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string^{cdc25} and cyclin E are required for patterned histone expression at different stages of *Drosophila* embryonic development

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Abstract

Metazoan replication-dependent histone mRNAs accumulate to high levels during S phase as a result of an increase in the rate of histone gene transcription, pre-mRNA processing, and mRNA stability at the G1–S transition. However, relatively little is known about the contribution of these processes to histone expression in the cell cycles of early development, which often lack a G1 phase. In post-blastoderm *Drosophila* embryos, zygotic expression of the stg^{cdc25} phosphatase in G2 activates cyclin/cdc2 kinases and triggers mitosis. Here we show that histone transcription initiates in late G2 of cycle 14 in response to stg^{cdc25} and in anticipation of S phase of the next cycle, which occurs immediately following mitosis. Mutation of *stg^{cdc25}* arrests cells in G2 and prevents histone transcription. Expression of a mutant form of Cdc2 that bypasses the requirement for stg^{cdc25} activates histone transcription during G2 in stg^{cdc25} mutant embryos. Thus, in these embryonic cycles, histone transcription is controlled by the principal G2–M regulators, string^{cdc25}, and cdc2 kinase, rather than solely by regulators of the G1–S transition. After the introduction of G1–S control midway through embryogenesis, histone expression depends on DNA replication and the function of cyclin E, and no longer requires stg^{cdc25}. Thus, during the altered cell cycles of early animal development, different cell cycle mechanisms are employed to ensure that the production of histones accompanies DNA synthesis.

Keywords: Cell cycle; SLBP; Histone mRNA; *Drosophila*; DNA replication; Cyclin E; stg^{cdc25}

Introduction

Metazoan replication-dependent histone mRNA biosynthesis is strictly coupled with cell cycle progression (Marzluff and Duronio, 2002). In mammalian cells, the steady-state level of histone mRNA increases between 35- and 50-fold as cells progress from G1 to S phase. Several pre- and posttranscriptional mechanisms cooperate to ensure that histone mRNA accumulates only during S phase. The rate of initiation of histone gene transcription increases 3- to 5-fold at the G1–S transition, and the efficiency of histone pre-mRNA processing increases 8- and 10-fold (Harris et al., 1991). Histone mRNA stability is also cell cycle regulated. Histone mRNA is relatively stable during S phase, but is rapidly destroyed when S phase is completed (Whitfield et al., 2000) or when DNA synthesis is inhibited (e.g., by aphidicolin) (Heintz et al., 1983; Sittman et al., 1983).

The basis for posttranscriptional cell cycle regulation of metazoan histone mRNA production lies in the unique structure of the mRNAs, which are not polyadenylated (Dominski and Marzluff, 1999). Histone mRNA instead terminates in a stem loop structure that is bound by a unique RNA binding protein termed the Stem Loop Binding Protein

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(SLBP) (Martin et al., 1997; Wang et al., 1996). Production of mature histone mRNA involves a single processing reaction that requires two cis-elements present at the 3′ end of the nascent pre-mRNA: the stem-loop sequence and a purine-rich sequence approximately 10 nucleotides downstream of the stem loop that is bound by U7 snRNP (Mowry and Steitz, 1987). The SLBP and U7 snRNP bind histone pre-mRNA resulting in recruitment of an endonuclease complex that cleaves the histone pre-mRNA 4–5 nucleotides (depending on species) after the stem loop (Dominski and Marzluff, 1999; Dominski et al., 1999). Depletion of either SLBP or inactivation of U7 snRNP in either mammalian or *Drosophila* nuclear extracts blocks processing of synthetic histone pre-mRNAs (Dominski et al., 1999, 2000). After pre-mRNA cleavage, SLBP remains bound to mature histone mRNA and accompanies the mRNA to the cytoplasm where it acts analogously to poly A binding protein (PABP) to regulate message turnover and translation (Sanchez and Marzluff, 2002). The SLBP protein accumulates predominantly during S phase and is destroyed at the end of S phase in mammalian cells, accounting for much of the cell cycle regulation of histone mRNA production (Whitfield et al., 2000; Zheng et al., 2003).

The paradigm of coupling histone mRNA expression to DNA replication has been established primarily from studies in mammalian cell culture. However, the mechanisms controlling histone mRNA biosynthesis during early animal development are less well understood. We have been examining histone mRNA expression in *Drosophila* embryos, which execute a well described and essentially invariant cell cycle program that deviates substantially from the canonical G1–S–G2–M cycles of typical diploid cells (Foe, 1989; Foe et al., 1993). An interesting feature of this program is that the first 16 embryonic cycles following fertilization lack a G1 phase, with S phase beginning immediately after completion of mitosis. One of our goals is to characterize the mechanisms of histone mRNA production during these early cell cycles.

After fertilization, 13 rapid, maternally controlled S–M nuclear division cycles that occur in a syncytium provide approximately 6000 blastoderm nuclei in approximately 2 h. There is no synthesis of histone protein (P. Fort, W.F.M. and R.J.D., unpublished) during this time and the embryo relies on stored histone protein to assemble newly replicated chromatin. Cellularization occurs during interphase 14, at which time these blastoderm nuclei synchronously enter G2 phase for the first time and initiate transcription. Shortly thereafter, specific groups of cells called mitotic domains (MD) enter M_{14} at different times, generating a stereotypic spatial–temporal pattern that coordinates mitosis with cell movements that occur during gastrulation (Foe, 1989). Entry into mitosis 14 is dependent on zygotic expression of *string*^{cdc25} (*stg*), which encodes a phosphatase that activates the mitotic cyclin-dependent kinase (Cdc2) (Edgar and O'Farrell, 1989, 1990). *stg* transcription coincides with the mitotic domains and is controlled by an extensive array of enhancers that integrate spatial and temporal information during development through the activity of numerous transcription factors (Edgar et al., 1994; Lehman et al., 1999). Cell cycles 15 and 16 also lack a G1 and are regulated at the G2–M transition by *stg* expression. In cycle 17, cells enter the G1 phase for the first time and their subsequent behavior depends on cell type. Most epidermal cells remain arrested in $G1_{17}$, while cells in the CNS and PNS continue to divide in cycles regulated by *stg* at G2–M. The cells in several tissues such as the midgut begin endocycles (Smith and Orr-Weaver, 1991). Cyclin E is necessary for DNA replication and cell cycle progression in both dividing and endocycling cells after cycle 16 (Knoblich et al., 1994). In this report, we analyze the contribution of stg^{cdc25} and cyclin E function to embryonic histone expression.

Most of the maternal histone mRNA is destroyed at the end of the syncytial stage and zygotic histone mRNA accumulates rapidly during cycle 14 (Edgar and Schubiger, 1986; Lanzotti et al., 2002). There is no maternal SLBP protein present in the 0- to 2-h embryo, and zygotic SLBP also accumulates during cycle 14 (Lanzotti et al., 2002). Mutation of the *Drosophila Slbp* gene results in the production of histone mRNAs that are not processed normally at the 3′ end (Sullivan et al., 2001). Instead, histone mRNA produced in *Slbp* mutants is polyadenylated via the use of cryptic poly A-signal sequences downstream of the normal processing site. Because the SLBP protein is not supplied maternally, this phenotype occurs as soon as high-level zygotic histone transcription begins (Lanzotti et al., 2002). Interestingly, the initial appearance of misprocessed histone message in *Slbp* mutant embryos emerges in the stereotypic pattern of the mitotic domains. Furthermore, by directly detecting nascent histone pre-mRNA, we established that histone transcription occurs during G2 precisely in the mitotic domain pattern (Lanzotti et al., 2002).

