the level of overproduction is relatively modest, the underlying pattern of endogenous ORC1 (i.e. the stripe posterior to the morphogenetic furrow) is still evident (Figure 6D and E). Thus, cells that otherwise would contain a low level of ORC1 contain an amount of ORC1 similar to the peak level seen in S phase cells of wild-type discs. Taken together, these observations suggest that the amount of excess protein in cells of *HS-ORC1* discs is within the

physiological range of ORC1 concentrations found in cells of wild-type discs.

The pattern of S phase cells in wild-type and *HS-ORC1* discs is shown in Figure 6F and G. In wild-type discs, cells within the morphogenetic furrow never incorporate BrdU, and cells in the posterior region of the disc do so only rarely at this stage of development. However, in *HS-ORC1* discs, some cells in both of these regions incorporate BrdU and thus appear to have entered S phase either prematurely or inappropriately. In principle, cells within the furrow might incorporate BrdU because the ectopic ORC1 delays S phase entry or retards transit through S phase. However, several lines of evidence argue against this idea. Ectopic ORC1 has no effect on either the onset or duration of the synchronous S phase among cells that emerge from the furrow (Figure 6). Nor does ectopic ORC1 have any noticeable effect on the proliferation of imaginal discs, the intensity of labeling at different BrdU concentrations or the growth rate of transgenic animals. Furthermore, the observation that endogenous ORC1 levels rise in anticipation of entry into S phase in the eye disc (Figure 5) is consistent with the idea that high levels of ORC1 promote DNA synthesis rather than the opposite.

Ectopic ORC1 alters origin utilization in the ovary

We first examined regulation of the *ORC1* promoter in the ovary. As shown in Figure 7A, the *ORC1* promoter is active in the nurse cells and the somatic follicle cells that envelop each egg chamber during the early stages of oogenesis. Activity of the promoter persists in follicle cells until stage 8, when it is extinguished; activity persists into later stages in the highly polyploid nurse cells (Figure 7B). In contrast, activity of the *ORC1* promoter bearing mutant E2F binding sites (Figure 3) is undetectable in follicle cells and substantially reduced in nurse cells (Figure 7C and D). Thus, as is the case in the imaginal discs (Figure 3), activity of the *ORC1* promoter is E2F-dependent in the ovary.

The distribution of ORC1 in wild-type and *HS-ORC1* ovaries is shown in Figure 7E–J. In wild-type ovaries, ORC1 is present throughout the nuclei of follicle cells in stages 1–7, but falls to relatively low levels in stage 8 (Figure 7E). Strikingly, in stage 10B when a developmentally regulated switch in the pattern of DNA replication takes place, ORC1 is redistributed into sub-nuclear foci (Figure 7F and G). As discussed in more detail below, these foci appear to correspond to gene amplification replicons. In the *HS-ORC1* ovaries, ORC1 is present at somewhat higher levels in the follicle cell nuclei throughout development (Figure 7H–J). Thus, there are two major differences between the pattern of ORC1 in wild-type and *HS-ORC1* ovaries. First, protein persists at relatively high levels beyond stage 7. Secondly, the protein is distributed throughout each follicle cell nucleus rather than being confined to sub-nuclear foci in stage 10B and beyond (Figure 7I and J).

Follicle cells undergo three distinct modes of DNA replication during ovarian development (Lilly and Spradling, 1996; Calvi *et al.*, 1998). During ovarian stages 1–6, they cycle mitotically as two precursor cells proliferate to form the ~650 cells that encapsulate each egg chamber. During stages 7–10A, they undergo three rounds of endoreplication. DNA synthesis during this

Fig. 6. Ectopic ORC1 alters the pattern of S phase in the eye imaginal disc. (A and B) Scanning electron micrographs of wild-type (A) and transgenic *HS-ORC1* (B) eyes. (C) Western blot showing the relative level of ORC1 in eye-antennal disc samples prepared from the isogenic wild-type strain (lane 1) and two different *HS-ORC1* transgenic lines (lanes 2 and 3). The prominent band of 120 kDa corresponds to ORC1. Below are shown the distributions of ORC1 (D and E) and incorporated BrdU (F and G) in wild-type (left) and *HS-ORC1* (right) eye-antennal imaginal discs. Note the presence of additional S phase cells in the posterior region of the eye disc and within the morphogenetic furrow in (G), in comparison with (F). The morphogenetic furrow is marked with an arrowhead in (D–G). These are bright-field images to allow simultaneous visualization of nuclei in different focal planes.

