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Developmental control of a G_1-S transcriptional program in *Drosophila*

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Summary

We have defined a coordinate program of transcription of S-phase genes (DNA polymerase α, *PCNA* and the two ribonucleotide reductase subunits) that can be induced by the G_1 cyclin, cyclin E. In *Drosophila* embryos, this program drives an intricate spatial and temporal pattern of gene expression that perfectly parallels the embryonic program of S-phase control. This dynamic pattern of expression is not disrupted by a mutation, *string*, that blocks the cell cycle. Thus, the transcriptional program is not a secondary consequence of cell cycle progression. We suggest that developmental signals control this transcriptional program and that its activation either directly or indirectly drives transition from G_1 to S phase in the stereotyped embryonic pattern.

Keywords

Drosophila; G_1-S phase; cell cycle; transcription; endoreduplication; cyclin E

Introduction

The cell cycle is not a relentless oscillator. It is regulated by a myriad of inputs such as cell size, nutritional status and, importantly, signals from other cells (O'Farrell, 1992; Pardee, 1989). Dramatic cell cycle regulation occurs during embryogenesis where precise and stereotyped programs of cell division guide orderly morphogenesis (Foe et al., 1993). In *Drosophila*, the mother supplies the egg with a plentiful reservoir of cell cycle components so that the earliest cycles are relatively unconstrained and lack the gap phases, G_1 and G_2. However, the early rapid divisions are soon curtailed as constraints are added. The constraints appear one at a time as developmental events remove or inhibit activities that were previously constitutive. Progress of subsequent cycles depends on reprovision of the limiting component. This reprovision is rigidly controlled by the developmental program (O'Farrell et al., 1989).

The first major constraint appears with the introduction of a G_2 phase in cycle 14 (Edgar and O'Farrell, 1990). Like G_2 of the fission yeast, *Schizosaccharomyces pombe* (Gould and Nurse, 1989; Lundgren et al., 1991; Moreno et al., 1990), this G_2 is regulated by inhibitory phosphorylation of Cdc2 kinase. After the first 13 rapid cell cycles, destruction of the maternal supplies of a phosphatase allows inactivation of the Cdc2 kinase by inhibitory phosphorylation in cycle 14 (Edgar and O'Farrell, 1989; Edgar et al., 1994). Mitosis then awaits new expression of the phosphatase and reactivation of Cdc2 kinase. Expression of the phosphatase, which is encoded by the *string* gene (Edgar and O'Farrell, 1989; Gautier et al., 1991), is regulated in an intricate spatiotemporal program that guides the spatiotemporal program of mitoses throughout the embryo (Edgar and O'Farrell, 1990; Foe, 1989). But all regulation of the cell cycle does not occur at the transition from G_2 to mitosis. After three *string* regulated cycles (14, 15 and 16), many of the cells acquire a G_1 phase in cycle 17 (Edgar and O'Farrell, 1990). Thereafter, the length of the G_1 phase is developmentally controlled in a stereotyped pattern. We have

sought to define cell cycle events associated with the appearance of G_1 and its subsequent developmental programming.

Numerous regulatory signals, including the mating pheromones of *Saccharomyces cerevisiae* and growth factors of mammalian cells, influence a decision whether to arrest the cell cycle prior to S phase (Pardee, 1989; Chang and Herskowitz, 1990; Ewen et al., 1993; Koff et al., 1993). These various signals appear to be integrated prior to the activation of a G_1 regulatory decision which commits the cell to progress into S phase and divide (Pringle and Hartwell, 1981; Pardee, 1989). A transcriptional program that drives expression of a number of replication functions such as ribonucleotide reductase (RNR) accompanies the transition to S phase in diverse organisms (Andrews and Herskowitz, 1990; Lowndes et al., 1992b; Nevins, 1992).

We show here that transcription of four S phase genes that are constitutively expressed during the early cycles is extinguished upon appearance of a G_1. Subsequent expression of each gene occurs in an identical, complex spatiotemporal pattern that is precisely coincident with the onset of S phase. This transcription program can be provoked by the G_1 cyclin, cyclin E. We suggest that this coordinate expression of S phase genes is analogous to the START program of transcriptional control described in yeast and that, in *Drosophila* embryos, the program is developmentally regulated.

Materials and Methods

Fly stocks

Wild-type embryos and DNA were of the Oregon R strain. *stg*^{7B69}/TM3 *Sb* and *stg*^{AR2}/TM3 *Sb* (a transcription null allele; Bruce Edgar, personal communication) stocks were used for the analysis of homozygous embryos lacking string function. Mutant embryos were unambiguously identified from their altered morphology and from the reduced cell number as determined by staining nuclei with the DNA-binding dye Hoechst 33258. All embryos were fixed in a 1:1 mixture of heptane:7% formaldehyde/PBS for 25 minutes and devitellinized. Immunostained embryos were mounted for microscopy in Fluoromount G (Fisher).

Probes used for whole-mount in situ hybridization analysis

All probe sequences were obtained by PCR amplification of genomic DNA prepared from adult Oregon R flies. A degenerate 5′ *DmRNR1* primer (5′AAGGTACCTA(T/C)TT(T/C)GGNTT(T/C)AA(A/G)ACN(C/T)TNGA3′) was derived from the conserved RNR large subunit amino acid sequence YFGFKTLE. A degenerate 3′ primer (5′-CCGAATTCCAT(A/G)AANA(A/G)(A/G)TCNGGNATCCA3′) was derived from the conserved RNR large subunit sequence WIPDLF. (N=A/C/T/G) The 5′ and 3′ primers for *DmRNR2* were 5′AAGGTACCGA(T/C)GGNAT(T/C/A)GTNAA(T/C)GA(A/G)AA3′ and 5′CCGAATTC(C/T)TT(C/T)TC(A/G)AA(A/G)AA(A/G)TTNGT3′, derived from conserved RNR small subunit amino acid sequences DGIVNEN and TNFFEK, respectively. *Eco*RI + *Kpn*I-digested PCR products were cloned into pBLUESCRIPT SKII–. *PCNA* probe sequences encompassing the entire open reading frame including the single intron (Yamaguchi et al., 1990) were obtained using the primers 5′CGGAATTCATGTTCGAGGCACGCCTGGGTCAA3′ (5′) and 5′CGTCTAGATTATGTCTCGTTGTCCTCGATCTT3′ (3′). DNA *POLa* probe sequences encompassing 900 bp of exon 2 (Hirose et al., 1991) were obtained using the primers 5′CGTCTAGAGGTTATGCAGAAGATCTTCGG3′ (5′) and 5′GCGAATTCTACTGGATCCTCATAGGCCTC3′ (3′). *Eco*RI + *Xba*I-digested *PCNA* and *POLa* PCR products were cloned into pBLUESCRIPT SKII–.

In situ hybridizations using digoxigenin-labeled DNA or RNA probes were performed as described (Tautz and Pfeifle, 1989; Lehner and O'Farrell, 1990b) except that xylene treatment was omitted. Digoxigenin-labeled DNA was made by either of two methods; random priming (Lehner and O'Farrell, 1990b) or inclusion of Dig-dUTP in PCR amplification of cloned DNA using the above primers. The final nucleotide concentrations in these reactions were 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 60 μM dTTP, and 40 μM Dig-dUTP (Boehringer). Dig-PCR products were digested with *Hae*III and *Alu*I before hybridization to facilitate probe penetration into fixed embryos. Digoxigenin-labeled RNA (Boehringer kit) was made with 350 μM Dig-UTP, 650 μM TTP, and 1 mM each of ATP, CTP and GTP in T3 or T7 RNA polymerase transcription reactions using linearized pBLUESCRIPT clones as templates. RNA-RNA hybridizations were performed at 70°C in pH 4.5 hybridization solution (Lehner and O'Farrell, 1990b). Embryos were photographed using a Nikon 20× objective and Kodak Ektachrome 160T or Technical Pan film.

