The Drosophila Suppressor of Underreplication Protein Binds to Late-Replicating Regions of Polytene Chromosomes

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ABSTRACT

In many late-replicating euchromatic regions of salivary gland polytene chromosomes, DNA is underrepresented. A mutation in the *SuUR* gene suppresses underreplication and leads to normal levels of DNA polytenization in these regions. We identified the *SuUR* gene and determined its structure. In the *SuUR* mutant stock a 6-kb insertion was found in the fourth exon of the gene. A single *SuUR* transcript is present at all stages of Drosophila development and is most abundant in adult females and embryos. The *SuUR* gene encodes a protein of 962 amino acids whose putative sequence is similar to the N-terminal part of SNF2/SWI2 proteins. Staining of salivary gland polytene chromosomes with antibodies directed against the SuUR protein shows that the protein is localized mainly in late-replicating regions and in regions of intercalary and pericentric heterochromatin.

POLYPLOID cells are commonly found in many different organisms. In Drosophila, polyploid tissues are present in almost all developmental stages and most larval tissues are polyploid. Polyploid cells undergo a modified cell cycle called the endocycle in which DNA replication is not followed by cell division. The endocycle lacks G2 and M phases of the cell-division cycle and the S phase alternates with a modified G1 phase. The G1 cyclin E in complex with cyclin-dependent kinase 2 is thought to be a key regulator of the endocycle and particularly of the S phase. The S phase depends also on a dE2F/dDP complex that is required for the expression of the *cyclin E* gene as well as other genes encoding essential replication factors (for review see Duronio 1999; Edgar and Orr-Weaver 2001). In Drosophila, salivary gland nuclei undergo the first endocycle between 8.5 and 10 hr of embryogenesis and replicate again only in first instar larvae, 7 hr after hatching (Smith and Orr-Weaver 1991). The final level of ploidy varies depending on the tissue; *e.g.*, salivary gland nuclei reach 1024-2048C, midgut has 64C, and follicle cells go up to 16C (for review see Zhimulev 1996). In some tissues, like the salivary gland, the chromatids remain associated to form visible polytene chromosomes.

DNA replication occurs in distinct temporal and spatial patterns. In particular, pericentric heterochromatin and certain euchromatic regions lag behind the rest of the genome and replicate late in S phase. In some polyploid tissues the lag is such that the next S phase starts before late-replicating regions complete the previous round. As a result, these regions become underrepresented (Lilly and Spradling 1996). Late replication is often associated with a heterochromatic chromatin state (for review see Allshire and Bickmore 2000). Euchromatic regions translocate to pericentric heterochromatin, where they are subject to position-effect variegation, replicate late during S phase, and become underreplicated in polytene tissues (Zhimulev 1998). In salivary glands, DNA underreplication is detectable from the first endocycle (Smith and Orr-Weaver 1991) while in nurse cells, the first four endocycles complete DNA replication and underreplication begins at the fifth endocycle (Dej and Spradling 1999).

From the time salivary gland cells are specified to the end of the third instar stage, their nuclei undergo 10 endocycles. Within heterochromatin, different sequences are underrepresented to different extents, ranging from almost diploid for satellite DNA to almost 1000C for complex DNA sequences within the heterochromatin (Devlin *et al.* 1990; Zhang and Spradling 1995; Zhimulev 1998). In addition to pericentric heterochromatin, certain euchromatic regions at specific sites on the polytene chromosome arms are also underreplicated (Zhimulev *et al.* 1982; Lamb and Laird 1987). For example, at 89E, the underreplicated region spans 300 kb of genomic DNA coinciding with the *Bithorax-Complex* (*BX-C*). The level of polytenization of this region, determined by Southern blot hybridization, drops 10-fold within 60–80 kb of the edge. The underreplicated region at 89E coincides with the interval containing the binding sites of Polycomb complexes responsible for

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silencing expression of the *BX-C* in salivary glands (Moshkin *et al.* 2001). Underreplicated euchromatic sites correspond to visible constrictions and tend to form breaks in salivary gland polytene chromosomes (Zhimulev 1998). In addition, these euchromatic sites resemble pericentric heterochromatin in that they replicate late during the endocycles and often form ectopic contacts with each other and with the chromocenter. These sites were named intercalary heterochromatin (IH) regions to reflect their "heterochromatic" features (Kaufmann 1939; for details see Zhimulev *et al.* 1982). Knowledge of the genes associated with IH features would be very useful to understand the mechanism of late replication.

The *Su*ppressor of *U*nder-*R*eplication (*SuUR*) mutation [previously known as *Su(UR)ES*] affects DNA replication both in IH and pericentric heterochromatin regions. Polytene chromosomes of the *SuUR* stock have no breaks or constrictions at all and the IH regions are fully polytenized (Belyaeva *et al.* 1998; Moshkin *et al.* 2001). The frequency of ectopic contacts between IH regions is also dramatically reduced. In addition, new banded chromosome regions become visible in the pericentric heterochromatin (Belyaeva *et al.* 1998).

In this article we describe the cloning of the *SuUR* gene. The putative SuUR protein possesses similarity to the ATP-binding domain of the SNF2/SWI2 protein family. Antibodies against the SuUR protein stain late-replicating regions in salivary gland polytene chromosomes.

MATERIALS AND METHODS

Stocks: We used deficiencies *Df(3L)vin2*, *Df(3L) vin4*, *Df(3L)vin5* (Akam *et al.* 1978), *Df(3L)h76* (Campbell *et al.* 1986), *Df(3L)lxd8*, *Df(3L)lxd9* (Schott *et al.* 1986), *Df(3L) X-61*, *Df(3L)X-66* (Staveley *et al.* 1991), and *Df(3L)klu*^{XR19} (Klein and Campos-Ortega 1997) and mutations from the 68AB region: *P*-element lethals *P{ry*$^{+t7.2}$ *= PZ} l(3)01239 ry*^{506} (late *P01239*), *P{ry*$^{+t7.2}$ *= PZ} l(3)09036 ry*^{506} (*P09036*), *P{ry*$^{+t7.2}$ *= PZ} l(3)02727 ry*^{506} (*P02727*) [Berkeley *Drosophila* Genome Project (BDGP)], EMS-lethals *l(3)68Ae*^{l58}, *l(3)68Ad*^{l55}, *l(3)68Ag*^{l1} (Campbell *et al.* 1986), the *klu*^{R5} allele (Klein and Campos-Ortega 1997), and the *Sod*^{h1} allele (Lindsley and Zimm 1992). As a source of transposase we used the *P* element in *y w; CyO Δ2-3/Bc Elp* stock kindly provided by Ernst Hafen.

Complementation analyses of *SuUR* mutation: The *SuUR* mutation leads to the disappearance of breaks in intercalary heterochromatin regions of salivary gland polytenes. The complementation between *SuUR* and deficiencies or mutations from the 68A region was determined by crossing *SuUR/SuUR* females and males bearing the aberrant chromosome. Complementation results in the appearance of breaks in intercalary heterochromatin regions in *trans*-heterozygote larvae. The absence of breaks was considered to indicate no complementation.

PCR analysis of recombinants: Two pairs of primers were used for analysis of flanking DNA fragments in the recombinant stocks: primers 20-42 and 3012 for the distal border and 559-539 and 3077 for the proximal border of the *P01239* insertion. Primer 3012 is from the *P*-element 3′ end (out of the transposon; CGTTAAGTGGATGTCTCTTGCCGAC) and primer 3077 is from the *P*-element 5′ end (out of the transposon; CCTTTCCTCTCAACAAGCAAACGTG); primers 20-42 (ACGTTTATCGCATAGTCCTCGTG; into the transposon) and 559-539 (AGCTCACTGCCCTTTTTGCTC; into the transposon) from the proximal and distal flanks of the *P*-element insertion in the *P01239* stock. After 30 cycles of amplification (94°, 40 sec; 69°, 40 sec; 72°, 1 min) samples were analyzed by electrophoresis in a 1.5% agarose gel.

