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The *Drosophila chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle

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SUMMARY

The *Drosophila* chorion genes encode the major protein components of the chorion (eggshell) and are arranged in two clusters in the genome. To meet the demand for rapid chorion synthesis, *Drosophila* ovary follicle cells amplify the chorion gene clusters ~80-fold. Amplification proceeds through repeated firing of one or more DNA replication origins located near the center of each gene cluster. Hypomorphic mutant alleles of the *chiffon* gene cause thin, fragile chorions and female sterility, and were found to eliminate chorion gene amplification. Null alleles of *chiffon* had the additional phenotypes of rough eyes and thin thoracic bristles: phenotypes often associated with disruption of normal cell cycle. The *chiffon* locus was cloned by chromosomal walking from the nearby *cactus* locus. A 6.5 kb transcript was identified and confirmed to be *chiffon* by sequencing of mutant alleles and by phenotypic rescue with genomic transformation constructs. The protein predicted by translation of the 5.1 kb *chiffon* ORF contains two domains related to the *S. cerevisiae* Dbf4 regulator of DNA replication origin firing and cell cycle progression: a 44 residue domain designated CDDN1 (43% identical) and a 41 residue domain designated CDDN2 (12% identical). The CDDN domains were also found in the *S. pombe* homolog of Dbf4, Dfp1, as well as in the proteins predicted by translation of the *Aspergillus nimO* gene and specific human and mouse clones. The data suggest a family of eukaryotic proteins related to Dbf4 and involved in initiation of DNA replication.

Key words: Origin, DNA replication, Replicator, ORC, *chiffon*, *Drosophila*, Dbf4, Cell cycle

INTRODUCTION

To meet demand for rapid synthesis of chorion (eggshell) proteins, *Drosophila* ovary follicle cells amplify the two chromosomal clusters of major chorion genes ~80-fold (Calvi and Spradling, 1998; Orr-Weaver, 1991; Spradling and Mahowald, 1980). Amplification occurs through repeated firing of one or more DNA replication origins located near the center of the gene clusters. Chorion gene amplification is amenable to both molecular and genetic analysis, making it an ideal model system for the study of metazoan chromosomal DNA replication origins and their regulation. Constructs derived from the third chromosome chorion gene cluster will amplify with normal tissue and temporal specificity when reinserted into the *Drosophila* genome by P element-mediated transformation (deCicco and Spradling, 1984; Lu and Tower, 1997). This assay has allowed mapping of a *cis*-acting control element required for high levels of amplification, called ACE3 (Amplification Control Element, 3rd chromosome), as well as several stimulatory regions (Delidakis and Kafatos, 1987; Orr-Weaver et al., 1989). Two-dimensional gel analysis of DNA replication intermediates originating from the endogenous locus and from transgenic constructs has allowed mapping of the major origin of replication, called Ori-β, as well as one or two more minor origin regions (Delidakis and Kafatos, 1989; Heck and Spradling, 1990).

Mutations that disrupt genes required in *trans* for amplification cause female sterility (Orr et al., 1984; Underwood et al., 1990). The sterility is due, at least in part, to underproduction of chorion proteins and synthesis of thin, fragile chorions. Twelve genes have been identified that are required for amplification (Calvi and Spradling, 1998; Royzman et al., 1999). The first of these to be cloned (*k43*), was found to encode the *Drosophila* homolog of the *S. cerevisiae* Origin Recognition Complex subunit 2 (ORC2) (Gossen et al., 1995; Landis et al., 1997). The ORC is a complex of six proteins (Orc1p-Orc6p) that bind to *S. cerevisiae* chromosomal DNA replication origins in vivo and in vitro (Bell and Stillman, 1992; Diffley and Cocker, 1992; Diffley et al., 1994). The phenotypes of mutations in yeast ORC subunit genes demonstrate that ORC is required for origin function, as well as for transcriptional silencing of the yeast mating-type loci (Bell et al., 1993; Foss et al., 1993). ORC is associated with the origin throughout the cell cycle and activation of the origin appears to result from association of additional proteins with the ORC. One of the last steps in activation of the yeast origin appears to be phosphorylation of one or more origin-associated proteins by the Cdc7-Dbf4

protein kinase (Jackson et al., 1993; Lei et al., 1997; Owens et al., 1997). Cdc7 is a cdk-like serine/threonine protein kinase whose activity is regulated in a cell-cycle-dependent manner by association with its regulatory subunit Dbf4. Dbf4 binds to origin-associated proteins, thus providing a likely mechanism for recruitment of Cdc7 to target(s) at the origin (Dowell et al., 1994).

The identification of *Drosophila k43* as the homolog of *S. cerevisiae* ORC2 suggested that all or part of the mechanism of origin regulation may be conserved between yeast chromosomal origins and the chorion gene origins. The *chiffon* gene was originally identified by female sterile mutants, which lay eggs with thin eggshells (Schüpbach and Wieschaus, 1991). Experiments presented here demonstrate that *chiffon* is required in *trans* for chorion gene amplification. Cloning and characterization of the *Drosophila chiffon* gene revealed that the predicted chiffon protein contains two domains (designated CDDN: for Chiffon, Dbf4 and Dfp1 and NimO) which are related to regulators of DNA replication and cell cycle in lower eukaryotes.

MATERIALS AND METHODS

Drosophila strains

EMS *chiffon* alleles *QW16, QY42, WD18, WF24, PS55* and *DB23* were obtained from Trudy Schüpbach (Schüpbach and Wieschaus, 1991). *Df(2L)RA5* {35E1.2;35F1.2} was generated by Yasushi Hiromi (Ashburner et al., 1990). *Df(2L)chif*^{64} {35F,36A;36D} was generated by local transposition/imprecise excision of a P element insert in *cactus* (*cactus*^{255}) (Tower et al., 1993). Other strains are as described (Lindsley and Zimm, 1992).

P element mobilization

Starting stock *PW62* was generated by Kiss and coworkers (Torok et al., 1993), and provided by John Roote and Michael Ashburner. The *PW62* chromosome was wild type for *chiffon* and *l(2)35Fe*, and mutant for *cactus*. Three P element insertions on the *PW62* chromosome (PW62-1, PW62-2 and PW62-3) were mapped within the *chiffon* walk by genomic Southern (Fig. 5A). The elements were mobilized by crossing to Δ2-3(99B) transposase source (Robertson et al., 1988) and new *chiffon* alleles identified by failure to compliment *chif*^{WD18}. 29 new alleles were identified out of 12,000 chromosomes tested. The majority were also mutant for *l(2)35Fe* and contained deletions extending from P element PW62-1 to P element PW62-3 (data not shown). In contrast, one new *chiffon* allele, *chif*^{ETBE3}, was wild type for *l(2)35Fe*. *chif*^{ETBE3} behaved genetically as a *chiffon* null: *chif*^{ETBE3}*/chif*^{WD18} and *chif*^{ETBE3}*/chif*^{WF24} both gave the severe phenotype. The *chif*^{ETBE3} molecular structure was analyzed by genomic Southern and revealed a deletion of ~6 kb extending distally from the PW62-3 P element (Fig. 5B). No other changes in the ETBE3 chromosome were detected.