Detection of replicating nuclei

Dechorionated embryos were permeabilized with octane and pulse labeled with 1 mg/ml BrdU (Sigma) for 15-30 minutes in Schneider's medium and immediately fixed as described above. Permeabilization and BrdU detection was performed according to Edgar and O'Farrell (1990). Mouse anti-BrdU monoclonal antibody was purchase from Becton Dickinson. Texas Red- or rhodamine-conjugated goat anti-mouse secondary antibodies were from Jackson. For histochemistry, a biotin-conjugated donkey anti-mouse secondary (Jackson) and streptavidin conjugated to horse radish peroxidase (Vector) were used. Conjugates were detected by incubation in 0.5 mg/ml 3,3′-diaminobenzidine (DAB), 0.08% $NiCl_2$ and 0.003% H_2O_2. Simultaneous BrdU labeling and in situ hybridization was performed exactly as in Richardson et al. (1993) except that incorporated BrdU was detected with fluorescently labeled secondary antibodies.

Heat-shock experiments

Overnight collections of embryos from hsp70-cyclin E/CyO or hsp70-cyclin E chromosome two homozygous stocks were heat shocked for 30 minutes by floating the small grape juice/agar egg collection plates on the surface of a 37°C water bath. After a 30-60 minute recovery period at room temperature (23°C), the embryos were prepared for in situ hybridization or labeled with BrdU for 15-45 minutes and immediately fixed as described above.

DNA sequencing and polytene chromosome in situ hybridization

Both strands of the cloned *DmRNR1* and *DmRNR2* PCR products were sequenced using nested oligonucleotide primers. Digoxigenin-labeled *DmRNR1* and *DmRNR2* probes were hybridized to salivary gland polytene chromosomes as follows. Salivary glands were dissected from late third instar larvae, fixed for 30 seconds in 45% acetic acid, transferred to a 1:2:3 mixture of lactic acid:water:acetic acid and squashed between a slide and a siliconized glass coverslip. The spread polytene chromosomes were subsequently dehydrated by soaking for 10 minutes in 95% ethanol and air drying. Dried chromosomes were denatured by rehydrating at 65°C in 2× SSC for 30 minutes and soaking in 70 mM NaOH for 3 minutes. After a brief wash in 2× SSC the denatured chromosomes were dehydrated with an ethanol series and air dried. Hybridization was performed overnight at 42°C with DIG-labelled DNA (prepared as described above) in 2× SSC, 50% deionized formamide, 12.3 mM Tris pH 7.5, 600 mM NaCl, 5× Denhardt's, 1 mM EDTA, 30 μg/ml salmon sperm DNA and 10% dextran sulfate. Hybridized chromosomes were washed 3× 20 minutes in 2× SSC at 53°C followed by 3× 5 minutes in PBS. After a 1 hour incubation with anti-DIG antibodies coupled to horse radish peroxidase (Boehringer) followed by three 5 minute PBS washes, hybrids were detected by

incubation in 0.5 mg/ml DAB, 0.01% H_2O_2, 0.08% $NiCl_2$. Polytene bands were visualized by Giemsa staining and chromosomes were mounted in Permount (Sigma).

Results

Developmental programming of G_1

Visualization of mitotic spindles by tubulin staining and immunological detection of S-phase nuclei labeled with 5-bromodeoxyuridine (BrdU) have provided a detailed description of the cell cycle program in early *Drosophila* embryos (Edgar and O'Farrell, 1990; Foe, 1989). There is no detectable lag between mitosis and S phase during the first 16 cell cycles. However, following mitosis 16, most cells enter a prolonged G_1 arrest in cycle 17. Many of these G_1-arrested cells are destined to polytenize their genomes. Following S phase 17, these cells enter amitotic endoreduplication cycles in which rounds of DNA replication are interrupted by periods of quiescence. Although we will focus on the populations of cells that acquire a G_1 and begin endoreduplication cycles, the still rapidly proliferating cells of the nervous system (Bodmer et al., 1989) and Malpighian tubules (Skaer, 1989; Skaer and Martinez-Arias, 1992) continue to label intensely with BrdU (e.g. see Fig. 3). It has not yet been determined if these more rapidly dividing cells have a G_1 phase.

S17 and the subsequent endoreduplication S phases occur in a stereotyped pattern (Smith and Orr-Weaver, 1991). The salient features of this pattern can be illustrated in the developing midgut. Three regions of the midgut, the anterior midgut (AMG), the central midgut (CMG) and the posterior midgut (PMG), initiate S phase at different times and thus define distinct 'replication domains' (see diagrams in Fig. 3). The CMG enters S17 early during germband retraction (stage 12; stages according to Campos-Ortega and Hartenstein, 1985), and is nearly completed by the time the AMG and PMG enter S17 (stage 13). Following S17 of the AMG and PMG, the CMG again enters S phase (stage 14), an endoreduplication S phase (S18). Although less well characterized, this alternating pattern of midgut S phases continues at least for one more cycle.

We define a program of coordinate transcription of four S-phase genes whose regulation parallels the three principle features of the S-phase program: (i) the initial appearance of a G_1 in cycle 17, (ii) the arrest of some cells (e.g. the epidermal cells) in G_1 throughout the remainder of embryogenesis and, most importantly, (iii) the re-entry of a number of the G_1-arrested cells into S phase according to a complex developmental schedule.

Cloning of the *Drosophila* ribonucleotide reductase genes

Of the numerous S-phase genes whose expression is coupled to entry into S phase, only ribonucleotide reductase (RNR) has shown this coupling in all species examined, from bacteria to mammals (Bjorklund et al., 1990; Elledge and Davis, 1990; Standart et al., 1985). Active RNR is a tetramer composed of two large and two small subunits encoded by separate genes (Reichard, 1993). Degenerate primers were designed from highly conserved regions of each RNR subunit (see Materials and methods). PCR amplification of *Drosophila* genomic DNA yielded unique fragments representing each of the two genes. One product, a ~500 bp fragment (*DmRNR1*), has an open reading frame whose sequence is 43% identical to the expected region of the large subunit of eukaryotic RNRs (Fig. 1A). A second product, a ~650 bp fragment (*DmRNR2*), has an open reading frame whose sequence is 51% identical to the expected region of the small subunit of eukaryotic RNRs (Fig. 1B). Hybridization to genomic blots and salivary gland polytene chromosomes indicated that *DmRNR1* and *DmRNR2* are single copy genes mapping to 31D and 48D, respectively (data not shown).

