Identification of Genomic Regions Required for DNA Replication During Drosophila Embryogenesis

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Manuscript received April 7, 1993
Accepted for publication July 17, 1993

ABSTRACT

A collection of Drosophila deficiency stocks was examined by bromodeoxyuridine (BrdU) labeling of embryos to analyze the DNA replication patterns in late embryogenesis. This permitted us to screen 34% of the genome for genes that when absent in homozygous deficiencies affect the cell cycle or DNA replication. We found three genomic intervals that when deleted result in cessation of DNA replication in the embryo, 39D2-3;E2-F1, 51E and 75C5-7;F1. Embryos deleted for the 75C5-7;F1 region stop DNA replication at the time in embryogenesis when a G_1 phase is added to the mitotic cell cycle and the larval tissues begin to become polytene. Thus, this interval may contain a gene controlling these cell cycle transitions. DNA replication arrests earlier in embryos homozygous for deletions for the other two regions. Analysis of the effects of deletions in the 39D2-3;E2-F1 region on DNA replication showed that the block to DNA replication correlates with deletion of the histone genes. We were able to identify a single, lethal complementation group in 51E, *l(2)51Ec*, that is responsible for the cessation of replication observed in this interval. Deficiencies that removed one of the Drosophila *cdc2* genes and the *cyclin A* gene had no effect on replication during embryogenesis. Additionally, our analysis identified a gene, *pimples*, that is required for the proper completion of mitosis in the post-blastoderm divisions of the embryo.

THE cell cycle goes through several dramatic changes during *Drosophila melanogaster* embryogenesis. In the initial 13 cycles S phase rapidly alternates with M phase, then a G_2 phase is added for cycles 14–16. After cycle 16, a G_1 phase is added to the cell cycle in the nervous system, while the larval tissues undertake a cycle that has an S and G phase but no mitosis (EDGAR and O'FARRELL 1990; FOE 1989; FOE and ALBERTS 1983; SMITH and ORR-WEAVER 1991). These cell cycle changes are under developmental control and occur at specific times of embryogenesis. The first major transition takes place at cellularization when the 13, rapid, syncytial divisions under the control of maternal gene products give way to slower divisions controlled by zygotic gene products. At this transition the changes to the cell cycle are multifaceted: a lengthening of S phase from 3–4 min to ~30 min is observed (BLUMENTHAL, KRIEGSTEIN and HOGNESS 1973; MCKNIGHT and MILLER 1977); centric heterochromatin first appears (VLASSOVA *et al.* 1991); and G_2 is added to the cell cycle (FOE 1989; FOE and ALBERTS 1983).

As the zygotic genome is expressed following cellularization, the morphogenetic events of gastrulation, germ band extension, and germ band retraction take place. Several cell cycle changes occur during these developmental stages. In contrast to the synchronous, preblastoderm mitoses, the three mitotic cycles that follow cellularization (cycles 14–16) have S, G_2 and M phases, and they are spatially and temporally regulated (FOE 1989). These divisions take place during gastrulation, germ band extension and immediately before germ band retraction. Following germ band retraction, the cells of the larval tissues commence a cell cycle consisting of alternating gap and synthesis phases that leads to the production of polytene chromosomes, a cycle we refer to as the endo cell cycle (SMITH and ORR-WEAVER 1991). Increasing polytenization by endoreplication is the primary mechanism responsible for the increase in larval size during the instar stages, since mitosis is dispensable for larval growth (GATTI and BAKER 1989). The cells in the nervous system continue to divide mitotically during embryogenesis and the larval stages, utilizing a cell cycle that has both a G_1 and G_2 gap phase (CAMPOS-ORTEGA and HARTENSTEIN 1985). The G_1 phase is added after the third post-blastoderm division, at about the same time that the first larval tissue begins endoreplication (EDGAR and O'FARRELL 1990).

Although the developmental timing and dynamics of these cell cycle transitions are well characterized, there have been no mutations identified that affect control genes for either the addition of G_1 to the cell cycle, for the transition from mitotic division to sub-

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sequent growth by the endo cell cycle, or for the cycling during endoreplication. This is due largely to the fact that no systematic search has been performed to isolate such mutations. The endo cell cycle seems likely to be actively regulated because of the precise developmental specificity observed in the endoreplication pattern (SMITH and ORR-WEAVER 1991). We have previously shown that cells that are differentiating into the same tissues undergo polytene replication together as a domain at specific times in late embryogenesis. This has permitted us to construct a developmental map of the replication domains (SMITH and ORR-WEAVER 1991).

Several key cell cycle regulators have been identified in Drosophila either by a mutant phenotype or by cloning homologs to known cell cycle genes. Mutations causing defects in the early cleavage divisions have been isolated from collections of maternal effect mutants (GLOVER 1989). Few zygotic mutants affecting the post-blastoderm divisions have been identified. *string* has been shown to be a key positive regulator required for the the G_2/M transition in all post-cellularization mitoses, and *pebble* mutants undergo normal nuclear division, but fail to undergo cytokinesis (EDGAR and O'FARRELL 1989; HIME and SAINT 1992). In contrast, many putative cell cycle mutants have been identified from collections of late larval mutants by their effects on mitotic divisions in the larval imaginal tissues (GLOVER 1989). An alternative approach to isolating mutants has been to clone Drosophila homologs to known cell cycle regulatory genes identified in other organisms. Two Drosophila homologs for the key regulator *cdc2* have been identified (JIMENEZ *et al.* 1990; LEHNER and O'FARRELL 1990a); there are Drosophila homologs of *cyclins A, B* and *C* (LAHUE, SMITH and ORR-WEAVER 1991; LEHNER AND O'FARRELL 1989, 1990b; LEOPOLD and O'FARRELL 1991), and two Drosophila *cdc25* homologs have been isolated, *string* and *twine* (ALPHEY *et al.* 1992; EDGAR and O'FARRELL 1989).

A powerful initial approach to screen for zygotic genes affecting endoreplication in embryos, or the mitotic cell cycle in the nervous system, is to examine homozygous deficiency embryos labeled with bromodeoxyuridine (BrdU) for alterations in the DNA replication pattern. Such an analysis on staged embryos permits the screening of the genome for genes whose absence alters the control of DNA replication in the cell cycle. Potentially, several phenotypes could be observed, depending on whether genes that function in both mitotic and endo cell cycle regulation are deleted in the deficiency, or whether independent regulators of DNA replication in either of these two cell cycles are deficient. Deletion of genes essential for activating DNA replication in the three post-blastoderm mitotic divisions (cycles 14–16) would result in a lack of replication throughout the embryo prior to germ band retraction. If a common regulator controlled the gap/synthesis transition in both the mitotic and endo cell cycles, then in its absence no replication would be observed in the nervous system and larval tissues after germ band retraction. Deletion of specific activators of either the mitotic G_1/S transition or the G/S endo cell cycle transition would result in a block to DNA replication in mitotic and endoreplicating tissues, respectively. If general components required for DNA replication were deficient, then a cessation of DNA replication throughout the embryo would be observed following the exhaustion of maternal supplies of these components. Since homozygous deficiency embryos are examined for DNA replication phenotypes, only those genes whose maternal contributions are depleted during embryogenesis would be detected by this screen.

Examination of homo- or hemizygous deficiency embryos provides a method for the rapid characterization and identification of phenotypes resulting from the loss of several complementation groups. A potentially limiting factor in using deficiencies for such an analysis is that some deficiencies can result in defects that mask the process of interest. For example, a severe mutant phenotype may appear early in embryogenesis and thereby prevent the full description of the phenotypes related to the process under study. As a general strategy, the effective use of deficiencies to analyze a process requires that it occurs early in development and will not be masked by large numbers of genes in the genome. This has the corollary that the earlier the process to be examined, the larger the number and size of deficiencies that can be examined. In a study to identify genes controlling cellularization, whole chromosome arm deficiencies were examined, since this process is one of the earliest to require zygotic gene expression (MERRILL, SWEETON and WIESCHAUS 1988; WIESCHAUS and SWEETON 1988).