We now demonstrate that this early zygotic histone transcription requires *stg*. Moreover, a form of Cdc2, Cdc2^{AF}, that is able to bypass the requirement for the phosphatase action of stg^{cdc25} can also trigger histone transcription during G2 of cycle 14. After the introduction of G1 regulation in cycle 17, histone synthesis depends on zygotic expression of cyclin E and does not require *stg*^{cdc25}. Thus, multiple cell cycle mechanisms control histone expression during *Drosophila* embryogenesis, and the unexpected coupling of histone transcription and to the G2–M regulatory machinery may ensure that sufficient histones are synthesized during early embryonic cell cycles that lack a G1 phase.

Materials and methods

Fly stocks and genetics

The loss of function alleles *stg*^{7B} and *Df(3R)stg*^{AR2} were previously described (Edgar and O'Farrell, 1989;

Jurgens et al., 1984; Lehman et al., 1999). stg^{7B} is an ethylmethanesulfonate-induced point mutant and *Df(3R)-stg*^{AR2} is a deletion created by a P-element excision. The *Df(3R)stg*^{AR2} deletion extends from directly upstream of the *stg* transcription start through the *Slbp* locus located 102 kb away. We verified that *Df(3R)stg*^{AR2} removes *Slbp* by PCR analysis using primers (5′-GGAGATGGCATTT-GAGTTCCTGGAT-3′ and 5′-GGTGGATCATAGATGTG-GAGGCTTT-3′) and genomic DNA prepared from single, GFP-selected *Df(3R)stg*^{AR2} mutant or heterozygous sibling embryos. As controls, the same genomic template DNA was amplified with the *stg*-specific primers (5′-CTCG CCCATCCAGAGCAAA-3′ and 5′-CGAGGAGAATTCG-CAGTGGAA-3′) or with the *rp49*-specific primers (5′-TGCTAAGCTGTCGCACAAATG-3′ and 5′-CGA-TGTTGGGCATCAGATAC-3′). In situ hybridization of *Df(3R)stg*^{AR2} mutant embryos also revealed a complete loss of *Slbp* mRNA in comparison to sibling controls (not shown). $Slbp^{15}$, $E2f1^{91}$, and $CycE^{AR95}$ are previously described null alleles (Duronio et al., 1995; Knoblich et al., 1994; Sullivan et al., 2001). *Df(3R)3450* is a deletion that completely removes the *Slbp* gene (Sullivan et al., 2001). P[w^+, hsp70-CycE] is described in Richardson et al. (1995). Recombinant lines made for these experiments include: (1) *Df(3R)3450*: P[w^+, hsp70-CycE]/TM3, (2) *Df(3R)stg*^{AR2}: P[w^+, arm-GAL4]x2/TM3, (3) *Df(3R)stg*^{AR2}: P[w^+, UAS-Stg]/TM3, (4) *Df(3R)stg*^{AR2}: P[w^+, hsp70-Cdc2^{AF}]/TM3.

Embryo in situ hybridization, immunostaining, and BrdU detection

For in situ hybridization, staged embryos were dechorionated and fixed in 37% formaldehyde (Sigma) for 5 min. Histone (H3 and H2bH4) and SLBP mRNAs were detected in situ by digoxigenin-labeled RNA probes (NEB) complementary to the coding region of these genes or to the 3′ flanking region of histone H3 (H3-ds) as previously described (Lanzotti et al., 2002). For immunodetection of mitotic nuclei, staged embryos were dechorionated and fixed in 10% formaldehyde for 10 min. Embryos were then labeled with rabbit anti-phosphohistone H3 (Upstate Biotechnology) and goat anti-rabbit cyanine 2 (Jackson Immunoresearch) antibodies. BrdU incorporation was performed as described (Duronio and O'Farrell, 1994). Briefly, permeabilized embryos were allowed to incorporate BrdU at 1 mg/ml for 15 min in Schneider's culture medium (Gibco) and then immediately fixed in 37% formaldehyde for 5 min. Following depurination by 2 N HCl, embryos were labeled with mouse monoclonal anti-BrdU (Becton Dickinson) and goat anti-mouse rhodamine (Molecular Probes) antibodies. To perform heat shock treatments, embryo collection plates were floated in a 37°C bath for 30 min and allowed to recover for 30, 45, or 60 min before fixation.

Results

string is required for histone transcription in the early embryo

To test whether stg^{cdc25} activity affected histone expression, we studied the effects of manipulating *stg* function on histone H3 mRNA accumulation in the early embryo. The total steady-state H3 mRNA level was detected using an antisense RNA probe derived from the coding region. In wild-type embryos, the signal generated by the H3 coding probe is most intense in the cycle 14 mitotic domain pattern, which can be visualized with anti-phosphohistone H3 staining (Figs. 1A and B). In *stg* null embryos, in which all cells arrest in G2 of cycle 14 and do not progress through mitotic cycles 15 and 16, the total H3 mRNA was reduced relative to wild type and was no longer detected in the mitotic domain pattern (Figs. 1C–F). The intensity of staining in the *stg* mutants varied somewhat from embryo to embryo. This may reflect different amounts of perdurance of maternal histone message, because similar results were observed in embryos homozygous for a deletion (*Df(2L)DS5*) that removes the histone locus (not shown).

To determine the mechanism by which histone H3 mRNA was reduced in *stg* mutant embryos, we took advantage of the *Slbp* mutant phenotype to directly measure histone gene transcription cytologically. To do this, a probe complementary to sequence downstream of the normal processing site for histone H3 mRNA was generated. This probe (called H3-ds) does not hybridize to any histone mRNA in wild-type embryos, but specifically detects the longer, polyadenylated H3 mRNAs that are generated as a result of the failure of normal pre-mRNA processing in *Slbp* mutant embryos (Lanzotti et al., 2002; Sullivan et al., 2001). This probe also detects nascent H3 pre-mRNA as intense focal points of stain within nuclei (i.e., "transcription dots", Fig. 2A), thereby providing a useful assay for directly detecting H3 transcription in vivo (Lanzotti et al., 2002). Normally, the termination of histone transcripts is coupled to 3′ end processing (Chodchoy et al., 1987), and so the prominent H3 transcription dots in *Slbp* mutants likely result from the accumulation of nascent transcripts due to inefficient transcription termination as well as from a polymerase pause site located just downstream of the stem loop (Adamson and Price, 2003).

