A New Enhancer of Position-Effect Variegation in *Drosophila melanogaster* Encodes a Putative RNA Helicase That Binds Chromosomes and Is Regulated by the Cell Cycle

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ABSTRACT

In *Drosophila melanogaster*, position-effect variegation of the *white* gene has been a useful phenomenon by which to study chromosome structure and the genes that modify it. We have identified a new enhancer of variegation locus, *Dmrnahel* (*hel*). Deletion or mutation of *hel* enhances *white* variegation, and this can be reversed by a transformed copy of hel^+. In the presence of two endogenous copies, the transformed hel^+ behaves as a suppressor of variegation. *hel* is an essential gene and functions both maternally and zygotically. The HEL protein is similar to known RNA helicases, but contains an unusual variant (DECD) of the DEAD motif common to these proteins. Potential HEL homologues have been found in mammals, yeast and worms. HEL protein associates with salivary gland chromosomes and locates to nuclei of embryos and ovaries, but disappears in mitotic domains of embryos as chromosomes condense. We propose that the HEL protein promotes an open chromatin structure that favors transcription during development by regulating the spread of heterochromatin, and that HEL is regulated by, and may have a role in, the mitotic cell cycle during embryogenesis.

POSITION-EFFECT variegation (PEV) of euchromatic gene expression occurs when chromosome rearrangements place euchromatic genes next to heterochromatic regions. Variable spreading of heterochromatin into the juxtaposed euchromatic genes during development is thought to inactivate the genes, creating a clonally inherited variegated, or mottled, pattern of gene expression (EISSENBERG 1989; TARTOF *et al.* 1989; HENIKOFF 1990; REUTER and SPIERER 1992). PEV is manifested as a complete inactivation of gene expression in individual cells and is thought to occur at the level of transcription (HENIKOFF 1981; RUSHLOW *et al.* 1984; KORNHER and KAUFFMAN 1986).

The product of the *white* gene of Drosophila is required for red eye pigmentation and is sensitive to PEV. A chromosomal inversion, $In(1)w^{m4}$, places *white* next to the heterochromatic region of the *X* chromosome and causes variegation in *white* gene expression, resulting in a mosaic pattern of red eye pigmentation (SCHULTZ 1936). Deletions and mutations in a number of loci dominantly suppress or enhance $In(1)w^{m4}$ variegation. These genetic properties facilitate the identification of loci that are involved in PEV. Several genes that act as haplo-suppressors of PEV have been cloned, and most, if not all, appear to encode proteins that associate with or modify chromatin (EISSENBERG *et al.* 1990, 1992; REUTER *et al.* 1990; GARZINO *et al.* 1992; TSCHIERSCH *et al.* 1994). HP-1 is a heterochromatin-associated protein encoded by *Su(var)205* (also known as *Suvar(2)5*); *Su(var)7* (also called *Suvar(3)7*) encodes a putative zinc-finger protein and the *modulo* gene produces a DNA-binding protein. *Su(var)3-9* contains regions of similarity to both negative and positive transcription regulators. In addition, a protein phosphatase encoded by *Suvar(3)6* acts as a haplo-suppressor of PEV and has been postulated to modify structural components of heterochromatin, such as the zinc-finger protein encoded by *Su(var)7* (BAKSA *et al.* 1993). Two cloned haplo-enhancers of PEV also appear to encode chromatin-associated proteins. *E(var)3-93D* encodes a protein that contains a domain found in transcriptional regulators and that has been localized to polytene chromosomes (DORN *et al.* 1993a), and *Trithorax-like* encodes another putative zinc-finger protein that most likely is the Drosophila GAGA factor (FARKAS *et al.* 1994). The GAGA factor, initially deemed a transcription factor, has been implicated in ATP-dependent chromatin remodeling (LU *et al.* 1993; TSUKIYAMA *et al.* 1994). Recently, *modifier of mdg4*, which turns out to be identical to *E(var)3-93D*, was shown to interact with *suppressor of Hairy wing* (*su(Hw)*) and to impart directionality on the chromatin insulating ability of

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su(Hw) (GERASIMOVA *et al.* 1995). Another enhancer of PEV, *Evar1*, encodes a ubiquitin-specific protease (HENCHOZ *et al.* 1996).

Here we describe the cloning and identification of *Dmrnahel* (*hel*), encoding a protein, HEL, with extensive similarity to known ATP-dependent RNA helicases. We demonstrate that the *hel* gene is a new haplo-enhancer of PEV in Drosophila, and we further show that the HEL protein locates to the nucleus and associates with chromosomes, intimating a role for a putative RNA helicase in chromatin structure. These results suggest that the HEL protein promotes an open chromatin structure that favors transcription by preventing the spread of heterochromatin. Furthermore, we have found that the HEL protein is present in transcriptionally competent nuclei (during interphase), but disappears as chromosomes condense and enter mitosis. The remarkable disappearance of the HEL protein in mitotic domains of embryos is of particular interest in light of the finding by WARBRICK and GLOVER (1994) that a *hel* cDNA (called WM6) can suppress premature mitosis caused by loss of *wee1* and *mik1* function in the fission yeast, *Schizosaccharomyces pombe*. *wee1* and *mik1* encode inhibitory kinases of the CDC2 protein kinase, which, together with an associated cyclin, play a central role in controlling entry into mitosis.

MATERIALS AND METHODS

Genetic stocks: Deficiency stocks containing $Df(2L)cl^{h1}$ and $Df(2L)cl^{h4}$ (SZIDONYA and REUTER 1988), and the $In(1)w^{m4}$ mutant stock were provided by the Mid-America Drosophila Stock Center in Bowling Green, Ohio. The balancer chromosome in the deficiency stocks was replaced by the *CyO*, wg^{en11} balancer chromosome (KASSIS *et al.* 1992), which expresses β-galactosidase in the *wingless* pattern. EMS- and X ray-induced lethal stocks in region 25E (SZIDONYA and REUTER 1988) were provided by J. SZABAD. The following alleles from complementation groups jf25–jf30 of the SZIDONYA and REUTER lethal mutant collection were confirmed by genetic complementation to lie within $Df(2L)cl^{h4}$: *b24*, *sz15′*, *sz9*, *sz18′*, *a7*, *sz63*, *b2′*, *b7*, *a26* and *b23*. These alleles were tested as heterozygotes over $Df(2L)cl^{h4}$ for rescue of lethality by transformed *hel* since several of the mutant chromosomes contain additional lethal mutations positioned outside of $Df(2L)cl^{h4}$ *sz15′* also was tested over $Df(2L)cl^{h4}$ for modification of PEV with the $In(1)w^{m4}$ chromosome. The $hel^{sz15'}$ lesion in *hel* was recombined away from a second *sz15* lesion in the *tkv* gene (see below) and recombinant $hel^{sz15'}$ recombinants were balanced with the *CyO*,wg^{en11} balancer chromosome.

Molecular cloning and localization of the *hel* gene: A cDNA clone encoding *nuclear lamin* (GRUENBAUM *et al.* 1988) was provided by P. FISHER and used to screen a *dp cn bw* genomic DNA library in the cosmid vector pWE16 (R. PADGETT and W. GELBART, unpublished results). Two cosmid clones, cosmids 4 and 8, were isolated and used to screen a 4- to 8-hr embryonic cDNA library (BROWN and KAFATOS 1988). A 1.6-kb cDNA encoding the *hel* gene was recovered.

The *hel* cDNA was used to probe genomic Southern blots containing DNA from wild-type flies and from flies heterozygous for deficiencies $Df(2L)cl^{h1}$, $Df(2L)cl^{h2}$, and $Df(2L)cl^{h4}$ to locate the *hel* gene by dosage comparison.

Sequencing and sequence analysis: Genomic and cDNA clones of *D. hel* were sequenced using Sequenase (United States Biochemical) as described by DEL SAL *et al.* (1989). Similarities to DEAD box family members, the rat homologue, and recently, the human and pig homologues were found by searching protein databases at NCBI using the BLASTP program (ALTSCHUL *et al.* 1990). D. MILLER discovered the homology between HEL and the yeast *Saccharomyces cerevisiae* protein, HER1, during a homology search of GenBank. He also pointed us toward a possible homologue in the worm, *Caenorhabditis elegans*. A partial sequence of the worm homologue was available as an expressed sequence tag under GenBank accession no. Z14797. The corresponding *C. elegans* cDNA, cm5d4 (WATERSTON *et al.* 1992), was provided by R. WATERSTON, and sequencing of the cm5d4 cDNA was completed for this study. Comparisons of homologues were done using the GCG Sequence Analysis Software Package (GENETICS COMPUTER GROUP 1991). The Drosophila and complete *C. elegans* sequences can be found under GenBank accession nos. L06018 and U08102, respectively.