In the analysis of zygotic segmentation, NÜSSLEIN-VOLHARD, WIESCHAUS and coworkers found that most of the homo- and hemizygous deficiency embryos derived from Drosophila deficiency stocks complete embryogenesis far enough to secrete a larval cuticle, an event that takes place late in embryogenesis after most morphogenesis and differentiation (JÜRGENS *et al.* 1984; NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984; WIESCHAUS, NÜSSLEIN-VOLHARD and JÜRGENS 1984). Consequently, the examination of deficiency stocks for cell cycle phenotypes is a viable methodology, since the production of the larval cuticle also follows the major cell cycle transitions that occur during embryogenesis. We have conducted a screen of the effect of Drosophila deficiencies on DNA replication domains in the embryo, and have identified

three regions of the genome that are required for DNA replication during late embryogenesis.

MATERIALS AND METHODS

Drosophila strains: Except as noted, all fly strains were obtained from the Drosophila stock center, Bloomington, Indiana, and except as noted are described in LINDSLEY and ZIMM (1992). Embryos were collected from the balanced stocks with the exception of the *X*-chromosome deficiency stocks that contained duplications covering the deficiency intervals (noted in Table 1). Those stocks were out crossed to a *C(1)RM, y^2 su(w^a) w^a* stock to remove the complementing duplication and allow the identification of deficiency phenotypes. In these cases the mutant fraction would be one-eighth and not one-quarter as for all the other stocks. We did not cytologically check the breakpoints of the deficiencies surveyed in our screen, but we did confirm *Df(2L)Jder2*, *Df(3L)vin7* and *Df(3L)Cat*. The percentage of the genome screened was calculated from the number of lettered cytological divisions uncovered by each deficiency relative to the total in the genome.

BrdU labeling of embryos: Drosophila embryos were labeled following the protocol outlined in SMITH and ORR-WEAVER (1991). In the initial characterization of deficiency phenotypes, embryos were collected from the balanced stocks for 4 hr at 25°, aged for 8 hr and subsequently labeled by an incubation for 30–40 min in BrdU at room temperature.

Scoring of embryos: We examined BrdU labeled embryos for alterations in the replication patterns detectable in 25% of the embryos (except in the *X* deficiency stocks described above). At least 100 well-labeled embryos were scored for each deficiency stock. In stocks in which the deficiency also uncovered a visible embryonic mutation, there was good correlation between the embryos showing a replication phenotype and those having a cuticle or morphological phenotype. Since the mutant phenotypes were characterized from balanced lethal stocks, the described phenotypes theoretically could have arisen from either the balancer or the deficiency-bearing chromosome. However, this is unlikely because in comparing multiple stocks bearing the same balancer chromosomes, no replication phenotype was observed for the balancer chromosomes.

Staining with DAPI and anti-tubulin antibody: Embryos were stained with DAPI (4′, 6-diamidino-2phenylindole) as described previously (SHAMANSKI and ORR-WEAVER 1991). A monoclonal antibody against β-tubulin (Amersham) at a 1:200 dilution was used to examine the microtubules during mitosis, and staining was done as described (SHAMANSKI and ORR-WEAVER 1991).

Microscopy: A Zeiss Axiophot microscope equipped with Nomarski optics and fluorescence was used to observe the embryos using Plan-Neofluar 10×, 20×, 25×, 40× and 100× objectives.

Calculation of nuclear density and percent mitotic figures: Embryos stained with anti β-tubulin or DAPI were photographed. To calculate nuclear density, nuclei were circled and the number in a region corresponding to a 50 μm × 50 μm area on the embryo were counted. Several of these areas were counted so that approximately 100–200 nuclei were scored. To compare mutant and wild-type embryos, nuclei from the same part of the epidermis were scored. To calculate the percent mitotic figures, embryos were stained with anti β-tubulin, photographed and the number of cells with mitotic spindles was counted relative to the total number of cells.

RESULTS

Identification of DNA replication phenotypes: To identify genes required for cell cycle progression or proper DNA replication late in embryogenesis, we screened deficiency stocks that in sum covered 41% of the lettered divisions of the genome (Table 1, Figure 1). As described below, we were able to score about 34% of the genome conclusively for DNA replication. Embryos were collected from balanced deficiency stocks, permeabilized, pulse labeled with BrdU, then fixed and stained with an antibody against BrdU. BrdU is a thymidine analog that is incorporated into DNA, thus its presence in DNA following a pulse can be used to monitor DNA replication during that period. Since we were particularly interested in the transition that occurs when most of the cells stop dividing mitotically and enter an endo cell cycle to become polytene, embryos were aged to 8–12 hr following fertilization. This period in development is characterized by extensive endoreplication in a specific temporal and spatial developmental pattern, as well as significant levels of mitotic cell division in the neural tissues (CAMPOS-ORTEGA and HARTENSTEIN 1985; SMITH and ORR-WEAVER 1991). Examination of DNA replication patterns at these developmental ages would permit the identification of mutant phenotypes in which the timing and/or tissue specificity of endoreplication domains was affected, as well as defects in mitotic divisions in the nervous system. This analysis allowed detection of alterations in the number of replicating cells, the size of replicating nuclei and the boundaries of replication domains, all modifications that would reflect changes in cell cycle regulation. In the cases in which the homozygous deficiency removed a gene that resulted in a visible embryonic phenotype, homozygous deficiency embryos were identified by that phenotype and scored for their BrdU-labeling pattern. In those deficiencies without a visible phenotype, we looked for BrdU labeling alterations detectable in 25% of the embryos, the percentage predicted to be homozygous for the deficiency.

Three deficiency intervals gave a striking defect in that homozygosity of the deficiency caused the cessation of DNA replication throughout the Drosophila embryo (Figure 1, Table 1). This phenotype is consistent with the deletion of genes that are required for both mitotic and endo cell cycle progression, or that provide essential replication functions. An overlap of control genes for the mitotic and endo cell cycles might be expected, since after the addition of G_1 to the mitotic cycle, both cell cycles have a gap/synthesis phase transition, and both cell cycles may utilize a set of the same key regulators to control entry into S phase. These three deficiencies will be described in more detail in the next section.