Oscillations in *DmRNR2* RNA mark the onset of G_1 cycles

In situ hybridization of whole embryos revealed the spatiotemporal pattern of *DmRNR2* RNA accumulation during embryogenesis. A strong signal detected prior to initiation of zygotic transcription indicates that there is a maternal supply of the *DmRNR2* message (Fig. 2A). The maternal RNA declines steadily during cycles 10-13 to near background levels early in cycle 14 (blastoderm stage; Fig. 2B). About 30 minutes into cycle 14 (mid cellularization) zygotic transcription drives reaccumulation of *DmRNR2* RNA. Initial accumulation of *DmRNR2* RNA appears faintly striped along the anterior-posterior axis, perhaps suggesting that the promoter is sensitive to regulators of early pattern formation (data not shown). Through the remainder of cycle 14, all of cycle 15 (data not shown) and into early cycle 16 (Fig. 2E) *DmRNR2* RNA is ubiquitous and nearly uniform. The faint patterns evident at these stages reflect only quantitative differences in accumulation of transcript in different cells, and these differences bare no apparent relation to cell cycle events.

Double label experiments reveal that *DmRNR2* RNA begins to disappear as cells complete cycle 15. Although the elaborate spatial and temporal program of cell cycles 15 and 16 have not been characterized throughout the embryo, the timing of these cell cycles within the dorsal epidermis is relatively simple, and S phases 15 and 16 can be recognized by the pattern of labeling in precisely staged embryos (Foe, 1989; Edgar and O'Farrell, 1990; Lehner and O'Farrell, 1990a; Hartenstein and Campos-Ortega, 1985). BrdU labeling shows that dorsal epidermis of the embryo in Fig. 2G is in S phase 16, and hybridization with *DmRNR2* probe shows that the RNA has begun to decline (Fig. 2E). In a slightly older embryo, the cells of the dorsal epidermis have completed S phase 16, and are in G_2 (Fig. 2H and data not shown). *DmRNR2* RNA is no longer detectable in these cells (Fig. 2F). Ventral epidermal cells enter S phase 16 later (Edgar and O'Farrell, 1990; also compare Fig. 2G and H) and lose *DmRNR2* RNA correspondingly later (Fig. 2E and F). A strong signal is retained in rapidly proliferating tissues, such as the nervous system (Fig. 3B,E,H). Thus, *DmRNR2* RNA declines towards the end of S phase 16 in those cells destined to arrest in G_1 in the next cell cycle.

In contrast to early ubiquitous expression, the levels of *DmRNR2* RNA are patterned from late cycle 16 onward. *DmRNR2* RNA is undetectable in G_1-arrested cells (e.g. see WT embryo in Fig. 5), except for brief bursts of accumulation in cells that re-enter S phase. The bursts of *DmRNR2* expression occur in a spatiotemporal pattern that precisely parallels the pattern of S phases in these tissues. For example, *DmRNR2* RNA signal seen in the CMG early in stage 12 embryos, corresponds to the early S phase 17 characteristic of this region of the midgut (Fig. 3A-C). In an older embryo (stage 13), the central region of the midgut has no signal while the AMG and PMG have a strong signal, corresponding to the later S17 in these regions (Fig. 3D-F). During stage 14, the CMG enters its first endoreduplication S phase (S18), and the signals reverse again: the CMG has a strong signal and the flanking AMG and PMG are again unstained (Fig. 3G-I). This correlation of patterns of *DmRNR2* RNA expression and BrdU incorporation holds throughout the embryo. Thus, onset of a G_1 phase is anticipated by extinction of *DmRNR2* expression, and there is pulse of RNA expression associated with each subsequent S phase.

The relative timing of the pulse of *DmRNR2* expression and DNA synthesis is illustrated by the cells of the anal pads located at the end of the retracting germ band. To verify that these cells are in G_1 during the early stages of germ band shortening, we showed that they fail to incorporate BrdU (i.e. they are not in S phase; Fig. 4C, see inset), and that they fail to stain with antibodies to cyclin A or cyclin B, indicating recent destruction of these proteins in mitosis 16 (data not shown). Early during this G_1 phase there is no *DmRNR2* signal (Fig. 4B). In slightly older embryos that have not yet begun incorporating BrdU into these cells (Fig. 4F) a *DmRNR2* RNA signal is detected (Fig. 4E). *DmRNR2* RNA remains at high levels during early S phase after the completion of germ band retraction (Fig. 4H,I). Thus, the burst of expression

precedes and overlaps S phase. Expression declines before the completion of S phase (data for the anal pads are not shown, but see CMG in Fig. 3E,F).

Together these data reveal three important features of the dynamic *DmRNR2* expression pattern. First, early zygotic transcription is constitutive in cell cycles that lack a G_1 phase. Second, *DmRNR2* expression ceases prior to, and is thus not a consequence of, entry into the first G_1. Third, re-accumulation of *DmRNR2* RNA occurs shortly before DNA synthesis begins, presumably in response to signals promoting the onset of S phase.

A coordinate program of G_1-S transcriptional control

The START transcriptional program of *S. cerevisiae* includes a number of genes encoding enzymes involved in DNA replication (Andrews and Herskowitz, 1990). To test if this is also the case in *Drosophila*, we examined expression of the other subunit of ribonucleotide reductase, *DmRNR1*, as well as DNA polymerase *α*(Hirose et al., 1991) and the DNA polymerase δ accessory subunit, *PCNA* (Yamaguchi et al., 1990). Each of these genes has a spatiotemporal program of expression that is identical to that of *DmRNR2*, except for minor differences in the extent of the decline in maternal signal prior to zygotic cycle 14 expression (data not shown). Fig. 3E,J,K,L illustrates the patterns of expression observed in stage 13 embryos and provides a striking example of the parallel transcriptional activation of these genes. This coincidence holds for the entirety of the detailed program of G_1 to S control at least through stage 16 (the last stage we have examined). The coordination of transcription of *DmRNR1*, *DmRNR2*, *PCNA* and *POLα* presumably involves common transactivation mechanisms, since these genes map to widely separated sites: 31D, 48D, 56EF and 93E, respectively (this work and Yamaguchi et al., 1990; Melov et al., 1992).

Cyclin E will activate the G_1-S transcriptional program

G_1 cyclins activate the START transcriptional program of *S. cerevisiae* (Dirick et al., 1992; Marini and Reed, 1992; Nasmyth and Dirick, 1991; Ogas et al., 1991). Several observations suggested that cyclin E might play an analogous role in metazoans (Cao et al., 1992; Hinds et al., 1992; Lees et al., 1992). Since induction of cyclin E expression in *Drosophila* can drive G_1 cells into S phase (H. Richardson and R. Saint, personal communication; Knoblich et al., 1994), we tested whether it would activate transcription of the S-phase genes. Embryos were collected from a stock carrying a homozygous cyclin E construct under the control of a heat-shock promoter (provided by H. Richardson and R. Saint). A 30 minute heat shock (37°C) resulted in induction of S phase in cells arrested in G_1 as revealed by BrdU incorporation into the epidermis of a stage 14 embryo (Fig. 5). Parallel heat shocks of embryos lacking the cyclin E transgene showed no alteration in S-phase pattern (Fig. 5 WT panels). Heat-shock-induced cyclin E expression also activated widespread expression of *DmRNR1*, *DmRNR2*, *PCNA* and *Pol α* (shown for *DmRNR2* in Fig. 5). This activation includes tissues that are normally silent for the remainder of embryogenesis after entry into G_1 (e.g. see the epidermis in Fig. 5). However, the induced expression is not uniform and the responsiveness of given tissues changes at different times. Heat treatment of embryos collected from a stock heterozygous for the cyclin E transgene caused more pronounced patterns (not shown), suggesting that the degree of the response is sensitive to the dosage of cyclin E. Apparently, the ability of cyclin E to activate the G_1-S transcriptional program is modulated by additional factors that we have not yet characterized.