Cloning: Genomic P1 clones from the 68A regions DS06480, DS01412, DS00731, DS04602, DS03354, DS02089, and DS01690 were provided by Marek Mlodzik (EMBL, Heidelberg). P1 DNA was isolated according to the BDGP protocol. DNAs were digested with restriction enzymes, separated on agarose gel, and blotted onto a nylon membrane. The membrane was hybridized with the digoxigenin (DIG)-labeled PCR fragments flanking the *P01239* insertion (starting point of cloning) or with fragments of DS01690 and DS04602 P1 clones subcloned in pBluescript KS−. DNA around the insertion in the *P01239* stock was PCR amplified from genomic DNA of Canton-S flies with primers 20-42 and 559-539 (see above) and labeled with DIG by polymerase chain reaction.

Rescue of the mutation with genomic fragments: The position of genomic fragments used for transformation is shown in Figure 2C. A 7.9-kb *Sal*I fragment was subcloned from the DS01690 P1 clone into pBluescript KS− to give S23. The S23 clone was digested with *Sal*I and *Pst*I and religated to give clones P92 and P42 containing the 4.1-kb *Sal*I-*Pst*I and 3.8-kb *Pst*I-*Sal*I fragments, respectively. The proximal part of the 7.9-kb *Sal*I fragment was removed by cutting with *Bam*HI. The resulting B13 clone contains a 5.7-kb *Sal*I-*Bam*HI DNA fragment. The 4.9-kb *Xba*I-*Sal*I DNA fragment from the DS04602 P1 clone was cloned into pBluescript KS−, resulting in X6S1. All clones were digested with *Not*I and *Acc*65I and the inserts were ligated into pWhiteRabbit (constructed by Nicholas Brown) cut with *Not*I-*Acc* 65I to produce the transposons for germline transformation. Standard germline transformation was performed in the *y w*^{67} host strain. Cytological analysis of polytene chromosomes was carried out as described in Belyaeva *et al.* (1998).

Constructs containing SuUR transcript under the control of the *hsp70* promoter: The 5′ untranslated region (UTR) was removed by inserting a *Pst*I site upstream of the AUG codon by PCR. The LD04285 cDNA clone was digested with *Xho*I, religated, and used as a template for PCR with the T7 standard primer and 5Pst primer (GCTGCAGC**ATG**TATCACTTTGT ATC; the *Pst*I site is underlined and the AUG codon is shown in boldface type). The PCR product was cut with *Pst*I and *Hin*dIII and ligated into pBluescript SK− to produce clone 31. The sequence was verified from T3 and T7 primers. The *Pst*I-*Hin*dIII fragment from the 5′ end of LD04285 was excised and replaced with the *Pst*I-*Hin*dIII fragment from clone 31, resulting in clone f40, which contains *SuUR* cDNA with a *Pst*I site replacing the 5′ UTR. Clone f40 was digested with *Xba*I and *Hpa*II and the insertion was ligated into the *Spe*I-*Sma*I-cut C4 Yellow hs2 (Poux *et al.* 2001) between the *hsp70* promoter and the *hsp70* 3′ UTR, resulting in the H7 transposon for germline transformation. This construct contains the entire open reading frame and part of the 3′ UTR of the *SuUR* gene under control of the *hsp70* promoter.

Sequencing: The expressed sequence tag (EST) clone LD04285 was obtained from Research Genetics and sequenced at the sequencing facilities of Geneva University. The sequence was submitted to GenBank under accession no. AJ277592. Sequence analysis was performed on the ExPASY server (http://www.expasy.ch) and BLAST (Altschul *et al.* 1997) was carried out at http://www.ncbi.nlm.nih.gov/BLAST/.

RNA isolation and Northern blot analysis: Total RNA was isolated from Drosophila embryos, larvae, pupae, or adult flies according to Maes and Messens (1992). Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose columns and 1.5 μg of poly(A)$^+$ RNA was loaded per lane of the agarose gel. After electro-

TABLE 1

Complementation of deletions with the *SuUR* mutation

Deletions	Breakpoints	Complementation with SuUR
Df(3L)vin4	68B4; 68F	+
Df(3L)X61	68B1-3; 68C15	+
Df(3L)X66	68A8-B3; 68C5	+
Df(3L)h76	68A8-9	+
Df(3L)vin5	68A3; 68F	−
Df(3L)vin2	68A1-2; 68C15	−
Df(3L)klu^{XR19}	68A1-2; 68A9	−
Df(3L)lxd8	68A3; 68A5-6	−
Df(3L)lxd9	68A3-4; 68B4-C1	−

phoresis, the RNA was transferred to a Hybond-N membrane (Amersham, Buckinghamshire, UK) and hybridized at 50° in high SDS-formamid buffer [7% SDS; 50% formamide; 5× SSC; 2% blocking reagent (Boehringer Mannheim, Indianapolis); 50 mM sodium phosphate, pH 7.0; 0.1% sarcosyl] overnight. The ^{32}P-labeled DNA probes were obtained by random priming. Membranes were washed two times in 0.1% SDS, 1× SSC at room temperature for 10 min, and then for 20 min in 0.1% SDS, 0.2× SSC at 65° and exposed to Kodak BioMax MS film with Kodak BioMax MS intensifying screen for 2–4 hr. The RNA bands were quantitated with a PhosphorImager.

***In situ* hybridization of tissue sections:** The flies were fixed in Carnoy's for 1 hr at room temperature. Paraffin embedding and preparation of 7-μm sections were done according to standard procedures (Ashburner 1989). Digoxigenin labeling of sense and antisense RNA and hybridization were performed according to the protocols for detection of mRNA with DIG-labeled RNA probes (Boehringer Mannheim).

Antibodies: Rabbit polyclonal antibodies were raised using a glutathione S-transferase (GST) fusion protein containing amino acid residues 371–578 of the SuUR protein. Fusion protein from BL21 *Escherichia coli* cells was purified on glutathione-Sepharose (Pharmacia, Piscataway, NJ) and the antibodies were affinity purified on a column containing the SuUR-GST protein coupled with CNBr-activated Sepharose according to the manufacturer's instructions (Pharmacia). Western blots and polytene chromosome staining were performed as described in Poux *et al.* (2001). The antibody was used at 1:1000 or 1:1500 dilution for Western blots and at 1:30 or 1:50 for polytene chromosomes.

RESULTS

Cytogenetic mapping of the *SuUR* mutation: Previous studies located the *SuUR* mutation in the 68A3-B4 region at 34.8 cM (Belyaeva *et al.* 1998). We mapped the breakpoints of deficiencies in this region and complementation analysis located the *SuUR* mutation in the 68A3-5 region between the distal breakpoint of *Df(3L)lxd9* and the proximal breakpoint of *Df(3L)lxd8* (Table 1). Three other genes fall in the same genetic interval, two of which are *low xanthine dehydrogenase* (*lxd*), *l(3)68Ad* (Campbell *et al.* 1986; Crosby and Meyerowitz 1986) and *l(3)01239* (also called *P01239*). The *SuUR* mutation complements mutant alleles of these genes. To determine the relative position of these mutations, we generated additional deficiencies in this interval using the method of Preston *et al.* (1996) and Preston and Engels (1996). Starting with a stock containing the *P*-element insertion *P01239* within the interval of interest, we mobilized the *P* element to induce male recombination, which is often accompanied by deletions flanking the inserted *P* element. The recombination events were detected using a tester chromosome marked with *ru* (*rough*) and *h* (*hairy*) distal to the *P01239* insertion and *th* (*thread*) and *st* (*scarlet*) on the proximal side. Females *P01239 ry/TM3, Sb ry* were crossed to males *CyO Δ2-3/+; ru h th st* bearing a source of transposase. The *P*-element-induced mitotic recombination occurs in males with genotype *CyO Δ2-3/+; P01239 ry/ru h th st*; these males were collected and crossed according to the scheme in Figure 1. Recombinant males with genotype *ru h Pr* (left recombinants) or *th st Pr* (right recombinants) were individually crossed to females *ru h th st cu sr Pr e ca/TM6, Tb Hu e ca* to balance the recombinant chromosome and establish individual stocks *rl* and *rr*, respectively.