TABLE 1

Deficiencies screened

Deficiency	Breakpoints	Replication phenotype	Reference	Source	Visible embryonic mutations[a]
Df(1)sc[J4][b]	01B;03A3	Endoreplication normal, no neural tissue	*a*	*e*	*ACS*
Df(1)S39	01E1-2;02B5-6	Normal*	*a*	*e*	
Df(1)A94	01E4-5;02B11-12	Normal	*a*	*e*	
Df(1)Pgd35	02C2-4;02E2-F1	Normal	*a*	*e*	
Df(1)N-71h	03C4;03D5	Normal	*a*	*e*	*N*
Df(1)HF366	03E7-8;05A7	Morphological defect obscures	*a*	*d*	*hnt*
Df(1)C149	05A8-9;05C5-6	Normal	*a*	*e*	
Df(1)N73	05C2;05D5-6	Normal	*a*	*d*	
Df(1)JF5	05E6; 05E7	Normal	*a*	*d*	
Df(1)HA32	06E4-5;07A6	Normal	*a*	*d*	
In(1)dl-49, Df(1)ct-J4[b]	07A2-03;07C1	Normal*	*a*	*d*	
Df(1)ct268-42	07A5-6;07B8-C1	Normal	*a*	*d*	
Df(1)C128	07D1;07D5-6	Normal	*a*	*d*	
Df(1)RA2	07D10;08A4-5	Normal	*a*	*d*	
Df(1)KA14	07F1-2;08C6	Normal	*a*	*d*	
Df(1)v-L15	09B1-2;10A1-2	Normal	*a*	*d*	
Df(1)v-L3[b]	09F10;10A7-8	Normal, ventral cord split	*a*	*d*	
Df(1)N71	10B2-8;10D3-8	Normal	*a*	*d*	
Df(1)N105	10F7;11D1	Normal, poor gut differentiation	*a*	*d*	*tsg*
Df(1)C246	11D-E;12A1-2	Normal	*a*	*d*	
Df(1)g-1, fB	12A;12E	Normal	*a*	*d*	
Df(1)N19	17A1;18A2	Normal, sloppy ventral cord	*a*	*d*	
Df(1)JA27	18A5;18D1-2	Morphological defect obscures	*a*	*d*	
Df(1)JC4	20A1;20E-F	Normal	*a*	*d*	*fog*
Df(2L)S2	21C6-D1;22A6-B1	Normal*	*a*	*d*	*S*
Df(2L)ast-l	21C7-8;23A1-2	Normal, midline defective	*a*	*f*	*S*
Df(2L)dp79b	22A2-3;22D5-E1	Normal, reduced brain	*a*	*e*	
Df(2L)M-zB	24D6-E13;24F7-25A1	Normal, larger ventral cord cells	*a*	*d*	
Df(2L)GpdhA	25D7-E01;26A8-9	Normal*	*a*	*d*	
Df(2L)2802	25F2-3;25F4-26A1	Normal	*a*	*e*	
Df(2L)wgCX3	28A	Normal	*c*	*d*	*wg*
Df(2L)J-der2	31B;32A	Fewer, larger nuclei, replication proceeds	*a*	*f*	*pim, bsk*
Df(2L)esc10	33A8-B1;33B2-3	Normal*	*a*	*e*	
Df(2L)b80k	34D2-4;35B10-C1	Normal*	*a*	*d*	
Df(2L)TW137	36C2-4;37B9-C1	Morphological defect obscures	*a*	*f*	
Df(2L)TW50	36E4-F1;38A6-7	Normal, severe morphological defect	*a*	*f*	
Df(2L)TW2	37D2-E1;38E6-9	Morphological defect obscures	*a*	*f*	
Df(2L)TW161	38A6-B1;40A4-B1	Replication defective, poorly differentiated	*a*	*f*	
Df(2L)C'PR31	40, 2L Heterochromatin	Normal	*a*	*f*	
Df(2R)M41A4	41A	Normal	*a*	*d*	
Df(2R)M41A4	41A	Normal	*a*	*e*	
Df(2R)cn88b	42A;42E	Normal	*a*	*d*	
Df(2R)pk78s	42C1-7;43F5-8	Normal, larger ventral cord cells	*a*	*d*	
Df(2R)P32	43A3;43F6	Normal, larger ventral cord cells	*a*	*e*	
Df(2R)cn83c	43C5-D1;44B6-C1	Normal, larger ventral cord cells	*a*	*d*	*eve*
Df(2R)evel.27	46C3-4;46C9-11	Normal, reduced ventral cord	*a*	*d*	*en, shn, sha*
Df(2R)enB	47E3;48B2	Normal, unsegmented ventral cord	*a*	*d*	
Df(2R)vg-C	49B2-3;49E7-F1	Normal	*a*	*d*	
Df(2R)vg-B	49D3-4;49F15-50A3	Normal*	*a*	*d*	
Df(2R)L-R+48	50F;51B	Normal	*a*	*d*	
Df(2R)trix	51A1-2;51B6	Normal			

Only one of the three Drosophila homologs of established replication genes that have been localized cytologically (topoisomerase II, the 73-kD subunit of the DNA polymerase α primase, and PCNA) was uncovered in this collection of deficiencies (COTTERILL, LEHMAN and MCLACHLAN 1992; NOLAN *et al.* 1986; YAMAGUCHI *et al.* 1990). In embryos homozygous for *Df(2L)TW50*, which uncovers the topoisomerase II gene, we observed morphological defects but DNA replication was normal (Table 1). The *mus* (mutagen sensitive) and several of the *mei* loci are defective in DNA repair, and thus it is likely that some of these loci are involved in DNA replication. Embryos homozygous for deficiencies uncovering the reported cytological locations of *mus101, 102, 105, 112* and *mei41* and *9* were examined (BAKER, SMITH and GATTI 1982; GATTI, SMITH and BAKER 1983; LINDSLEY and ZIMM 1992). All of these had normal DNA replication

TABLE 1

Continued

Deficiency	Breakpoints	Replication phenotype	Reference	Source	Visible embryonic mutations[a]
Df(2R)XTE18	51E3;52C9-D1	Replication defective	*a*	*d*	
Df(2R)WMG	52A;52D	Normal	*a*	*d*	
Df(2R)Pc111B	54F6-55A1;55C1-3	Fewer, larger nuclei, replication proceeds	*a*	*e*	*thr*
Df(2R)Pc4	55A;55F	Fewer, larger nuclei, replication proceeds	*a*	*d*	*thr*
Df(2R)PuD17	57B4;58B	Normal, severe morphological defect	*a*	*d*	*flb*
Df(2R)bwD23	59D4-5;60A1-2	Normal	*a*	*d*	
Df(3L)R	62B7;62B12	Morphological defect obscures	*a*	*e*	
DF(3L)h-i22	66D10-11;66E1-2	Normal, poor ventral cord organization	*c*	*e*	*h*
Df(3L)29A6	66F5;67B1	Normal	*a*	*d*	
Df(3L)AC1	67A;67D	Normal	*a*	*e*	
Df(3L)vin7	68C8-11;69B4-5	Normal	*a*	*d*	
Df(3L)fzGF3b	70B;70C6	Normal	*a*	*e*	
Df(3L)fzD21	70D;71F	Normal, severe morphological defect	*a*	*e*	*shd?*
Df(3L)st-f13	71C1-D1;73A3-4	Normal	*a*	*d*	
Df(3L)W10	75A6-7;75C1-2	Normal	*a*	*e*	
Df(3L)Cat	75B8;75F1	Replication defective	*a*	*e*	
Df(3L)w[+R4]	75B8-11;75C5-7	Normal	*a*	*d*	
Df(3L)VW3	76A3;76B2	Normal	*a*	*d*	
Df(3L)in61	76F;77D	Normal*	*a*	*d*	
Df(3L)ri79C	77B-C;77F-78A	Normal, severe morphological defect	*a*	*e*	*kni*
Df(3R)Scr	84A1-2;84B1-2	Normal, severe morphological defect	*a*	*d*	*ftz, Antp, Scr*
Df(3R)D6	84D2-3;84F13-16	Normal	*a*	*e*	
Df(3R)p40	84E8-9;85B6	Normal	*c*	*d*	
Df(3R)by62	85D11-14;85F6	Normal	*a*	*d*	
Df(3R)by10	85D8-12;85E7-F1	Normal, poor gut differentiation	*a*	*e*	*hth?, knk?*
Df(3R)M-Kx1	86C1;87B1-5	Normal*	*c*	*e*	
Df(3R)kar1W	87A6-7;87D12-13	Normal	*a*	*d*	
Df(3R)ry615	87B12-15;87E8-11	Normal, sloppy ventral cord	*a*	*d*	*yrt?*
Df(3R)ry[506-85C]	87D1-2;88E5-6	Morphological defect obscures	*c*	*d*	*yrt, ems*
Df(3R)red-P93	88A10-B1;88C2-3	Normal	*a*	*d*	
Df(3R)sbd105	88F9-89A1;89B9-10	Morphological defect obscures	*a*	*e*	*srp*
Df(3R)C4	89E;90A	Normal	*a*	*e*	
Df(3R)ChaM7	91A;91F3	Normal	*a*	*e*	
Df(3R)XS, Dp(3R)xs	96A1-7;96A21-26	Normal, sloppy ventral cord	*a*	*e*	
Df(3R)Tl-P	97A;98A1-2	Normal	*a*	*d*	
Df(3R)X3F	99D1-2;99E1	Normal	*a*	*f*	
Df(3R)tll[e]	100A1-2;100B5-9	Morphological defect obscures	*a*	*f*	*tll*
Df(4)M62F	101E;102B16-17	Normal	*a*	*e*	

* Reaction had 5–10% unlabeled or unevenly labeled embryos.
[a] LINDSLEY and ZIMM (1992).
[b] Crossed to *C(1)RM*.
[c] FLYBASE (1993).
[d] HERMANN STELLER.
[e] Bloomington Drosophila Stock Center.
[f] ALLAN SPRADLING.
? = Gene that may reside within interval.

in late embryogenesis except *Df(1)HF366*, which should uncover *mei9*. A severe morphological defect in embryos homozygous for this deficiency prevented analysis of the pattern of DNA replication domains, although DNA replication was not blocked.