The time course of the cyclin E response is consistent with an involvement of the transcription program in progress to S phase. In stage 14 and older embryos, short BrdU pulses (15 minutes) begin detecting ectopic S phase between 45 and 60 minutes after the heat-shock treatment. In contrast, ectopic *DmRNR2* RNA accumulation has already occurred by 45 minutes after heat

shock (not shown). Therefore, the transcription program may be required to restore sufficient levels of S-phase functions after prolonged G_1 arrest (see Discussion).

Developmental signals rather than cell cycle progress activates the G_1-S transcriptional program

To examine whether the transcriptional program that we have characterized is only a secondary outcome of cell cycle progress toward S phase, we tested whether the program operates following cell cycle arrest. The cell cycle of *string* mutant embryos is blocked in G_2 of cycle 14 (Edgar and O'Farrell, 1989). The patterns of expression revealed by probes for *DmRNR2* (Fig. 6) and *PCNA* (not shown) were virtually identical in wild-type and *string* mutant embryos. Constitutive early expression terminates normally, and subsequent bursts of expression mimic the wild-type pattern (cf. Fig. 6A with Fig. 3E and Fig. 6C with Fig. 3H). Note that S17 is blocked in the string mutant (Fig. 6B), while the programmed transcription of *DmRNR2* is unabated (Fig. 6A). Thus, the program of G_1- to S-phase transcription operates independently of cell cycle events. Apparently, developmental controls trigger this program at the appropriate times and in the appropriate cells.

A developmental program that overcomes the block to re-replication

Endoreduplicating cells must bypass the usual requirement of S phase for a preceding mitosis. Since S phase 18 is the first S phase that occurs without a preceding mitosis, we expect the bypass to be induced in those cells undergoing this round of DNA replication. This bypass was detected in our analysis of DNA synthesis in *string* mutant embryos. As expected, BrdU labeling failed to detect DNA synthesis in *string* embryos at the times of normal S phase 15 or 16 (not shown). Similarly, despite the accurate timing of the bursts of *DmRNR2* expression, *string* null mutant embryos exhibited no BrdU incorporation in the S phase 17 pattern (Fig. 6B). However, BrdU incorporation was detected in *string* embryos at the time and position of the first endoreduplication S phase (S18 in the CMG, Fig. 6D). Note that, while our results are not unlike those of Smith and Orr-Weaver (1991), we discriminate between S17 (which follows mitosis) and S18 (an endoreduplication S phase). Only those S phases that normally occur without a preceding mitosis occur in a *string* mutant. Thus, an ability to enter S phase without passing through mitosis (checkpoint bypass) is acquired in particular cells (those exhibiting endoreduplication) just before they progress to S phase 18. The observed renewal of replication in a *string* mutant suggests that an event leading to checkpoint bypass is programmed by developmental controls independent of cell cycle processes.

Discussion

We have shown that coordinate transcription of genes involved in DNA replication occurs prior to the G_1- to S-phase transition in *Drosophila* embryos. This transcriptional program is governed by developmental signals, as expected if its regulation were a means of developmental control of cell cycle progress.

Introduction of developmental constraints on the cell cycle

Development begins with abundant supplies of maternally provided cell cycle components and few cell cycle constraints. Constraints are added by changes that make previously constitutive components rate limiting. The introduction of a delay between mitosis and S phase (a G_1 phase) at cycle 17 requires introduction of a constraint that prevents immediate progress from mitosis to S phase. Reduction in one or more activities required for DNA replication could provide such a constraint. It is our premise that it is a change in gene expression, from constitutive to regulated, that introduces a G_1 phase into the cell cycle and that regulates its length.

In diverse species, transcriptional activation of a number of genes accompanies entry into S phase. Our analysis of the expression of the small subunit of ribonucleotide reductase, *DmRNR2*, uncovered such a G_1 to S phase program of transcription in *Drosophila* embryos. *DmRNR2* RNA is expressed constitutively prior to cycle 17, extinguished in anticipation of appearance of a G_1, and re-expressed in abrupt bursts at subsequent transitions from G_1 to S phase.

Ribonucleotide reductase is, perhaps, an unlikely candidate for the key regulator of the G_1- to S-phase transition. Indeed, it is a marker of a larger regulatory program rather than the focus of it. In addition to *DmRNR2*, we have examined three other genes encoding products involved in DNA synthesis, *DmRNR1*, polymerase *α* and *PCNA*. Expression of each of these genes, but not other genes less directly involved in DNA synthesis such as topoisomerase II (R. J. D and P. H. O'F., unpublished observations), parallels that of *DmRNR2*. In addition, cyclin E appears to be similarly regulated (Knoblich et al., 1994; R. J. D and P. H. O'F., unpublished observations). Thus, a coordinate program of G_1- to S-phase transcription drives expression of at least five genes. Upon introduction of a G_1, we suggest that one or more of the products under the control of the coordinate G_1- to S-phase transcriptional program becomes limiting. But which if any of the regulated genes limits progress to S phase?

In *S. cerevisiae*, the products of all CDC genes, except for CDC4, are sufficient for more than one cell cycle, and overexpression of Cdc4 provides a supply sufficient for several cell cycles without disturbing the cycle (Breck Byers, personal communication). This suggests that periodic expression of these genes is not crucial to the cell cycle. In contrast, periodic expression of the G_1 cyclins encoded by the CLN genes appears to necessary for the *S. cerevisiae* cell cycle (Nasmyth and Dirick, 1991; Ogas et al., 1991), and constitutive expression disturbs the cycle and drives premature S phase (Hadwiger et al., 1989). Similarly, in *Drosophila Cyclin* E mutant embryos S phase is blocked once maternal cyclin E stores are depleted (Knoblich et al. 1994), and heat-shock induction of cyclin E can induce S phase in G_1-arrested cells (H. Richardson and R. Saint, personal communication; Knoblick et al., 1994; and the present work). Thus, cyclin E limits progress to S phase, but it is not clear whether it acts directly.

Our work shows that cyclin E expression induces a coordinate program of gene expression of S-phase genes. This introduces, but does not resolve the question of whether or not this transcriptional induction is instrumental in driving progress from G_1 to S phase. Analysis of mutations in the induced genes (e.g. RNR2) will determine whether new expression of these genes is required for the progress to S phase. Additionally, analysis of RNR expression in cyclin E mutant embryos will reveal whether or not the transcriptional program requires cyclin E as might be suggested by the induction of this program by heat-shock-induced expression of cyclin E. Although we must await these experiments to resolve these issues, analogies with yeast suggest that the essential role of the S-phase transcriptional program is to induce G_1 cyclins, and that the remainder of the transcriptional program is of secondary importance (Koch et al., 1993).

Cyclin E and an S-phase trigger

Entry into S phase is a decisive event for a cell because it usually represents a commitment to progress through an entire cycle and to divide. The control system governing this commitment step ought to generate an unambiguous on/off signal. In *S. cerevisiae*, this type of signalling is accomplished via a positive feedback loop. The *S. cerevisiae* G_1 cyclins, Cln1 and Cln2, stimulate the Cdc28 kinase to activate two heteromeric transcription factors that each include a Swi6 subunit (Swi4/Swi6 and Mbp1/Swi6) (Andrews and Herskowitz, 1989; Dirick et al., 1992; Koch et al., 1993; Lowndes et al., 1992a; Primig et al., 1992; Verma et al., 1992). These factors drive the program of G_1-S transcription which includes expression of CLN1 and CLN2.