The $hel^{sz15'}$ mutation was cloned from homozygous $hel^{sz15'}$ mutant pupae (identified as animals that failed to differentiate abdominal structures) by PCR. Three *hel* clones were sequenced as described above or according to the Sequenase manual provided by United States Biochemical.

***P* element-mediated transformation:** The *hel* gene is included in a 4.5-kb *Eag*I/*Bgl*II genomic DNA fragment. This fragment first was cloned from cosmid 8 into the *Not*I/*Bam*HI sites of pCaSpeR2 (THUMMEL *et al.* 1988) for *P*-element transformation of Drosophila. The transformation construct contains ~700 bp of genomic DNA 5′ to the putative translation initiation site of the HEL protein, and ~1300 bp 3′ to the putative translation termination site. Flies were transformed by injection of *y w* embryos containing the endogenously expressed Δ2-3 transposase (ROBERTSON *et al.* 1988) and recovered as described (SPRADLING 1986). One of three transformant lines contained a second chromosome insertion of the pCaSpeR2 construct suitable for studying effects of PEV in concert with the $In(1)w^{m4}$ chromosome. The pCaSpeR2 vector expresses low levels of *white* from a *miniwhite* gene construct (w^{+mC}) as a transformation marker, and produces an orange background throughout the eye in this transformant line. Visual observation of PEV was possible with the transformant line containing pCaSpeR2, but because the orange background made spectrophotometric quantitation of PEV difficult, a second transformation construct was made using the Y.E.S. vector (PATTON *et al.* 1992), which expresses the *yellow* body pigment gene (y^+) as a transformation marker. For this, the 4.5-kb *Eag*I/*Bgl*II genomic DNA fragment was removed from pCaSpeR2 as an *Eag*I/*Xba*I fragment and cloned into the *Not*I/*Xba*I sites of the Y.E.S. vector to obtain transformants without background eye color from the transformation vector. One transformant line containing the Y.E.S. construct was used to measure PEV effects of *hel* on *white* gene expression from $In(1)w^{m4}$. Similar visual results were obtained using the pCaSpeR2 transformation vector, expressing low levels of w^{+mC} as a transformation marker and yielding orange eyes (not shown), as were obtained with the Y.E.S. vector, which expresses the y^+ body pigmentation gene as a marker (shown in Figure 3).

Genetic crosses and quantitation of PEV: Transformants containing the Y.E.S. construct (see above) were used to quantitate effects of *hel* on PEV. To assay the gene dosage effect of $Df(2L)cl^{h4}$ on PEV, we used spectrophotometric quantitation of the eye pigments in female offspring from reciprocal crosses of *y* $In(1)w^{m4}$; $Df(2L)cl^{h4}$/*CyO* with *y w*; *P*{*hel*, y^+}//+. To test the effect of $hel^{sz15'}$ on PEV we assayed the eye pigment levels in the female offspring of $In(1)w^{m4}$; *l(2)sz15*/*CyO* males crossed with *y w*; *P*{*hel*, y^+}//+ females. Mean OD_{485nm} readings

for four ($hel^{sz15'}$) or five ($Df(2L)cl^{h4}$) separate eye pigment extractions per genotype were determined. For each extraction, heads of 12 flies aged 5–7 days were homogenized in 200 μl chloroform and 200 μl 0.1% ammonium hydroxide and centrifuged for 2 min, and 100 μl of the aqueous phase was used for spectrophotometry without dilution (ASHBURNER 1989). The extracts were normalized relative to the two dose value in each of the four or five trials after subtracting the basal reading (from *y w* heads).

Generation of mutant *hel* germline clones: Homozygous mutant *hel* clones were generated in the germline using the autosomal FLP recombinase-dominant female sterile technique (CHOU and PERRIMON 1996). This technique employs the yeast *FLP* recombinase, which induces recombination at homozygous *FRT* sites, and the ovo^{D1} dominant female sterile mutation for selection of clones. Because the original *hel* mutant chromosome, *sz15*, also carries a mutation in the closely linked *thick veins* (*tkv*) gene, we used three independent $hel^{sz15'}$ recombinants, which remove the tkv^{sz15} mutation, to put onto the FRT^{40A} chromosome. To generate clones, females of genotype $hel^{sz15'}$ *P{neo FRT40A}/CyO* were crossed to males of genotype *y w* FLP^{12}*/Y;* $P\{w^{+mC}$ $ovo^{D1}\}$ $P\{neo$ $FRT^{40A}\}$*/CyO*. Progeny were heat shocked for 1 hr at 37° during the third larval instar, and females of genotype *y w* FLP^{12}*/+;* $hel^{sz15'}$ $P\{neo$ $FRT^{40A}\}$*/* $P\{w^{+mC}$ $ovo^{D1}\}$ $P\{neo$ $FRT^{40A}\}$ were tested for the presence of germline clones.

Antibody generation, tissue immunodetection and Western blot analysis: To produce HEL protein in bacteria, a 1.4-kb restriction fragment of the *hel* cDNA extending from a *Spe*I site (bp 429–434 in Figure 2A) to a *Not*I site in the pNB40 vector was subcloned into Bluescript SK^+ (Stratagene). This fragment was then excised with *Sma*I and *Not*I and subcloned into the glutathione-S-transferase (GST) expression vector pGEX-5X-1 (Pharmacia). Fusion protein was purified from *Escherichia coli* lysates by affinity chromatography to glutathione-Sepharose-4B and elution with 10 mM glutathione. The 76-kD fusion protein, containing GST, 18 novel amino acids (nine plasmid-encoded, nine from the *hel* 5′ UTR) and the entire HEL polypeptide was used to immunize a rabbit. Injections were 100 mg of purified fusion protein in Freund's incomplete adjuvant at biweekly intervals.

For enzymatic immunodetection of HEL, larval salivary glands were dissected and gently squashed in 50% acetic acid, 3.7% formaldehyde over a period of ~2–5 min. Embryos were fixed in 50% acetic acid, 3.7% formaldehyde:heptane (1:1) for 5 min and devitellinized by shaking embryos in heptane with methanol (1:2); ovaries were fixed for 5 min in 50% acetic acid, 3.7% formaldehyde. All antibody incubations and washes were done in phosphate-buffered saline (PBS) with 0.1% Tween-20. A 1:50 or 1:100 anti-HEL antibody dilution gave optimal results. Salivary glands were incubated with anti-HEL (or with preimmune serum for control experiments) for 1 hr at room temperature; embryos and ovaries were incubated for 15–30 min (embryos) for 1 hr (ovaries) at room temperature and then overnight at 4°. Biotinylated secondary antibody (anti-rabbit IgG, Vector Laboratories, Inc.) was diluted to 1:200 and incubated with all tissues for 2 hr at room temperature. Secondary antibodies were detected using an avidin/biotinylated-HRP reagent system (Vectastain Elite, Vector Laboratories, Inc.) with DAB and NiCl for color development.

For fluorescent images, embryos were fixed as described for enzymatic immunodetection (except it was necessary to omit acetic acid to see microtubule staining with anti-tubulin; HEL detection was unaffected by the omission of acetic acid), labeled with anti-HEL and/or an anti-tubulin monoclonal antibody (Boehringer Mannheim, 1:50 dilution) and detected with fluorescein-conjugated and Texas Red-conjugated secondary antibodies (Vector Laboratories, Inc.; 1:250 dilutions).

Conventional color and fluorescent images were photographed with a Zeiss Axiophot fluorescent microscope. Resulting photographic slides were computer-scanned and processed using Adobe Photoshop software and assembled for printing with Deneba Canvas software. Confocal imaging was done with a BioRad MRC-600 Laser Scanning System, enhanced with Adobe Photoshop and assembled using Deneba Canvas.