Two of the regions examined encoded known cell cycle regulatory genes, allowing analysis of the roles that these genes play in Drosophila cell cycles. A deficiency that uncovered the *cyclinA* gene, *Df(3L)vin7* [68C8-11;69B4-5] (LEHNER and O'FARRELL 1989), was striking for its lack of effect on DNA replication in the embryo. The deficiency *Df(2L)J-der2* [31B;32A] uncovered one of the Drosophila *cdc2* homologs (JIMENEZ *et al.* 1990; LEHNER and O'FARRELL 1990a), yet did not block DNA replication during the time periods examined (see below).

Deletion of two genomic intervals, *Df(2L)M-zB* [24D6-E13;24F7-25A1] and the overlap of *Df(2R)pk78s* and *Df(2R)cn83c* [43C5-D1;43F5-8], resulted in homozygous deficiency embryos with fewer ventral cord nuclei that were larger and more disorganized than the nuclei of phenotypically wild-type embryos (data not shown). In addition to pattern defects, deficiencies that uncovered *kni* (*Df(3L)ri79C*)

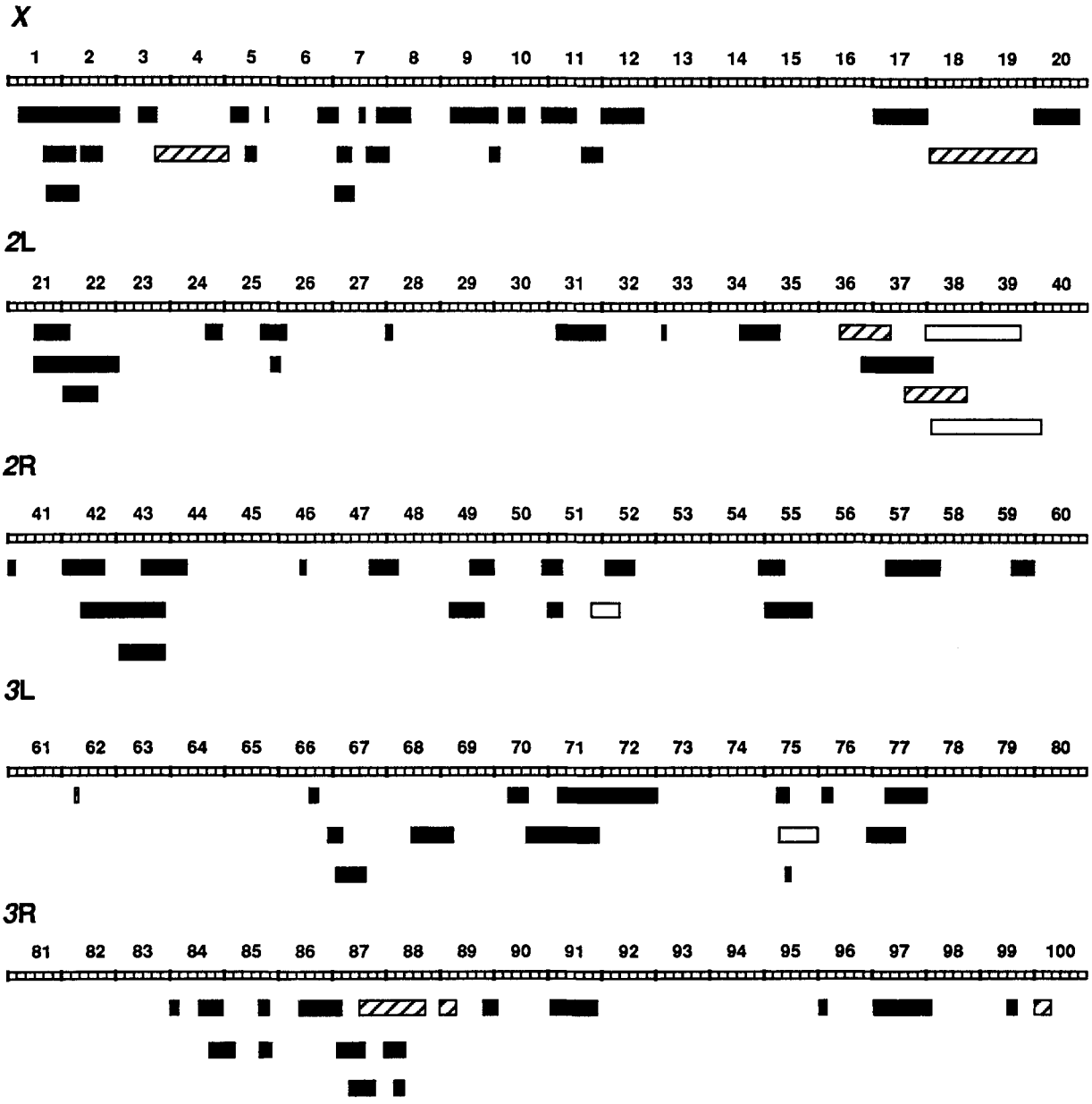

FIGURE 1.—Summary of deficiency intervals screened. The numbered divisions of the salivary gland polytene chromosomes are marked, and each lettered subdivision is shown. The boxes indicate the cytological extent of each deficiency; exact breakpoints are given in Table 1. The deficiencies for which the embryos labeled poorly are not shown on this figure, but they are listed in Table 1. Black boxes, normal developmental DNA replication pattern; hatched boxes, early defect obscures the identification of mitotic and polytene tissues; open boxes, cessation of DNA replication.

and *eve* (*Df(2R)eve1.27*) resulted in fewer cells in the ventral cord with larger nuclei, although the nuclei were not as large as in deficiencies for the 24D-F and 43C-F intervals. Further investigation will be required to determine whether these deficiencies produce mitotic defects and possibly polytenization in the nervous system.

Of the 41% of the genome that was screened, ~4% contained genes that when deleted had phenotypes that may have obscured the detection of certain classes of cell cycle defects. Eight deficiencies (Figure 1, Table 1) produced homozygous embryos with severe defects in the overall morphology of the embryo. Most of the morphological defects were attributable to the deletion of known genes in the interval (Table 1). The defects made it difficult to distinguish between mitotic and endoreplicating tissues and prevented us from determining whether the pattern of DNA replication was normal. However, within these regions of the genome there are not any genes that are required for DNA replication in both mitotic and endoreplicating tissues, since BrdU labeling was observed in the mutant embryos. In addition, the replication was cyclic, since dynamic patterns of DNA replication were observed in the homozygous deficiency embryos despite the mutant morphology. Thus, although the mutant

phenotypes seen in these deficiencies would have prevented observation of defects related to the decision between the mitotic and endo cell cycle, it is unlikely that these deficiencies uncover regulators common to both cell cycles.