To confirm the structure of *chif*^{ETBE3}, a genomic DNA fragment containing the predicted deletion was amplified by PCR. *chif*^{ETBE3} is homozygous lethal and is lethal over deficiencies uncovering this region, due to the *cactus* mutation on the *chif*^{ETBE3} chromosome. Therefore DNA from heterozygous *chif*^{ETBE3}*/CyO* flies was isolated. PCR was carried out using primers flanking the *chif*^{ETBE3} deletion predicted by Southern analysis (primers #45283 and #37094; Fig. 5B). A unique PCR product of the predicted size of 2.3 kb was obtained, subcloned into pBluescript and sequenced. Sequencing confirmed a deletion from position 37488 to 44728, numbering in accord with BDGP cosmid DS009218.1. Left behind was a nine base pair novel sequence (GTGGCGCCC) and a 1.42 kb fragment of the PW62-2 P element extending from position 9271 to 10698 (numbering according to FlyBase ID FBmc0000231 compiled sequence for pP{LacW}).

Chromosome walking

Genomic lambda phage clones containing the *cactus* gene were obtained from David Marcey and Christianne Nüsslein-Volhard (Geisler et al., 1992). The walk was extended proximally by isolating overlapping genomic cosmid clone 10-1 (Tower et al., 1993), and genomic lambda clones #22 and #37 (Fig. 5A) from a Canton S genomic library in Lambda Fix vector (Stratagene).

Northern analysis

mRNA was isolated from dissected *Drosophila* ovaries using Micro Fast Track kit (Invitrogen), fractionated on 1.0% agarose/formaldehyde gels and transferred to GeneScreen membranes (DuPont/NEN). DNA probes were ^{32}P-labeled by random oligomer priming, using the Prime-It II kit (Stratagene). Hybridization signals were visualized by autoradiography. Transcript size was determined by comparison with 1 kb RNA ladder (Gibco-BRL) per manufacturers instructions.

In situ hybridization to egg chambers

Whole-mount in situ hybridization to egg chamber (EC) RNA was performed using digoxigenin-labelled probes (Tautz and Pfeifel, 1989; Cooley et al., 1992). The *bicoid* probe was *bicoid* cDNA clone c53.46.7 (Berleth et al., 1988).

cDNA cloning

cDNA clones X4 and Y4 were isolated from an adult *Drosophila* Canton S cDNA library in lambda gt10 vector (Clonetech). The probe used to identify cDNA X4 was the 9 kb *Xba*I-*Sal*I genomic fragment X from cosmid 10-1. The probe used to identify cDNA Y4 was the 4.8 kb *Xba*I-*Sal*I genomic fragment Y from lambda phage λ 22 (Fig. 5A). Additional cDNA clones LD19808, LD14373, HL02831 and GM06264 were obtained from Genome Systems, Inc. (‘I.M.A.G.E. Consortium LLNL cDNA clones’) (Lennon et al., 1996), based upon homology to genomic sequence. Genomic sequence of the ‘DS90218.1’ cosmid was obtained from Berkeley Drosophila Genome Project (BDGP).

Measurement of chorion gene amplification

Amplification was assayed as previously described (Underwood et al., 1990). Briefly, DNA was isolated from stage 13 egg chambers (ECs) and transferred to duplicate Southern slot blots. Blots were hybridized with a radiolabelled restriction fragment from the third chromosome chorion gene cluster, the p302.77 subclone (Spradling, 1981), and with rDNA probe pDmrY22 (Dawid et al., 1978) as a control for amount of DNA loaded. Signals were quantitated by phosphoimager and amplification level was calculated by comparing the signal for chorion probe in EC DNA to the signal for chorion probe in male DNA (non-amplifying control), using the following formula: ‘fold amplification’ = (chorion probeEC/chorion probeMale) / (rDNAprobeEC/rDNAprobeMale). No amplification yields a value of 1.

Electron microscopy

Scanning electron microscopy of adult *Drosophila* and laid *Drosophila* eggs was carried out at the University of Southern California Center for Electron Microscopy and Microanalysis, using a Cambridge 360 SEM. Samples were prepared using standard methods, except that critical point drying was replaced by a 15 minute treatment with hexamethyldisilazane (Adams et al., 1987).

DNA sequencing

Dideoxy sequencing was performed with the Sequenase version 2.0 kit (United States Biochemical). cDNAs were subcloned into pBlueScript vector (Stratagene). For mutant *chif*^{WF24}, overlapping

segments of the *chif*^{WF24}-coding region were amplified by PCR from genomic DNA isolated from *chif*^{WF24}*/Df(2)RA5* flies and subcloned into pBluescript. An equimolar pool of six independent clones, three each from two independent PCR reactions was sequenced for each region. The *chif*^{WF24} mutant sequence was then confirmed by sequencing a clone from a third independent PCR reaction.

For the *chif*^{ETBE3} mutation, primers on both sides of the deletion (37094-tgtcacatttgccactagatgc and 45283-gagcgattttggaatagtccga, Fig. 5B) were used to PCR-amplify a 2.3 kb fragment, which was cloned into pBluescript and sequenced. The sequence of the region immediately spanning the junction was confirmed by sequencing two additional clones, one each from two additional independent PCR reactions.

P element-mediated transformation

Restriction fragments from the genomic walk (Fig. 5A) were cloned into pY.E.S. transformation vector (Patton et al., 1992), as follows: The 4.8 kb *Sal*I-*Xba*I genomic fragment ('Y') was subcloned into the unique *Xba*I-*Sal*I sites of the pY.E.S. vector, to generate construct pYES5. pYES5 was digested with *Xba*I and a 12 kb *Xba*I-*Xba*I restriction fragment ('XC') was cloned into this site, to generate pYES512. Restriction analysis identified the correct orientation of fragment XC with respect to fragment Y. Construct pYES9 was generated by removing an 8 kb *Sal*I-*Sal*I fragment from pYES512 and religating. Multiple independent germline transformants were generated for each of the rescue constructs (Rubin and Spradling, 1982), using the *y-ac-w*^{1118} recipient strain (Patton et al., 1992). Multiple independent insertions for each construct were crossed into the *chif*^{WD18}*/Df(2L)RA5* mutant background and assayed for phenotypic rescue.

chiffon homology comparisons

The 5.1 kb *chiffon* predicted ORF was used to query National Center for Biotechnology Information (NCBI) databases using BLASTP program on the NCBI Blast web site page (www.ncbi.nlm.nih.gov/). The known or predicted protein sequences identified were aligned with the chiffon predicted protein sequence using the ClustalW program and default settings (Thompson et al., 1994).