In two experiments, 22,952 progeny were analyzed and 57 "left" and 40 "right" recombinants were found. Among stocks established from left recombinant males, two, *rl-3* and *rl-5*, failed to complement *SuUR*, *klu*^{R5}, and *P09036* but complemented *68Ad*^{l55} (Figure 2A). The integrity of the DNA fragments immediately flanking

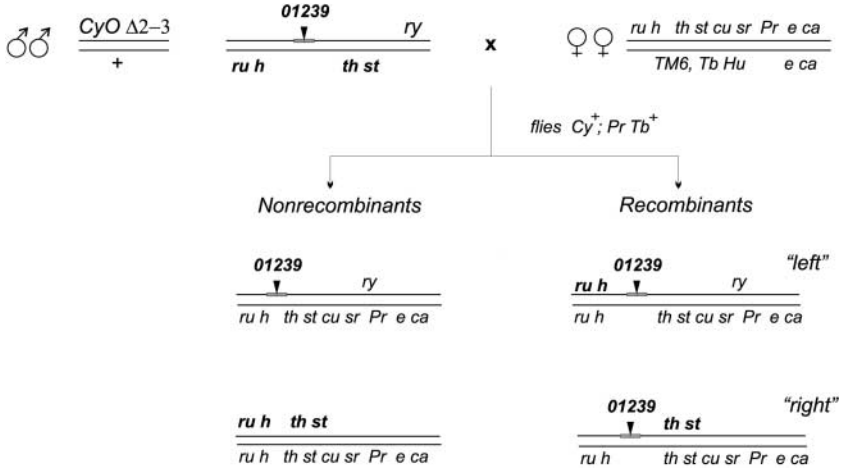
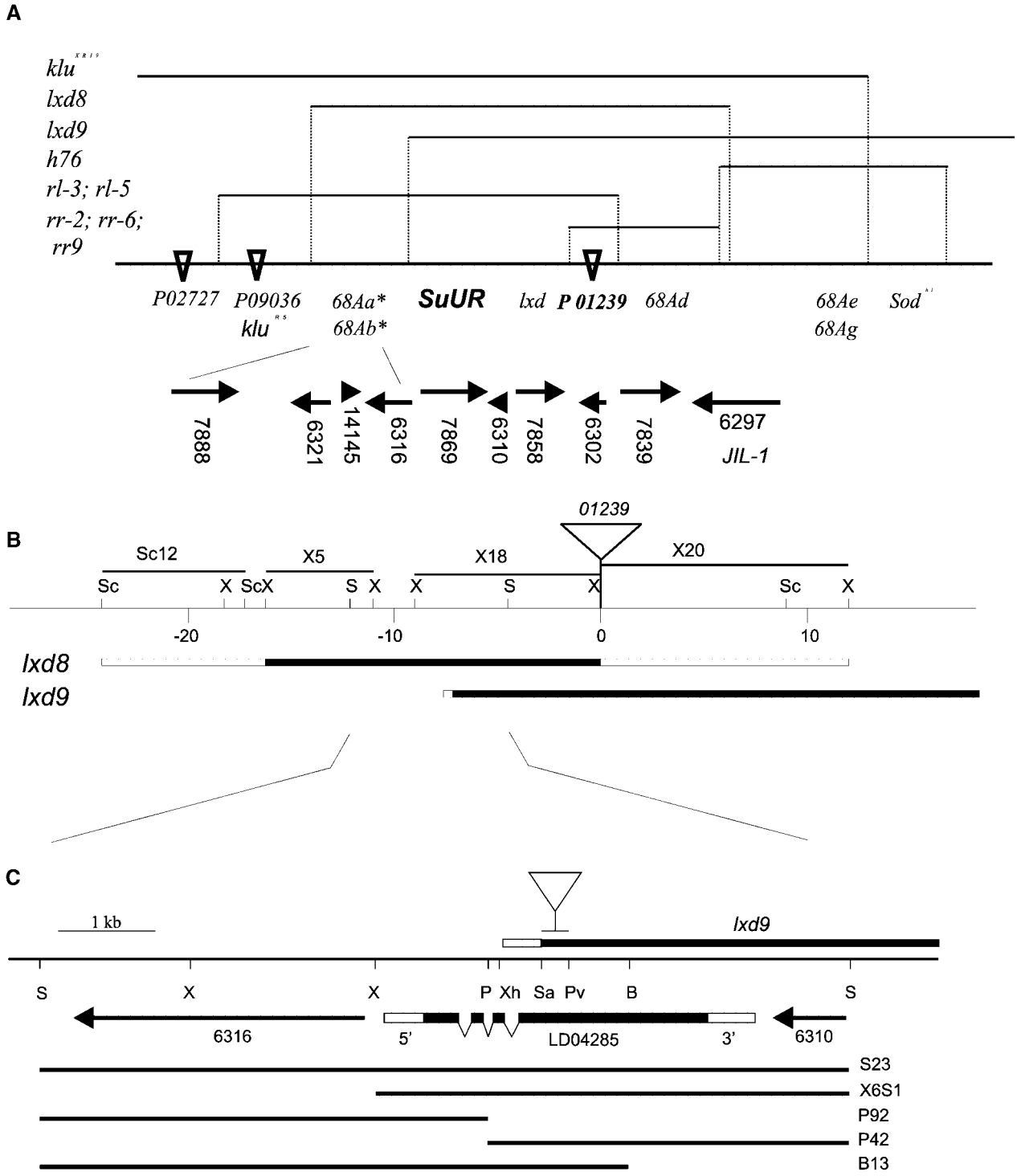


Figure 1.—Scheme of isolation of recombinant stocks. Only recombinant males were used for establishing recombinant stocks to avoid further recombination.

the *P01239* insertion site was checked by PCR with two pairs of primers for the distal and proximal edges of the *P* element. In each pair one primer was from the *P*-element terminus, and the other was from the neighboring genomic DNA, as described by Preston *et al.* (1996). No boundary fragments were amplified by PCR for the distal side, whereas PCR products from the proximal boundary coincided with those amplified from the initial stock (data not shown), indicating that some rearrangement had occurred on the distal side of *P01239* in the *rl-3* and *rl-5* stocks. The failure to complement klu^{R5} and *P09036* on the distal side of *P01239* and the loss of PCR products from the distal boundary of the insertion indicate that the *rl-3* and *rl-5* stocks have deficiencies removing DNA distally from *P01239*. This allowed us to locate the *SuUR* gene on the distal side of the *P01239* insertion. Both *Df(3L)rl-3* and *Df(3L)rl-5* have additional modifications. Cytological analysis revealed the



presence of an additional inversion, *In(3LR)* 67E; 86E, in the *Df(3L)rl-3* stock, and the *Df(3L)rl-5* stock displayed dominant female sterility. In heterozygous *Df(3L)rl-5* females the germarium contained dead and degraded nurse cells, an abnormal number of nurse cells, and the eggs produced were defective.

Three stocks established from the right recombinant males, *rr-2*, *rr-6*, and *rr-9*, failed to complement $68Ad^{l55}$ but complemented *SuUR*, $68Ae^{l58}$, and $68Ag^{l1}$. No PCR products were obtained for the proximal boundary of *P01239* in *rr-2*, *rr-6*, and *rr-9*, while PCR fragments corresponding to the distal boundary were the same as in the parental *P01239* strain (data not shown). These data indicate that the changes in *rr-2*, *rr-6*, and *rr-9* stocks were proximal to the insertion and suggest that $68Ad^{l55}$ is located proximally to the *P01239* insertion. A genetic map of the region obtained from this analysis is shown in Figure 2A.