Activation of this self re-enforcing transcriptional loop appears to be the pivotal event in the START decision (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Koch et al., 1993; Nasmyth and Dirick, 1991; Ogas et al., 1991).

G_1 cyclins also play an important role in advancing animal cells to S phase (Baldin et al., 1993; Ohtsubo and Roberts, 1993; Sherr, 1993), and increasing evidence suggests that G_1 cyclins are involved in transcriptional activation. For instance, cyclin E complexes with the cdk2 kinase (Dulic et al., 1992; Koff et al., 1992), promoting phosphorylation of the tumor suppressor gene product Rb (Hinds et al., 1992). Phosphorylation of Rb is thought to induce transcription by disrupting the inhibitory interaction of Rb with E2F-type transcription factors (Cao et al., 1992; Chellappan et al., 1991; Devoto et al., 1992; Mudryj et al., 1991; Shirodkar et al., 1992; Weintraub et al., 1992; Zamanian and La Thangue, 1993; Zhu et al., 1993). Complexes containing E2F have been implicated in transcriptional regulation of replication functions at G_1-S (Blake and Azizkhan, 1989; Mudryj et al., 1990; Pearson et al., 1991; Slansky et al., 1993). Furthermore, overexpression of transcriptionally active forms of E2F can drive quiescent mammalian cells into S phase (Johnson et al., 1993).

Consistent with the suggestions from mammalian systems, cyclin E in *Drosophila* induces both S phase and the G_1-S transcriptional program. If the transcriptional program includes activation of cyclin E expression, then a switch-like positive feedback loop analogous to *S. cerevisiae* may drive the G_1-S transition in *Drosophila* embryos. While no analog of Rb has yet been identified in flies, other components of the presumed positive feedback loop have been defined. *Drosophila* Cdc2c appears to be the analog of the mammalian cdk2 (Lehner and O'Farrell, 1990a; Stern et al., 1993; and C. Lehner personal communication) and a *Drosophila* homolog of E2F has recently been cloned (Dynlacht et al., 1994). Thus, based on our demonstration that cyclin E induces a transcriptional program, together with the analogies to yeast and mammals, we suggest that cyclin E induces a positive feedback loop to turn on its own expression and consequently progress to S phase.

While G_1 cyclins might play the major role in the G_1-S transition, evolutionary conservation suggests that the broader program of coordinate expression of replication functions is also important. We suggest that new expression of genes such as RNR and DNA polymerase adopts increasing importance during growth arrest. Early in a G_1 arrest only the more unstable gene products (perhaps only cyclin E) will have decayed to a point requiring renewed expression for entry into S phase. However, with increasing time during a G_1 arrest the more stable gene products will decline, eventually falling below a threshold required for S phase. Consequently, re-entry into S phase from a prolonged G_1 would require activation of the full coordinate program of gene expression.

Developmental programming of G_1

Whatever the mechanistic basis of the G_1- to S-phase transition, the timing of the transition is regulated during development. Orderly morphogenesis is associated with rigid developmental programs and stereotyped times of mitosis and S phase in each tissue (O'Farrell, 1992; O'Farrell et al., 1989). While developmental inputs control earlier cycles at the G_2 to M transition, once a G_1 appears in cycle 17, its length is regulated, and the stereotyped pattern of endoreduplication S phases that follow must be controlled by developmental inputs governing entry into S phase.

The G_1 to S transcriptional program might represent part of the signaling system that drives the programmed S phases, or it might be coupled to and a consequence of transit through a step in the cell cycle. To address this, we tested the influence of cell cycle arrest on the G_1- to S-phase transcriptional program. In embryos mutant for *string*, the cell cycle arrests in G_2 of cycle 14, but development continues with few defects beyond those attributable to the shortage of cells (Edgar and O'Farrell, 1989; Gould et al., 1990; Hartenstein and Posakony, 1990).

Analysis of the expression of genes involved in embryonic pattern formation, such as *Ubx*, show a fairly normal spatiotemporal program (Gould et al., 1990). Similarly, analysis of the expression of *DmRNR2* and *PCNA* revealed that these genes also followed their normal spatiotemporal program of expression. For example, at the time that S phase 17 would ordinarily occur, *string* embryos exhibit the normal pattern of pulses of RNR2 expression without an associated S phase. The failure of the *string* mutation to disrupt the G_1- to S-phase transcriptional program demonstrates that it is not driven by cell cycle events, and the detailed spatiotemporal pattern of this program indicates that it is controlled by developmental signals. We suggest that this transcriptional program is coupled to the developmental signal that triggers reactivation of the cycle in G_1 cells.

Modifying the cycle

In a typical cell cycle, DNA replication follows mitosis. Indeed, work in several systems has suggested that a ubiquitous checkpoint mechanism prevents re-entry into S phase until completion of mitosis, and thus prevents re-replication (Hartwell and Weinert, 1989; Murray, 1992; Li and Deshaies, 1993). In *Drosophila* such a dependency of DNA synthesis on mitosis also seems to operate, as suggested by the finding that *string*, which is required for entry into mitosis, prevents S phases after the mitotic block (Edgar and O'Farrell, 1989, 1990).

Following S phase 17, many of the larval cells evade this checkpoint and re-replicate their genomes in a series of ‘endocycles’. These endocycles generate polytene nuclei, such as the well-known salivary gland nuclei, which have an especially high degree of polytenization. To initiate endocycles, the checkpoint must be bypassed. We have detected this ‘checkpoint bypass’ by examining DNA replication in *string* mutants that are blocked in G_2 of cycle 14. Such G_2-arrested mutants cannot initiate S phase until the checkpoint is bypassed. At the time and positions at which the first endocycles S phases begin, replication is again detected in the mutant. Thus, the spatial and temporal program of checkpoint bypass occurs independent of cycle progression. We suggest that developmental programs direct the spatial and temporal pattern of expression of a ‘checkpoint bypass gene’, that directs this modification of the cell cycle (e.g. see Moreno and Nurse, 1994).

Initiation of each endocycle S phase is associated with a pulse of expression of *DmRNR2* RNA as well as expression of the other genes of the G_1- to S-phase transcriptional program. These endocycle S phases also require cyclin E function (Knoblich et al., 1994). Thus, it seems likely that initiation of endocycle S phases is controlled in manner similar to a typical G_1 to S transition, and that the checkpoint bypass is only a permissive modification of the cycle that removes a constraint on the normal progress to S phase without modifying the mechanism that triggers this progress.