RESULTS

chiffon mutants were generated by Schüpbach and co-workers as part of a screen for female sterile mutations on the *Drosophila* second chromosome (Schüpbach and Wieschaus, 1991). *chiffon* was genetically mapped to location 2-53, and mapped by complementation tests with chromosomal deficiencies to cytological location 35F1; 36A1, near the *cactus* locus (Ashburner et al., 1990; Schüpbach and Wieschaus, 1991). The homozygous *chiffon* phenotype was female sterility characterized by a thin, fragile chorion (eggshell) structure, which suggested the name for the gene.

The similarity of the *chiffon* thin chorion phenotype to the thin chorion of *k43* and other genes disrupting chorion gene amplification suggested that *chiffon* might also disrupt amplification. The phenotypes of multiple *chiffon* alleles were characterized (Table 1). *chiffon* phenotypes fell into two general classes: a mild phenotype where defects were limited to thin chorions, and a severe phenotype characterized by thin chorions (Fig. 1), as well as rough eyes (Fig. 2) and thin

Table 1. *chiffon* alleles and phenotypes

		Allele/deficiency phenotype			
Allele	Mutagen	Egg chamber	Eye	Scutellar bristles	Amplification
chif^{QW16}	EMS	Thin	Normal	Normal	<7% of normal
chif^{DB23}	EMS	Thin	Normal	Normal	<7% of normal
chif^{QY42}	EMS	Thin	Normal	Normal	<7% of normal
chif^{PS55}	EMS	Thin	Intermediate	Intermediate	<7% of normal
chif^{WD18}	EMS	Thin	Rough	Thin	<7% of normal
chif^{WF24} null	EMS	Thin	Rough	Thin	<7% of normal

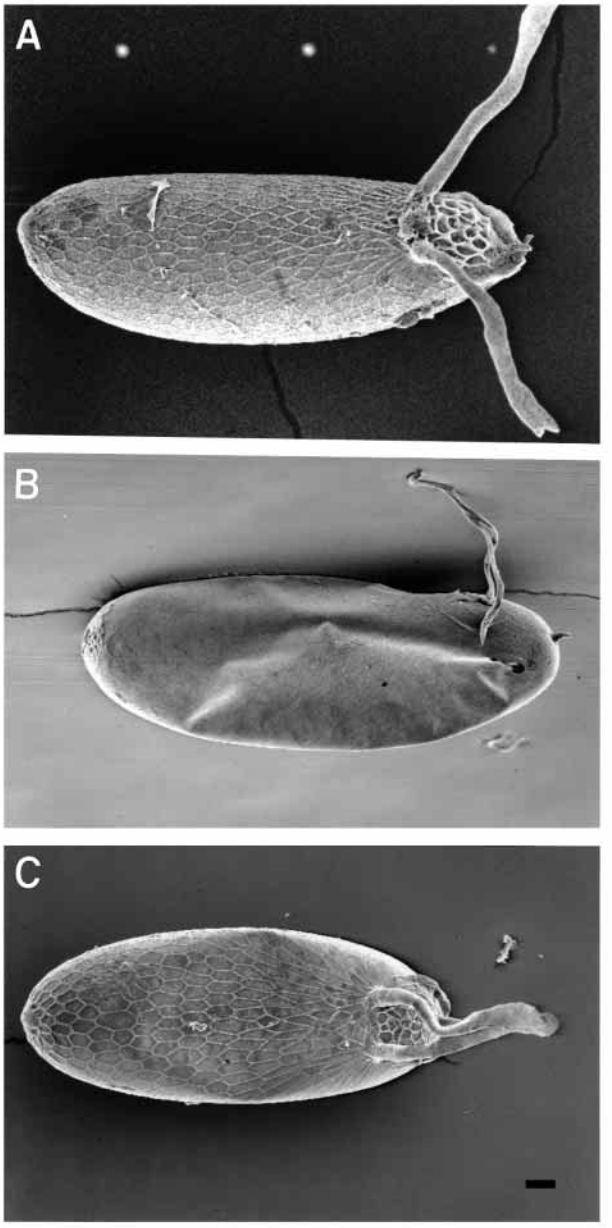


Fig. 1. *chiffon* chorion phenotype and phenotypic rescue. Laid eggs were analyzed by scanning electron microscopy for chorion morphology and are presented in a dorsal view, with anterior to the right. The two large chorion dorsal appendages are apparent at the dorsal anterior (right). (A) Wild-type Oregon R strain. (B) *chiffon* mutant *chif*^{WD18}*/Df(2L)RA5*. (C) *chiffon* mutant plus rescue construct, genotype: *y ac w; chif*^{WD18}*/Df(2L)RA5; p[yellow+; YES512]/+*. The honey comb-like pattern on the egg surface is the outline of chorion material deposited by each individual follicle cell. Note that in the *chiffon* mutant (B) the amount of chorion material present in the dorsal appendages and surrounding the egg is greatly reduced, and the mutant egg is slightly collapsed. All defects are corrected in the rescue strain (C). Bar, 32 μm.

thoracic bristles (Fig. 3). All homozygous alleles exhibited the mild phenotype with the exception of *chiffon*^{WF24} homozygotes, which exhibited the severe phenotype. When placed over deficiency, the *chiffon*^{WD18} mild allele then exhibited the severe phenotype, characterized by rough eyes and thin thoracic bristles. Thus the mild phenotype appears to be hypomorphic. The phenotype of *chiffon*^{WF24} did not change over deficiency, suggesting that the *chiffon*^{WF24} severe phenotype represents the null. This was subsequently confirmed by molecular characterization of *chiffon*^{WF24} and another null allele, *chiffon*^{ETBE3}, as described below.

The effect of *chiffon* mutations on chorion gene amplification was determined by Southern analysis of DNA isolated from mutant egg chambers (Fig. 4). DNA was isolated from mutant and control stage 13 egg chambers, and the samples were divided equally onto identical slot blots. One blot was hybridized with a probe specific for the amplified third chromosome chorion gene cluster, and the other with a probe specific for the non-amplified rDNA genes. DNA isolated from male flies, where amplification does not occur, yielded approximately equal signal with both probes in the autoradiographic exposure presented (Fig. 4A-C). In contrast, DNA isolated from non-mutant control egg chambers exhibited greatly increased signal with the chorion probe, due to amplification (Fig. 4D). In DNA isolated from *chiffon* mutant egg chambers, hybridization to the chorion probe is reduced to nearly non-amplified levels (Fig. 4F-K).

chiffon was mapped by complementation tests to the overlap between the chromosomal deficiencies *Df(2L)chif*^{64} and *Df(2L)RA5* (Fig. 5A). The overlap was found to uncover *chiffon*, *cactus* and one other mutation *l(2)35Fe*. The order of *chiffon* and *l(2)35Fe* could not be determined.