Researchers have observed that the *P* element remained in place in the majority of their recombinant stocks (Preston *et al.* 1996; Preston and Engels 1996). In our case, among 26 "left recombinant" chromosomes analyzed, 16 lacked the *P*-element marker ry^+. PCR with sets of primers for distal and proximal boundary fragments for four such stocks gave no amplification of a boundary fragment in these recombinant stocks in contrast to the control *P01239* stock. This suggests that in our experiments a significant fraction of recombinant stocks lost or altered the original *P*-element insertion.

Cloning of DNA surrounding the *P01239* insertion: The complementation analysis places the *SuUR* gene between the distal edge of *P01239* and the distal edge of *Df(3L)lxd9* (Figure 2A). We used the *P01239* insertion as a starting point for cloning. DNA around the insertion site was amplified by PCR and used for hybridization with genomic P1 phage clones from the 68A region (obtained from BDGP). The P1 phage clones were assembled in a 150-kb contig and a detailed restriction map was obtained for ~50 kb around the insertion site (Figure 2B and data not shown). DNA fragments from genomic P1 clones were subcloned in pBluescript and used for mapping the *Df(3L)lxd8* and *Df(3L)lxd9* breakpoints by *in situ* hybridization to polytene chromosomes (data not shown). The distal breakpoint of *Df(3L)lxd9* was mapped more precisely by Southern blot analysis (data not shown) between *Xho*I and *Sca*I sites in the distal part of a 7.9-kb *Xba*I fragment. These results are summarized in the map shown in Figure 2B.

Relationship between Celera transcripts and genes from 68A region: The genomic DNA sequence (Adams *et al.* 2000), together with a map of deficiency breakpoints in 68A, allowed us to localize genes mapped earlier in this region (Campbell *et al.* 1986; Crosby and Meyerowitz 1986; Staveley *et al.* 1991) as well as the genes predicted from the genomic DNA sequence in the Celera Genomics "Annotation Jamboree" and to narrow the localization of the *SuUR* gene. The precise identification of *SuUR* is described in the next section. Lethals *l(3)68Aa* and *l(3)68Ab* complemented with *Df(3L)lxd9* but not with *Df(3L)lxd8* (Crosby and Meyerowitz 1986; see Figure 2A). The genomic sequence predicts the presence of four genes in the region between distal breaks of *Df(3L)lxd8* and *Df(3L)lxd9* (Figure 2B). Two of these are most probably *l(3)68Aa* and *l(3)68Ab*. The *lxd* gene probably corresponds to CG7858. The CG7858 sequence predicts a protein with high degree of similarity to molybdenum-binding proteins (data from GadFly on the BDGP website), and the gene was accordingly named *molybdenum coenzyme synthase I* (*mocsI*; Gray and Nicholls 2000). Mutations in *lxd* fail to complement both *Df(3L)lxd8* and *Df(3L)lxd9* deficiencies (Crosby and Meyerowitz 1986) and homozygous *lxd* flies have only 10% of the normal molybdenum cofactor activity (Schott *et al.* 1986). We suppose therefore that CG7858/*mocsI* corresponds to *lxd* but additional analysis will be needed to verify this conclusion. Gene CG6302 is disrupted by the *P*-element in-

Figure 2.—Genetic and molecular map of the 68A region. (A) Relationship between known genes from the 68A region and genes predicted from the genomic sequence in the Celera Genomics "Annotation Jamboree." Solid lines above the map correspond to deficiencies whose name is given on the left. The genetic map is based on complementation analyses. Lethals *l(3)68Aa* and *l(3)68Ab* (marked by *) are not available and their position is based on the original study (Crosby and Meyerowitz 1986). $Df(3L)klu^{XR19}$ places *l(3)68Ae* and *l(3)Ag* distally to *Sod* but the relative position of these lethals is unknown. Triangles correspond to *P*-element insertions. Arrows indicate the direction of transcription of predicted genes from Celera. The position and size of transcription units corresponds approximately to the scale in B except for *JIL-1*. (B) Physical map in the vicinity of the *P01239* insertion used as a starting point for cloning. Restriction enzymes used are the following: *Xba*I (X), *Sac*I (Sc), and *Sal*I (S; not all *Sal*I sites are shown). Numbers below indicate distance in kilobase pairs. The triangle corresponds to the *P*-element insertion in the *P01239* stock. Fragments used to map deficiency breakpoints by *in situ* hybridization are shown above the restriction map. The *Df(3L)lxd8* (lxd8) and *Df(3L)lxd9* (lxd9) deficiencies are shown below, with open boxes indicating the uncertainty of breakpoint mapping and solid lines corresponding to the DNA deleted. (C) Map of the *SuUR* gene. Restriction sites are given for the following: *Pst*I (P), *Pvu*I (Pv), *Sal*I (S), *Sca*I (Sa), *Xba*I (X), and *Xho*I (Xh). The triangle corresponds to the 6-kb insertion in the *SuUR* mutant stock with the mapping uncertainty indicated by the line below. DNA removed by deficiency *Df(3L)lxd9* is shown as a solid line above the map with the open box indicating the distal breakpoint. The exon structure of *SuUR*, shown below the map, is based on the sequence of EST clone LD04285. Open and solid boxes correspond to UTRs and coding regions. The position of neighboring predicted genes is given by arrows. Only 115 bp separate EST clone LD04285 and the divergent clone LD47413 corresponding to the CG6316 gene. Genomic fragments used for rescue experiments are shown below the transcript.

TABLE 2

Frequency of breaks in intercalary heterochromatin regions in salivary gland polytene chromosomes

Genotype		Frequencies of breaks (%) in the regions					
		11A	19E	39E	42B	75C	89E
1	Oregon-R (wild stock)	96 ± 2.8	87 ± 3.8	100	80 ± 3.7	92 ± 2.6	68 ± 3.1
2	*w; SuUR*	0	0	0	0	0	0
3	*X6S1; SuUR*	94 ± 3.1	88 ± 3.7	100	90 ± 2.9	90 ± 3.2	75 ± 4.3
4	*P42; SuUR*						
	P92; SuUR	0	0	0	0	0	0
	B13; SuUR						
5	*H7; SuUR* without heat shock	12 ± 3.6	5 ± 2.0	100	3 ± 1.6	5 ± 2.1	1 ± 0.7
6	40 min heat shock 3–5 hr embryos	96 ± 2.0	88 ± 3.5	100	89 ± 3.4	94 ± 2.4	78 ± 4.7
7	24–26-hr first instar larvae	93 ± 2.3	76 ± 3.8	100	75 ± 3.7	94 ± 1.9	45 ± 4.5
8	44–54-hr second instar larvae	84 ± 2.3	61 ± 4.0	100	58 ± 3.9	76 ± 3.2	30 ± 3.9
9	67–77-hr second instar larvae	60 ± 4.0	37 ± 4.1	100	23 ± 6.2	46 ± 4.4	9 ± 2.4
10	80–90-hr third instar larvae	6 ± 3.6	2 ± 2.1	100	3 ± 1.6	18 ± 3.1	2 ± 1.2

Rows 6–10 show results for the *H7; SuUR* stock after a 40-min heat-shock treatment administered to embryos or larvae of the indicated age (hours after egg deposition).

sertions in the *P01239* and the *l(3)j9B4* stocks (BDGP). The *l(3)68Ad* gene was mapped proximally to the *P01239* insertion by complementation with *Df(3L)rl-3* and *Df(3L)rl-5* and failure to complement *Df(3L)rr-2*, *Df(3L)rr-6*, and *Df(3L)rr-9* (Figure 2A). Only one gene, CG7839, was predicted between CG6302 (*P01239*) and the recently described *JIL-1* kinase CG6297 (Jin *et al.* 1999; Wang *et al.* 2001). The CG7839 is therefore the best candidate to be *l(3)68Ad*. The CG7839 gene encodes a protein with strong similarity to the CCAAT-box-binding transcription factor from different organisms, including human and mouse. Two lethals, *l(3)68Ae* and *l(3)68Ag*, were mapped between the proximal breaks of *Df(3L)lxd8* and $Df(3l)klu^{XR19}$. Although we did not map precisely the proximal breakpoint of $Df(3L)klu^{XR19}$, we expect it to be distal to the *Sod* gene, located 38–40 kb on the proximal side of the *P01239* insertion, according to the Celera sequence. The genomic sequence predicts nine genes in the 19-kb interval between *JIL-1* and *Sod*, two of which are most likely *l(3)68Ae* and *l(3)68Ag*.