For Western blot analyses, total protein from 0–4-hr embryos was recovered by homogenization in PBS containing 1 mM PMSF and 0.1% SDS. Proteins were separated on 10% polyacrylamide SDS gels and electroblotted to BioTrans nitrocellulose membranes for immunodetection. Membranes were blocked with 5% nonfat dry milk in PBS with 0.1% Tween-20 for 15 min at room temperature before incubation with anti-HEL antibody (or preimmune serum) at a 1:100 dilution for 15 min at room temperature and then 4° overnight. Membranes were reblocked and antibody detection was accomplished as described for tissue analyses (see above) except secondary antibody incubations were for 1 hr. Developed blots were computer-scanned using Adobe Photoshop and labeled for printing with Deneba Canvas.

RESULTS

Molecular cloning of the *hel* gene: Genomic and cDNA clones encoding *hel* were isolated as part of our efforts to characterize RNA transcripts in the 25E-F region of the second chromosome of Drosophila. A chromosomal walk from the *nuclear lamin* locus at 25F1 (GRUENBAUM *et al.* 1988; see Figure 1) was initiated using a *nuclear lamin* cDNA to screen a *dp cn bw* genomic DNA library in the cosmid vector pWE16 (R. PADGETT and W. GELBART, unpublished results). Two cosmid clones, cosmids 4 and 8, ~35 kb in length and overlapping by ~17 kb (data not shown), were isolated. A 1.6-kb cDNA was recovered from a 4- to 8-hr Drosophila embryonic cDNA library (BROWN and KAFATOS 1988), using both cosmids 4 and 8 as probes. Sequencing of the cDNA clone and of genomic DNA encompassing the cDNA indicated that a mature mRNA transcript is created from the splicing together of eight exons (Figure 2, A and B). The cDNA hybridized to two mRNAs of ~1.6 and 1.8 kb upon Northern blot analysis of polyadenylated RNA from various stages of embryonic development (data not shown), indicating that the recovered cDNA is full-length or nearly full-length.

An apparent RNA helicase with an unusual DEAD box (DECD): A long open reading frame can be found beginning within the second exon of the transcript encoded by the 1.6-kb cDNA. Encoded within the open reading frame is a protein of 425 amino acids (Figure 2A) with extensive similarity to RNA helicases. The protein, HEL, is similar to the murine translation initiation factor eIF4A and other DEAD box-containing RNA helicases in eight regions conserved between members of this family (Figure 2C; SCHMID and LINDER 1992). However, HEL contains a cysteine in place of the more common alanine in the third position of the DEAD box

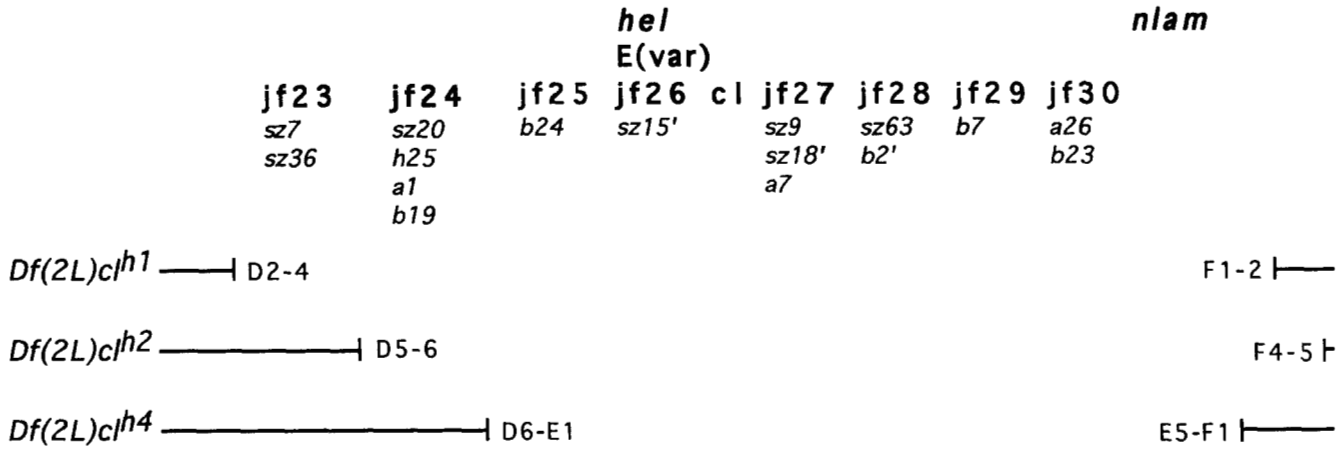

FIGURE 1.—Genetic organization of the 25D-F region that contains the *hel* gene. Complementation groups are printed in bold and mutant alleles of each group are listed in italic. Complementation mapping of the jf series (SZIDONYA and REUTER 1988) with deficiencies (*Df*) that encompass the *hel* gene was confirmed. Placement of nuclear lamin (*nlam*) was based on molecular analyses (data not shown). The relative order of complementation groups within $Df(2L)cl^{h4}$ is undetermined.

(DECD rather than DEAD). At least one other helicase-like protein, the plum pox virus C1 protein, has cysteine in this position (LAIN *et al.* 1989), but it also contains a histidine in place of the second aspartic acid (DE**CH**), and may fall into the DEAH family of helicases (BORK and KOONIN 1993). At least four members of the DEAD box family, eIF4A, p68, An3 and VASA, and the viral C1 protein have been shown to possess RNA helicase activity (HIRLING *et al. 1989;* ROZEN *et al.* 1990; LAIN *et al.* 1990; GURURAJAN *et al.* 1994; LIANG *et al.* 1994). Other members are assumed to unwind RNA on the basis of sequence comparison to the proven helicases.

HEL homologues in other organisms: The amino acid sequence of the HEL protein was used to search databases for homologues in other organisms. A possible functional homologue of HEL (based on sequence similarity) from the rat was found (p47, 82% amino acid identity; NAIR *et al.* 1992). After submitting the HEL sequence to Genbank, a second putative homologue from the budding yeast, *S. cerevisiae,* surfaced (HER1, 63% identity; D. MILLER, personal communication). In addition, partial sequence of a cDNA recovered from the nematode worm, *C. elegans* (Cernahel; WATERSTON *et al.* 1992), suggested the existence of another homologue of HEL. We completed the sequencing of the *C. elegans* cDNA (see MATERIALS AND METHODS), revealing a high degree of similarity between the encoded protein and HEL (79% identity). A comparison of the four protein sequences is shown in Figure 2D. A recent BLAST search for homologues of HEL has identified additional apparent homologues in humans and pigs (BAT1, 83% identity; PEELMAN *et al.* 1995).

***hel* modifies PEV:** Previously, SZIDONYA and REUTER (1988) described two loci in region 25F-26B of the second chromosome that behave as haplo-enhancers and one triplo-suppressor of $In(1)w^{m4}$ variegation in the presence of a strong suppressor of PEV, $Su\text{-}var(2)1^{01}$. We found that two deficiencies that delete region 25E, $Df(2L)cl^{h4}$ and $Df(2L)cl^{h1}$, also act as haplo-enhancers of variegation from $In(1)w^{m4}$ in the absence of any suppressors (data not shown, see Figure 1). Southern blot analyses revealed that the *hel* gene is removed by the two deficiencies (data not shown), so we tested whether *hel* could influence PEV.

Flies were transformed with genomic DNA encoding *hel* by *P* element-mediated transformation. Transformation constructs contained ~700 bp of genomic DNA 5′ to the putative translation initiation site of the HEL protein, and ~1300 bp 3′ to the putative translation termination site. Crosses were made to see whether transformed copies of *hel* could rescue reduced *white* gene expression from $In(1)w^{m4}$ caused by $Df(2L)cl^{h4}$ enhancement of PEV. Transformed *hel* was capable of restoring *white* expression to unmodified $In(1)w^{m4}$ levels in the presence of $Df(2L)cl^{h4}$ (Figure 3; cross A in Table 1). The eye pigment level in single dose flies was significantly lower than in flies with two *hel* gene doses (paired *t*-test; $P < 0.001$). The enhancement of variegation was rescued by a transformed copy of *hel* to a level not significantly different from that of two endogenous copies. Therefore, *hel* encodes a gene that, when deleted, causes enhancement of PEV. We have identified a mutation in *hel* (see next section) that behaves in a similar way as the deficiency. In addition, transformed *hel* mediates an increase in eye pigment level over that of two endogenous copies ($P < 0.05$), suggesting that *hel* also behaves as a triplo-suppressor of PEV (Table 1).