period is asynchronous, and only a fraction of the follicle cells incorporates BrdU during a brief pulse-labeling (Figure 8A). This endoreplication also ends asynchronously, and during a brief window in stage 10A, only a few follicle cells incorporate BrdU (Figure 8B). Immediately thereafter in stage 10B, follicle cells over the oocyte synchronously reinitiate DNA synthesis at two chorion gene loci and two as yet unidentified loci (Figure 8C). This phase of selective gene amplification continues through stage 13, and can be visualized by the incorpor-

ation of BrdU into distinct sub-nuclear foci (Figure 8D and E).

The pattern of DNA replication is specifically altered during the latter stages of oogenesis in the *HS-ORC1* ovaries (Figure 8F–J). During follicle cell proliferation and endoreplication (stages 1–10A), the pattern of DNA replication is essentially identical in wild-type and *HS-ORC1* ovaries (Figure 8F and G; data not shown). In particular, the follicle cells cease endoreplicating on schedule such that almost no DNA synthesis is detectable

Fig. 7. ORC1 is restricted to sub-nuclear foci at the onset of chorion gene amplification. At the top, activity of the wild-type (A and B) and mutant (C and D) *ORC1* promoter fusions (schematic drawings on the left) is revealed by the accumulation of Ftz-GFP-Myc tag protein in confocal mid-line images of egg chambers at various stages of development. The large egg chambers in (A) and (C) are at developmental stage 6–7, and the egg chambers in (B) and (D) are at stage 10B. Note the presence of small follicle cell nuclei at the periphery (arrow) and much larger nurse cell nuclei (arrowhead) inside each egg chamber. The oocyte, which grows to occupy virtually the entire egg chamber by stage 13, is the large unstained cavity that occupies ~50% of the stage 10B egg chambers. These and all successive egg chambers are oriented anterior to the left. At the bottom, the accumulation of ORC1 in wild-type (E–G) and *HS-ORC1* (H–J) egg chambers is shown. (G and H) High magnification surface views of the stage 10B egg chambers shown in (F) and (I). The largest egg chambers in (E) and (H) are at stages 7 and 8, respectively. Note that each follicle cell nucleus contains four ‘dots’ of ORC1 in (G), although some of these are out of the focal plane and hence not visible. The boundary of a single nucleus in (G) is outlined.

in stage 10A, just as in wild-type ovaries. However, upon entering stage 10B, each follicle cell over the oocyte in *HS-ORC1* ovaries begins to synchronously incorporate BrdU throughout its nucleus, rather than in discrete sub-nuclear foci as in wild-type ovaries (Figure 8H). This pattern of DNA synthesis persists through stage 13 (Figure 8I and J).

The simplest interpretation of the observations described above is that ectopic ORC1 activates otherwise quiescent origins throughout the genome in the *HS-ORC1* follicle cells. According to this idea, the level and sub-nuclear distribution of ORC1 play a critical role in regulating origin utilization during the switch from endoreplication to gene amplification. We also considered the idea that the aberrant replication observed in stage 10B–14 *HS-ORC1* egg chambers (Figure 8H–J) represents delayed endoreplication that is normally completed by stage 10A in wild-type ovaries. According to such a model, ectopic ORC1 might suppress the activity of the endogenous protein, competing for a common partner, for example.