As we previously described (Lanzotti et al., 2002), the H3-ds probe detects the de novo transcription of aberrant poly A+ H3 mRNA in the mitotic domain pattern in *Slbp* mutant embryos (Figs. 2A and C). This transcription begins in $G2_{14}$ and is aborted during mitosis 14, as observed by staining the H3-ds in situ preparations with the DNA binding dye DAPI (Fig. 2B). The difference in overall staining intensity between the H3 coding probe in WT (Fig. 1A) and the H3-ds probe in *Slbp* mutant embryos (Fig. 2C) is because at this stage the H3-ds probe primarily detects nascent transcripts due to a delay

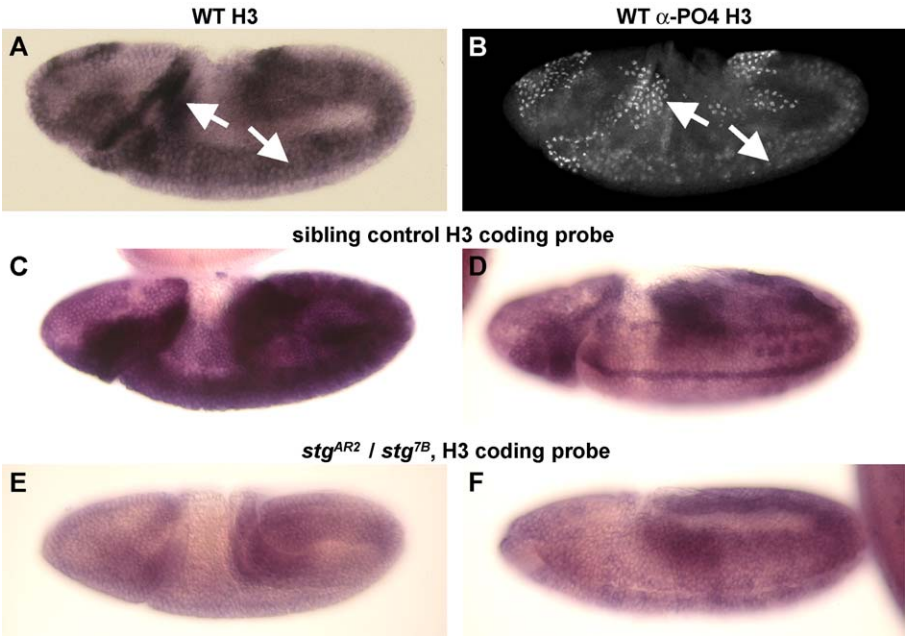


Fig. 1. Early histone expression in the mitotic domains is dependent on Stg^{cdc25}. (A) Stage 8 wild-type embryo hybridized with the H3 coding probe. High-level H3 expressions in mitotic domains 6 and 10 are indicated with arrows as an example. (B) Stage 8 wild-type embryo labeled with anti-phosphohistone H3 to detect mitotic domains (arrows as in A). (C and D) Stages 8 and 9 (C and D, respectively) embryos hybridized with H3-coding probe. (E and F) *Df(3R)stg*^{AR2}/*stg*^{7B} mutant embryos that are staged-matched siblings to those in panels C and D, respectively, hybridized with H3-coding probe. Note the loss of patterned H3 accumulation in *stg* mutant embryos in panels.

in cytoplasmic accumulation of the mis-processed poly A+ H3 mRNA: older *Slbp* mutant embryos stain much more intensely in the cytoplasm with the H3-ds probe (e.g., see Figs. 3C and E) (Lanzotti et al., 2002). To monitor H3 transcription directly in *stg* mutants, we used a deletion allele of *stg* (*Df(3R)stg*^{AR2}) that simultaneously removes the nearby *Slbp* locus (see Materials and methods). Because there is no maternal contribution of SLBP protein, the *Df(3R)stg*^{AR2} mutants are also null for SLBP (Lanzotti et al., 2002). Neither transcription dots nor accumulation of unprocessed message was detected by the H3-ds probe in these *stg-Slbp* double mutant embryos during gastrulation and germ band extended stages, indicating that stg^{cdc25} is required for histone transcription (Fig. 2D). Ubiquitous expression of UAS-stg using the *arm*-GAL4 driver in embryos homozygous for the *Df(3R)stg*^{AR2} deletion caused the appearance of transcription dots and the accumulation of unprocessed H3 message (Figs. 2E and F, arrowhead). Notably, expression occurred in many cells throughout the embryo and was no longer restricted to the mitotic domain pattern, as would be expected from loss of patterned *stg* expression. These data indicate that *stg* is required for transcription of histone H3 mRNA during G2 of cycle 14 in the early embryo.

Histone expression does not require stg^{cdc25} at all embryonic stages

In later embryonic stages (e.g., during germ band retraction), H3 expression is detected in some tissues of *Df(3R)stg*^{AR2} mutant embryos with either the coding (Fig. 3A) or the H3-ds probe (not shown). Expression occurs in the midgut (arrowhead, Fig. 3A) and in cells along the ventral portion of the embryo (arrow, Fig. 3A). Midgut cells in wild-type embryos are post-mitotic and begin endocycling at this stage, and have previously been shown to incorporate BrdU in *stg* mutant embryos (Duronio and O'Farrell, 1994). Thus, *stg* is not required for histone expression in the midgut, consistent with the observation that *stg* is not normally expressed in this non-dividing tissue (Edgar et al., 1994). The ability of midgut cells to escape G2 cell cycle arrest imposed by mutation of *stg* and enter endo-S phase may be due to the expression of *fizzy-related* (*fzr*), which targets cyclins A and B for APC-dependent proteolysis and relieves the block to re-replication (Sigrist and Lehner, 1997). It is difficult to unambiguously determine the identity of the other H3-expressing cells due to the disorganization of the embryo. These cells also incorporate BrdU and are therefore replicating. They may represent other cells entering an endocycle or they could correspond to cells of the ventral CNS that normally continue to divide and express histone mRNA. We occasionally observed phospho-H3-positive mitotic cells in this region of *Df(3R)stg*^{AR2} mutant embryos (data not shown), indicating that at later stages of development, some cells are able to escape *stg*-induced G2 arrest and re-enter the cell cycle. How this occurs is not clear. These data indicate that stg^{cdc25} is not required for embryonic histone gene expression in all cells, and in particular is not required in endocycling cells.