***hel* is an essential gene:** In addition to the haplo-enhancer of variegation function we describe here, at least five homozygous lethal complementation groups are uncovered by $Df(2L)cl^{h4}$ (SZIDONYA and REUTER 1988; see Figure 1). We have found that *P*-element transformation constructs containing a genomic copy of the *hel* gene fully rescue lethality caused by a single homozygous recessive mutation, *sz15′*, indicating that the *sz15′* mutation resides in the *hel* gene and that *hel*

is an essential gene. We also have determined that the $hel^{sz15'}$ mutation acts as a haplo-enhancer of $In(1)w^{m4}$ variegation in $hel^{sz15'}/+$ heterozygotes (cross B in Table 1; $P < 0.05$), indicating a loss-of-function phenotype similar to that seen with $Df(2L)cl^{h4}$. Furthermore, the enhancement of variegation caused by one copy of the $hel^{sz15'}$ mutation is reversed by a transformed copy of *hel* (Table 1). In cross B, we again saw the triplo-suppressor effect of *hel* on PEV (Table 1; $P < 0.01$). Thus, heterozygosity of *hel* (in $hel^{sz15'}/+$ animals) results in enhancement of PEV, while homozygosity of *hel* (in $hel^{sz15'}/Df(2L)cl^{h4}$ animals) is lethal. Lethality caused by a loss of *hel* occurs during embryonic, larval and pupal stages of development. This variability is most likely related to the strong maternal expression of *hel* (next section). It is also possible that the $hel^{sz15'}$ mutation is not a complete null mutation since $hel^{sz15'}$ may enhance PEV to a lesser extent than does $Df(2L)cl^{h4}$ (Table 1; we recommend caution in comparing the results between these crosses done at different times). This could alternatively be explained if more than one enhancer of PEV lies within $Df(2L)cl^{h4}$. A small percentage of mutant embryos show signs of morphological defects such as incomplete involution of the head and gut. $hel^{sz15'}$ homozygotes surviving to the pupal stage develop small heads and appendages, and thoraces that often are truncated posteriorly, but most or all abdominal structures fail to differentiate. These characteristics were used to identify $hel^{sz15'}/hel^{sz15'}$ homozygous pupae for PCR cloning of the mutant *hel* gene. Upon DNA sequencing, a glutamic acid to lysine substitution was identified at position 245 of the mutant HEL protein.

Effects on PEV by *hel* include a maternal component: The influence of *hel* on $In(1)w^{m4}$ variegation includes a maternal effect. Male offspring of $In(1)w^{m4}$; $Df(2L)cl^{h4}/+$ females (with one dose of *hel*) express little *white*, whether or not they receive the $Df(2L)cl^{h4}$ chromosome (data not shown), and the female progeny express less *white* than female progeny of $In(1)w^{m4}$ females (with both doses of *hel*). Different overall levels of eye pigments in female offspring from the two reciprocal crosses shown in Table 1 (cross A) illustrate the maternal effect associated with *hel*. *HEL* mRNA is supplied to embryos maternally and is present throughout embryogenesis (data not shown). These results suggest that maternal HEL protein, in addition to zygotically expressed HEL, promotes transcriptional competence during development.

***hel* is required during oogenesis:** Homozygous mutant *hel* clones were generated in female germlines using the autosomal FLP recombinase-dominant female sterile technique (CHOU and PERRIMON 1996) and the *sz15'* mutation in *hel* (see MATERIALS AND METHODS). Females with $hel^{sz15'}/hel^{sz15'}$ germlines lay very few eggs, and the eggs that are laid are small, often collapsed and sometimes have fused dorsal appendages. No obvious defect before late oogenesis was detected upon examination of dissected ovaries with germline clones. Egg chambers develop to late stages before showing signs of degeneration and resorption. Similar results were obtained with three independent $hel^{sz15'}$ recombinants. These results indicate that wild-type *hel* activity is required during oogenesis and are consistent with the maternal effect on PEV.

The HEL protein is nuclear and associates with chromosomes: HEL protein was expressed in bacteria and used to generate a rabbit polyclonal antibody against HEL. The polyclonal anti-HEL antiserum was used on Western blots of total protein from 0- to 4-hr-old embryos (Figure 4). A strongly reacting, major band of the expected molecular weight of the endogenous HEL protein (~47 kD) was seen with immune serum, but not with preimmune serum. A few weakly reacting, minor bands were seen with both immune and preimmune sera.

The antibody then was used to determine the cellular location of endogenous HEL protein in embryos, larval salivary glands and adult ovaries (Figure 5). In all three cases HEL protein was found in the nucleus in cells that express it. In no case was nuclear staining detected when preimmune serum was substituted for antibody-containing serum. A relatively low cytoplasmic background color reaction occurred with both preimmune and immune sera.

HEL protein clearly is found in association with chromosomes in salivary glands, although it sometimes appears to be located around the chromosomes as well (Figure 5A). It appears to be associated with all regions of the chromosomes; there is no evidence that HEL binds only at particular loci. Strong nuclear staining is apparent in most cells on the outer, largely epidermal, surface of embryos (Figure 5B), but notable areas of much lower staining intensity, corresponding to mitotic domains described by FOE (1989) and by FOE *et al. (1993)*, can be seen in early-mid embryonic stages when mitotic cell cycles become asynchronous in the Drosophila embryo (Figure 6). HEL also is expressed in ovaries where heavy staining is seen in large, polyploid nurse cell nuclei and in smaller follicle cell nuclei as the follicle cells migrate between the nurse cells and across each egg chamber (Figure 5C).

Nuclear expression of HEL protein disappears during mitosis: Because we observe regionally reduced HEL staining in the mitotic domains of asynchronously dividing embryos (described by FOE 1989 and FOE *et al.* 1993), we examined in more detail the relationship between HEL and the mitotic cell cycle. To visualize areas of mitosis in developing embryos more clearly, we used confocal microscopy on embryos double-stained with the HEL antibody and an antibody against β-tubulin to detect disintegration of cytoplasmic microtubule networks and subsequent formation of nuclear mitotic spindles (see FOE 1989 and FOE *et al.* 1993). In individual and composite images generated from the

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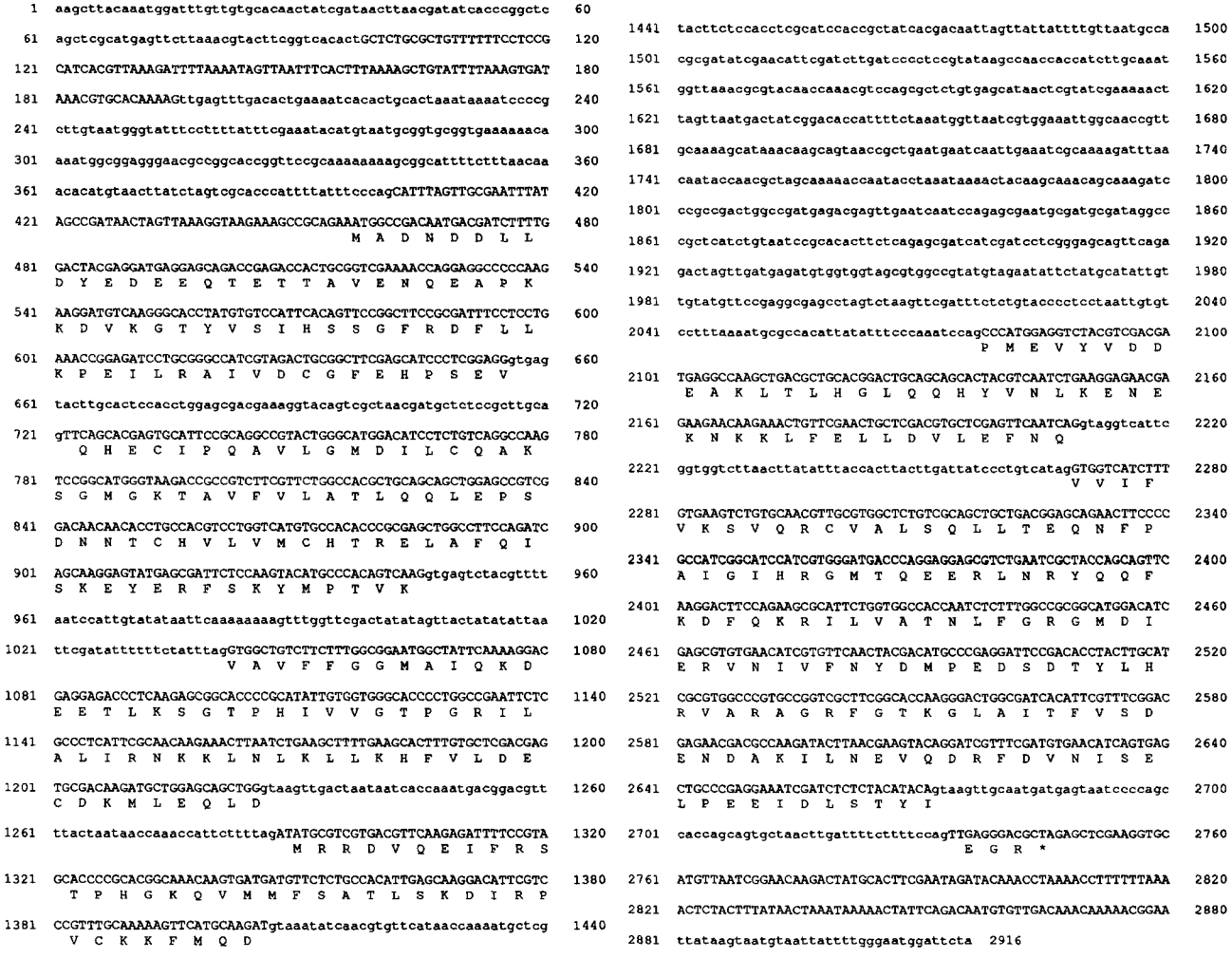