In seven deficiencies, which removed another 3% of the genome, 5–10% of the embryos were weakly or unevenly labeled due to variability in the BrdU labeling reaction. Since 90–95% of the embryos from these stocks showed a normal BrdU pattern, they were designated as normal with the caveat that the labeling reaction was suboptimal (Table 1).

Three Intervals Required for DNA Replication

Timing of the defects: To investigate further the role of the genes deleted in the three intervals that block DNA replication, the time of onset of the replication defect was examined by BrdU labeling of embryos at different developmental stages. We wished to determine whether the block in replication correlated with the timing of G_1 addition to the cell cycle or happened earlier. A defect manifest at G_1 addition would be consistent with the hypothesis that the deficiency contained a specific regulator of DNA replication that is added to the cell cycle at this time.

The developmental onset of the block in DNA synthesis was different for the three deficiencies. The defect resulting from the absence of region 39, as evidenced by *Df(2L)TW65* [37F5-38A1;39E2-F1], was the earliest. Embryos deficient for this region ceased replication midway through the extended germ band stage (Figure 2A). The homozygous deficiency embryos were easily identified because they failed to undergo germ band retraction, the gut was not formed, and there was no evidence of patterning in the epidermis. Comparison of the nuclear density of anti-tubulin stained homozygous deficiency embryos to wild-type embryos showed that embryos homozygous for the deficiency had about half the nuclear density on the epidermis. We cannot be certain that the nuclear density in these deficiency embryos is influenced only by the number of post-blastoderm divisions completed, because the germ band was considerably more narrow than in wild type. Therefore, if cells were restricted to a smaller spatial interval in the germ band, the nuclear density might have appeared higher than would be predicted based solely on the number of post-blastoderm divisions completed. Consequently, we conclude that in these deficiency embryos cell cycle arrest occurs prior to mitosis 16, but we cannot ascertain the exact time of the block relative to the 15th S phase, the 15th mitosis, or the 16th S phase.

Embryos deficient for the 51-52 region (*Df(2R)-XTE18*, [51E3;52C9-D1]) maintained normal DNA replication and cell division later in embryogenesis than did those deficient for region 39 (Figure 2B).

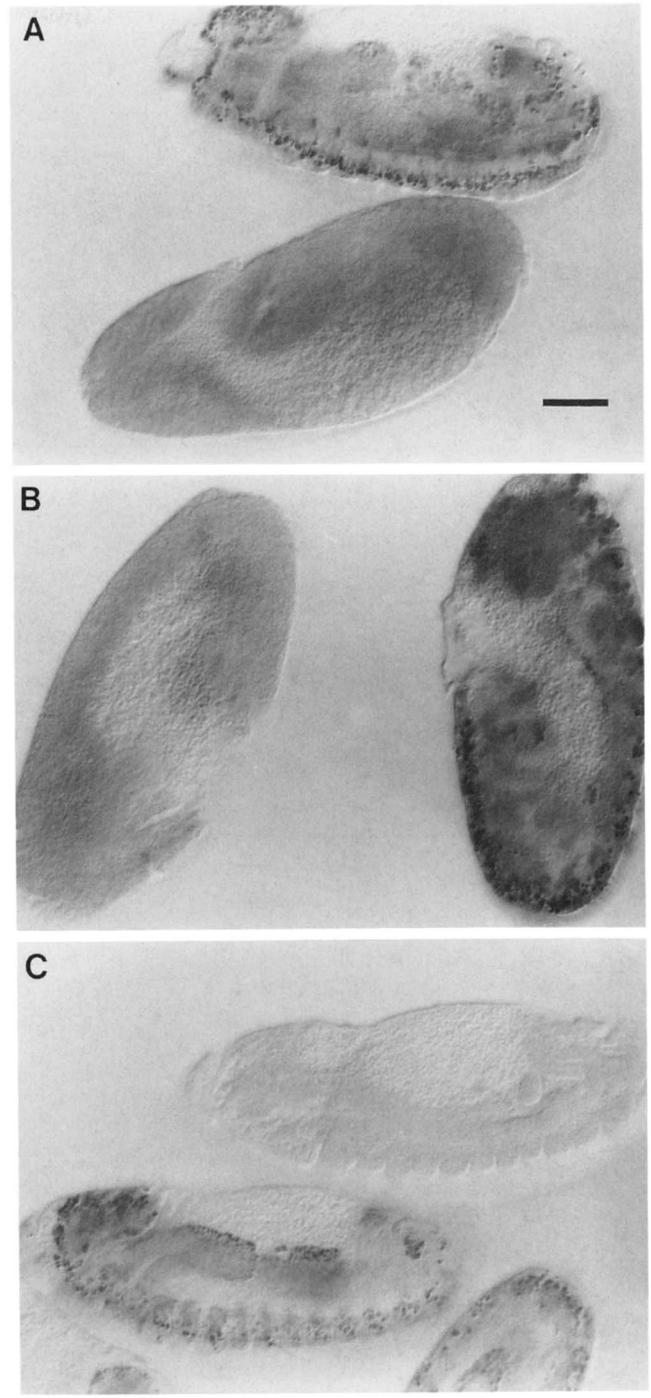

FIGURE 2.—Three genomic intervals are required for replication during embryogenesis. (A) Embryos from an 8- to 12-hr collection of *Df(2L)TW65*. The homozygous mutant embryo on the bottom can be identified by its failure to undergo germ band retraction and differentiation. Moreover, no BrdU incorporation is detected. Both embryos are oriented with anterior to the left and dorsal up. (B) Embryos from an 8- to 12-hr collection of *Df(2L)XTE18*. The homozygous mutant embryo on the left is not undergoing DNA replication. The embryo on the left is oriented with anterior down and dorsal to the right. The wild-type embryo is anterior up and dorsal left. (C) An 8- 12-hr collection of embryos from *Df(3L)Cat*; 25% of the embryos failed to label with BrdU like the one in the top of the photograph. Both embryos are shown with anterior to the left and dorsal up. The scale bar is 50 μm.

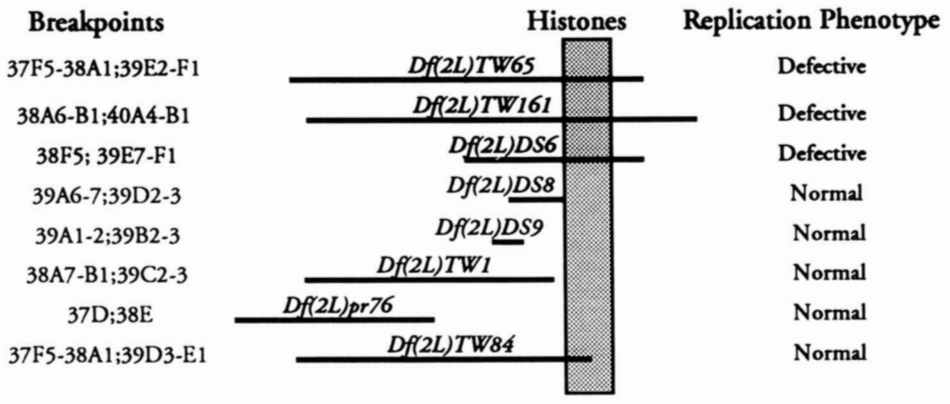

FIGURE 3.—Delineation of the 38-39 interval. The deficiencies shown were tested for embryonic DNA replication by BrdU labeling of 8- to 12-hr embryos. Those deficiencies that when homozygous resulted in a block in embryonic DNA replication are listed as "defective." The deficiencies are described in MOORE, SINCLAIR and GRIGLIATTI (1983). *Df(2L)DS8* and *Df(2L)DS9* were obtained from STEPHANIE PAINE-SAUNDERS (Massachusetts Institute of Technology). The lines indicate the deleted region. The location of the histone genes was determined by *in situ* hybridization (PARDUE *et al.* 1977).