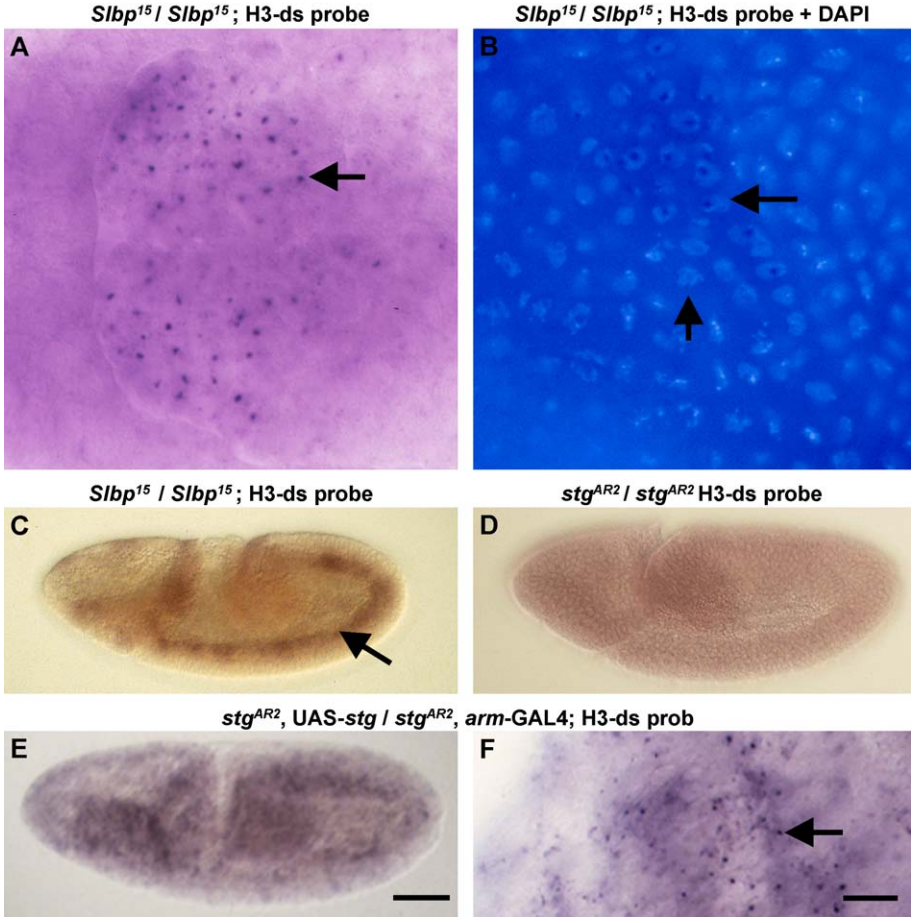
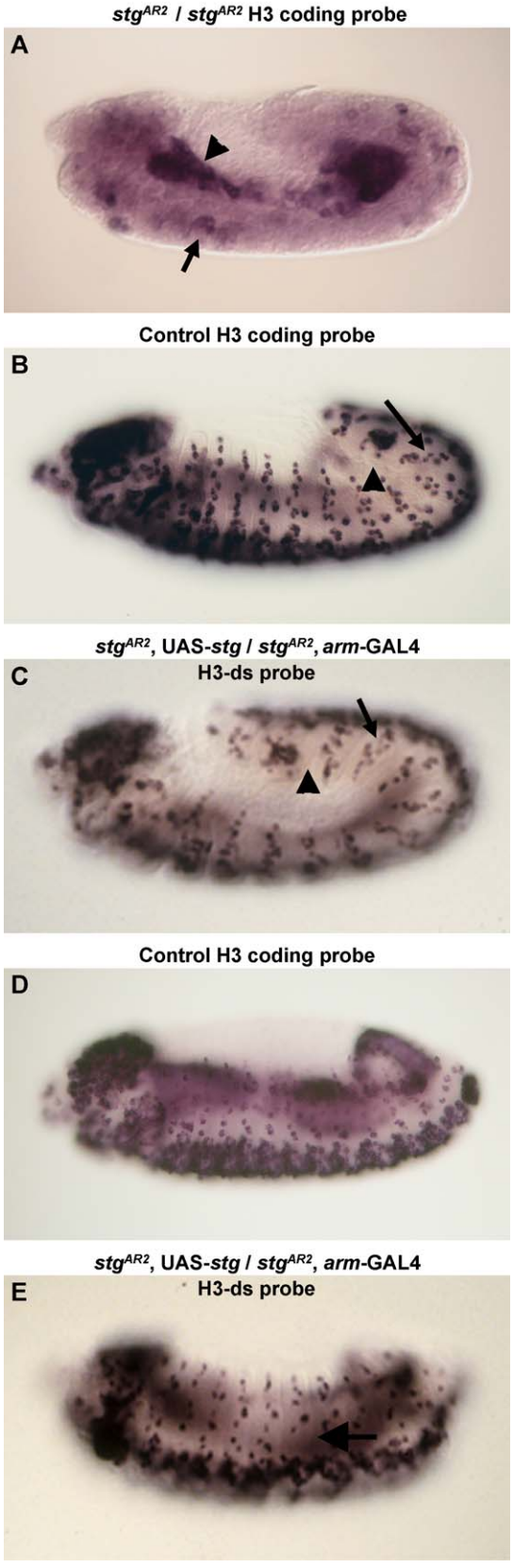


Fig. 2. Histone H3 transcription requires stg^{cdc25}. (A) A gastrulating *Slbp*^{15}/*Slbp*^{15} mutant embryo hybridized with the H3-ds probe. Nascent transcripts (arrow), which arose in G2 of cycle 14, are detected in S phase 15 of domain 4 in this image. (B) Mitotic domain 2 of an *Slbp*^{15}/*Slbp*^{15} mutant hybridized with the H3-ds probe and stained with DAPI. The horizontal arrow indicates a nucleus that is still in $G2_{14}$ with a prominent transcription dot. The vertical arrow indicates condensed chromosomes in a mitosis 14 nucleus without a transcription dot due to abortion of nascent transcripts during mitosis (Shermoen and O'Farrell, 1991). (C) Stage 8 *Slbp*^{15}/*Slbp*^{15} mutant embryo hybridized with the H3-ds probe. The arrow indicates accumulation of mis-processed H3 mRNA in mitotic domain 10. (D) Stage 8 *Df(3R)stg*^{AR2} homozygous mutant embryo hybridized with H3-ds probe. Note the lack of signal in this *stg Slbp* double mutant. (E) Constitutive expression of UAS-Stg by *arm*-GAL4 results in re-accumulation of histone message detected with the H3-ds probe in early *Df(3R)stg*^{AR2} mutant embryos. Scale bar indicates 50 μm. (F) Higher magnification of embryo in panel H showing transcription dots (arrow) in histone-expressing cells. Scale bar indicates 17 μm.

Constitutive stg expression restores near normal histone mRNA expression and embryo morphology to stg mutants

The aberrant morphology of *stg* mutant embryos is due to precocious arrest in cell cycle 14 rather than cell cycle 17, reducing the number of cells in the embryo. The complex spatiotemporal pattern of post-blastoderm, *stg*-dependent cell cycles is thought to be important for morphogenesis because it coordinates entry into mitosis with cell movements during gastrulation. However, Edgar and O'Farrell (1990) showed several years ago that expression of wild-type *stg* using three separate heat shocked-induced transient pulses can substantially rescue the morphological defects of *stg* mutant embryos. Interestingly, both the overall embryo morphology and the pattern of histone H3 expression appear remarkably normal even after constitutive expression of UAS-stg in *Df(3R)stg*^{AR2} mutant embryos (Figs. 3B and C). Histone synthesis in UAS-stg-rescued embryos ceases on schedule in quiescent cells (arrowhead Figs. 3B and C) and continues in cells of the developing CNS and PNS (arrow, Figs. 3B and C). However, histone expression does eventually show signs of disorganization in the PNS, which becomes more obvious at later stages of development (Figs. 3D and E, arrow). While this may be caused by the absence of the normal timing of *stg* expression, we cannot exclude the possibility that other genes deleted by *Df(3R)stg*^{AR2} could be responsible for this effect. Nevertheless, in the face of unregulated, continuous *stg* transcription, which disrupts the post-blastoderm mitotic domain pattern, the embryo is apparently able to rely on other cell cycle regulatory cues to produce the correct number of cell divisions and restore near normal morphology to the embryo.