FIGURE 2.—The *hel* gene encodes a protein with similarity to RNA helicases. (A) DNA sequence and deduced protein sequence of the Drosophila *hel* gene (GenBank accession number L06018). DNA sequence found in both the cDNA and genomic clones (representing exons) is in uppercase, sequence found only in genomic clones (representing introns) is in lowercase. The deduced protein sequence is shown below the DNA sequence. (B) Schematic of the exon/intron structure of the *hel* gene. Horizontal lines represent exons, the splicing out of introns is illustrated by v-shaped lines connecting the exons, and protein-encoding regions are indicated as boxes. Positions of motifs commonly found in RNA helicases are shown in shaded and hatched boxes within exons. Alternative nomenclatures for ATPase A and B domains and domains I–VI (WALKER *et al.* 1982; HODGMAN 1988) are shown. (C) Comparison of conserved regions in the DEAD family (modeled after PAUSE and SONENBERG 1992; PAUSE *et al.* 1993) and a proposed DECD subfamily of putative RNA helicases based on HEL and apparent homologues. The correspondence among conserved amino acids within the ATPase A and B domains/domains I–VI is shown. (D) Similarity among the Drosophila HEL protein and worm (*C. elegans*), yeast (*S. cerevisiae*) and rat homologues. Divergent regions are shaded. The Drosophila protein is 79% identical to the *C. elegans* protein (Cernahel; WATERSTON 1992 and this study; GenBank accession number U08102), 63% identical to the yeast protein (HER1; D. MILLER, personal communication; GenBank accession no. Z74132; ORF YDL084W) and 82% identical to the rat protein (p47; NAIR *et al.* 1992; GenBank accession no. M75168).

anterior (head) portion of an embryo in early mitotic cycle 14 (Figure 6), one can see, by comparing β-tubulin patterns with those of HEL, that HEL disappears within mitotic domains. Although cells within a mitotic domain traverse mitosis somewhat synchronously, a closer look at each domain reveals that different cells within a domain can be at various stages of mitosis. Cells near the center of a domain often are among the first to enter mitosis and then mitosis often spreads toward the periphery of a domain (FOE *et al.* 1993; Figure 6), though a slightly scattered pattern can be seen upon careful inspection. Various states of microtubule breakdown and spindle formation are apparent (Figure 6). Triple staining for HEL, β-tubulin and DAPI (data not shown) has allowed us to interpret the mitotic stages of these structures under our experimental conditions. In preparation for mitosis, depolymerization of cytoplasmic microtubules can be identified by loss of β-tubulin immunoreactivity in the cytoplasm. This occurs before detectable formation of mitotic spindles within nuclei and is most evident at the periphery of mitotic domains in Figure 6, in cells that in general are the last

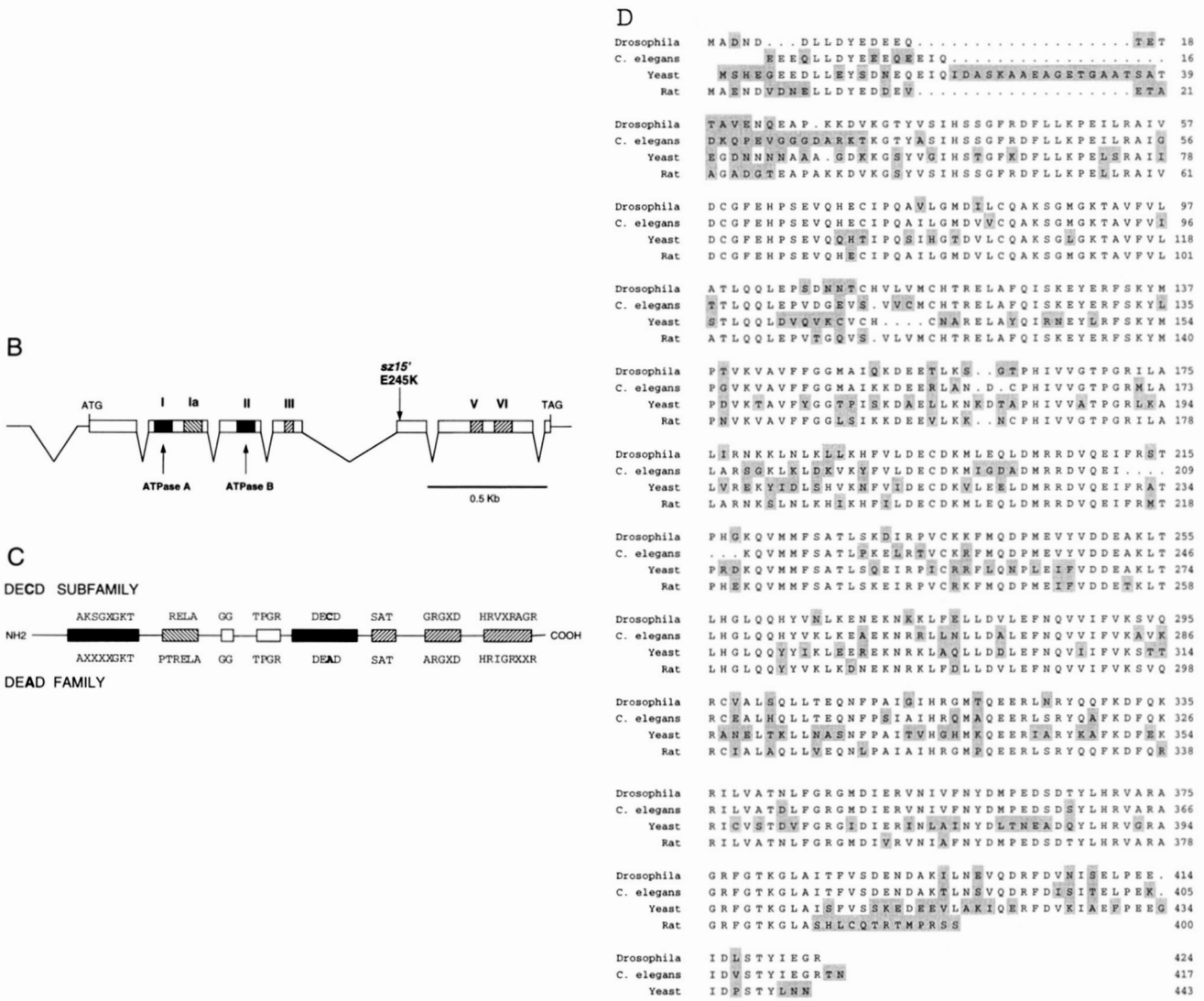

FIGURE 2.—*Continued*

to enter mitosis, and thus are at relatively early mitotic stages. Robust HEL protein expression is seen in nuclei as these cells enter mitosis. Moving slightly more interiorly into the domain (Figure 6), HEL still is detectable as spindles begin to form, although at reduced intensity. As the spindle continues to form, condensing chromosomes move toward the metaphase plate, and HEL seems to follow this movement as it fades. By the time the chromosomes gather on the metaphase plate, HEL immunoreactivity is extinguished. HEL remains absent during anaphase, when β-tubulin detection reveals expanded spindles with characteristic gapping at the equator, and in telophase, as evidenced by lingering β-tubulin staining of what appears to be the slowly disintegrating spindle overlap microtubules between parting cells (described by FOE *et al.* 1993 and also seen in recent data of SIGRIST *et al.* 1995). HEL immunoreactivity resumes in interphase as cytoplasmic microtubules reform (data not shown). We note that HEL protein disappears in all mitotic domains described by FOE (1989) that we have thus far been able to identify.