Identification of the *SuUR* gene: Analysis of the genes predicted to be in the correct interval between the *P01239* insertion and the distal breakpoint of *Df(3L)lxd9* indicated that CG7858/*lxd* and CG6302 are not likely to be the *SuUR* gene, leaving CG6316, CG7869, and CG6310. To identify the *SuUR* gene, we made five constructs for *P*-element-mediated transformation with different overlapping genomic fragments from this region (Figure 2C). The constructs were injected in $y\ w^{67}$ embryos and several independent transformants were obtained for each construct. Two of them, S23 and X6S1, rescue the *SuUR* mutation by restoring to wild-type level the frequency of breaks in intercalary heterochromatin regions of polytene chromosomes of Tn; *SuUR* stocks (Table 2). No breaks were observed with constructs B13, P42, or P92 in presence of the *SuUR* mutation. Hence the *SuUR* gene is entirely contained within the 4.9-kb *Xba*I-*Sal*I fragment, ruling out CG6316 as a candidate. Furthermore, CG6310 is also ruled out because the P42 construct fails to rescue *SuUR*.

The 4.9-kb *Xba*I-*Sal*I genomic fragment was used to probe a Northern blot prepared with total embryonic RNA. The probe detects a single band of 3.6–4.0 kb in $y\ w^{67}$ RNA that is absent in the *SuUR* mutant (Figure 3A). The size of transcript indicates that the corresponding gene spans almost the entire length of the 4.9-kb *Xba*I-*Sal*I fragment (Figure 2C). Several EST clones reported from this fragment (BDGP; Rubin *et al.* 2000) were ordered from Research Genetics and the sequence of clone LD04285 was determined. The length of the cDNA (3771 bp) corresponds to the size of the transcript observed in the Northern blot (Figure 3A). The comparison of the cDNA and the genomic sequences reveals three introns 107, 66, and 86 bp long. The genetic analysis, the rescue experiments, and the agreement between cDNA length and transcript size in Northern blot hybridization and the disappearance of the corresponding transcript in the *SuUR* mutant indicate that the EST clone LD04285 and the corresponding gene CG7869 represent the *SuUR* gene.

The *Xba*I-*Sal*I genomic fragment in the X6S1 construct includes the *SuUR* gene but it might also contain other transcription units. To determine whether *SuUR* is in fact CG7869 we made an additional rescue construct, H7, in which the LD04285 cDNA is expressed under control of the *hsp70* promoter. The H7 construct, containing the longest open reading frame of the LD04285 cDNA fused to the *hsp70* promoter in the C4Yellow hs2 vector, was injected into $y\ w^{67}$ embryos, and several independent transformant stocks were established and tested in a *SuUR* mutant background. The H7; *SuUR* embryos were collected for 2 hr, aged for various times,

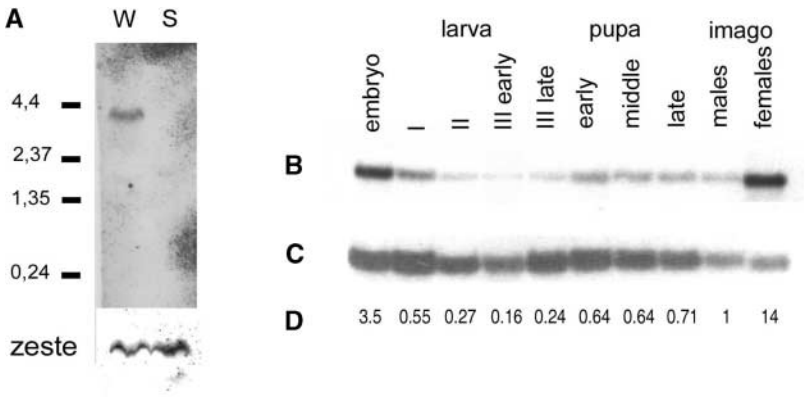


FIGURE 3.—Northern blot hybridization. (A) Total embryonic RNA from Oregon-R (W) and the *SuUR* (S) stock were blotted and hybridized with the DIG-labeled 4.9-kb *Xba*I-*Sal*I fragment containing the entire SuUR coding region. The single major transcript of 3.6–4 kb was observed in the wild-type stock but not in the *SuUR* mutant. Hybridization with *zeste* cDNA was used as a control. (B) Wild-type poly(A)$^+$ RNA from various developmental stages was hybridized with the ^{32}P-labeled LD04285 clone. The same single transcript is present in all stages. (C) The same blot hybridized with the *Ras2* probe. (D) Relative levels of *SuUR* RNA. The Northern blots were quantitated with a PhosphorImager; signals were normalized to the *Ras2* band (for details see SOLDATOV *et al.* 1999). The level of transcription in adult males was taken as 1.

heat-shocked for 40 min, and allowed to develop at 25° to the third instar stage for cytological analysis of polytene chromosomes. We determined the frequency of breaks for a set of six IH regions that display a high frequency of breaks in wild-type stocks. This set reliably reflects the effect of heat-shock-induced SuUR protein on all IH regions during ontogeny. A single embryonic heat shock restores the frequency of breaks to that of the wild-type Oregon-R stock (Table 2, line 6). The frequency of breaks observed decreased when expression of the *hs-SuUR* transgene was induced late in development. The frequency of breaks in the 89E region decreased significantly when the heat shock was administered to first instar larvae. The break frequency decreased for all regions except 39E when the heat shock was given during the second instar stage. Induction during the third instar stage results in a very weak (if any) effect on break frequency (compare lines 5 and 10 in Table 2). Without heat shock, the frequency of breaks in H7; *SuUR* chromosomes is very low for the majority of regions except for 39E where it is 100%. This region is very sensitive to the presence of SuUR product. Breaks at 39E occur at 100% frequency even in the presence of one zygotic dose *SuUR*$^+$ (BELYAEVA *et al.* 1998) and the basal transcription from the *hsp70* promoter in the H7 construct is probably sufficient to induce breaks at this site (Table 2). Regions 89E and 42B are the least sensitive to the SuUR product. Heat-shock treatment of the *SuUR* stock in the absence of the H7 transposon did not result in the appearance of breaks (data not shown).

Southern blot analysis of genomic DNA from the *SuUR* mutant stock revealed the presence of a 6-kb insertion within the 4.9-kb *Xba*I-*Sal*I fragment, compared to Oregon-R or Canton-S stocks. More detailed analysis with subfragments of the 4.9-kb *Xba*I-*Sal*I fragment shows that the insertion site is in the 268-bp fragment between *Sca*I and *Pvu*I sites in the last exon of the *SuUR* gene (data not shown). This insertion is most probably the cause of the mutation in *SuUR* stock.

The LD04385 cDNA was used to probe Northern blots of wild-type poly(A)$^+$ mRNA isolated from different developmental stages, from embryo to imago (Figure 3B). A single transcript of ~4 kb was detected in all stages but was most abundant in adult females and embryos. This agrees well with the maternal effect of the *SuUR* gene described previously (BELYAEVA *et al.* 1998). The distribution of the *SuUR* transcript in adult females was determined by *in situ* hybridization of tissue sections using sense and antisense riboprobes obtained from the LD04285 cDNA (Figure 4). As expected, the *SuUR* transcripts were detected in germline cells (both in nurse cells and oocytes) as well as in follicle cells.