CDC2 can activate histone transcription during G2 in the early embryo

Thus far, we have shown that histone gene transcription is dependent on *stg* and that restoring *stg* expression in *stg* mutant embryos reestablishes histone transcription. Because the only known biochemical function of stg^{cdc25} is to dephosphorylate and activate the mitotic CDK, Cdc2, we tested whether a constitutively activated form of Cdc2 ($Cdc2^{AF}$) could bypass the requirement for stg^{cdc25} activity and drive histone expression. $Cdc2^{AF}$ contains Ala and Phe substituted for Thr14 and Tyr15, the sites of inhibitory phosphorylation normally removed by stg^{cdc25}. We introduced a heat shock-inducible *hsp70-Cdc2^{AF}* transgene in a *Df(3R)stg^{AR2}* mutant background. Because the expression of $Cdc2^{AF}$ depends on heat shock treatment, both histone transcription and cell cycle progression can be followed after induction of $Cdc2^{AF}$. When *Df(3R)stg^{AR2}-hsp70-Cdc2^{AF}* embryos, aged to the time of G2 in cycle 14, were placed at 37°C for 30 min and allowed to recover for 30 min, ubiquitous accumulation of unprocessed histone H3 mRNA was detected with the H3-ds probe (Figs. 4A and B). No H3 expression was detected in these embryos in the absence of heat shock. As we observed with constitutive *stg* expression, higher magnification revealed the presence of transcription dots throughout the embryo (arrowhead, Fig. 4B). To determine whether the cells enter mitosis following $Cdc2^{AF}$ induction, embryos were labeled with anti-phosphohistone H3 to detect mitotic chromosomes. At 30 min following heat shock induction of $Cdc2^{AF}$, cells throughout the embryo contained phosphohistone H3 (Fig. 4C), and at 45 min the nuclei labeled even more intensely (Fig. 4D). Thus, *Df(3R)stg^{AR2}* mutant embryos that receive pulsed expression of $Cdc2^{AF}$ express histone mRNA within 30 min, which coincides with the beginning of mitotic entry. In addition, *Df(3R)stg^{AR2}* mutant embryos that received pulsed expression of $Cdc2^{AF}$ did not incorporate BrdU during a 15-min pulse spanning 25–40 min post-heat shock (Fig. 4E), whereas at this stage wild-type embryos label with BrdU in the expected mitotic domain pattern (Fig. 4F). This indicates

Fig. 3. Constitutive Stg expression restores nearly normal morphology and pattern of histone expression to *stg* mutant embryos. (A) Germ band retracting *Df(3R)stg^{AR2}* homozygous mutant embryo hybridized with H3 coding probe. Histone mRNA synthesis occurs in endocycling midgut cells (arrowhead) and unspecified ventral cells (arrow). (B) Wild-type stage 12 embryo hybridized with the H3 coding probe. The obvious segmental pattern results from lack of expression in quiescent epidermal cells (arrowhead) and continued expression in the developing PNS (arrow). (C) Stage 12 *Df(3R)stg^{AR2}*, UAS-*stg^{cdc25}*/*arm*-GAL4, *Df(3R)stg^{AR2}* embryo hybridized with the H3-ds probe. Note that the pattern of misprocessed H3 accumulation in the *stg*-rescued embryo is similar to that in wild type (arrow and arrowhead as in panel B). (D) Phenotypically wild-type stage 13 embryo from a *stg* mutant cross hybridized with the H3 coding probe. (E) Stage 13 *Df(3R)stg^{AR2}*, UAS-*stg^{cdc25}*/*arm*-GAL4, *Df(3R)stg^{AR2}* embryo hybridized with the H3-ds probe. At this later stage, the pattern of H3 expression in *stg*-rescued embryos appears somewhat disorganized in the PNS in some segments (arrow).