Four lines of evidence argue against such a model (Figure 9). First, we examined the DNA content in the population of follicle cell nuclei prepared from wild-type and *HS-ORC1* ovaries by FACS analysis. As described

by Calvi *et al.* (1998), wild-type follicle nuclei are distributed in four peaks that correspond to 2C, 4C, 8C and 16C DNA content, the latter three peaks resulting from successive rounds of endoreplication (Figure 9A). The distribution of *HS-ORC1* follicle cell nuclei is essentially identical, suggesting that endoreplication is not substantially inhibited by ectopic ORC1. Taken with the observations that, in *HS-ORC1* ovaries, the switch from asynchronous to synchronous DNA synthesis occurs normally (Figure 8) and the pattern of BrdU incorporation in stages 1–10A is normal, we conclude that replication of the follicle cell genome is not significantly delayed by ectopic ORC1. Secondly, as measured by Western blot, ORC1 is overproduced by a factor of only two-to-three in *HS-ORC1* ovaries (Figure 9B). This modest level of excess protein is unlikely to compete effectively for interactions with an ORC1 partner or as a substrate for an ORC1-modifying enzyme. Moreover, according to such ‘titration’ models, excess inactive protein should compete more effectively than excess wild-type protein for a limiting target or as a substrate. To test this idea, we prepared transgenic flies that direct the synthesis of a mutant form of ORC1 under the control of the *hsp70* promoter. The mutant protein bears a substitution in the

Fig. 8. Ectopic ORC1 alters the utilization of DNA replication origins during the latter stages of oogenesis. The distribution of BrdU incorporated during a 60 min pulse-label is shown in surface views for wild-type (A–E) and *HS-ORC1* (F–J) egg chambers of various developmental stages, as indicated on the left. The subnuclear foci of BrdU which correspond to gene amplification replicons in wild-type egg chambers are most easily seen in the high magnification view of (E), where the boundary of a single nucleus is outlined. At the corresponding stage in *HS-ORC1* egg chambers, BrdU is incorporated throughout each follicle cell nucleus, except in a small zone that may comprise the nucleolus (J). Note also that incorporated BrdU is visible in nurse cell nuclei out of the focal plane in many of the earlier egg chambers and at the anterior of the egg chambers in (D) and (I).

conserved Walker A motif that is thought to mediate ATP-binding, K604E. The same substitution at the analogous position in the yeast ORC1 and CDC6 abolishes their function (Klemm *et al.*, 1997; Perkins and Diffley, 1998). Flies bearing four copies of the *HS-ORC1*^{K604E} gene exhibit none of the phenotypes seen in *HS-ORC1*⁺ flies. In particular, DNA replication is unperturbed in stage 10B follicle cells (and in other stages) even though relatively high levels of mutant ORC1 fill the nuclei of these cells (Figure 9C). Thus, rather than inhibiting DNA replication, the ectopic K604E mutant protein is inert, supporting the idea that the ectopic wild-type ORC1 promotes rather than inhibits DNA synthesis. Finally, a specialized form of the substrate titration model is suggested by the work of Calvi *et al.* (1998), who proposed that Cyclin E plays a role in inhibiting genomic origins in the latter stages of oogenesis. Since ORC1 has five consensus CDK phosphorylation sites [TPX (K/R)], overexpression of ORC1 might inhibit Cyclin E and thereby de-repress these origins. To address this issue, we examined the activity of Cyclin E in

HS-ORC1 egg chambers using the MPM-2 monoclonal antibody which recognizes an unknown phospho-epitope, accumulation of which is dependent on Cyclin E activity (Calvi *et al.*, 1998). According to this assay, there is no detectable difference between the Cyclin E activity in wild-type and *HS-ORC1* egg ovaries (Figure 9D). In summary, all of these observations are consistent with the idea that the level and sub-nuclear localization of ORC1 is limiting during the switch from endoreplication to gene amplification.