Analysis of the promoter region and deduced SuUR protein: Only 115 bp separate the 5′ end of the longest *SuUR* EST clone LD04285 from the 5′ end of the LD47413

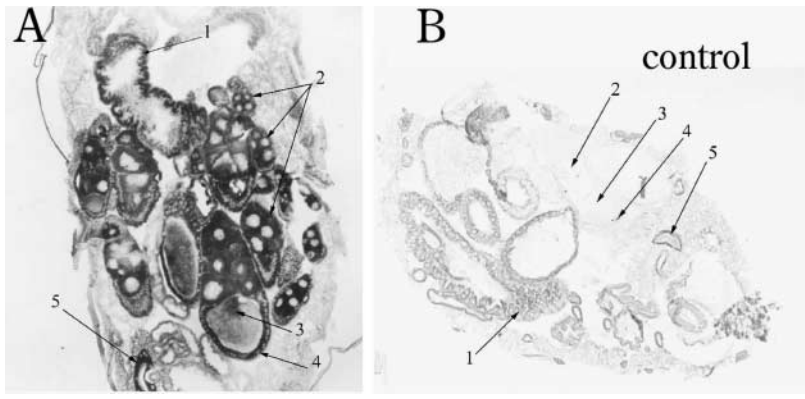


FIGURE 4.—Spatial localization of *SuUR* transcripts. *In situ* hybridization of a digoxigenin-labeled *SuUR* antisense (A) and control sense (B) riboprobe to paraffin-embedded tissue sections prepared from wild-type female fly. Immature oocytes (3) give a weaker signal compared to nurse cells (2) and follicle cells (4). Hybridization signals are visible in the gut (1) and the seminal receptacle (5). No signal was detected when tissue sections were hybridized with sense *SuUR*-RNA control probe (B).

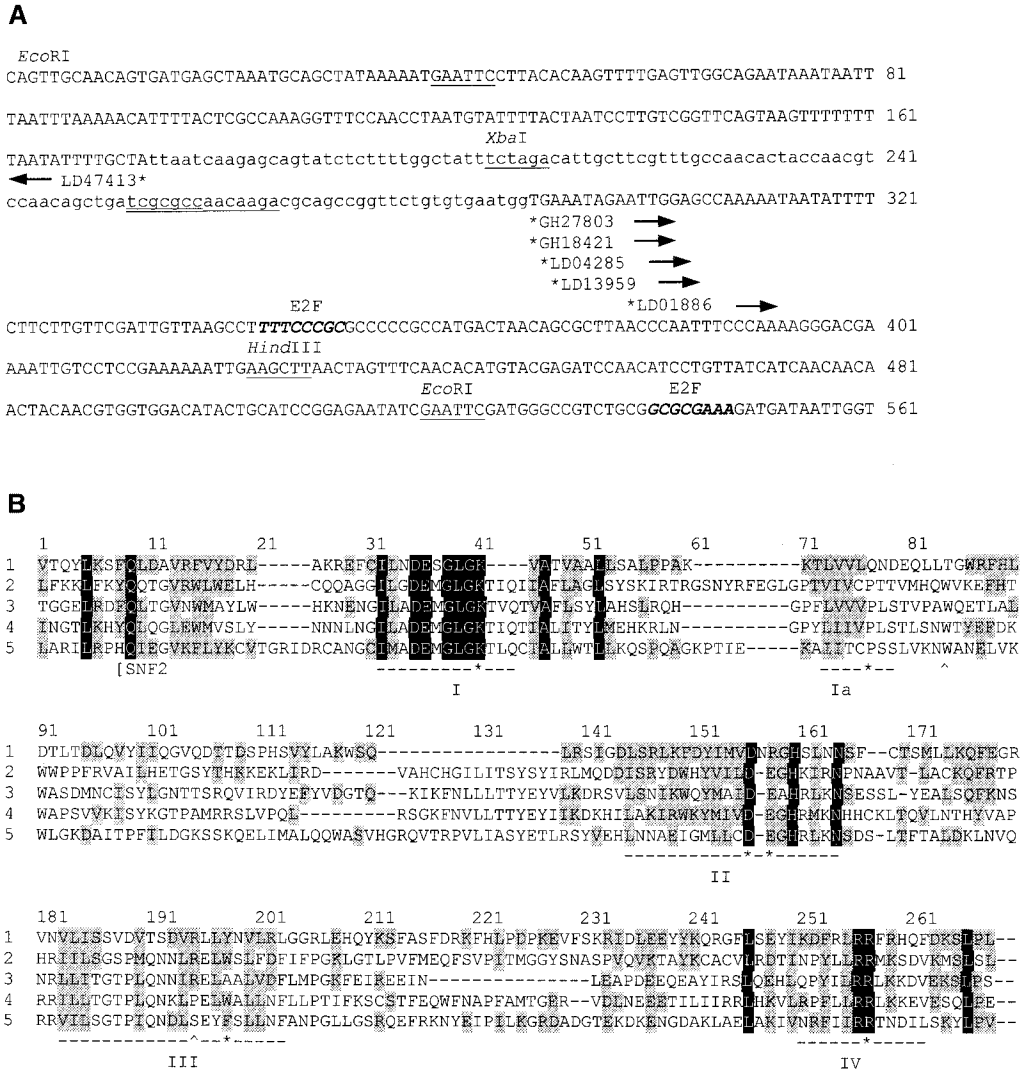


FIGURE 5.—(A) The promoter region of the *SuUR* gene. Uppercase letters correspond to the sequences present in the EST cDNA clones, with the name of the clone given below the sequence. Asterisks indicate the 5′ end of the clones and arrows mark the direction of transcription. Restriction enzyme sites are underlined and noted above the sequence. The BRE consensus G/C-G/C-G/A-C-G-C-C, which is thought to increase the affinity of TFIIB for the promoter (LAGRANGE *et al.* 1998), is doubly underlined; an A-rich sequence where a TATA box might be expected, at position −25 from the putative *SuUR* transcription START site, is singly underlined. The putative E2F binding sites are shown in bold italics. (B) Amino acid sequence alignment of the SuUR protein and four members of the SNF2 protein family. Sequences: 1, SuUR (24–253 amino acid residues), *Drosophila melanogaster*; 2, ERCC6 (503–756 aa), *Homo sapiens* (accession no. Q03468); 3, HRP3 (371–603 aa), *Schizosaccharomyces pombe* (accession no. O14139); 4, SNF2A or SNF2-ALPHA(716–956 aa), *H. sapiens* (accession no. P51531); and 5, RPR54, RAD54 homolog (260–520 aa), *S. pombe* (accession no. P41410). Multiple alignment was done using the VOSTORG program developed in the Institute of Cytology and Genetics (Novosibirsk, Russia). Amino acid residues identical in all five sequences are shown against a black background, and less conservative residues are shaded. Substitutions between aromatic (YWF), charged (RKDE), and aliphatic (IVL + M) amino acid residues are considered as similar. The first five conserved motifs within the SNF2 region are underlined and marked with roman numerals; amino acid substitutions at positions marked with an asterisk (*) disrupt SWI2 function *in vivo*, and substitutions at positions marked with a caret (^) do not affect SWI2 function (RICHMOND and PETERSON 1996). The position marked "[SNF2" corresponds to the N terminus of the SNF2-N motif (from the BLAST server at the National Center for Biotechnology Information).

EST clone corresponding to the adjacent CG6316 gene (Figure 5A). Since clone LD04285 and several other EST clones start only 85 bp from the *Xba*I site in the X6S1 construct that rescues the *SuUR* mutation, the *SuUR* promoter probably resides within this 85 bp sequence. Since for transcripts <4 kb, the 5′ ends of EST clones very often coincide with full-length cDNAs (RUBIN *et al.* 2000), we expect the transcription start of the *SuUR* gene to be close to the 5′ end of LD47413. Judging from the sequence, the promoter region of the *SuUR* gene has poor matches for all three common promoter motifs: TATA box, Inr, and DPE elements (ARKHIPOVA 1995; KUTACH and KADONAGA 2000). Two putative E2F-binding sites (YAMAGUCHI *et al.* 1997) occur within the first exon of the *SuUR* gene (Figure 5A).