A genomic walk was initiated from clones of the *cactus* locus (Geisler et al., 1992) to clone the ~40 kb interval between the breakpoints of *Df(2L)chif*^{64} and *Df(2L)RA5* (Fig. 5A). The locations of the chromosomal breakpoints within the cloned fragments were determined by Southern analyses. Both *Df(2L)chif*^{64} and *Df(2L)RA5* were originally derived from a P element inserted in *cactus* (*cactus*^{255}), and both deficiencies were found to have distal breakpoints at the site of the original *cactus*^{255} P element insertion. The ~40 kb interval containing *chiffon* and *l(2)35Fe* was mapped by restriction enzyme digestion. Three transcripts within the region were identified by hybridization of various radiolabeled restriction fragments with northern blots of ovary RNA, and are diagrammed (Fig.

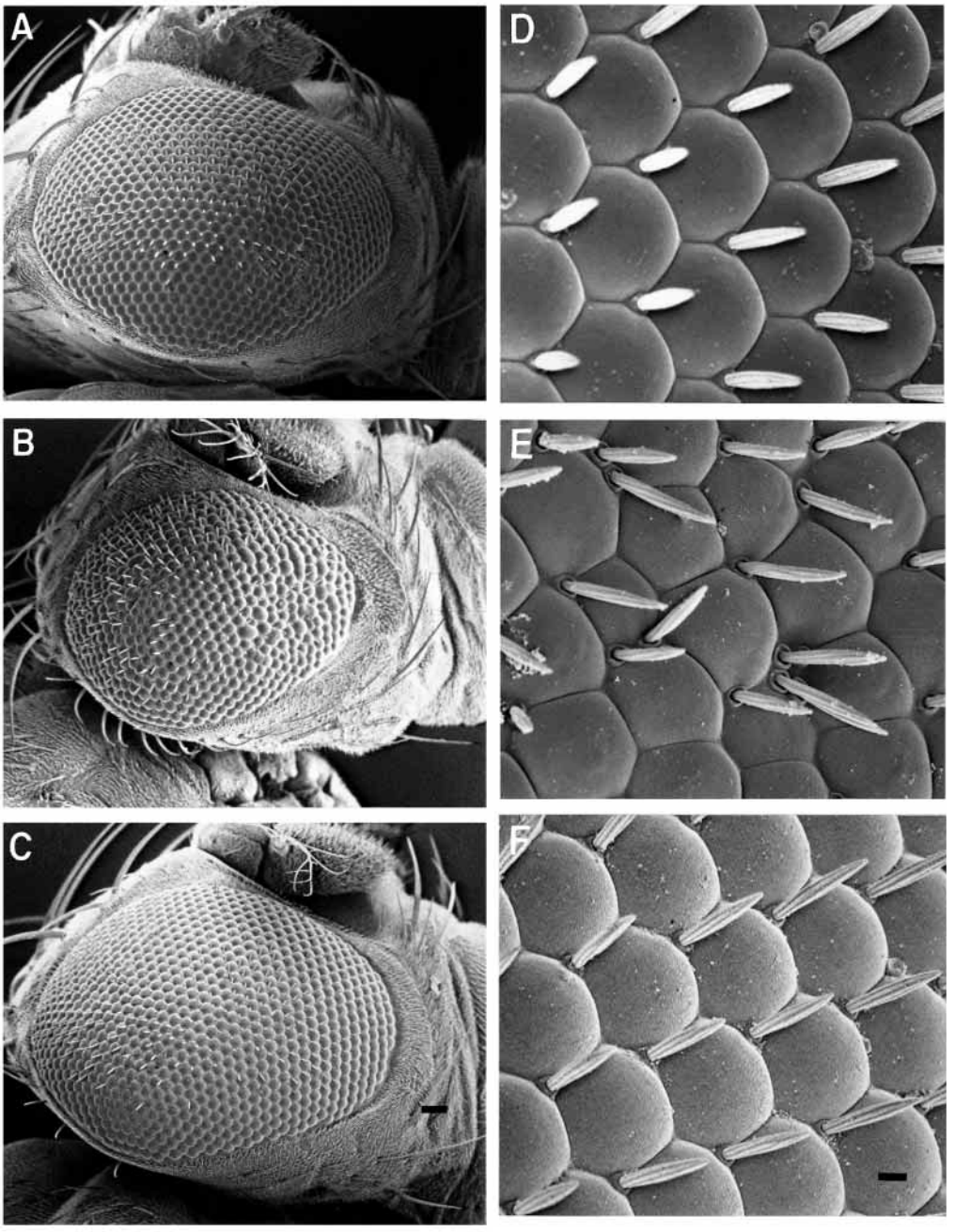


Fig. 2. *chiffon* eye phenotype and phenotypic rescue. Adult fly eyes (A-C), and portions of the eye surface (D-F), were analyzed by scanning electron microscopy for morphology, and are presented in a dorsal view, with anterior to the right. (A,D) Wild-type Oregon R strain; (B,E) *chiffon* mutant *chif*^{WD18}*/Df(2)RA5*; (C,F) *chiffon* mutant plus rescue construct, genotype: *y ac w; chif*^{WD18}*/Df(2L)RA5; p[yellow+; YES512]/+*. Bar, 33 μm (A-C), and 3.7 μm (D-F).

5A), including a transcript of 6.5 kb subsequently identified as *chiffon* (Fig. 5C).

To determine which transcript was *chiffon*, new alleles of *chiffon* were generated by P element mobilization as described in Materials and Methods. One new *chiffon* allele generated, $chif^{ETBE3}$, behaved genetically as a null, and was wild type for *l(2)35Fe*. Molecular characterization of $chif^{ETBE3}$ revealed a deletion of a region contained entirely within the 6.5 kb transcript (Fig. 5B), indicating that the 6.5 kb transcript is *chiffon*.