Why does aberrant replication of the follicle cell genome during late oogenesis cause female sterility? The eggs deposited by *HS-ORC1* females are flaccid, and have thin, fragile chorions with defective dorsal appendages (data not shown). This phenotype is very similar to that associated with the *ORC2*^{fs293} mis-sense allele which selectively affects chorion gene amplification (Landis *et al.*, 1997). Therefore, we asked whether inappropriate genomic replication interferes with chorion gene amplification. Total DNA was prepared from stage 12 and 13 egg chambers

Fig. 9. Ectopic ORC1 inhibits neither genomic replication nor Cyclin E activity in the follicle cells. (A) FACS profiles of nuclear preparations from wild-type (left) and *HS-ORC1* (right) ovaries. The four prominent peaks that correspond to mitotic (2C DNA content) and endoreplicated (4C, 8C and 16C) follicle cell nuclei are labeled. Although the size of the unlabeled peak with an apparent DNA content of 32C is slightly larger in the *HS-ORC1* sample, this is not observed reproducibly. Note that the number of nuclei in this sample was ~50% greater than in the wild-type sample, and that the scale on the vertical axis is different (0–1050 versus 0–1600). (B) Western blot of total protein prepared from isogenic wild-type (1) or 4× *HS-ORC1* ovaries (2–4) from three different transgenic lines. The prominent band of ~120 kDa corresponds to ORC1. (C) Distribution of ORC1 (left) and BrdU incorporated during a pulse-label (right) in stage 10B follicle cells from 4× *HS-ORC1*^{K604E} flies. (D) The distribution of MPM-2 reactive antigen is shown in follicle cells of wild-type (left) and 4× *HS-ORC1* (right) stage 10B egg chambers. Every nucleus in these fields contains a sphere of approximately equivalent intensity, although some of these are out of the focal plane.

dissected from three different 4× *HS-ORC1* lines, otherwise isogenic wild-type flies, and *ORC2*^{fs293} flies. Aliquots of the DNA were hybridized separately to probes for the third chromosome chorion cluster and for a single copy gene elsewhere in the genome. With respect to the wild-type egg chambers, chorion gene amplification is depressed 1.7- to 3.5-fold in *HS-ORC1* egg chambers (Figure 10A). In this assay, chorion gene amplification is depressed by a similar factor in the *ORC2*^{fs293} egg chambers. Thus, the activation of genomic origins depresses chorion gene amplification.

While we do not understand the molecular basis of this inhibition, it appears to be a secondary consequence of genomic origin activation, based on the following observations. Since the macroscopic phenotypes associated with ectopic expression of ORC1 are clearly dose dependent, we asked whether the molecular consequences of ectopic ORC1 expression on DNA synthesis are also dose-dependent. Flies carrying two copies of the *HS-ORC1* transgene exhibit no macroscopic phenotypes. In particular, females are fertile and deposit eggs with normal chorions. Consistent with these observations, chorion gene amplification is normal in 2× *HS-ORC1* egg chambers (Figure 10A). However, the ectopic ORC1 in these flies activates genomic origins in follicle cells beginning in stage 10B, albeit to a lesser extent than in 4× *HS-ORC1* flies (Figure 10B). Apparently, the ectopic genomic replication in 2× *HS-ORC1* follicle cells is insufficient to inhibit the chorion gene replicons. The progression of replication forks into the amplification loci from surrounding DNA may inhibit amplification. Alternatively,

Fig. 10. High level genomic replication inhibits chorion gene amplification. (A) Slot blot to measure chorion gene amplification in stage 13 egg chambers dissected from *ORC2*^{fs293}, wild-type (wt) and three different 4× *HS-ORC1*⁺ flies, as indicated. Duplicate filters were hybridized with a single copy gene probe (*RNR2*, left) or a third chromosome chorion gene cluster probe (right) and the chorion/*RNR2* signal intensity measured using the PhosphorImager. The level of chorion gene amplification relative to that observed in wild-type samples is shown at the right (wt = 1.0). In the lower panel, samples were prepared independently from wild-type flies and the second 4× *HS-ORC1*⁺ transgenic line shown in the upper panel, as well as 2× *HS-ORC1*⁺ and 4× *HS-ORC1*^{K604E} flies. The slight (<10%) depression of chorion gene amplification apparent in the 4× *HS-ORC1*^{K604E} sample is not reproducible. (B) Distribution of BrdU incorporated during a pulse-label in stage 10B 2× *HS-ORC1*⁺ follicle cells.

activation of origins throughout the genome may simply starve the amplification loci for scarce replication factors during the brief period (stage 10B–14) when chorion biosynthesis occurs. In either case, limiting the level and sub-nuclear distribution of ORC1 evidently is essential for proper chorion assembly and fertility.