DNA replication stopped at the end of the extended germ band stage, just prior to germ band retraction. Embryos that were homozygous for this deficiency had morphological defects; although most underwent germ band retraction, there was only some gut formation and epidermal patterning that did not appear normal. Examination of the nuclear density of homozygous deficiency embryos following DAPI staining revealed a reduction in nuclear density relative to wild type but not by a factor of two. While it seems likely that these embryos complete the 15th S phase, we were unable to determine an exact arrest point relative to the 15th mitosis, the 16th S phase or the 16th mitosis.

In the deficiency for the 75 interval (*Df(3L)Cat* [75B8;75F1]), the cessation of replication followed the time of the 16th mitosis, correlating with the addition of G_1 to the mitotic cycle in the nervous system and the onset of the endo cell cycle in the larval polytene tissues (Figure 2C). There was no morphological effect associated with this deficiency, but 25% of the embryos failed to label with BrdU. To show that this was not simply a consequence of failure of BrdU permeabilization, we injected embryos from the balanced stock with BrdU. Following injection, 25% of the embryos were not labeled. The normal morphology of the unlabeled embryos suggests that all three post-blastoderm divisions were completed. The interval that blocks DNA replication can be further limited to 75C5-7;75F1 because *Df(3L)*w^{+R4} showed normal replication (Table 1).

Demarcation of the 38-39 interval: A collection of deficiencies in the 38-39 region was examined in order to define further the location of the gene(s) essential for DNA replication. The deficiencies examined limited the region responsible for the phenotype to 39D2-3;E2-F1, since *Df(2L)DS8* did not display the absence of replication phenotype whereas *Df(2L)TW65* did (Figure 3). This interval is coincident with the chromosomal location of the histone genes, which had previously been localized to 39D3-E1,2 by salivary gland *in situ* analysis as well as deficiency analysis (MOORE, SINCLAIR and GRIGLIATTI 1983; PARDUE *et al.* 1977). *Df(2L)TW84* showed normal DNA replication, and it breaks within the histone gene cluster, but about 60% of the histone genes are not removed by this deficiency (MOORE, SINCLAIR and GRIGLIATTI 1983). Although the defect observed is consistent with absence of the histone genes, we cannot rule out the possibility that another locus within 39D-E is essential for proper DNA replication.

The replication defect in the 51-52 interval maps to a single complementation group: The 51-52 region has been extensively characterized genetically. A number of deficiencies exist for the region, and screens for lethals and maternal effect mutations in the region have been performed (UNDERWOOD *et al.* 1990). Deficiencies in this interval were examined for the defective replication phenotype, and the phenotype mapped to a small deficiency, *Df(2L)l13* (Figure 4). Although *Df(2L)l13* is not cytologically visible, it has been classified as a deficiency due to its inability to complement two distinct complementation groups, *fs(2)PA77* and *l(2)51Ec*. The *l(2)51Ec* complementation group was essential for proper DNA replication, because both *l(2)51Ec* homozygous and *l(2)51Ec/Df(2L)l13* embryos exhibited cessation of DNA replication (Figure 4). Examination of BrdU labeled *l(2)51Ec* embryos from a time course of increasing age revealed similar labeling defects as had been observed in the larger deficiency, *Df(2L)XTE18*. Moreover, *l(2)51Ec* mutant embryos have the same morphological defects as those homozygous for the deficiency. Thus, mutation of a single, zygotic lethal complementation group caused a block in DNA replication during embryogenesis.

The other mutation that fails to complement *Df(2L)l13*, *fs(2)PA77*, has previously been demonstrated to be a female sterile mutation; females homozygous for this mutation produce ovaries in which the chorion gene clusters fail to amplify to normal levels (UNDERWOOD *et al.* 1990). *fs(2)PA77* is lethal over a

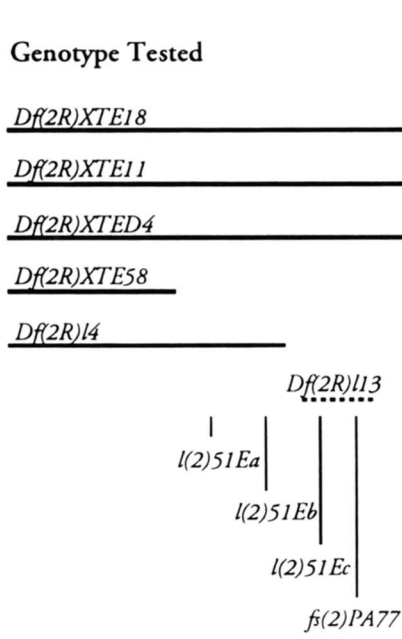

FIGURE 4.—Identification of the lethal complementation group responsible for the replication defect in the 51E-52A deficiency. The deficiencies and complementation groups shown were obtained from EILEEN UNDERWOOD (Bowling Green State University) and are described (UNDERWOOD *et al.* 1990); 8- to 12-hr embryos were collected from these stocks, labeled with BrdU, and the replication phenotype was checked. *fs(2)PA77* was tested by crossing to *Df(2L)XTE11* and examining the progeny embryos for the replication phenotypes. For *l(2)51Ea* and *l(2)51Eb* a single allele of each was tested (*l7* and *l12*, respectively).

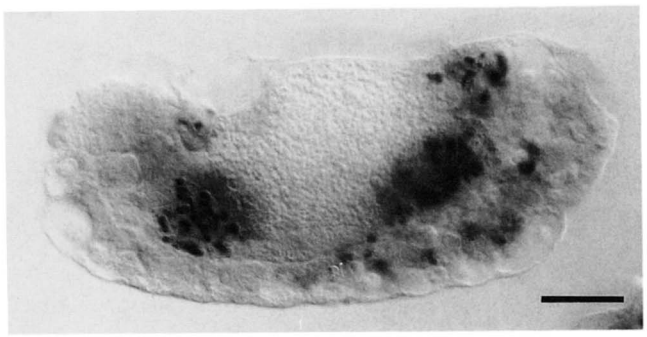

FIGURE 5.—An embryo homozygous for *Df(2L)J-der2* from an 8- to 12-hr collection labeled with BrdU. Fewer cells are visible, and the BrdU-labeled nuclei are larger than in wild-type embryos. The embryo is oriented with anterior to the right and dorsal up. Scale bar is 50 μm.

deficiency, suggesting perhaps that *PA77* is a hypomorphic allele of a gene required for DNA replication at times other than just amplification of the chorion gene clusters in the follicle cells. However, examination of *fs(2)PA77/Df(2L)XTE11* embryos revealed no reduction in DNA replication relative to wild type embryos (data not shown). *l(2)51Ec* was fully viable in trans to *fs(2)PA77*, thereby making it unlikely that these two mutations are alleles of the same gene.

A deficiency that uncovers a gene required for mitosis and *cdc2*: Embryos deficient for *Df(2L)J-der2* (region 31B-32A) had fewer cells, and the nuclei that were labeled with BrdU were much larger than those in wild-type embryos (Figure 5). This suggested that either mitosis was defective but DNA replication continued to produce polyploid cells, or that the onset of normal endoreplication might be occurring early. Since the gene *pimples* (*pim*) was known to reside in this interval and a *pim* mutation produces a cuticular phenotype similar to that of the homozygous deficiency embryos (NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984), we tested the single existing allele of *pim* for its DNA replication phenotype. The DNA replication phenotype of pim^{IL} embryos was indistinguishable from that of homozygous deficiency embryos (data not shown). Examination of *pim* embryos that had been stained with DAPI and an antibody against β-tubulin revealed that many of the nuclei throughout the embryo at 8.5 hr following fertilization had condensed DNA (Figure 6). The nuclear density in *pim* mutant embryos was about half that of sibling controls, and the frequency of mitotic figures was considerably higher in *pim* mutant embryos than in controls (Table 2). Thus, it seems likely that the nuclei in *pim* embryos enter mitosis but fail to complete it properly. Some of the cells that fail to complete mitosis may progress to the next S phase to produce the polyploid nuclei observed with BrdU labeling.