The HEL protein appears to be present throughout interphase in embryos. HEL must be present in G2 since it is evident in nuclei as cells prepare for mitosis at an embryonic stage that includes a G2 phase (preceding M), and because HEL protein persists in nondividing cells that later become the amnioserosa and that arrest in G2 (see FOE *et al.* 1993; data not shown). HEL also is found in germ cells as they become mitotically quiescent and migrate into the embryo and in nuclei throughout the outer surface of embryos (data not shown) when most of these cells have stopped dividing and have settled in G1. HEL protein accumulates soon after mitosis (when cytoplasmic microtubule networks begin to reassemble) in the epidermis of embryos completing cycle 14, before acquisition of a G1 phase (see FOE *et al.* 1993), and gaps in HEL immunoreactivity in the epidermis of embryos other than in the mitotic domains do not arise, suggesting that HEL also is present during DNA synthesis or S phase. Polytene salivary gland cells, which alternate between G and S cycles, contain HEL protein, although we do not yet know

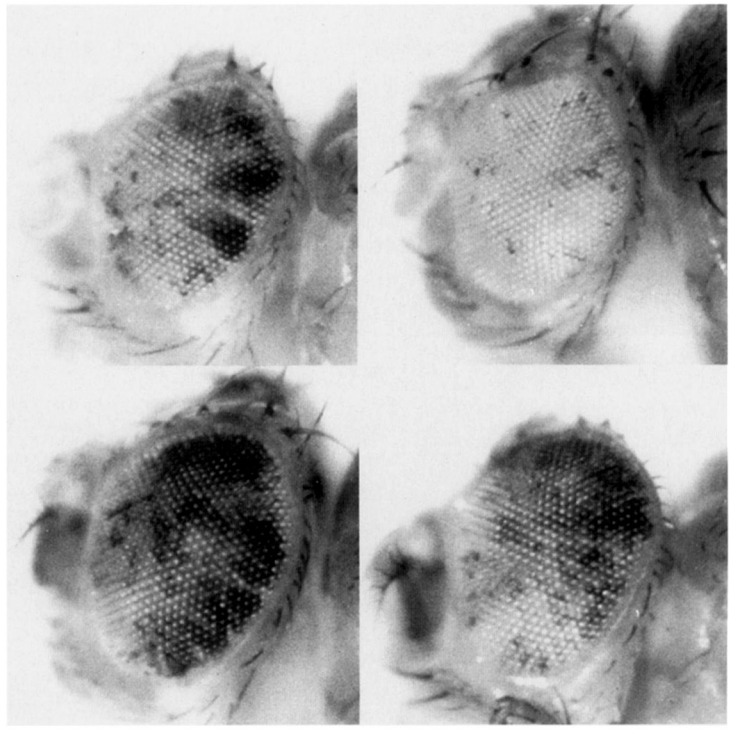

FIGURE 3.—*hel* is a modifier of PEV. Transformation with *hel* rescues the $Df(2L)cl^{h4}$-induced enhancement of *white* variegation. Shown are eyes of selected flies with identical *X* chromosome genotypes ($y\ In(1)w^{m4}/y\ w$) but different *hel* gene dosages. $In(1)w^{m4}$ induces variegation of the *white* gene in the presence of the two endogenous copies of *hel* ($+/CyO$) but this variegation is enhanced if one copy of *hel* is deleted ($+/Df(2L)cl^{h4}$). Addition of a transformed copy of *hel* ($P\{hel\ y^+\}/Df(2L)cl^{h4}$) restores the variegation to the original level. Three copies of *hel* ($P\{hel\ y^+\}/CyO$) moderately suppress the variegation. See Table 1 for quantitation of the eye pigments in these genotypes.

whether it is during the G or the S phase (or both) that HEL associates with the chromosomes in these cells. Taken together, our results suggest that HEL protein is expressed throughout G1, S and G2 phases of the cell cycle in Drosophila.

DISCUSSION

We have identified a new gene of Drosophila, *hel*, that encodes a protein that is similar to members of a family of ATP-dependent RNA helicases. We have shown that *hel* is both an enhancer of PEV and an essential gene. The maternal contribution of *hel* also influences PEV, and examination of mutant *hel* germline clones indicates that *hel* is required for oogenesis. An antibody generated against bacterially expressed HEL protein recognizes a protein of expected size on Western blots, and reveals that HEL is expressed in nuclei and associates with salivary gland chromosomes. The HEL protein disappears in mitotic domains of embryos, indicating that HEL is regulated by the cell cycle and opening the intriguing question of whether HEL is involved in cell-cycle control during development.

HEL shares several motifs with known RNA helicases: Several of the regions conserved between HEL and members of the DEAD box-containing RNA helicase family (see Figure 2C) have been implicated in particular molecular functions (refer to LINDER *et al.* 1989; PAUSE and SONENBERG 1992; PAUSE *et al.* 1993). The ATPase A/I domain (AXXXXGKT) is required for ATP binding, and the ATPase B/II motif (DEAD) is involved in ATP hydrolysis and the coupling of ATPase and helicase activities. Motif III (SAT) has been implicated in RNA unwinding, and region VI (HRIGRXXR) is required for RNA binding and ATP hydrolysis. A mutagenesis study of the VASA protein of Drosophila has revealed that a glycine to glutamic acid change in the ARGXD domain abrogates the ability of this DEAD box-containing helicase to unwind RNA (LIANG *et al.* 1994). DORN *et al.* (1993b) have reported the cloning of the translation initiation factor eIF4A of Drosophila. The fly eIF4A protein is 73% identical to the mouse translation factor. HEL is ~35% identical to both mouse and fly eIF4A proteins.

HEL is likely to function as an ATPase: DNA encoding the potential homologue of HEL in the rat (p47) was obtained through screening of an expression library with antibodies against an ATPase (NAIR *et al.* 1992). ATP binding and hydrolysis domains (see LINDER *et al.* 1989; PAUSE and SONENBERG 1992) of all of the putative HEL homologues (including HEL itself) are conserved with members of the DEAD box family of ATP-dependent RNA helicases. One notable exception is the DECD variation itself, which is found in all HEL homologues. We suspect that this substitution would not preclude ATPase activity (see below).

Does HEL unwind RNA? Although variegation is thought to act through a change in chromatin structure, we consider the possibility that HEL may act on RNA, rather than DNA, because of its similarity to RNA

TABLE 1

Effect of *hel* gene dosage on variegation of *In(1)w^{m4}*

Cross	Genotype[a]	Gene dose: Endogenous	Gene dose: Transformed	*Df(2L)cl^{h4}* fathers[b]	*Df(2L)cl^{h4}* mothers[c]	Normalized[d]
A	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{+}{CyO}$	2	0	0.191 ± 0.0157	0.0855 ± 0.0078	1.000 ± 0
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{+}{Df(2L)cl^{h4}}$	1	0	0.095 ± 0.0055	0.0424 ± 0.0030	0.408 ± 0.0349***
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{P\{hel\ y^+\}}{Df(2L)cl^{h4}}$	1	1	0.167 ± 0.0129	0.0723 ± 0.0043	0.856 ± 0.0627
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{P\{hel\ y^+\}}{CyO}$	2	1	0.229 ± 0.0159	0.0997 ± 0.0075	1.270 ± 0.1116*
B	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{+}{CyO}$	2	0	0.206 ± 0.0111		1.000 ± 0
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{+}{l(2)sz15}$	1	0	0.169 ± 0.0197		0.805 ± 0.0729*
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{P\{hel\ y^+\}}{l(2)sz15}$	1	1	0.201 ± 0.0094		0.976 ± 0.0125
	$\frac{y\ In(1)w^{m4}}{y\ w}; \frac{P\{hel\ y^+\}}{CyO}$	2	1	0.256 ± 0.0224		1.246 ± 0.0432**

Female offspring[a] from reciprocal crosses[b,c] of *y In(1)w^{m4}; Df(2L)cl^{h4}/CyO* with *y w; P{hel, y$^+$}/+* in cross A, and from *y In(1)w^{m4}; l(2)sz15/CyO* males with *y w; P{hel, y$^+$}/+* females in cross B. The extracts were normalized[d] relative to the value for two endogenous gene doses in each of the five trials (four trials for cross B) after subtracting the basal reading (from *y w* heads); the normalized values for the two reciprocal crosses in cross A were then pooled for statistics. Statistical significance of the normalized values relative to the two dose values are indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

helicases, including only conservative changes in the HRIGRXXR block, which is indicative of RNA helicase activity and which is required for RNA binding (PAUSE *et al.* 1993). A second motif, SAT, which is critical for RNA unwinding (PAUSE and SONENBERG 1992), also is conserved in HEL. In addition, a highly conserved glycine in the RGXD domain, shown to be essential for RNA unwinding activity of the VASA protein (LIANG *et al.* 1994), is conserved in HEL.