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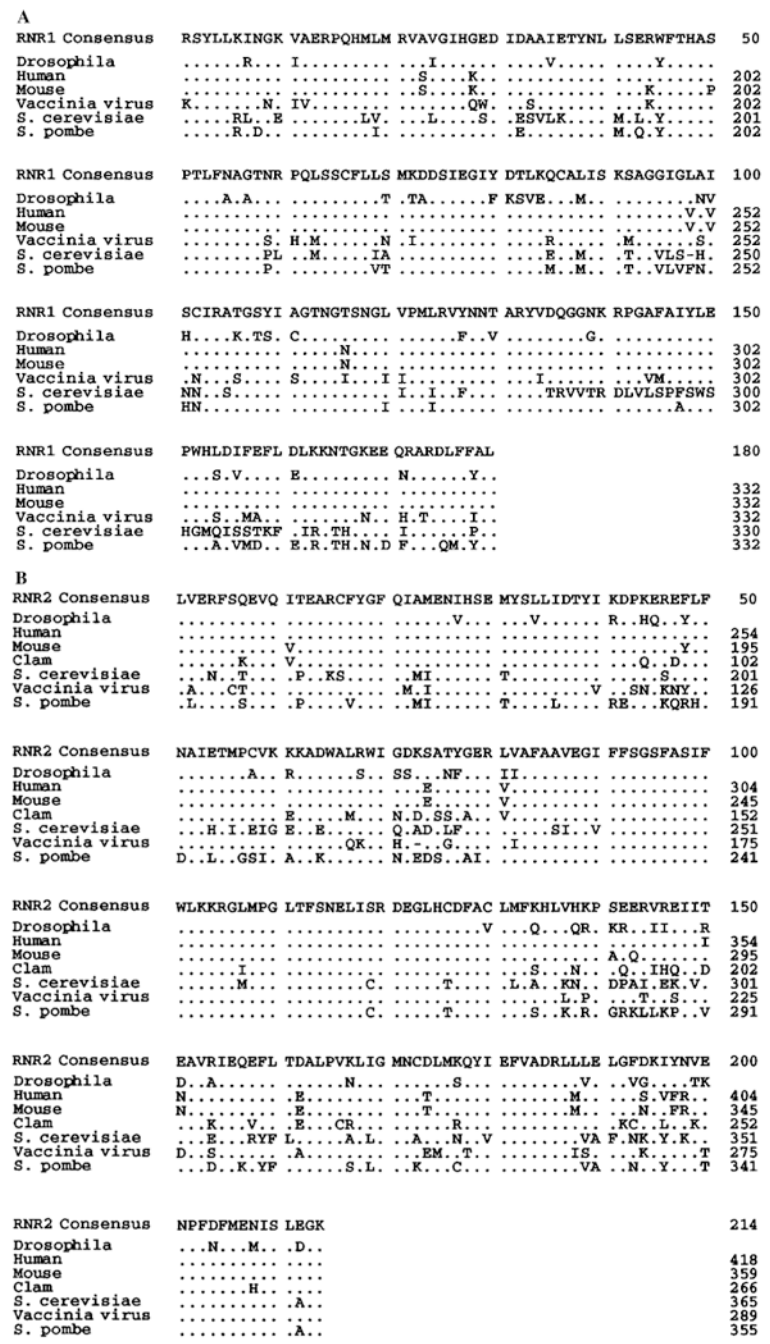

Fig. 1.

Predicted amino acid sequence of the *DmRNR1* and *DmRNR2* cloned PCR products with comparison to other eukaryotic and viral ribonucleotide reductases. Only sequence between and not including the PCR primer sites is shown. The alignments were performed using Geneworks. 'Consensus' amino acids appear in ≥2 of the sequences. Dots indicate identities with the consensus. Dashes indicate gaps introduced in a sequence to maximize the alignment. (A) RNR large subunit (RNR1) alignment. The *S. cerevisiae* genome contains two large subunit genes, RNR1 and RNR3 (Elledge and Davis, 1990), and sequence from the latter is shown in this alignment. (B) RNR small subunit (RNR2) alignment.

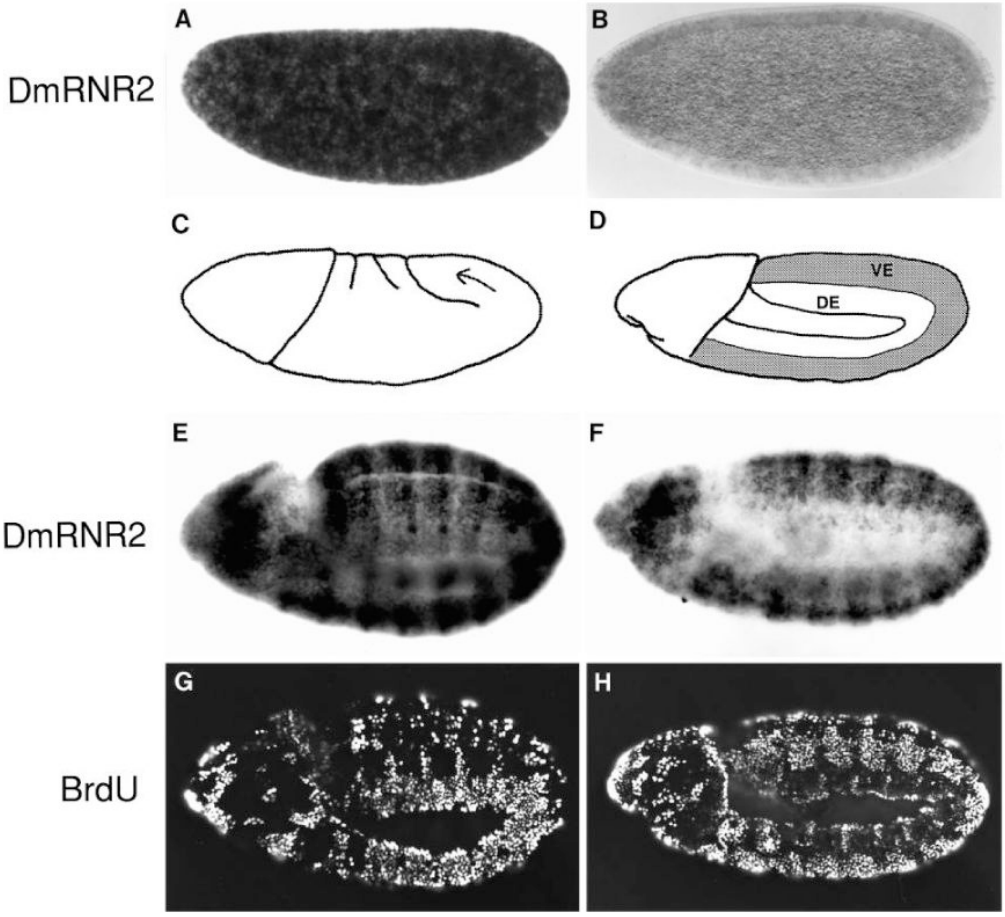

Fig. 2.

Embryonic expression of *DmRNR2* prior to the appearance of G_1-S control. *DmRNR2* mRNA was detected by whole embryo in situ hybridizations using a digoxigenin-labeled probe. (A) A preblastoderm embryo with high levels of maternal *DmRNR2* message. (B) The maternal message nearly disappears by blastoderm cellularization during early cycle 14. (C) Gastrulation begins shortly after cellularization is complete. Invagination of cells along the ventral midline creates a three-layered germ band. The arrow indicates the movement of cells of the germ band around the posterior tip of the embryo during germ band extension. (D) In a fully germ band extended embryo, the ventral epidermis (VE) extends around to the dorsal side of the embryo as well (shaded region), while the dorsal epidermis (DE) is located laterally. The embryos in E-H are at the germ band extended stage. They have been pulse labeled with BrdU and then hybridized with a *DmRNR2* probe prior to immunofluorescent detection of incorporated BrdU (G and H). (E) The constitutive and ubiquitous levels of *DmRNR2* that characterize the postblastoderm divisions are beginning to decline in the dorsal epidermis. This same embryo viewed with fluorescent illumination is shown in G. The heavily labeled nuclei in the dorsal epidermis are in early S16, while the unlabeled cells in the ventral epidermis have just completed S15. (F) *DmRNR2* expression in a slightly older embryo. Note that *DmRNR2* message is significantly reduced in the dorsal epidermis, while the level remains high in ventral epidermis. (H) Fluorescence view of the embryo in F shows the S-phase pattern. The ventral epidermal cells have finished G_2 and mitosis of cycle 15 and entered S16 without an intervening G_1. Cells in the dorsal epidermis have completed or nearly completed S16 and will enter G_1 after progressing through a short G_2 and mitosis. In each panel, dorsal is to the top and anterior

is to the left. The embryo in E and G is rotated slightly on its side relative to the embryo in F and H such that the ventral midline is apparent.