The *SuUR* cDNA encodes a deduced protein of 962 amino acids (aa) or 107.6 kD. Statistical analysis using the SAPS program (BRENDEL *et al.* 1992) showed a modest excess of positively charged residues (KR-ED = 148 − 103 = +45). A statistically significant (*P* value 0.0079) large spacing between positively charged residues is located at position 302–363 and the next largest spacing (*P* value 0.0176) is at 904–942, indicating a degree of clustering of positively charged residues. A majority of the positively charged residues resides in the middle part of the protein where they constitute multiple nu-

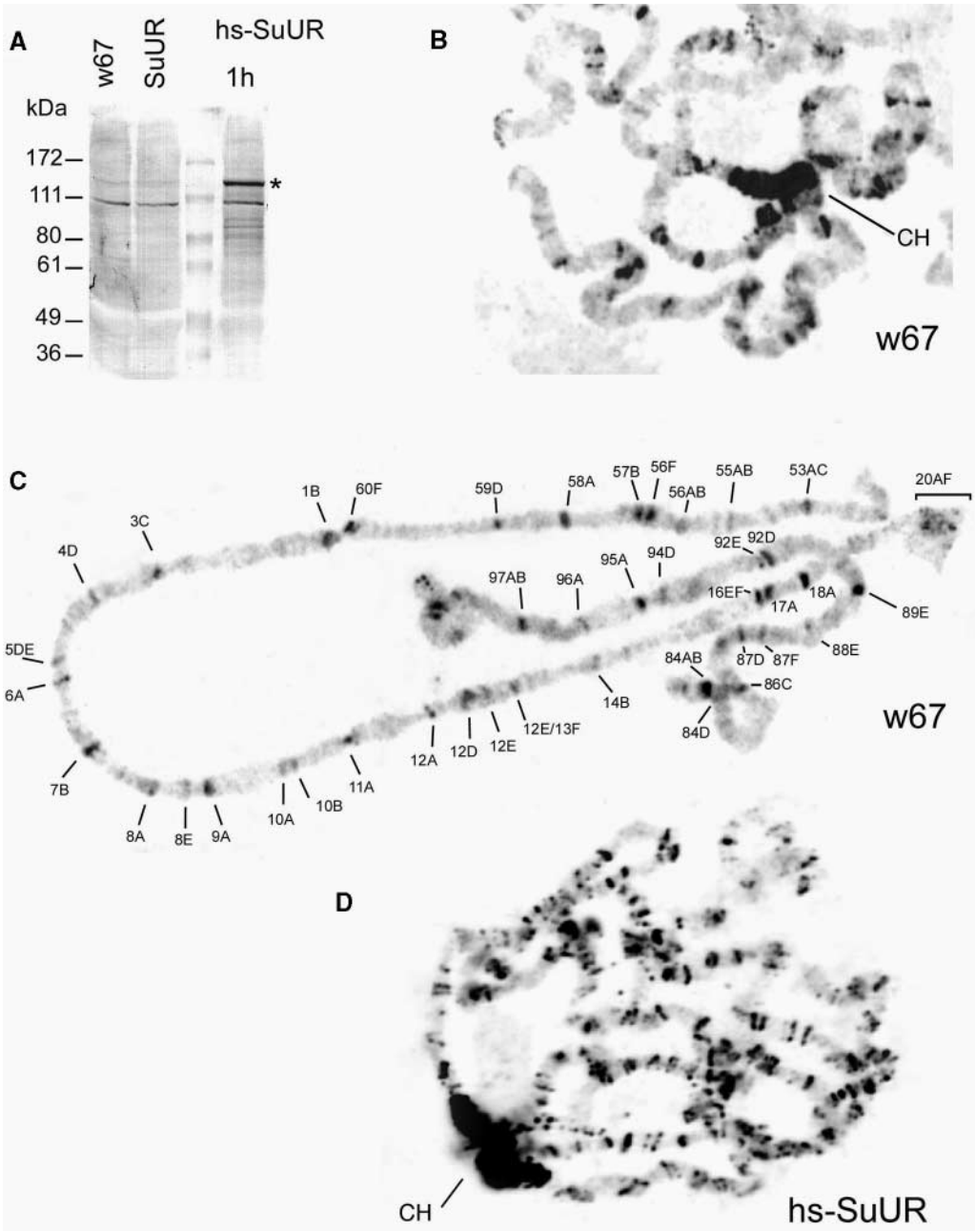


FIGURE 6.—Antibody staining of SuUR protein. (A) The antibody recognizes an additional band (*) in the *H7* stock (hs-SuUR) after heat shock. Adult H7 flies were heat-shocked for 45 min at 37° and allowed to recover for 1 or 2 hr before extraction. The endogenous SuUR band is extremely weak in the w^{67} extracts and a difference with the *SuUR* mutant is barely perceptible. The band of 110 kD visible in all extracts is due to nonspecific staining because it is not recognized by antibodies from a second rabbit. The sizes of molecular weight markers (middle lane) are shown on the left. (B and C) The anti-SuUR antibody stains the chromocenter (CH) and ~110 euchromatic sites on salivary gland polytene chromosomes of the *y, w*^{67} stock (shown in reversed contrast). The stained regions identified correspond well with late-replicating sites. (D) Overproduction of SuUR protein after heat shock of *H7* larvae results in appearance of additional strong signals (shown in reversed contrast). The chromocenter (CH) stains strongly.

clear localization signals (NLS). The SuUR protein contains 20 predicted type 1 NLSs and three (two of which are overlapping) type 2 or bipartite NLSs. Coupled with a high number of positive charges, this suggests that the SuUR protein is localized in the nucleus.

A BLAST search (ALTSCHUL *et al.* 1997) of the protein databases did not reveal proteins with significant similarity for full-length SuUR protein. However, the first 250 aa from the N terminus show a moderate similarity to the N-terminal part of the ATPase/helicase domain found in the SNF2/SWI2 family of proteins (Figure 5B). The strongest similarity corresponds to the conserved motifs I, II, III, and IV within the SNF2 domain but SuUR contains substitutions, deletions, or insertions in conserved parts of motifs I and II, which make it difficult to predict whether it would have ATP-binding and hydrolysis activity. The N-terminal region of SuUR is separated from the positively charged middle region by a cluster of negative charges. The C-terminal region has a sequence predicted to induce instability, according to GURUPRASAD *et al.* (1990).

Staining of polytene chromosomes with antibodies against SuUR protein: Polyclonal antibodies were raised against the middle part of the SuUR protein fused to GST. The antibody was affinity purified and checked for ability to recognize the SuUR protein in a Western blot of fly extracts. After heat-shock induction to overexpress SuUR, a protein band with molecular weight of >110 kD was detected in H7 stock (Figure 6A). In a Western blot prepared from *y, w*^{67} adult flies, the anti-

TABLE 3

Localization of the SuUR antibodies binding sites in polytene chromosomes

X	2L	2R	3L	3R	4
1AB	21A	41A-C	61A	81F	101DF
3C	22A	41DE	64C	83D	102D
4A	22B	42B	65A	84AB	102F
4D	23A	43A	65D	84D	
4E	24D	44CD	65E	86C	
5DE	25A	44F	65F	86D	
6A	25E	47D	67A	87B	
7B	25F-26A	48E	67D	87D	
7C	26C	50A	67F	87E	
7E	30A	50C	68E	88E	
8B	32A	53A-C	70C	89E	
8E	32F-33A	54AB	71C	92D	
9A	33CD	55AB	72E	92E	
10A	34EF	56AB	73A	94A	
10B	35C-E	56F	74A	94D	
11A	36A*	57A	75A	95A	
12A	36D	58A	75C	96A	
12D*	38C	59D	77E	97AB	
12E1-2	39DE	60F	79E	98C	
12E7-8/13A	40AF		80A-C	100A1-2	
14B				100B1-2	
16F				100B4-5	
17A				100F	
17B					
18A					
19A					
19E					
20AF					
28	19	19	20	23	3

Asterisks mark regions in which late replication was not found (Zhimulev *et al.* 1982). The total number of sites for each chromosome arm is summarized in the last row.

body detects a very weak band of the same molecular weight but the signal is barely detectable. It is possible that adult flies contain very little SuUR protein and that the high level of *SuUR* RNA present in the ovaries is not translated. Alternatively, the protein has a short half-life and is rapidly degraded.