Another gene that resides within the *Df(2L)J-der2* interval is a Drosophila *cdc2* homolog (*Dm cdc2*) (JIMENEZ *et al.* 1990; LEHNER and O'FARRELL 1990a). *cdc2* is a key cell cycle regulator required for both the G_1/S and G_2/M cell cycle transitions in yeast (NURSE and BISSETT 1981; PIGGOT, RAI and CARTER 1982; REED and WITTENBERG 1990). On the basis of sequence homology Drosophila has two *cdc2* genes, localized to 31E and 92F, but only the 31E homolog functionally complements a *Schizosaccharoymyces pombe* strain that is mutant for *cdc2* (JIMENEZ *et al.* 1990; LEHNER and O'FARRELL 1990a). Despite the effect on cell number and nuclear size, homozygous *Df(2L)J-der2* embryos still showed dynamic patterns of cyclic replication in late stages (data not shown). Therefore, this *cdc2* gene is not essential for DNA replication during embryogenesis.

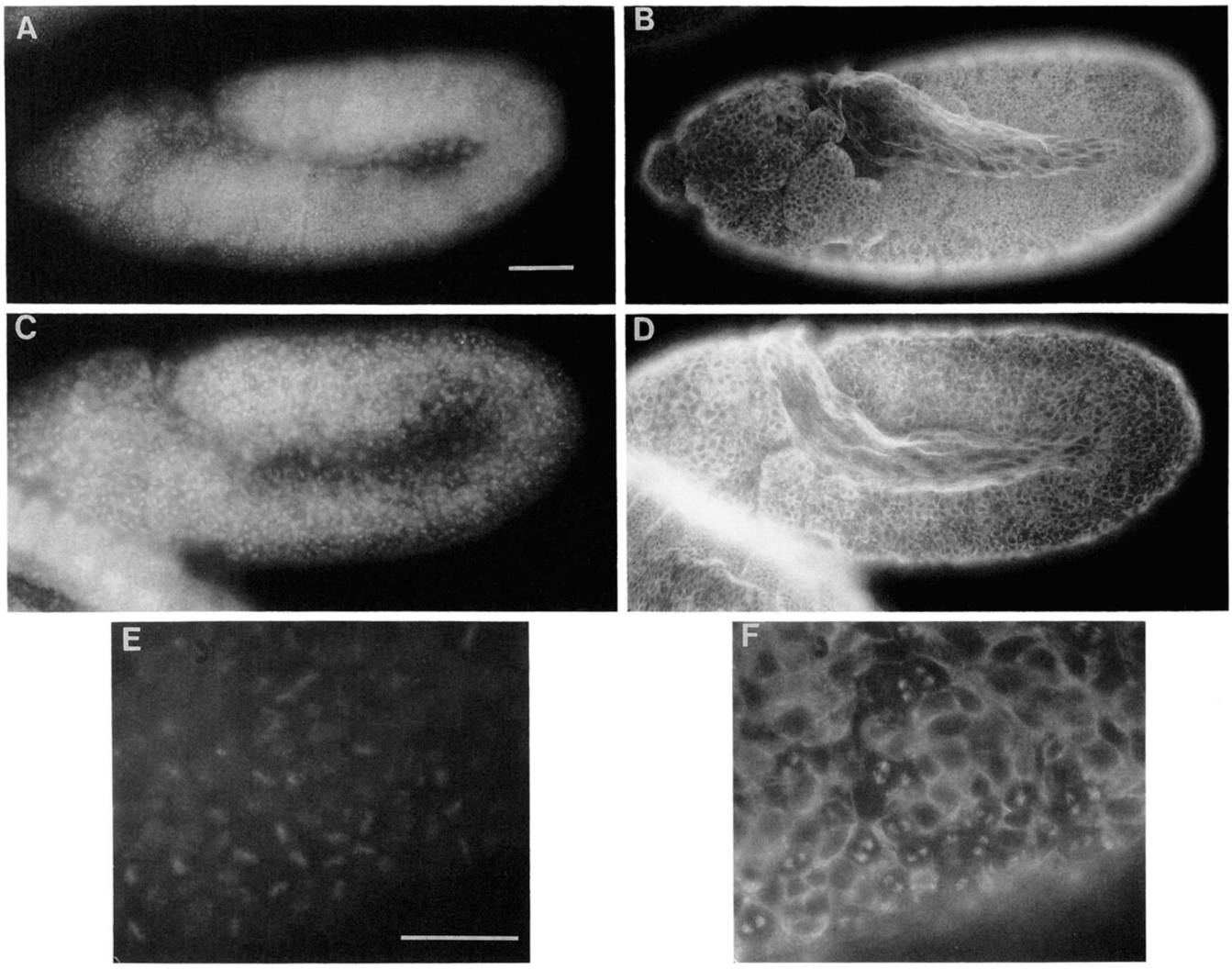

FIGURE 6.—Mitotic defect in *pim* mutant embryos. Wild-type germ band extended embryo stained with DAPI (A) or an antibody against β-tubulin (B). A pim^{IL} mutant embryo from the same stage and collection stained with DAPI (C) or anti-β-tubulin (D). There are many fewer cells in the *pim* mutant embryo and the anti-tubulin staining shows that a higher proportion of the cells are in mitosis. An enlarged region from a *pim* mutant embryo is shown stained with DAPI (E) or anti-β-tubulin (F). Many mitotic figures can be seen. In A–D the embryos are oriented with anterior to the left and dorsal up; the scale bar is 50 μm. In E the scale bar is 25 μm.

TABLE 2

Nuclear density and mitotic phenotype of *pim* mutant embryos

Genotype	Nuclear density (no. per 1000 μm^2)	Frequency of mitotic figures
pim^{IL}/pim^{IL}	16.2	24.1% ($n = 511$)[a]
$pim^{IL}/+$ or $+/+$	35.2	6.3% ($n = 410$)[a]

[a] Two embryos were examined, total number of nuclei scored is shown.

***Cyclin A* effect:** Another known cell cycle gene that was examined by deficiency analysis was *cyclin A*, which is uncovered by *Df(3L)vin7*(LEHNER and O'FARRELL 1989). Previous analysis of a mutant in *cyclin A* showed that during embryogenesis cells of the epidermis failed to undergo the third post-blastoderm mitotic division (LEHNER and O'FARRELL 1989). An allele of *cyclin A* that blocks cell division solely in adult sensory cells also has been identified (UEDA *et al.* 1992). Cyclin A has been proposed to have a role in DNA replication because injection of anti-sense *cyclin A* mRNA or cyclin A antibodies into mammalian cells inhibited DNA replication, and ablation of *cyclin A* mRNA in Xenopus extracts by an antisense oligonucleotide caused entry into mitosis before S phase was completed (GIRARD *et al.* 1991; PAGANO *et al.* 1992; WALKER and MALLER 1991). However, the DNA replication observed in *Df(3L)vin7* homozygous embryos was indistinguishable from that of wild-type embryos (data not shown). Replication proceeded normally in both the ventral cord and in endoreplicating tissues. A *P*-element allele that has been reported as failing to express cyclin A protein, *l(3)neo114* (LEHNER, YAKUBOVICH and O'FARRELL 1991), also did not exhibit any differences in the DNA replication pattern (data not shown). Similar observations have been reported

by LEHNER, YAKUBOVICH and O'FARRELL (1991). *Cyclin A* therefore is not required for the continued rounds of DNA replication in these tissues at this developmental time.