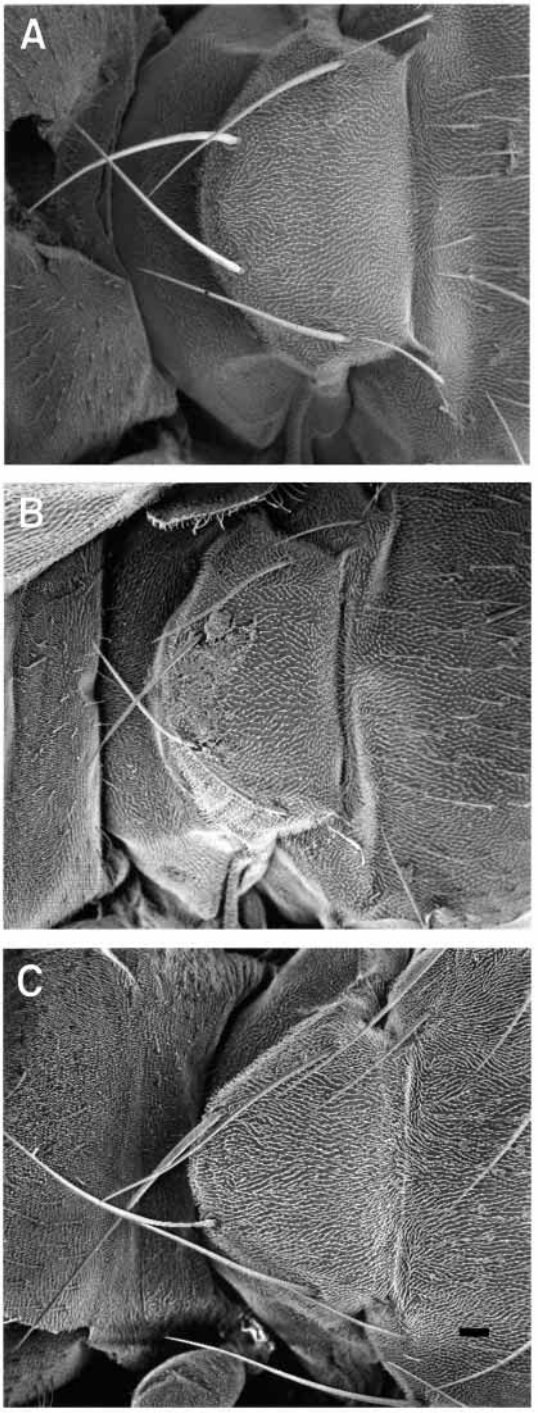


Fig. 3. *chiffon* bristle phenotype and phenotypic rescue. Adult fly thoraces were analyzed by scanning electron microscopy for scutellar bristle morphology, dorsal view, anterior to the right. (A) Wild-type Oregon R strain; (B) *chiffon* mutant $chif^{WD18}/Df(2L)RA5$; (C) *chiffon* mutant plus rescue construct, genotype: *y ac w;* $chif^{WD18}/Df(2L)RA5$*; p[yellow+; YES512]/+*. Bar, 37 μm.

To confirm the identification of the 6.5 kb transcript as *chiffon*, two genomic constructs were generated to attempt phenotypic rescue by germline transformation. Construct YES512 contained an ~17 kb genomic DNA fragment extending from the *Xba*I restriction site to the *Sal*I restriction site, which contains both the 6.5 kb transcript and the 1.8 kb transcript (Fig. 5A). Construct YES9 contained an ~9 kb fragment containing the complete 1.8 kb transcript and only a fragment of the 6.5 kb transcript (Fig. 5A). Three independently derived transgenic insertions of each of these constructs were tested for their ability to rescue both the mild and severe *chiffon* phenotypes. All three independent insertions of the YES512 construct were able to rescue all of the *chiffon* phenotypes: female sterility and chorion gene amplification (Fig. 4), thin chorion (Fig. 1), rough eyes (Fig. 2), and thin thoracic bristles (Fig. 3). In contrast, the three insertions of the smaller YES9 construct containing the 1.8 kb transcript and only part of the 6.5 kb transcript did not rescue any of the *chiffon* phenotypes. Thus the rescue experiments further support the identification of the 6.5 kb transcript as *chiffon*. As seen below, this identification was confirmed by sequencing of the $chiffon^{WF24}$ null allele.

The genetic analyses suggested that *chiffon* null mutants are viable. However, there might be a more general requirement for *chiffon* function that is masked by maternal supply of wild-type *chiffon* gene product to the mutant embryo. To begin to

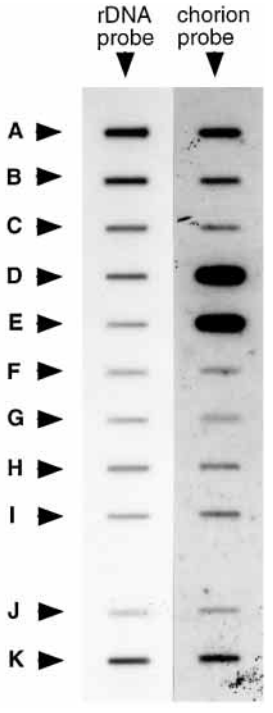


Fig. 4. Chorion gene amplification assay. Chorion gene amplification was quantitated by isolating DNA from stage 13 egg chambers and transferring equal portions to identical slot blots. One membrane was hybridized with radiolabelled rDNA probe, as indicated, which serves as a non-amplifying control for DNA concentration. The second membrane was hybridized with radiolabelled fragment from the third chromosome chorion gene locus, as indicated. DNA was isolated from whole adult males as a non-amplifying control (A-C) in amounts as described, or from stage 13 egg chambers, 0.25 μg each (D-K). (A) Male DNA, 1.2 μg. (B) Male DNA, 0.6 μg. (C) Male DNA, 0.3 μg. (D) Flies heterozygous for the recessive $chif^{WD18}$ mutation, genotype: *y ac w;* $chif^{WD18}/+$. (E) The transgenic YES512 rescue fragment (diagrammed in Fig. 5A) in the *chiffon* mutant background, genotype: *y ac w;* $chif^{WD18}/Df(2L)RA5$*; p[yellow+; YES512]/+*. (F) $chif^{QY42}/Df(2L)RA5$. (G) $chif^{QW16}/Df(2L)RA5$. (H) $chif^{DB23}/Df(2L)RA5$. (I) $chif^{PS55}/Df(2L)RA5$. (J) $chif^{WD18}/Df(2L)RA5$. (K) $chif^{WF24}/Df(2L)RA5$. Amplification was quantitated by phosphoimager in this and additional experiments, and was <7% of the heterozygous control for each the *chiffon* mutant genotypes (F-K). Amplification in the rescue strain (E) was equal to control.