When the affinity-purified anti-SuUR antibody was used to stain wild-type polytene chromosomes, the chromocenter stained very strongly, together with numerous sites on the euchromatic arms. About 110 euchromatic sites were identified (Figure 6, B and C; Table 3) and, with two exceptions, all coincided with regions of late replication mapped by Zhimulev *et al.* (1982). No or very weak staining was observed in the *w, SuUR* stock (data not shown). We also stained salivary gland polytene chromosomes of flies bearing the H7 transposon expressing the *SuUR* cDNA under control of the *hsp70* promoter. The H7 larvae were heat-shocked for 45 min at 37° and allowed to recover for 1–2 hr before preparation of polytene chromosomes. The SuUR overexpression resulted in the appearance of stronger and more numerous bands in the euchromatic arms (Figure 6D).

DISCUSSION

To identify the *SuUR* gene, we revised the localization of several deficiency breakpoints and genes from the 68A region. While our mapping of this region gives the most probable localization of a number of genes, this work stops short of definitive proof except for the case of the *SuUR* gene. Several arguments indicate that the *SuUR* gene corresponds to the Celera-predicted gene CG7869. The strongest evidence is the fact that the X6S1 transposon, but not transposons B13, P42, or P92, rescues the *SuUR* mutation, as does the cDNA sequence LD04285 expressed under control of the *hsp70* promoter. In addition, the *SuUR* mutant stock contains a 6-kb insertion in the predicted last exon of the gene and the RNA transcript detected by the LD04285 cDNA is absent. All these data indicate that CG7869 is the *SuUR* gene.

The rescue of the *SuUR* mutation with the X6S1 transposon shows that it contains a fully functional gene and that the upstream regulatory region of the *SuUR* gene is very small. The *SuUR* promoter has no canonical TATA box, Inr, or DPE elements, but the promoter region contains two putative binding sites for the E2F protein, an important regulator of DNA replication. The E2F/DP complex activates transcription of genes involved in DNA replication. Binding of retinoblastoma (RB) protein to E2F abolishes its activation function and represses transcription (for review see Dyson 1998). Mutations in the *Rbf* gene, the Drosophila RB homolog, cause defects in DNA replication and induce additional rounds of polytenization in a fraction of the follicle cells (Bosco *et al.* 2001). The presence of putative E2F binding sites in the *SuUR* promoter might reflect its regulation by genes controlling the cell cycle.

The N-terminal part of the SuUR protein possesses similarity to the N-terminal domain of the SWI2/SNF2 protein family. The SWI2/SNF2 polypeptides belong to a broad group of NTP-binding proteins, including RNA and DNA helicases and DNA-dependent ATPases. The SuUR protein shows a moderate level of similarity to members of different subfamilies of the SNF2-like protein family (Pazin and Kadonaga 1997), such as ERCC6, SNF2, and RAD54 (Figure 5B). These proteins share seven conserved motifs situated within a region of >400 amino acids. Mutations in all seven conserved motifs as well as in some other regions affect SNF2 function *in vivo* (Richmond and Peterson 1996). SWI2/SNF2-like polypeptides are found in a number of multiprotein complexes exhibiting a broad range of biological functions in the cell. Some of these complexes act as transcriptional activators or repressors and participate in chromatin remodeling and DNA repair (Pazin and Kadonaga 1997; Havas *et al.* 2001). No significant

homology was found between SuUR and SWI2/SNF2 proteins beyond the N-terminal domain containing the first five conserved motifs I, Ia, II, III, and IV (Figure 5B). The strongest similarity occurs within the putative nucleotide binding loop (motif I/ATPase A) and the DExH box (motif II/ATPase B) involved in ATP hydrolysis. This observed similarity with the SNF2/SWI2 proteins, although not a perfect consensus, raises the possibility that SuUR might bind and utilize ATP or GTP. Future experiments will be needed to shed light on this issue.

Despite the lack of more extensive homology, the SuUR protein might have some functional similarity with members of the SWI2/SNF2 group. The central part of the SuUR protein contains a cluster of positively charged amino acids, nuclear localization signals, and a AT-hook DNA-binding motif. The AT-hook motif is found in many CHD proteins that interact preferentially with AT-rich sequences (Stokes and Perry 1995).

In the *y, w*^{67} stock, the SuUR antibody strongly stains the chromocenter and ~110 euchromatic sites (Table 3) that correspond to the late-replicating IH sites (Zhimulev *et al.* 1982). These include IH regions that display breaks and high frequency of ectopic contacts (*e.g.*, 11A, 39E, and 89E) as well as late-replicating regions that lack these features (*e.g.*, 6A, 10A, 10B, and 56F). It is significant that increasing the dosage of the *SuUR* gene by adding X6S1 transgenes in a *SuUR*$^{+}$ background (four doses of *SuUR*$^{+}$ gene) leads to the appearance of breaks in virtually all late-replicating regions (Zhimulev *et al.* 2000). In contrast, no constrictions or breaks are observed in the *SuUR* mutant and the underreplication of IH regions is suppressed (Belyaeva *et al.* 1998; Moshkin *et al.* 2001). Most probably underreplication and induction of IH features start in early development because ectopic expression of the *hs-SuUR* transgene H7 in the embryo is sufficient to induce breaks in salivary gland polytene chromosomes in late third instar larvae (Table 2). Ectopic expression of *hs-SuUR* at later stages results in a lower frequency of breaks, probably because the appearance of SuUR protein after several endocycles have already occurred has a smaller effect on DNA polytenization and results in a lower degree of underreplication. Heat-shock induction of *hs-SuUR* in third instar larvae does not induce additional breaks (Table 2) or ectopic contacts. These results indicate that DNA underreplication depends on the quantity of SuUR protein bound to the IH regions during development and such IH features as break frequency and ability to form ectopic contacts depend on the level of DNA underreplication within the IH regions rather than on the presence of SuUR protein *per se*. Different intercalary and pericentric heterochromatin regions vary in their response to the presence and quantity of SuUR protein. This is shown by the fact that different IH regions respond with very different break frequencies. For example, the basal expression of the *hs-SuUR* transgene induces breaks at the 89E site in all chromosomes, while other regions show no breaks or breaks only at a low frequency (Table 2). Pericentric heterochromatin responds even more heterogeneously. The *SuUR* mutation suppresses underreplication only for a small fraction of pericentric heterochromatin (Belyaeva *et al.* 1998).

How could the *SuUR* gene affect DNA underreplication? We consider two mechanisms. The SuUR protein could affect DNA replication in IH regions by affecting chromatin structure or SuUR might be involved in the regulation of the endocycle. For example, shortening S phase would prevent the termination of late replication and result in underreplication. The fact that SuUR protein is associated with IH regions and that increasing doses of *SuUR* reduce the level of polytenization of these regions (A. A. Alekseyenko, personal communication) supports the chromatin-mediated model of *SuUR* action. In this case, the presence of SuUR protein at IH sites indicates that some specific feature of these sites recruits SuUR protein, which then impedes the progress of replication forks or affects the activation of late origins of replication.

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