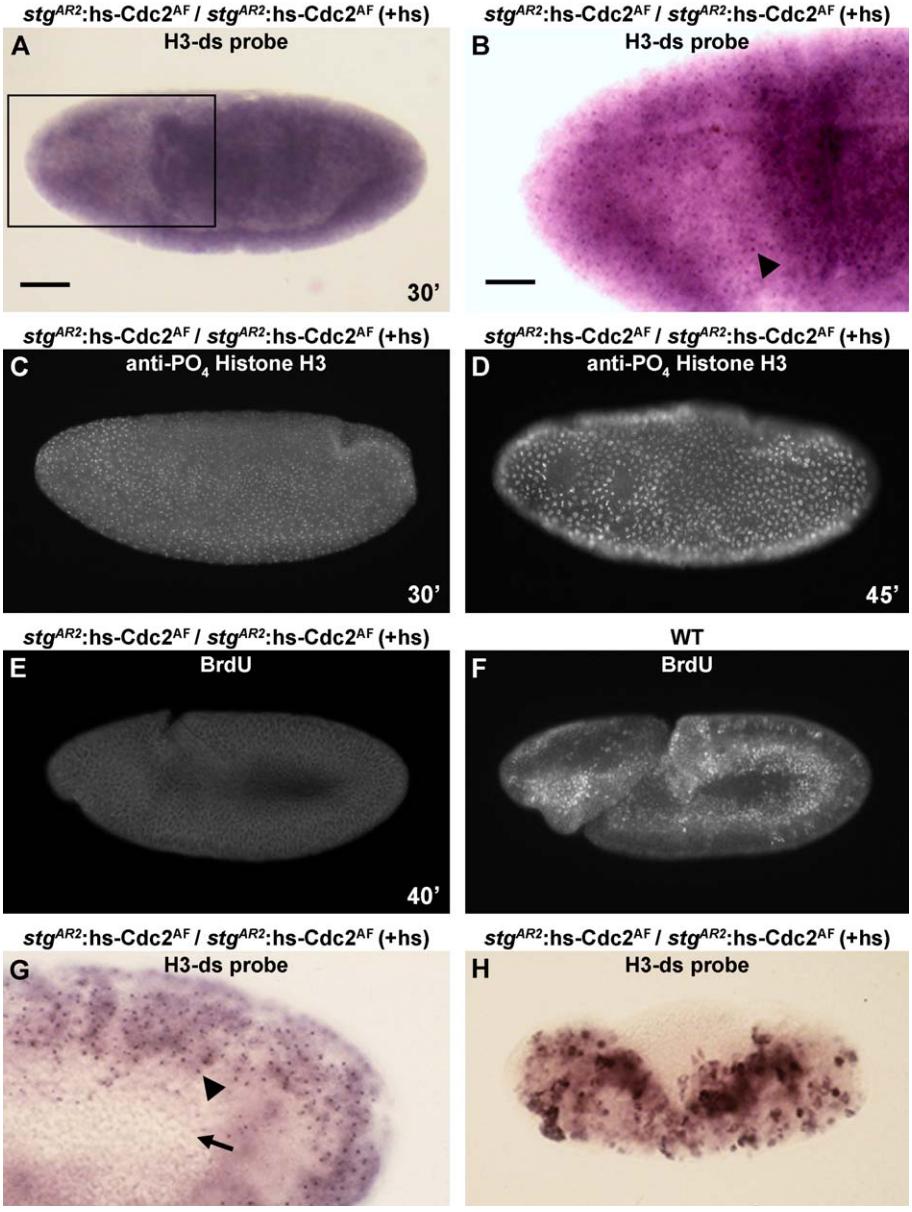


Fig. 4. Ectopic Cdc2^{AF} expression in *Df(3R)stg^{AR2}* mutants triggers histone transcription during G2. (A) *Df(3R)stg^{AR2}* stage 9 mutant embryo carrying a hsp70-Cdc2^{AF} transgene fixed 30 min after heat shock and hybridized with the H3-ds probe. Scale bar indicates 50 μm. (B) Higher magnification of the boxed area of the embryo in panel A allows visualization of transcription dots in histone-expressing cells (arrowhead). Scale bar indicates 25 μm. (C and D) *Df(3R)stg^{AR2}* stage 8 mutant embryos carrying a hsp70-Cdc2^{AF} transgene fixed 30 or 45 min, respectively, after heat shock and stained with anti-phosphohistone H3 antibody to detect mitotic cells. Note that cells are beginning to enter mitosis by 30 min and stain more intensely at 45 min after Cdc2^{AF} induction. (E) *Df(3R)stg^{AR2}* mutant embryo carrying a hsp70-Cdc2^{AF} transgene pulse labeled with BrdU from 25 to 40 min after heat shock. Note the lack of replicating cells, consistent with these cells being in mitosis at this time. (F) BrdU-labeled wild-type embryo, in which many cells have completed mitosis of cycle 14 and entered S phase 15 in the mitotic domain pattern. (G) Posterior of germ band-extended *Df(3R)stg^{AR2}* mutant embryo carrying a hsp70-Cdc2^{AF} transgene fixed 30 min after heat shock and hybridized with the H3-ds probe. The appearance of nascent transcripts (arrowhead) indicates the initiation of transcription by Cdc2. Transcription induction is not detected in all cells, such as those in the amnioserosa (arrow). (H) Germ band-retracted *Df(3R)stg^{AR2}* mutant embryo carrying a hsp70-Cdc2^{AF} transgene fixed 30 min after heat shock and hybridized with the H3-ds probe.

that the Cdc2^{AF}-induced histone transcription in *Df(3R)-stg^{AR2}* embryos occurs during G2 phase. We conclude from these data that the developmentally controlled activation of Cdc2 by *stg* expression causes the initiation of histone gene transcription in late G2 of cycle 14.

We also tested the effects of Cdc2^{AF} expression on histone mRNA synthesis in *Df(3R)stg^{AR2}* mutant embryos that were allowed to age further into development before the heat shock induction. Embryos heat shocked for 30 min at the germ band extension stage displayed clear induction of histone synthesis with obvious transcription dots appearing in a large number of cells (arrowhead, Fig. 4G). This demonstrates that many G2-arrested cells maintain their ability to respond to Cdc2^{AF} induction and initiate histone

transcription for several hours. However, the number of cells able to respond to this induction was progressively more restricted over time. For instance, cells of the amnioserosa did not respond to induction at the germ band extended stage (arrow, Fig. 4G), and by germ band retraction (approximately 9 h), many fewer cells displayed induction of histone mRNA accumulation (Fig. 4H) or transcription dots after the heat shock (data not shown). Because individual cells of *stg* mutant embryos appear healthy by several criteria and survive until the end of embryogenesis and secrete cuticle (Edgar and O'Farrell, 1989, 1990), one possible explanation for this result is that the responsiveness of histone transcription to cdc2 activity changes during embryogenesis, similar to the loss of dependency of H3 expression on *stg* function in endocycling tissues of older embryos (i.e., Figs. 2A and B).

Cyclin E is required for histone expression after the introduction of G1 regulation

After mitosis of cycle 16, cells arrest in G1 phase for the first time. In the epidermis, G1 arrest is established by downregulation of cyclin E transcription and by the expression of *dacapo*, a homolog of p27 that inhibits cyclin E/CDK2 kinase activity (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996). G1 quiescence is maintained by Rbf1, a *Drosophila* retinoblastoma homolog that represses the activity of the heterodimeric transcription factor E2f1/Dp and prevents the expression of genes required for S phase (Du and Dyson, 1999). Endocycling cells of the midgut and proliferating CNS cells depend on zygotic expression of *cyclin E* to continue replicating, and *cyclin E* mutant embryos do not incorporate BrdU after cycle 17 (Knoblich et al., 1994). To determine whether histone expression is dependent on continuing DNA synthesis and cell cycle progression, we analyzed the effect of the *cyclin E* mutation on histone expression. In germ band-retracted embryos, the pattern of histone mRNA expression directly reflects the pattern of S phase, with expression in endocycling cells such as the midgut (arrow, Figs. 5A and B) and in dividing cells of the CNS (arrowhead, Figs. 5A and B). In similarly staged *cyclin E* mutant embryos, the amount of histone H3 or H2b mRNA is dramatically reduced (Figs. 5C and D, respectively), indicating that histone mRNA synthesis is dependent on continued cyclin E activity or DNA synthesis. A few cells in the salivary glands, brain lobes, and ventral nerve cord continue to express detectable histone message, and this likely results from the small amount of DNA synthesis supported by residual maternal cyclin E activity in these cells (Knoblich et al., 1994). Conversely, ubiquitous overexpression of cyclin E using an *hsp70-cyclin E* transgene results in histone H3 expression throughout the embryo (Fig. 5E). Ectopic cyclin E induction by this transgene has previously been shown to drive G1-arrested embryonic cells into S phase as measured by BrdU incorporation (Duronio and O'Farrell, 1994, 1995; Knoblich et al., 1994). We conclude that histone expression requires cyclin E when G1–S regulation appears during embryogenesis.

Cyclin E activates SLBP expression

We are interested in determining the mechanisms by which cell cycle regulators affect the histone pre-mRNA processing machinery. To test whether the increased expression of histone H3 following induction of cyclin E represented appropriately processed mRNA, we hybridized these embryos with the histone H3-ds probe. No H3-ds signal was detected, indicating that the newly synthesized H3 mRNA was processed normally (Fig. 5F). This suggests that either the components of the processing machinery, including SLBP, are present at sufficient levels in G1 cells, or that cyclin E can induce the expression or activation of this machinery as part of its role in activating entry into S phase. To explore these possibilities, we carried out experiments to determine if reducing SLBP function results in the appearance of unprocessed histone mRNA after overexpression of cyclin E. In *Slbp*/+ heterozygous embryos, very little unprocessed histone message was detected after heat shock using either one (Fig. 6A) or two (Fig. 6B) copies of the hsp70-cyclin E transgene. With two copies of hsp70-cyclin E, a few cells began to accumulate mis-processed H3 mRNA (arrowhead, Fig. 6B), but this is a small fraction of the total as indicated by using a coding probe to detect all H3 mRNA (Fig. 5E). As expected, the *Slbp* mutant sibling embryos from this experiment accumulated mis-processed H3 mRNA in response to cyclin E induction, especially in the cells of the amnioserosa (Figs. 6C and D, arrowhead).