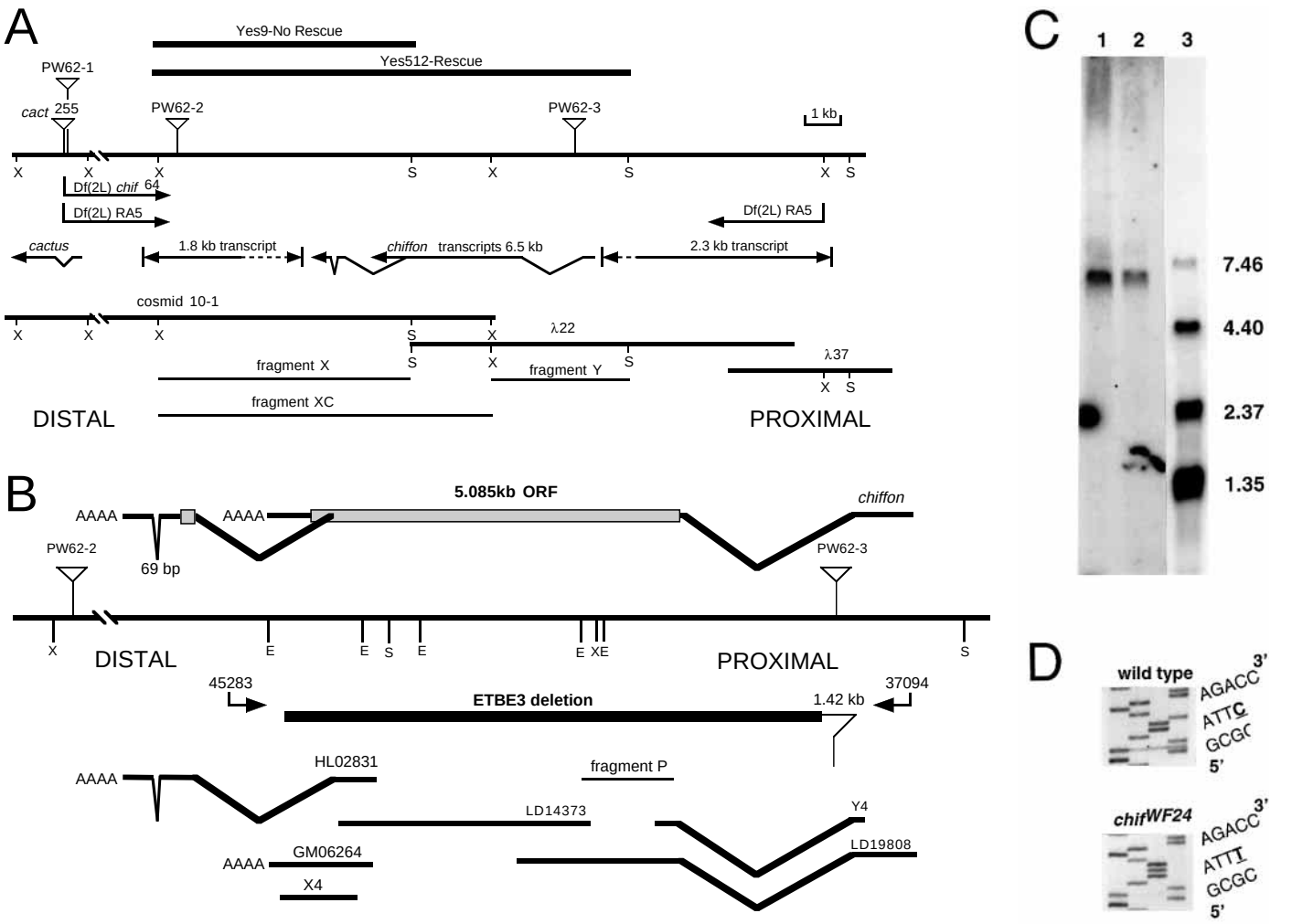


Fig. 5. (A) The ~40 kb chromosomal walk is diagrammed. The extent of the chromosomal deficiencies, *Df(2L)RA5* and *Df(2L)chif*^{64}, are indicated by arrows. Genomic lambda phage clones λ22 and λ37, and genomic cosmid clone 10-1 are indicated as thick lines below the map, as are the genomic fragments X, XC and Y. Approximate locations of the *cactus* and *chiffon* transcripts are indicated by arrows, and the *cact*^{255}, PW62-1, PW62-2 and PW62-3 P element insertions are indicated by triangles. Two other ovarian transcripts were identified in the region between the *Df(2L)RA5* breakpoint and the PW62-1 P element insertion, and are indicated by double arrows. The rescue constructs YES9 and YES512 are represented by thick lines above the genomic map. The YES512 construct was able to completely rescue all of the *chiffon* mutant phenotypes. S, *Sal*I; X, *Xba*I. (B) *chiffon* transcript map. The overlapping cDNAs HL02831, LD14373, LD19808, GM06264, Y4 and X4 were sequenced and their location is diagrammed. Two alternative *chiffon* transcripts are indicated above the restriction map as discussed in the text. The 5.1 kb *chiffon* ORF is indicated by a stippled line. The genomic fragment P used as a probe for northern analysis is indicated. The extent of the deletion in *chif*^{ETBE3} is indicated by a thick line below the restriction map along with the remaining fragment of the PW62-2 P element (not to scale). The primers 45283 and 37094, used to amplify the fragment containing the *chiffon*^{ETBE3} deletion, are indicated by arrows. E, *Eco*RI; S, *Sal*I; X, *Xba*I. (C) Northern analysis of the *chiffon* transcript. A northern blot with two independent preparations of total ovarian RNA (lanes 1 and 2) was hybridized with genomic fragment P and transcript size was determined using RNA molecular weight markers (lane 3). (D) Sequence analysis of the *chif*^{WF24} null mutant allele. The *chiffon*-coding region was sequenced in the control (Canton S) and *chif*^{WF24} mutant backgrounds. The sequence of the region from nucleotide 514 to 526 is presented (5′ GCGCATTTAGACC 3′), with numbering relative to 1 as the A of the ATG start codon for translation. The *chif*^{WF24} allele contains a substitution of T for the C at position 521, as indicated by underlining. This mutation thus causes a TAG stop termination of the predicted *chif*^{WF24} encoded protein at amino acid residue position #174.

examine this possibility, experiments were performed to determine if *chiffon* RNA was maternally synthesized and supplied to the embryo (Fig. 6). Whole-mount in situ hybridization to RNA in dissected ovaries was used to visualize patterns of RNA expression. Hybridization to the known maternally supplied *bicoid* RNA was used as a control (Fig. 6A). As expected, *bicoid* RNA was abundant in nurse cells, and exhibited a characteristic localization in a ring at the anterior of the oocyte (Berleth et al., 1988; St. Johnston et al., 1989). Abundant *chiffon* RNA expression was also detected in the germline nurse cells (Fig. 6B). Faint *chiffon* signal could sometimes be detected in the follicle cells of late stage ECs (Fig. 6B), however, we were unable to determine at what stage the follicle cell expression was initiated.

To confirm that the maternally synthesized *chiffon* RNA was supplied to the embryo, northern analysis was performed on RNA isolated from egg chambers and embryos. RNA was isolated from stage 10/11 egg chambers, which comprise nurse

cells, follicle cells and the oocyte (Fig. 6C, lane 1); stage 13/14 egg chambers, which comprise oocyte and follicle cells (lane 2); and 0-30 minute-old embryos (lane 3), which have not yet begun zygotic transcription. The northern blot was first hybridized with probes specific for several control RNAs. As expected, the maternally supplied *bicoid* RNA was present in all three samples. The follicle-cell-specific chorion gene RNAs S36, S15 and S18 were present only in the egg chamber samples, since the follicle cells are degraded prior to egg laying. Abundant S18 and S15 RNA synthesis in the follicle cells does not begin until after stage 11. Finally, the 6.5 kb *chiffon* transcript was detected in all three samples and therefore is maternally supplied to the embryo.

Multiple *chiffon* cDNA clones were identified by screening an adult Canton S cDNA library with genomic DNA probes. Additional cDNA clones were obtained from BDGP and Genome Systems, Inc., based upon sequence homology with genomic DNA. The indicated cDNAs were sequenced and assembly of overlapping cDNA sequences yielded a complete cDNA sequence of the 5.085 kb *chiffon* ORF and several hundred bp of 5′ and 3′ untranslated sequence (Fig. 5B and 7A). The large ORF matched the known *Drosophila* codon bias (data not shown). A search of the BDGP EST database identified GM06264, a cDNA from an adult brain and sensory organ cDNA library that indicated an alternatively spliced *chiffon* transcript containing a 5.133 kb ORF (Fig. 5B).