The DEAD box domain has been implicated in ATP hydrolysis and in coupling of ATPase and helicase activities (see LINDER *et al.* 1989; PAUSE and SONENBERG 1992). We suspect that the alanine to cysteine change in the DEAD box in HEL (to DECD) still would allow ATPase and helicase activity since the plum pox virus C1 protein also contains a cysteine in this position and has been shown to have ATP-dependent RNA helicase activity (LAIN *et al.* 1990). However, we have not been able to demonstrate helicase activity in an initial attempt with bacterially expressed glutathione S-transferase-HEL fusion protein when incubated *in vitro* with a double-stranded RNA template (provided by N. METHOT and N. SONENBERG). If HEL is a functional RNA helicase, it may require additional factor(s) and/or specific template(s).

How might HEL influence PEV? Strong association of HEL protein with salivary gland chromosomes and nuclear localization of HEL in embryonic and ovarian tissues intimate a direct role for HEL in chromatin structure. HEL may act at the level of the chromosomes, perhaps by unwinding or removing mRNA from the site of transcription, consequently keeping the chromosome accessible to further transcription. Another possibility is that HEL may discourage heterochromatin spreading by associating with chromosomes in a complex that may include an RNA component. Alternatively, HEL may affect PEV indirectly by regulating the expression of specific genes, such as chromosomal proteins.

Clues that implicate RNA in the regulation of transcriptional competence have been uncovered. The MALELESS (MLE) protein of Drosophila regulates dosage compensation by associating with the *X* chromosome in males, resulting in increased transcription from the single male *X* chromosome (KURODA *et al.* 1991). MLE is a likely homologue of human RNA helicase A (LEE and HURWITZ 1993), a demonstrated RNA helicase, and can be removed from chromosomes with RNase (BASHAW and BAKER 1996). Two recently identified Drosophila male-specific RNAs, *roX1* and *roX2*, associate with *X* chromosome chromatin (AMREIN and AXEL 1997; MELLER *et al.* 1997). Perhaps HEL affects PEV by acting on chromatin-associated RNAs such as these. Mammalian dosage compensation occurs via *X*-chromosome inactivation in females. A nuclear RNA transcribed from the *XIST/Xist* locus has been implicated

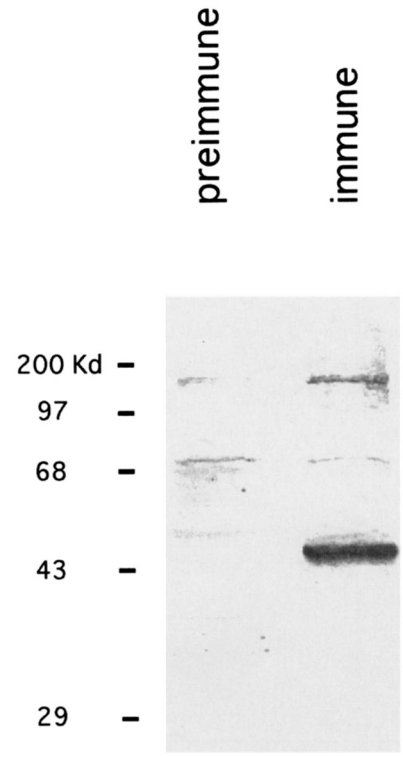

FIGURE 4.—Western blot analysis of HEL. Western blot analysis of protein from 0- to 4-hr wild-type embryos. Although both the preimmune serum (left) and the anti-HEL immune serum (right) recognize a few minor proteins, only the anti-HEL immune serum identifies a prominent protein of molecular weight expected for the endogenous HEL protein (~47 kD).

in *X* inactivation (BROCKDORFF *et al.* 1992; BROWN *et al.* 1992). The XIST RNA complexes with the inactive *X* chromosome during interphase (CLEMSON *et al.* 1996). One might expect that an RNA helicase (or RNA-dependent ATPase) might prevent such a complex from forming on the *X* chromosome that remains transcriptionally active. Further experiments are required to determine if HEL affects PEV by any of these mechanisms.

HEL and the cell cycle: We have begun to characterize the possible role of HEL in cell cycle regulation by looking for cell cycle defects in *hel* mutant embryos and by overexpression of HEL in wild-type embryos, but both of these approaches led to inconclusive results. We have found that $hel^{z15'}/Df(2L)cl^{h4}$ heterozygotes and $hel^{z15'}$ homozygotes sometimes suffer what appear to be problems in morphogenetic movements during embryogenesis. These may be significant because FOE *et al.* (1993) report that the timing of mitosis is important in cells that undergo complex morphogenetic movements. However, these *hel* mutant defects occur at very low frequencies; stronger effects are most likely masked by the strong maternal contribution of *HEL* mRNA. Overexpression of HEL during cycle 14 of embryogenesis, using a transformed *hel* cDNA driven by a heat shock promoter, has not prompted obvious passage through mitosis. Nor is it clear that mitosis is delayed in these overexpression experiments, because heat shock treatment of wild-type embryos can cause chromosome condensation, cell cycle delay and mitotic synchronization (MALDONADO-CODINA *et al.* 1993; L. J. LORENZ, personal observations), as well as microtubule breakdown (L. J.