In summary, modest overexpression of ORC1 in the follicle cells is sufficient to activate genomic origins, and thus the level of ORC1 normally limits their utilization. The *HS-ORC1* flies are female sterile, at least in part because genomic replication interferes with chorion gene amplification. *HS-ORC1* flies exhibit a number of other phenotypes, including male sterility and lethality. Although we have not investigated the molecular events underlying these phenotypes, apparently a number of other cell types are also sensitive to the level of ORC1.

Discussion

Based on the experiments reported here, we draw three main conclusions. First, E2F acts positively to stimulate transcription of *ORC1* *in vivo*. Secondly, the level of ORC1 is modulated throughout *Drosophila* development, particularly during a regulated cell cycle transition in the eye imaginal disc and during a switch in origin utilization in ovarian follicle cells. Thirdly, overexpression of ORC1 alters the pattern of DNA synthesis in these cell types. Taken together, these observations are consistent with the idea that the level of ORC1 is limiting for DNA synthesis, at least in some cell types, and that *ORC1* is a significant E2F regulatory target during the G₁–S transition in these cells.

ORC1 abundance and the regulation of origin activity

We find a correlation between the spatiotemporal accumulation of ORC1 and DNA synthesis at three different stages of development. In the embryo, ORC1 accumulates to high levels in proliferating cells (Figure 4). In the eye imaginal disc, ORC1 levels are high from a point late in G₁ through the end of S phase (Figure 5). And in the ovary, ORC1 is restricted to subnuclear foci within follicle cell nuclei, at a stage in development when DNA synthesis is restricted to foci of gene amplification (Figure 7). Moreover, overexpression of ORC1 alters the pattern of DNA synthesis in the morphogenetic furrow of the eye disc and in the latter stages of oogenesis, consistent with the idea that the level of ORC1 is limiting in these cell types.

The restriction of ORC to sub-nuclear foci during gene amplification represents a novel form of replication control. Although we have not shown directly that these foci correspond to the amplification replicons, we believe this to be the case for several reasons. (Detection of incorporated BrdU destroys ORC1 antigenicity, and thus we are unable to detect both simultaneously.) The sub-nuclear ORC1 foci appear only in follicle cells overlying the oocyte at precisely the stage of ovarian development that amplification commences. In addition, they are not detectable in the follicle cells of earlier stages, in the follicle cells that overlie the nurse cell complex in later stages, or in the nurse cells. Finally, our observations are supported by a recent report that ORC2 is restricted to

the amplification replicons (Royzman *et al.*, 1999). This localization is dependent on the normal activity of E2F, since hypomorphic alleles of *E2F* and the gene encoding its heterodimeric partner, *DP*, cause female sterility by mis-regulating the subnuclear localization of ORC2 in the follicle cells.

Based on the phenotypes we observe in the transgenic *HS-ORC1* flies, a number of other cell types apparently are also sensitive to the level of ORC1. We have examined the molecular consequences of ORC1 mis-expression in the eye imaginal disc, where we see that some of the cells normally held in an extended G₁ phase are driven into S phase (Figure 6). Several lines of evidence suggest that ectopic ORC1 has driven arrested cells prematurely into S phase, rather than delaying entry into or progression through S phase. First, the width of the (largely) S phase-free zone that includes the furrow is unaffected by ectopic ORC1. Secondly, the positions of ectopic labeled cells are random within the furrow, rather than skewed toward the anterior margin. Thirdly, the intensity of labeling within these cells is very similar to the corresponding intensity in cells elsewhere in either the *HS-ORC1* or wild-type discs. Taken together, these observations suggest that ectopic ORC1 promotes premature S phase entry in some of the cells that have entered the furrow, rather than delaying S phase in the asynchronous anterior cells until they enter the furrow. We currently do not understand why only some of the cells in the furrow are ORC1 responsive. Perhaps other replication factors are limiting in other cells.