To confirm the identification of *chiffon*, the coding region was PCR-amplified from genomic DNA isolated from $chif^{WF24}/Df(2L)RA5$ adult flies and sequenced. The numbering is started from the first nucleotide of the translation initiation codon, and the DNA sequence analysis indicated a substitution of T for the C at nucleotide position 521 in $chiffon^{WF24}$ (Fig. 5D). This mutation creates a stop codon near the beginning of the ORF, corresponding to amino acid residue #174/1695. This lesion is therefore consistent with the identification of the $chiffon^{WF24}$ allele as a null (Table 1), and confirms the identification of the 6.5 kb transcript as *chiffon*.

The predicted 5.085 kb chiffon ORF sequence (Fig. 5B) was used to search the NCBI databases for related proteins. The predicted chiffon protein was found to be related to a group of known or predicted proteins: *S. cerevisiae* Dbf4, *S. pombe* Dfp1 (which is the *S. pombe* homolog of *S. cerevisiae* Dbf4), *Aspergillus nidulans* NimO protein, SRAD35 (which stands for 'Similar to RAD35', where RAD35 is another name for Dfp1), and finally the protein predicted by translation of specific human and mouse clones. The protein sequences were aligned, and identity with chiffon highlighted in red, similarities with chiffon in yellow (Fig. 7B). The region of greatest similarity between chiffon and the other proteins was a 44 amino acid residue domain designated CDDN1 (for chiffon, Dbf4, Dfp1 and NimO), (Fig. 7B). In the CDDN1 domain, chiffon was 43% identical to Dbf4, 41% identical to Dfp1, 36% identical to NimO and 32% identical to the human BAC clone translation product. chiffon was also related to the other proteins in an amino terminal region designated CDDN2, although to a lesser extent than for CDDN1. The relative location of the CDDN domains in the various proteins is diagrammed (Fig. 7C).

DISCUSSION

Chorion gene amplification occurs through the repeated firing of one or a small number of origins located near the center of each of the chorion gene clusters. Amplification is expected to utilize all or part of the cell's general DNA replication machinery. In addition, there must be some mechanism making

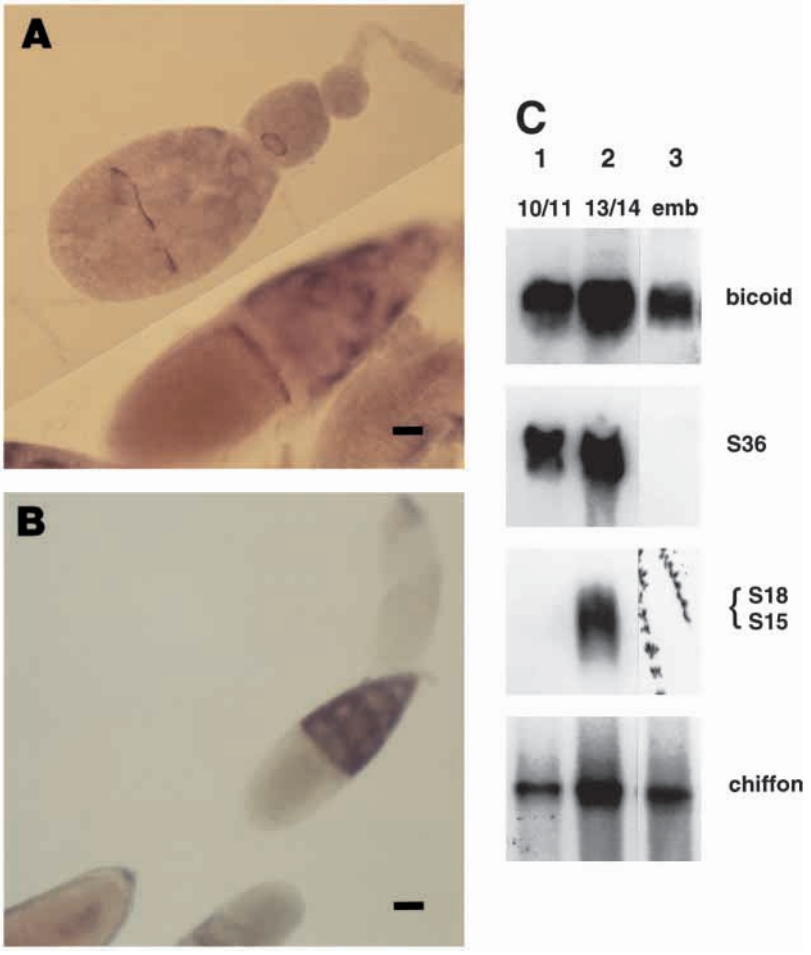
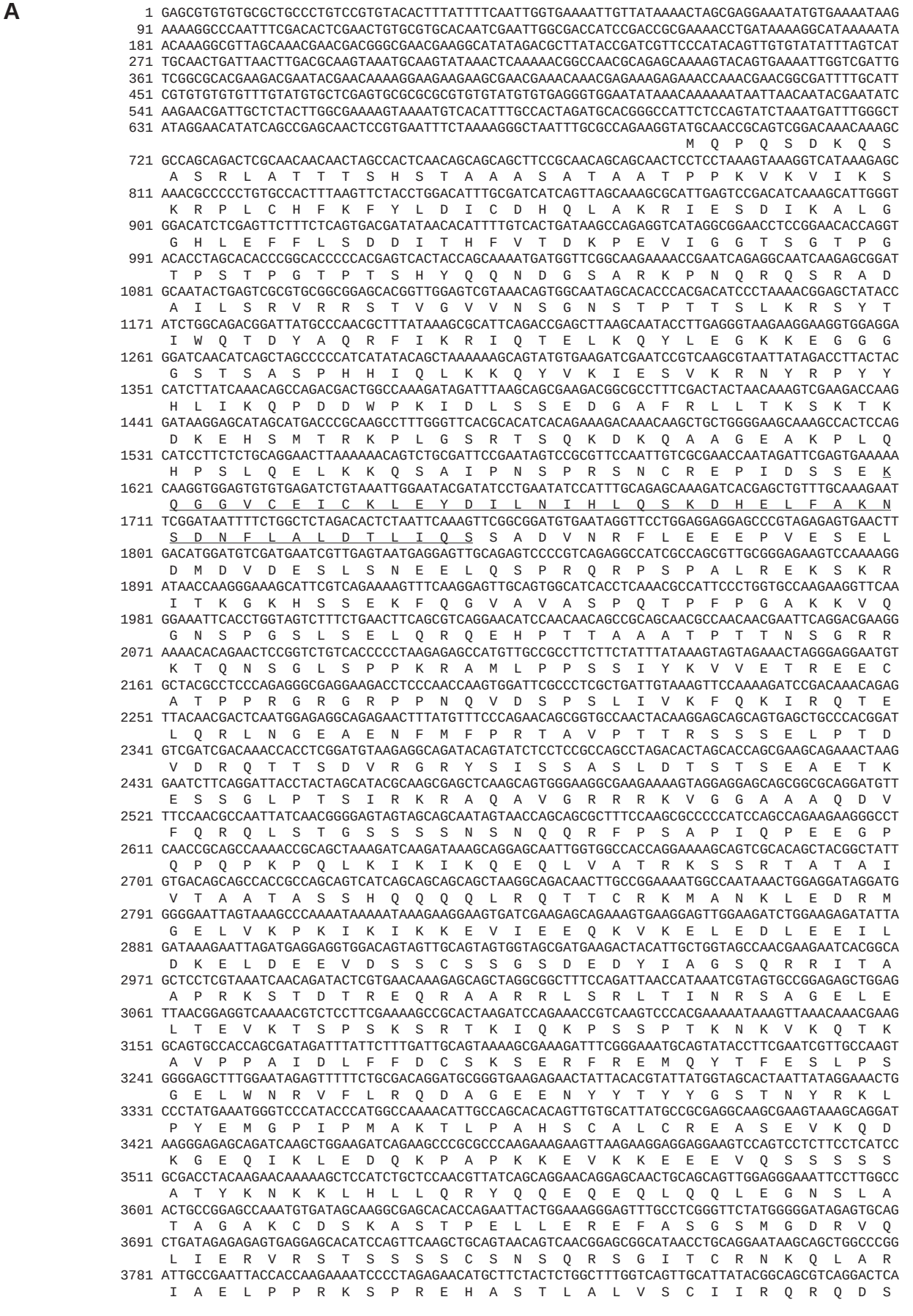