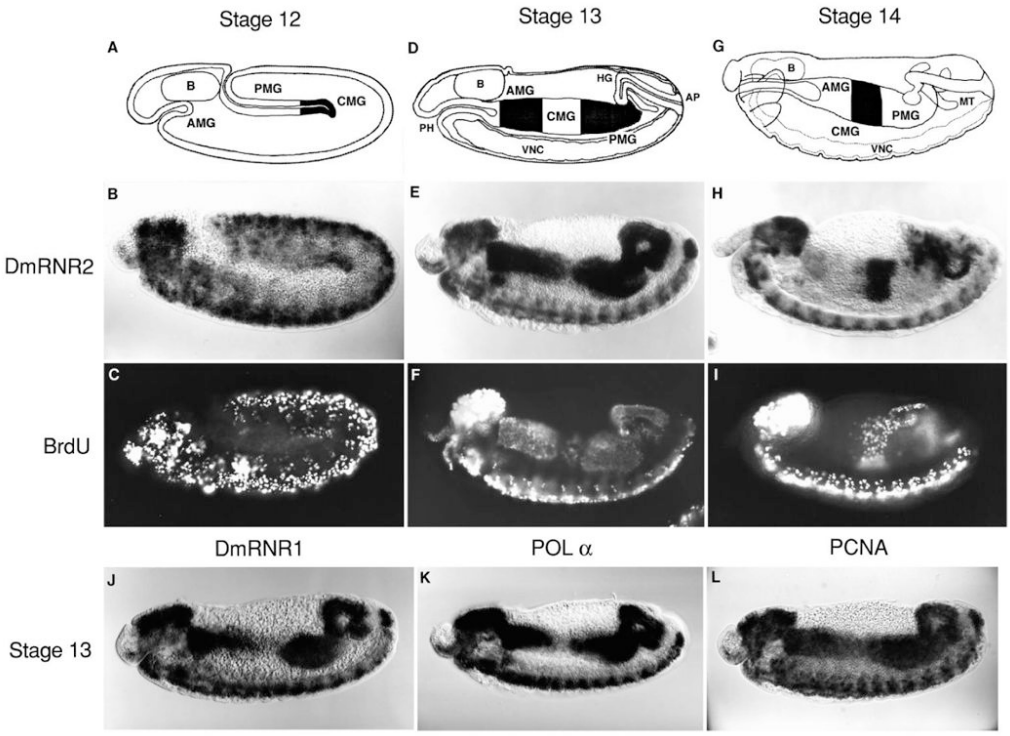

Fig. 3.

The patterned expression of S-phase genes correlates with the G_1-S transition. In each panel, an embryo is displayed in lateral view with dorsal up and anterior to the left. (A) During early stage 12 of embryogenesis, the distinct anterior and posterior invaginations of the midgut primordia (AMG and PMG, respectively) remain separated while germ band retraction initiates. (B) *DmRNR2* expression is extinguished in the G_1-arrested epidermal cells but persists in neural tissue along the ventral midline and in the brain (B in diagram). At this stage, DmRNR2 expression can be detected in large endodermal cells at the leading edge of the migrating posterior midgut invagination (shaded region in A). These cells will occupy the central portion of the midgut (CMG) after fusion of the midgut primordia. (C) An embryo pulse labeled with BrdU at the same stage as B reveals that the cells of the CMG have entered S17. Replicating nuclei can also be detected in the nervous system. (D) By stage 13, germ band retraction is complete and the midgut primordia have fused on either side of the yolk resulting in three distinct regions of the midgut along the anterior-posterior axis: the AMG, CMG and PMG. (E) In stage 13, *DmRNR2* expression has terminated in the CMG and initiated in the AMG and PMG. Several other tissues also accumulate *DmRNR2* RNA at this time. Among those visible in this embryo are the hindgut (HG), the anal pads (AP), the pharynx (PH), and the brain (B) and ventral nerve cord (VNC). (F) The S-phase pattern of a stage 13 embryo is identical to the *DmRNR2* expression pattern. Clearly visible is S17 in the AMG and PMG. Note that a few CMG cells near the end of S17 have incorporated BrdU (slightly out of focus). (G) The midgut cells begin migrating dorsally and ventrally on either side of a stage 14 embryo to eventually fuse and create a tube that envelopes the yolk. (H) The *DmRNR2* expression pattern has changed substantially by stage 14 (this embryo is ~1 hour older than the embryo in E). Expression has ceased in the AMG, PMG, HG, and PH and RNA reaccumulates in the CMG. Staining is also apparent in the Malpighian tubules (MT) and persists in the VNC. (I) The S-phase pattern at stage 14 changes in concert with the *DmRNR2* expression pattern. The CMG has reentered S phase (S18), which is the first endoreduplication S in the midgut. The BrdU incorporation in the Malpighian tubules (MT) at this stage is due to endoreduplication

S phases. The pattern of expression of *DmRNR1* (J), DNA polymerase α (K), and *PCNA* (L) during stage 13 is identical to that of *DmRNR2* (E).

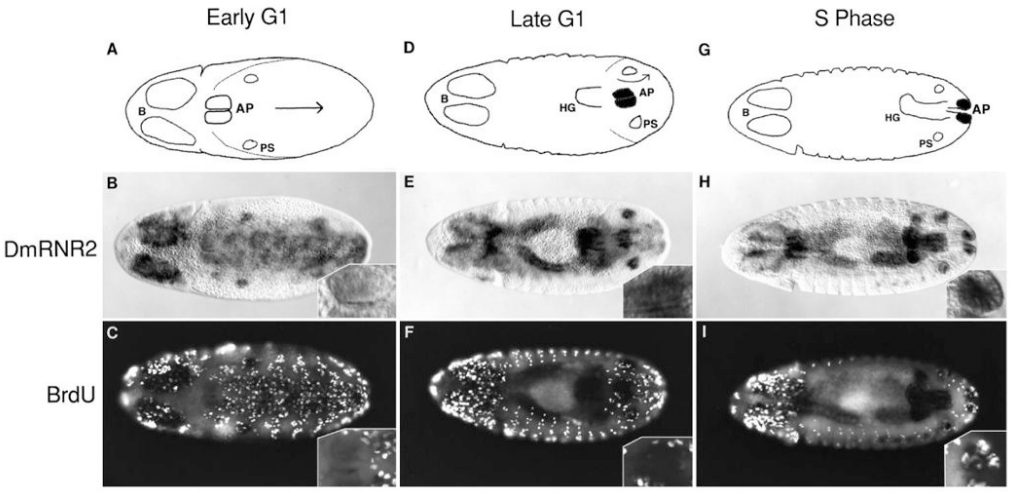

Fig. 4.