Whether ORC levels also are modulated during the development of other organisms remains to be determined. ORC expression is static in *S.cerevisiae*, where the ORC is bound constitutively to origins of replication throughout the cell cycle (Diffley and Cocker, 1992). Similarly, ORC1 levels are not regulated during the cell cycle in *S.pombe* (Muzi-Falconi and Kelly, 1995; Grallert and Nurse, 1996). ORC function has been studied primarily in these micro-organisms, as well as in *Xenopus* and *Drosophila*, where high levels of protein are found in embryos during the rapid cleavage cycles of early development (Gossen *et al.*, 1995; Rowles *et al.*, 1996; Pak *et al.*, 1997). To our knowledge, this work and that of Royzman *et al.* (1999) constitute the first demonstration that ORC levels vary *in vivo*.

Similarly, the idea that changes in ORC abundance can have regulatory consequences is largely untested in other organisms. Transfection of a human ORC1 expression construct into serum-starved rat embryo fibroblasts did not drive these cells into S phase (Ohtani *et al.*, 1998). The significance of this result is not clear, since the level of protein expression was not measured. Moreover, it is possible that ORC1 is simply not limiting in this cell type. Overexpression of human ORC1 in *S.pombe* interferes with cell cycle progression, blocking re-replication control and inhibiting entry into mitosis (Wolf *et al.*, 1996). However, expression of the human ORC1 in these experiments does not complement the lethality associated with an *ORC1* mutation in the native *S.pombe* gene, and the observed effects appear to be due to drastic overproduction of a non-functional protein that interferes with Cyclin function by substrate titration.

The mechanism by which ORC1 abundance regulates

origin utilization awaits the definition of replication origins and biochemical studies of replication initiation. Recent studies in yeast suggest that ORC1 may have a special role in regulating replication. First, ORC1 appears to mediate the ATP-dependence of origin binding in *S.cerevisiae* (Klemm *et al.*, 1997). Secondly, ORC1 interacts (directly or via bridging interactions) with the regulatory factor CDC18 in *S.pombe* (Grallert and Nurse, 1996). Thirdly, based on structural and functional homology, Perkins and Diffley (1998) have suggested that ORC1 is a member of an extended super-family of ATP binding proteins that includes CDC6/CDC18 and replication clamp-loading proteins. Thus, CDC6 and ORC1 may have similar biochemical and regulatory roles *in vivo*.

ORC1 as an E2F target

Despite a wealth of information about the mechanism of E2F action and the identification of a large number of E2F-regulated genes, it is not yet clear which E2F target genes are primarily responsible for mediating the S phase-promoting activity of E2F. In this report we show that expression of *ORC1* is dependent on E2F, that ORC1 levels are modulated as a result, and that constitutive mis-expression of ORC1 alters the pattern of DNA synthesis in at least two different cell types *in vivo*. Taken together these findings support the view that *ORC1* is a limiting E2F target, at least in some cells.

In contrast, preliminary evidence suggests that E2F-dependent regulation of the gene encoding another member of the replication complex, *ORC2*, is not important. Like *ORC1* mRNA, *ORC2* mRNA appears to be E2F-regulated in embryos and imaginal discs (i.e. as in Figures 1 and 2; data not shown). Moreover, the *ORC2* promoter bears a single presumptive E2F binding site (ATTCCCCG) immediately downstream from the start site of transcription. However, a modified *ORC2* transgene in which this site is mutated fully rescues homozygous flies bearing a lethal *ORC2*⁻ allele to fertile adulthood. [Similar experiments with a wild-type *ORC2* transgene are described by Landis *et al.* (1997)]. Thus, E2F-dependent regulation of *ORC2* does not appear to play an important role *in vivo*. This finding is consistent with the observations that ORC2 levels are not modulated during embryonic development, in follicle cells during the early stages of oogenesis, or in cultured cells (Pak *et al.*, 1997; Royzman *et al.*, 1999). Thus, while ORC1 and ORC2 presumably function together as a complex in *Drosophila* as they do in yeast, ORC1 abundance appears to be regulated whereas ORC2 abundance does not.