These data indicate that a single *Slbp* gene is able to supply sufficient SLBP to process the increased amounts of histone message resulting from ectopic cyclin E expression. Because SLBP protein levels begin to decline once cells enter G1 (Lanzotti et al., 2004), we hypothesized that cyclin E/CDK2 activates the function of SLBP, perhaps by stimulating de novo synthesis of SLBP to accommodate new histone synthesis during S phase. Consistent with this hypothesis, heat shock induction of cyclin E stimulated *Slbp* expression in epidermal cells that normally express no or very little amounts of SLBP mRNA (Figs. 6E and F). Thus, cyclin E activation can contribute to histone biosynthesis via the induction of *Slbp* expression.

Discussion

Eukaryotic cells have evolved sophisticated mechanisms to trigger the accumulation of histone mRNA at the G1–S transition of a canonical, four-phase G1–S–G2–M cell cycle. Here we show that specific cell cycle controls also regulate histone mRNA expression during the post-blastoderm embryonic cell cycles in *Drosophila* that lack a G1 phase. The primary control point for progression through

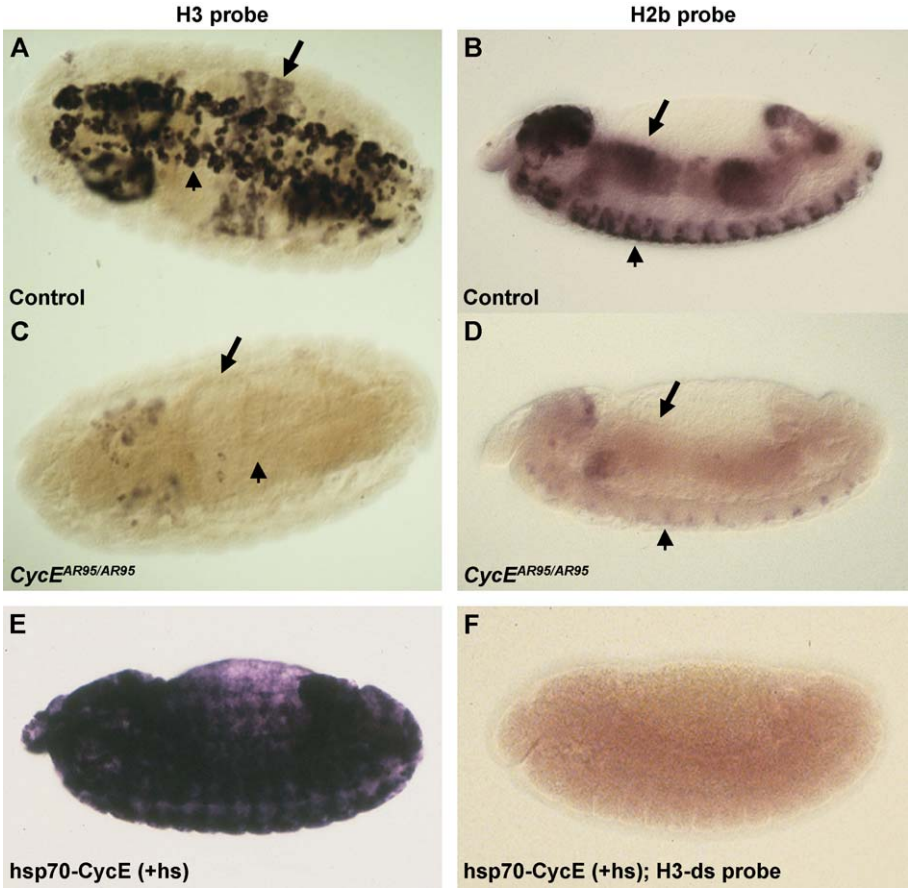


Fig. 5. Histone expression requires cyclin E. (A) Stage 14 control embryo hybridized with H3 coding probe. In this and the other panels, the arrow indicates endocycling midgut cells and the arrowhead indicates dividing cells in the CNS. (B) Stage 13 control embryo hybridized with a histone H2b probe. (C) Stage 14 *cyclin E*^{AR95} homozygous null mutant embryo (sibling to panel A) hybridized with a histone H3 coding probe. Note that the embryos in panels A and C were photographed in the same microscopic field to allow a direct comparison. (D) Stage 13 *cyclin E*^{AR95} homozygous null mutant embryo (sibling to panel B) hybridized with a histone H2b coding probe. (E) Stage 13 hsp70-CycE/hsp70-CycE embryo hybridized with an H3 coding probe 30 min following heat shock induction of cyclin E. (F) Stage 13 hsp70-CycE/hsp70-CycE embryo hybridized with an H3-ds probe 30 min following heat shock induction of cyclin E. The lack of staining indicates that all histone H3 mRNA produced in response to cyclin E induction was processed normally. In panels B and D–F, the embryos are oriented anterior at left and dorsal at right. In panels A and C, anterior is at left, but a ventral view is shown.

these cycles is the G2–M transition, which is regulated by zygotic expression of the stg^{cdc25} phosphatase. Our data indicate that histone transcription is triggered upon activation of Cdc2 kinase in response to the initiation of *stg* transcription during late G2. Because S phase begins immediately after completion of mitosis in these cycles, this transcriptional mechanism ensures the timely production of histones. New synthesis of histones is necessary at this stage of development, because homozygous deletion of the histone locus blocks DNA synthesis as early as cycle 15 or 16 (Smith et al., 1993).

The mechanism by which Cdc2 activity triggers histone gene transcription in cycle 14 is not known. Some possible scenarios are provided by recent studies in mammalian cells indicating that proteins directly phosphorylated by CDKs, such as NPAT and HIRA, may regulate histone gene transcription. NPAT localizes to the histone locus in cultured cells and can stimulate transcription from histone promoter-reporter constructs in transient transfection assays (Ma et al., 2000; Zhao et al., 2000). HIRA acts as transcriptional repressor and can downregulate histone expression and arrest cells in S phase when overexpressed (Hall et al., 2001; Nelson et al., 2002). While a *Drosophila* orthologue of NPAT has not been characterized, the single *Drosophila* HIRA gene is expressed during embryonic development (Kirov et al., 1998; Llevadot et al., 1998). Cyclin E or A/cdk2 phosphorylation of mammalian HIRA occurs during S phase in mammalian cells, presumably relieving repression by HIRA (Hall et al., 2001). However, cyclin E/cdk2 is constitutively active during the post-blastoderm cycles in *Drosophila* embryos (Sauer et al., 1995), making the regulation of histone transcription by HIRA unlikely at this stage of development. Perhaps the role of stg/cdc2 is to activate remodeling of the histone gene chromatin, allowing transcription of the histone genes. With the constitutive expression of cyclin E/cdk2, this could then result in transcriptional activation of the histone genes in G2 of cycle 14. Histone gene transcription persists into S phase of the next G2-regulated cell cycles, where cyclin E/cdk2 remains active. After the initial activation in G2 of cycle 14,