DISCUSSION

In screening the genome for deficiencies that affect DNA replication during embryogenesis, we identified three genomic intervals, 39D2-3;E2-F1, 51E and 75C5-7;F1, required zygotically for replication late in embryogenesis. We demonstrated that the defect in the 51E interval was associated with a single complementation group, *l(2)51Ec*. In addition, mutation of the gene *pimples* (*pim*) (31B-32A) leads to mitotic arrest. Our analysis would not have detected genes that may affect DNA replication but whose maternal contributions persisted throughout embryogenesis.

Regions required for embryonic DNA replication: The cessation of DNA replication observed in embryos homozygous for the three deficiency intervals could arise from deletion of genes specifically required to activate S phase of the cell cycle, or the deficiencies could uncover factors generally required for DNA replication. It is also possible that these deficiencies block DNA replication because they affect another step in the cell cycle on which DNA replication is dependent. However, there does not appear to be a defect in mitosis since no increase in mitotic figures was observed. The time DNA replication ceases in embryos deficient for the 75C5-7;F1 interval is coincident with two major transitions in cell cycle regulation, and therefore this interval is the most likely to contain a replication control function. During this period after the 16th division, G_1 is added to the mitotic cell cycle, while cells becoming polytene enter a cell cycle consisting of alternating gap and synthesis phases (EDGAR and O'FARRELL 1990; SMITH and ORR-WEAVER 1991). It is possible that late in embryogenesis, after the addition of G_1 to the mitotic cycle and the start of the endo cell cycle, regulatory controls for the entry into S phase are added, and that these are shared between the two types of cycles. The observed phenotype is consistent with the absence of a trigger for DNA synthesis that is added to both cell cycles at this time.

Although the timing of the block in DNA replication in the deletion 75C5-7;F1 is consistent with this interval containing a specific regulator of DNA replication, we cannot exclude the possibility that a function essential for general replication is present in the region. For the other genomic interval (39D2-3;E2-F1) whose deletion blocks DNA replication in the embryo and the *l(2)51Ec* complementation group, we also cannot distinguish whether the intervals contain a regulatory function or replication factor. However, the timing of the defects makes it more likely that these regions encode general DNA replication functions. No cell cycle change has been observed during the post-blastoderm divisions when replication ceases in embryos homozygous for the 39D2-3;E2-F1 deficiency or the *l(2)51Ec* lethal gene. If the defects in these intervals are due to the deletion of general DNA replication functions, then replication following cellularization probably occurs because the maternal stockpiles for these functions have not yet been depleted. Further analysis of the *l(2)51Ec* mutation will reveal whether it affects a regulator of DNA replication or a component required for DNA replication. As the cytological locations of additional genes for known DNA replication proteins are determined, it will be of interest to see if any map to these three intervals.

Embryos deficient for the 39D2-3;E2-F1 region cease DNA replication during the post-blastoderm divisions. The location of the histone gene cluster within this region is consistent with the hypothesis that deletion of the histone genes leads to arrest of DNA replication, but the possibility that another closely linked locus is responsible for the observed defect cannot be excluded. A reduction of histone proteins accompanying deletion of the genes may block DNA replication, either by directly impeding replication or by a feedback control mechanism. Alternatively, the absence of histones may cause a reduction in general gene expression due to an inability to form normal chromatin; this in turn could result in many indirect effects during embryogenesis including the cessation of DNA replication and inhibition of differentiation of the embryo. This is supported by the observation in *Saccharomyces cerevisiae* that alteration of histone gene dosage affects transcription levels (CLARK-ADAMS *et al.* 1988).

Embryonic effects of cell cycle genes: Deficiencies removing topoisomerase II and loci involved in DNA repair did not affect DNA replication late in embryogenesis. *Df(2L)J-der2* uncovers a Drosophila homolog of the key cell cycle regulator *cdc2*, yet it has no effect on DNA replication during embryogenesis. Recently, mutations in this *cdc2* gene at 31E have been identified (STERN *et al.* 1993). While these mutations show no effect on embryogenesis, they result in late larval/pupal lethality, presumably due to mitotic defects in the imaginal cells. Although maternal stores of *cdc2* are essential for proper mitotic divisions in the embryo, the *cdc2* gene at 31E is not required for endoreplication during embryogenesis or in the larval stages (STERN *et al.* 1993). It will be interesting to test the role of the other *cdc2* homolog in the endo cell cycle.

Both a deficiency that uncovers *cyclin A*, *Df(3R)vin-7* and a *P*-element mutation that fails to make any detectable cyclin A protein, *l(3)neo114*, do not alter

the replication patterns in either endoreplicating or mitotically dividing cells of late embryos (LEHNER and O'FARRELL 1989; LEHNER, YAKUBOVICH and O'FARRELL 1991). In contrast to these results in Drosophila, cyclin A has been implicated in the regulation of S phase in Xenopus and mammalian cells. One explanation for this apparent paradox is that maternally transcribed product persists and allows for DNA replication to proceed in *cyclin A* mutant embryos. An alternative possibility is that cyclin A is not required for S phase late in embryogenesis in either polytene cells or the mitotic cells of the ventral cord.

Failure to recover deficiencies affecting polytenization: In our screen we failed to detect a specific regulator for S phase in all endoreplicating tissues. Such a regulator may not have been in the 34% of the genome we could score conclusively. The possibility also remains that such a regulator does not exist. Polytenization would then be proposed to arise not as a direct consequence of a common endo cell cycle operating in all tissues but as some indirect effect of failure to go through mitosis. However, active regulation of the endo cell cycle is supported by a number of observations. Endoreplication domains follow precise developmental regulation during embryogenesis (SMITH and ORR-WEAVER 1991). In *string* mutants the developmental timing and pattern of endoreplication is normal, despite the fact that these mutants fail to undergo any post-blastoderm mitotic divisions (SMITH and ORR-WEAVER 1991). This indicates that endoreplication does not arise as a consequence of failure to enter mitosis. In addition to the precise regulation of the onset of endoreplication, two other aspects of endoreplication are developmentally regulated. The length of the gap phase during the embryonic endo cell cycles varies from tissue to tissue, and the final level of polytenization is tissue specific (SMITH and ORR-WEAVER 1991). This developmental regulation is most simply explained by active control of the endo cell cycle.

It is possible that rather than global regulators controlling endoreplication in all polytene tissues, regulators exist for individual replication domains. This model implies a greater number of regulators of endoreplication, since individual regulators are required for individual replication domains. In hypothesizing more regulators, the likelihood that they would have been scored is increased. Thus, we would have expected to detect this class of mutations in the 34% of the genome where we could see no alteration in the embryonic morphology.

Further delineation of the two intervals that when deleted result in the cessation of DNA replication in the embryo is necessary to identify the complementation group responsible for the DNA replication defect. Additional genetic analysis and phenotypic characterization of these genes together with *l(2)51Ec* will be required to determine whether the genes encode regulatory functions for replication or general replication factors. In either case, further investigation of these genes will lead to insights on how DNA replication is controlled during Drosophila embryogenesis.

This paper is dedicated to the memory of ALBERT J. SMITH. We thank ERIC WIESCHAUS for the suggestion to screen deficiency collections. We are grateful to KATHY MATTHEWS and the Bloomington Stock Center, EILEEN UNDERWOOD, STEPHANIE PAINE-SAUNDERS, ALLAN SPRADLING and HERMANN STELLER for providing stocks. We thank BRUCE REED for help with polytene chromosome cytology. MYLES AXTON, LIZ GAVIS, BRUCE REED and ALLAN SPRADLING provided helpful comments on the manuscript. A.S. was supported by NIH predoctoral training grant GM07287. This research was supported by NIH grant GM39341 and by a grant from the Lucille P. Markey Charitable Trust.

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Communicating editor: T. SCHÜPBACH