Cyclin E constitutes another significant regulatory target of E2F in mammalian cells (Ohtani *et al.*, 1995; Botz *et al.*, 1996; Geng *et al.*, 1996) and in flies (Duronio and O'Farrell, 1995). Indeed, recent experiments show that much of the activity of E2F in promoting wing imaginal disc cell proliferation can be mimicked by co-expression of Cyclin E, which drives the G₁-S transition, and String, which drives the G₂-M transition (Neufeld *et al.*, 1998). However, Cyclin E may not be a limiting E2F target in every cell type of the fly (Duronio and O'Farrell, 1995; Duronio *et al.*, 1996), nor is it clear that E2F-dependent regulation plays a major role in altering the abundance of Cyclin E protein through the cell cycle. Since overexpres-

sion of neither Cyclin E nor ORC1 reproduces the effects of overexpression of E2F, it seems likely that additional E2F targets are limiting in other cell types.

Our analysis of the regulation of the *ORC1* promoter indicates that E2F acts positively to activate transcription. Mutations in the E2F binding site of the *Drosophila ORC1* promoter abolish its activity in transgenic animals (Figures 3 and 7). In contrast, many studies have suggested that mammalian E2F acts negatively to repress transcription by recruiting a Rb family member (see Dyson, 1998; Nevins, 1998). When bound to E2F, Rb proteins both mask its activation domain and inhibit activation by factors bound elsewhere in the promoter. For example, mutations in the relevant E2F binding site of the human *ORC1* promoter enhance its activity in cells brought into quiescence by withdrawal of serum (Ohtani *et al.*, 1996). It is possible that most E2F-regulated genes in *Drosophila* are negatively regulated by E2F, and that *ORC1* is exceptional. Alternatively, E2F-mediated negative control may play a greater role in mammals than in flies. The analysis of additional promoters in transgenic flies and mice will be required to distinguish between these possibilities.

Materials and methods

Strains and reagents

In the experiments of Figures 1 and 4, *E2F*⁻ embryos were *trans*-heterozygotes of either *E2F*^{rm729}/*E2F*⁷¹⁷² or *E2F*⁹¹/*E2F*⁷¹⁷² (Duronio and O'Farrell, 1995). *dpp-lacZ* flies are described by Blackman *et al.* (1991), and *w*¹¹¹⁸ flies were used to generate transgenic animals by standard methods. Flies were reared at 25°C except in the heat shock experiment of Figure 2 where third instar larvae were heat shocked at 37°C for 30 min and allowed to recover at 25°C.

An *ORC1* cDNA clone was isolated by low stringency hybridization using a probe derived from the human *ORC1* gene. The sequence of the protein coding region of a full-length clone is identical to the published sequence (Pak *et al.*, 1997). A clone containing the *ORC1* promoter was isolated from a λEMBL3 genomic library. The *ORC1* promoter sequence has been deposited in the DDBJ/EMBL/GenBank data base under accession No. Y18882. Antibodies were raised against GST fusion proteins containing amino acids 144-492, 411-748 and 673-925 of ORC1. Several antisera raised against each antigen in both rabbits and rats specifically recognize ORC1 on Western blots and in fixed tissue by the following criteria. First, each detects the distribution of protein in embryos reported in Figure 4. Secondly, each detects primarily a protein of ~120 kDa on Western blots. Thirdly, the protein detected on such blots co-migrates with the protein prepared by *in vitro* translation of the *ORC1* cDNA and with the protein generated *in vivo* following administration of a heat shock to transgenic *hsp70-ORC1* flies. This ectopic protein is also readily detectable with standard immunohistochemical methods *in situ*. One of these antibodies was affinity-purified (Tang, 1993) and used as described below. The *ftz-GFP-Myc* fusion protein was detected either with mAb 9E10 or with affinity-purified rabbit anti-GFP, a gift from P.Silver. Incorporated BrdU was detected with a monoclonal antibody from Zymed, LacZ was detected with a rabbit antibody from Cappel and MPM-2 monoclonal antibody was from DAKO.