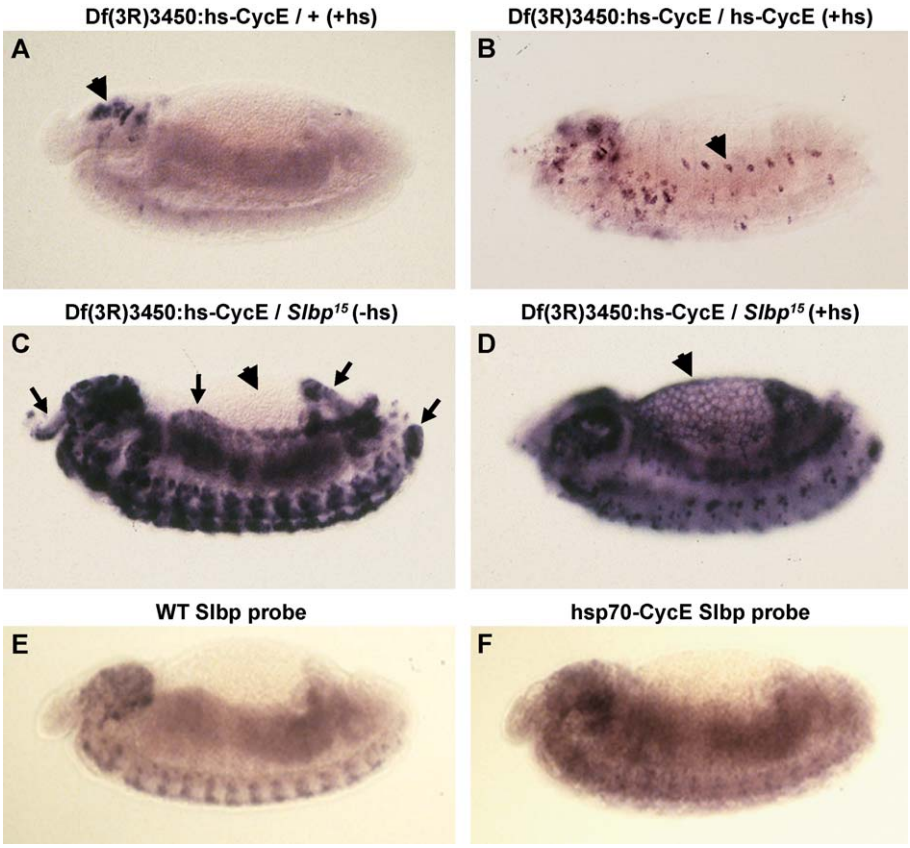


Fig. 6. Cyclin E induces *Slbp* expression. (A–D) Stage 14 embryos hybridized with H3-ds probe to detect mis-processed histone H3 mRNA. (A) A *Df(3R)3450*, *hsp70-Cyc E*/+ embryo subjected to heat shock induction of cyclin E and hybridized with the H3-ds probe (*Df(3R)3450* deletes the *Slbp* locus). The unprocessed H3 mRNA in the brain (arrowhead) was also observed in *Slbp*/+ heterozygotes in the absence of additional cyclin E expression, which we previously interpreted as an indication of SLBP haploinsufficiency in this particular tissue (Lanzotti et al., 2002). (B) A *Df(3R)3450*, hsp70-Cyc E/+, hsp70-Cyc E embryo subjected to heat shock induction of cyclin E from two copies of the transgene (as in Fig. 5E) and hybridized with the H3-ds probe. Some cells produce mis-processed H3 mRNA (arrowhead), although the majority of cells in the embryo do not. (C) *Df(3R)3450*, *hsp70-Cyc E*/*Slbp*^{15} embryo hybridized with the H3-ds probe. The embryo exhibits the previously described aberrant pattern of mis-processed histone mRNA expression (Lanzotti et al., 2002; Sullivan et al., 2001), which includes tissues that are not replicating (arrows). The arrowhead indicates the amnioserosa cells. (D) Following heat shock induction of cyclin E in the *Slbp* mutant embryos, additional unprocessed histone mRNA is observed, especially in the amnioserosa (arrowhead). (E) Stage 13 wild-type embryo hybridized with a probe synthesized from *Slbp* cDNA. (F) Stage 13 hsp70-CycE/hsp70-CycE embryo subjected to a 30-min heat shock and hybridized with a probe synthesized from *Slbp* cDNA. Note that ectopic SLBP mRNA expression is observed throughout the epidermis.

histone gene transcription may not continuously require *stg*, but may simply require active cyclin E/cdk2.

Our data indicate that multiple mechanisms regulate histone expression in the embryo. The mode of regulation of histone gene expression changes with the introduction of G1–S regulation during cell cycle 17. Histone expression no longer requires *stg* during midgut endocycles, but instead requires cyclin E. This is not a mechanism specific to endocycles, because mutation of cyclin E also blocks histone expression in mitotic cells of the CNS and PNS. Because cyclin E is a potent regulator of DNA synthesis and cell cycle progression, it is difficult to separate the effects of cyclin E manipulation on histone expression from effects on DNA replication. Data collected from work done in cell culture demonstrate that histone synthesis and DNA synthesis are interdependent. When cells are treated with inhibitors of DNA replication such as aphidicolin or cytosine arabinoside, replication stops and histone mRNAs are rapidly degraded (Graves and Marzluff, 1984; Sittman et al., 1983). Conversely, replication ceases when histone transcription is reduced in S phase cells by overexpression of HIRA (Nelson et al., 2002) or when chromatin assembly is blocked (Ye et al., 2003). These data argue that DNA synthesis requires sufficient levels of histone protein to assemble nucleosomes in newly duplicated DNA and that production of histone mRNA and protein is dependent on ongoing DNA synthesis. Nevertheless, at the G1–S transition, cyclin E may activate histone transcription independently of activating DNA synthesis. Overexpression of NPAT, a cyclin E/cdk2 substrate (Zhao et al., 1998), activates histone transcription by a mechanism independent of NPAT's ability to stimulate entry into S phase (Wei et al., 2003).

Cell cycle regulators may also contribute to posttranscriptional histone mRNA regulation. In mammalian cells, the level of SLBP protein is tightly cell cycle regulated and SLBP is the sole cell cycle-regulated factor required for histone pre-mRNA processing (Whitfield et al., 2000; Zheng et al., 2003). Microarray experiments using RNA

extracted from cultured mammalian cells show relatively small periodic changes in steady-state levels of SLBP mRNA during the cell cycle (Ishida et al., 2001; Muller et al., 2001; Whitfield et al., 2002). Our results indicate that in *Drosophila* embryos the *Slbp* gene is induced in response to cyclin E activation. The mechanism of this induction by cyclin E is unclear, although it may involve the E2F transcription factor, which is activated when cyclin E is overexpressed in embryos (Duronio and O'Farrell, 1995). *Slbp* was recently shown to be a transcriptional target of E2F in cultured S2 cells (Dimova et al., 2003). Such regulation may serve to stimulate histone processing concomitantly with increased histone transcription during the G1–S transition. Consistent with this hypothesis, the transcriptional regulator NPAT was recently shown to also be regulated by E2F in mammalian cells (Gao et al., 2003). Future work will be aimed at tackling such questions to determine potentially direct connections between cyclin/cdk activity and histone synthesis.

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