DmRNR2 message accumulation occurs late in G_1 prior to the initiation of DNA replication. Embryos were pulse labeled with BrdU and then hybridized with a *DmRNR2* probe prior to immunofluorescent detection of incorporated BrdU. All embryos are viewed from the dorsal perspective and anterior is to the left. The schematics indicate the changing position of the anal pads (AP), which occupy the tip of the germ band. In stage 12, the germ band retracts along the same pathway as when it extended. The arrow indicates the direction of cell movement during this process. PS indicates the bilaterally symmetric groups of cells that will form the posterior spiracles, which are already in S phase and express *DmRNR2* in the embryos shown. The B in the schematics indicates the two lobes of the brain. In the leftmost three panels (A-C), the cells of the AP are in early G_1, and they neither express *DmRNR2* (B) nor incorporate BrdU (C). The insets show a close-up view of the right (towards the top) anal pad. In the central panels (D-F), the AP cells are in late G_1. At this stage, segmentation is obvious and retraction of the germ band (75%) has revealed the hindgut (HG). The movements of the germ band (arrow in D) are such that the AP will eventually appear more posterior than the PS. The AP now expresses *DmRNR2* RNA (E, and indicated by shading in the diagrams), but still fails to incorporate BrdU (F). In the rightmost three panels (G-I), the cells of the AP are in S phase. At this stage, germ band retraction is complete, placing the AP at the end of the hindgut tube. *DmRNR2* RNA is still detected (H, and indicated by the shading in G). The fluorescence signal from BrdU labeling of the AP cells is partially quenched by the histochemical staining of the *DmRNR2* RNA, but it is nonetheless evident that these cells are in S phase (I, see inset). Note that most of the numerous isolated cells that incorporate BrdU and stain in C, F and I are rapidly dividing cells of the peripheral and central nervous systems.

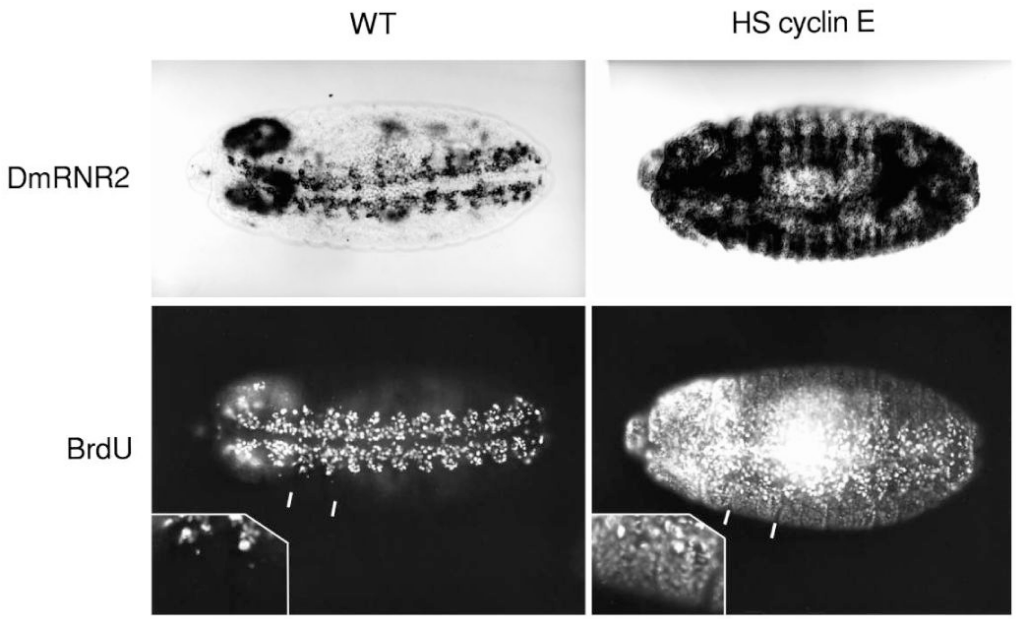

Fig. 5.

Ectopic production of *Drosophila* cyclin E induces the G_1-S transcriptional program and drives G_1 cells into S phase. All panels show a ventral view of a stage 14 embryo either stained for *DmRNR2* expression or BrdU incorporation. The VNC is clearly visible in the wild-type (WT) embryos. Note that the G_1-arrested epidermal cells on either side of the VNC neither incorporate BrdU nor express *DmRNR2*. Embryos homozygous for a *hsp70-cyclin E* transgene (HS cyclin E) were heat shocked at 37°C for 30 minutes. After a subsequent 45 minute recovery period at room temperature, they were either fixed and stained for *DmRNR2* expression or pulse labeled with BrdU. The insets represent enlargements of the two segments included between the white markings in the BrdU-labeled embryos.

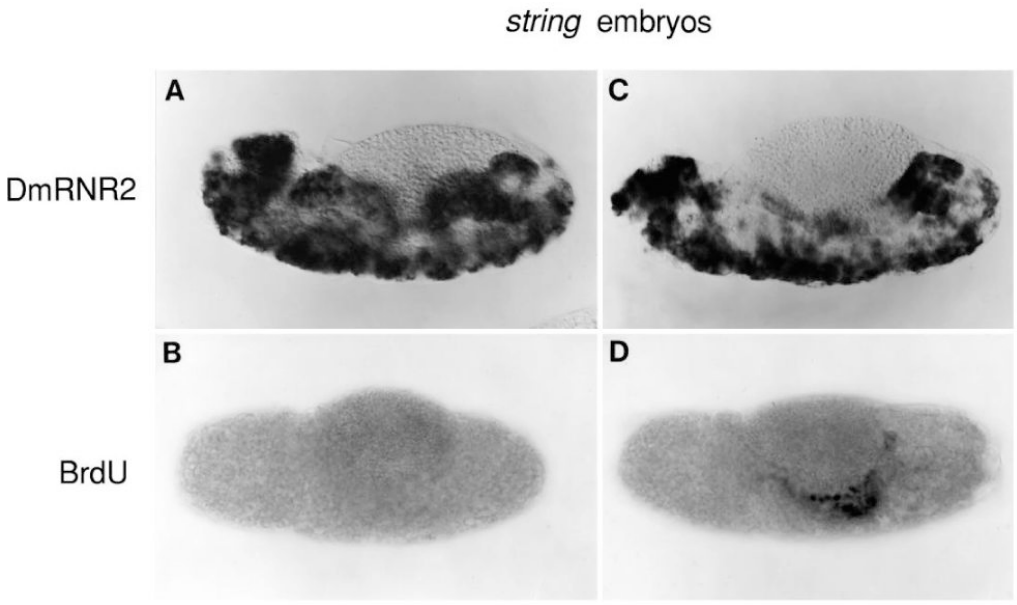

Fig. 6.

The G_1-S transcriptional program operates independently of cell cycle progress. Expression of *DmRNR2* (A,C) and BrdU incorporation (B,D) are shown in approximately stage 13 (A,B) and stage 14 (C,D) *string* mutant embryos. See Fig. 3 panels E, F, H and I for analogous staining of wild-type embryos (note that BrdU incorporation was detected histochemically in this experiment but immunofluorescently in the experiment shown in Fig. 3). In stage 13 *string* embryos, *DmRNR2* is expressed in a near normal pattern (A) despite the lack of detectable DNA replication (B). In stage 14 *string* embryos, *DmRNR2* is also expressed in a near normal pattern (C), and DNA replication is confined to a few cells (note the large nuclei in the center of the midgut in D). These replicating cells are apparently those that would undergo an endoreduplication S phase at this stage (see text).