RNA

In situ hybridization was performed essentially as described previously (Duronio and O'Farrell, 1994), using a 756 bp *KpnI-EcoRI* fragment from *ORC1* to generate digoxigenin-labeled probes by random priming. For the Northern blot of Figure 2, 5 µg samples of total RNA prepared from each eye-antennal imaginal disc sample were electrophoresed through a denaturing gel, transferred to nylon, and probed with ³²P-labeled fragments derived from each of the indicated genes. The bound radioactivity was measured using a Molecular Dynamics Phosphor-Imager, and induction ratios were calculated normalizing to the quantity of *rp49* mRNA in each lane. Transcription start sites were determined both by RNase protection and primer extension experiments; minor *ORC1* starts were seen at -10, -7, +7 and +10 nt (data not shown).

DNA

Gel mobility-shift experiments were performed essentially as described previously (Ohtani and Nevins, 1994). In each reaction, an aliquot of labeled synthetic duplex containing the *Drosophila* Pol α E2F-binding site was incubated with 7 μ g of nuclear protein from S2 cells. Competitor DNA was added at a 50-fold molar excess. The wild-type *Drosophila* ORC1 competitor is described in the text. The mutant ORC1 competitor was generated by changing the sequence of the E2F binding sites from ATTGCGCCAAA to ATTGATCCAAA. FACS analysis was performed essentially as described previously (Lilly and Spradling, 1996), using propidium iodide rather than DAPI. Slot blot hybridization was performed essentially as described previously (Lilly and Spradling, 1996). Total DNA was prepared from stage 13 egg chambers, and aliquots were applied to nitrocellulose filters which were subsequently hybridized with probes derived from *RNR2* (as a representative single copy gene) and from the third chromosome chorion cluster (a 3.8 kb *SalI* fragment from plasmid pt2 (Calvi *et al.*, 1998). Quantitation was performed using a Molecular Dynamics PhosphorImager, normalizing to the level of *RNR2* DNA.

Protein

Western blot analysis was performed following standard methods using ECL detection. Ten-microgram samples (measured with the Bio-Rad protein assay) were electrophoresed in each lane.

Plasmid constructions

The ORC1 promoter fusions to *ftz*-GFP-Myc were constructed with a *Bam*HI-MunI DNA fragment that includes ~2.3 kb upstream and 60 bp downstream of the transcription start site. The *ftz*-GFP-Myc gene, a gift from S.-K.Chan and G.Struhl, encodes a fusion protein that includes the first 273 amino acids of Ftz, the entirety of GFP, six copies of a 13 amino acid peptide recognized by mAb 9E10, and a nuclear localization signal. The *HS-ORC1* gene was made by fusing a 0.5 kb fragment bearing the *hsp70* promoter and leader, a 2.9 kb *Hind*III-PacI fragment bearing the entire ORC1 protein coding region, and a 0.8 kb fragment bearing the α -tubulin 84B 3' UTR and polyadenylation signal. To prepare the *HS-ORC1*^{K604E} gene, codon 604 was changed from AAA to GAA by site-directed mutagenesis of a *Nhe*I-StuI fragment which was subsequently substituted for the corresponding fragment of the *HS-ORC1*⁺ gene.

Histology

Embryos were fixed in either 1 or 2% formalin, 100 mM PIPES pH 6.9, 1 mM EGTA, 2 mM MgCl₂ under heptane; devitellinized into methanol, and rehydrated into phosphate-buffered saline (PBS). Imaginal discs were fixed in either 1 or 2% paraformaldehyde (PFA) in PBS as previously described (Asano *et al.*, 1996). Ovaries were fixed in 1% PFA in PBS and processed as described previously (Lin *et al.*, 1994). BrdU labeling was performed as described (Asano *et al.*, 1996; Lilly and Spradling, 1996). The double-labeling experiments of Figure 5 were performed using the SG reagents (Vector) as described by the manufacturer. Primary antibodies were used at the following concentrations: anti-ORC1, 1:300; anti-LacZ, 1:200; anti-BrdU, 1:3; 9E10 mAb, 1:5; anti-GFP, 1:1000; anti-MPM-2, 1:800. Secondary antibodies were from Jackson Laboratories. Samples were prepared for SEM as described previously (Asano *et al.*, 1996).

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