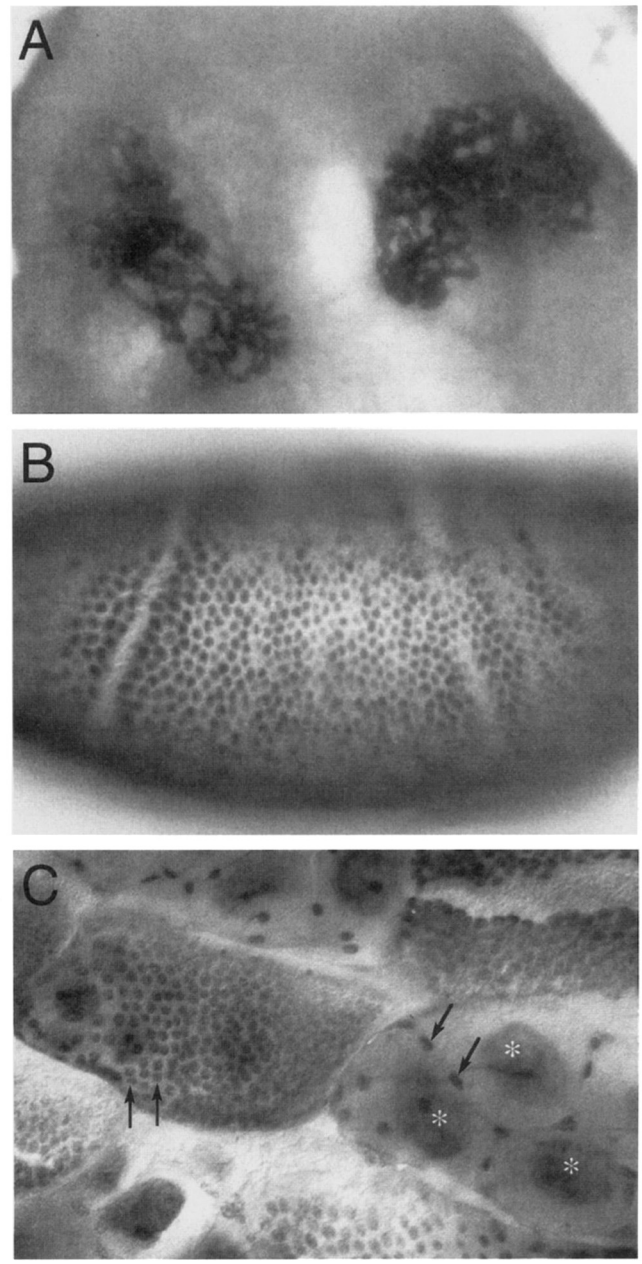

FIGURE 5.—HEL is a nuclear protein. HEL protein localization in nuclei of salivary glands, embryos and ovaries is seen using the anti-HEL polyclonal antibody and enzymatic detection. (A) Gently squashed polytene chromosomes with surrounding cytoplasm and nuclear envelopes show association of HEL protein with and around chromosomes. (B) HEL protein is expressed in nuclei of an embryo during early gastrulation. In this lateral view, one can see that the intensity of HEL protein expression begins to vary among different nuclei at this stage. (C) A closeup view of an intact ovary showing nuclear expression of HEL protein in large polyploid nurse cell nuclei (*) and smaller follicle cell nuclei (→), which migrate over egg chambers and between the nurse cells.

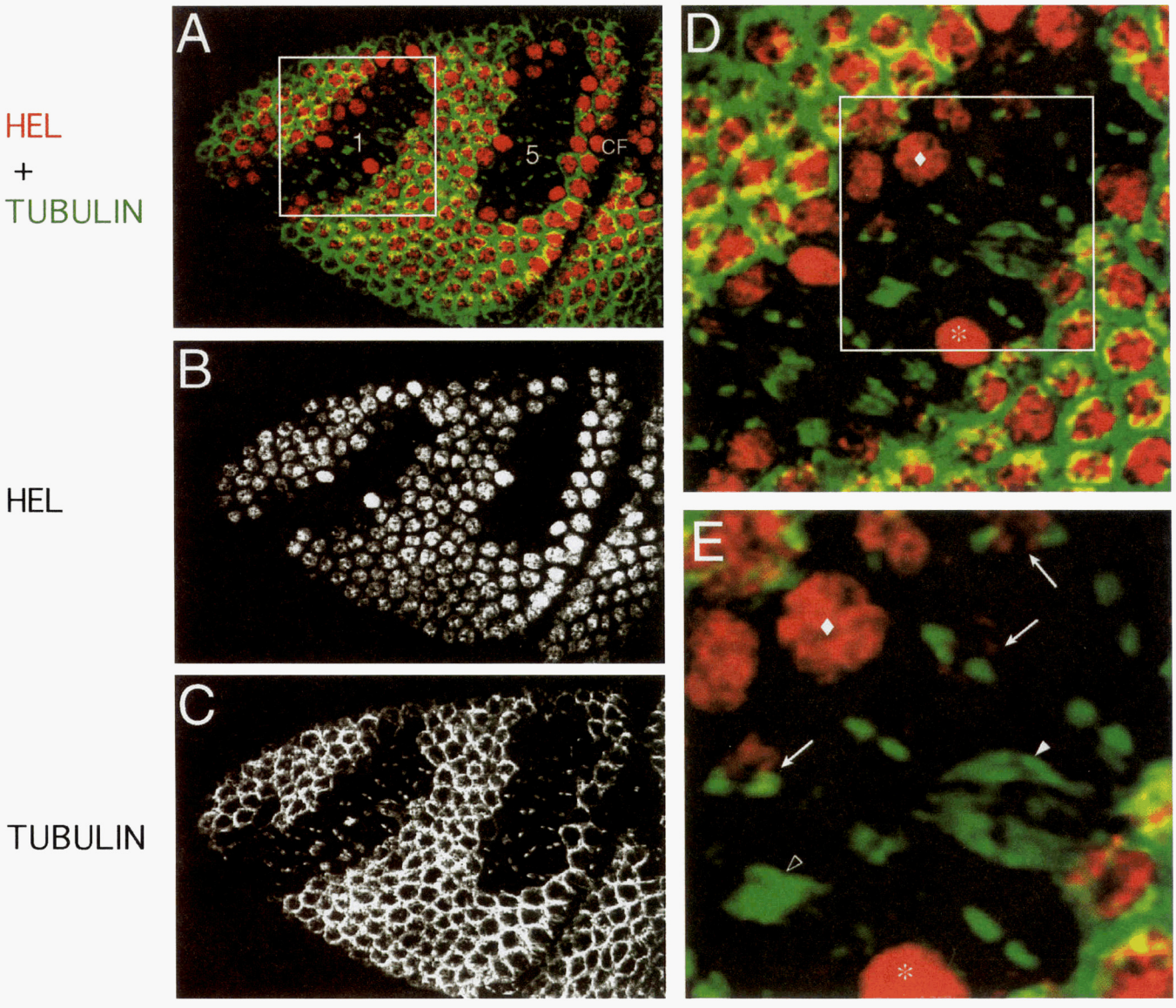

FIGURE 6.—HEL protein disappears during mitosis. Confocal images of mitotic domains (domains 1 and 5 as per FOE 1989) in the head region of an embryo, anterior to the cephalic furrow (CF) during early cell cycle 14 are shown. (A) A composite Z-series of optical sections through the outer lateral cell layer of an embryo double stained for HEL protein (red) and β-tubulin (green) clearly indicates that HEL disappears during mitosis. Bright HEL nuclear signals (red) are surrounded by cytoplasmic β-tubulin staining (green) in interphase cells, imparting a "pimento and green olive" appearance to these cells. (Yellow halos around nuclei are produced by the fusion into one dimension of two-dimensional cytoplasmic β-tubulin signals, slightly above and below nuclei, with nuclear HEL signals.) Mitotic domains 1 and 5 (numbered) appear as obvious dark regions (where HEL protein fades) containing β-tubulin positive mitotic spindles. Black and white photos show HEL alone (B) and β-tubulin (C). (D) Closeup view of domain 1 region boxed in A showing mitotic details. Cytoplasmic β-tubulin staining disappears before HEL is affected (*) and subsequently, HEL begins to disappear (diamond). (E) Magnified view of region boxed in D. Dynamics of HEL and β-tubulin staining progress in the following steps: (1) cytoplasmic microtubules break down (*), (2) HEL begins to fade (diamond), (3) spindle microtubules begin to appear when HEL is almost gone (→), (4) HEL is undetectable in anaphase (filled arrowhead) when the spindle is prominent with equatorial gapping and (5) in telophase (open arrowhead) when spindle remnants are seen between daughter nuclei.

LORENZ, personal observations), all of which complicate these studies. Nevertheless, HEL may have a regulatory role in the cell cycle that we have not yet been able to identify, because it has been shown that overexpression of a *hel* cDNA in the fission yeast, *S. pombe*, can suppress premature mitosis induced by loss of the two known inhibitory kinases of CDC2, Wee1 and Mik1, (WARBRICK and GLOVER 1994), though there is no effect on wild-type yeast. Perhaps we could uncover a role for HEL in cell cycle regulation in Drosophila embryos by overexpression of a stable form of HEL that cannot be degraded at mitosis. This approach was useful in characterizing the roles of the A and B CYCLINS (SIGRIST *et al.* 1995).

If there is a role for HEL in cell cycle regulation, it would help to consolidate the possibility of a link between PEV and cell cycle control. There is growing evidence that these two processes may be intimately associated. A cloned haplo-suppressor of PEV, *Su-var(3)6*, encodes a protein phosphatase involved in mitosis (AXTON *et al.* 1990; BAKSA *et al.* 1993). Mutations in *Su-var(3)6* can prompt overly condensed chromosomes and abnormal chromosome segregation. Furthermore, the transcriptional activator and cell cycle regulator, E2F, also influences PEV (SEUM *et al.* 1996). Finally, the Drosophila gene encoding PCNA is a recessive suppressor of PEV (HENDERSON *et al.* 1994). PCNA functions during DNA replication and repair, and exists in complexes with various cyclins and CDKS (cdc-like kinases), including CYCLINS A and B and CDC2 (ZHANG *et al.* 1993). HEL clearly affects PEV, and understanding the functional basis of the mitotic disappearance of HEL will further illuminate the relationship between these two processes.

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