Fig. 6. Maternal supply of *chiffon* RNA to the embryo. (A,B) RNA was visualized in wild-type egg chambers by whole-mount in situ hybridization with probes specific for the maternally supplied control *bicoid* (A), and for *chiffon* (B). The *bicoid* RNA localization pattern was as previously described, with RNA localized in the nurse cells and in a ring at the anterior of the oocyte (St. Johnston et al., 1989). *chiffon* RNA was detected in the nurse cells from approximately stage 10 onwards, and faintly visible in the follicle cell layer in late stage egg chambers. (C) Northern analysis of *chiffon* and control RNAs. Total RNA was isolated from stage 10 and 11 egg chambers (lane 1; 10 μg), stage 13 egg chambers (lane 2; 10 μg), and 0-30 minute embryos (lane 3; 10 μg). The northern blot was hybridized successively with probes specific for the maternally supplied *bicoid* RNA, the follicle-cell-specific chorion gene RNAs S36, S15 and S18, and the *chiffon* RNA, as indicated.

A


1 GAGCGTGTGTGCGCTGCCCTGTCCGTGTACACTTTATTTTCAATTGGTGAAAATTGTTATAAAACTAGCGAGGAAATATGTGAAAATAAG
91 AAAAGGCCCAATTTCGACACTCGAACTGTGCGTGCACAATCGAATTGGCGACCATCCGACCGCGAAAACCTGATAAAAGGCATAAAAATA
181 ACAAAGGCGTTAGCAAACGAACGACGGGCGAACGAAGGCATATAGACGCTTATACCGATCGTTCCCATACAGTTGTGTATATTTAGTCAT
271 TGCAACTGATTAACTTGACGCAAGTAAATGCAAGTATAAACTCAAAAACGGCCAACGCAGAGCAAAAGTACAGTGAAAATTGGTCGATTG
361 TCGGCGCACGAAGACGAATACGAACAAAAGGAAGAAGAAGCGAACGAAACAAACGAGAAAGAGAAACCAAACGAACGGCGATTTTGCATT
451 CGTGTGTGTGTTTGTATGTGCTCGAGTGCGCGCGCGTGTGTATGTGTGAGGGTGGAATATAAACAAAAAATAATTAACAATACGAATATC
541 AAGAACGATTGCTCTACTTGGCGAAAAGTAAAATGTCACATTTGCCACTAGATGCACGGGCCATTCTCCAGTATCTAAATGATTTGGGCT
631 ATAGGAACATATCAGCCGAGCAACTCCGTGAATTTCTAAAAGGGCTAATTTGCGCCAGAAGGTATGCAACCGCAGTCGGACAAACAAAGC
M Q P Q S D K Q S
721 GCCAGCAGACTCGCAACAACAACTAGCCACTCAACAGCAGCAGCTTCCGCAACAGCAGCAACTCCTCCTAAAGTAAAGGTCATAAAGAGC
A S R L A T T T S H S T A A A S A T A A T P P K V K V I K S
811 AAACGCCCCCTGTGCCACTTTAAGTTCTACCTGGACATTTGCGATCATCAGTTAGCAAAGCGCATTGAGTCCGACATCAAAGCATTGGGT
K R P L C H F K F Y L D I C D H Q L A K R I E S D I K A L G
901 GGACATCTCGAGTTCTTTCTCAGTGACGATATAACACATTTTGTCACTGATAAGCCAGAGGTCATAGGCGGAACCTCCGGAACACCAGGT
G H L E F F L S D D I T H F V T D K P E V I G G T S G T P G
991 ACACCTAGCACACCCGGCACCCCCACGAGTCACTACCAGCAAAATGATGGTTCGGCAAGAAAACCGAATCAGAGGCAATCAAGAGCGGAT
T P S T P G T P T S H Y Q Q N D G S A R K P N Q R Q S R A D
1081 GCAATACTGAGTCGCGTGCGGCGGAGCACGGTTGGAGTCGTAAACAGTGGCAATAGCACACCCACGACATCCCTAAAACGGAGCTATACC
A I L S R V R R S T V G V V N S G N S T P T T S L K R S Y T
1171 ATCTGGCAGACGGATTATGCCCAACGCTTTATAAAGCGCATTCAGACCGAGCTTAAGCAATACCTTGAGGGTAAGAAGGAAGGTGGAGGA
I W Q T D Y A Q R F I K R I Q T E L K Q Y L E G K K E G G G
1261 GGATCAACATCAGCTAGCCCCCATCATATACAGCTAAAAAAGCAGTATGTGAAGATCGAATCCGTCAAGCGTAATTATAGACCTTACTAC
G S T S A S P H H I Q L K K Q Y V K I E S V K R N Y R P Y Y
1351 CATCTTATCAAACAGCCAGACGACTGGCCAAAGATAGATTTAAGCAGCGAAGACGGCGCCTTTCGACTACTAACAAAGTCGAAGACCAAG
H L I K Q P D D W P K I D L S S E D G A F R L L T K S K T K
1441 GATAAGGAGCATAGCATGACCCGCAAGCCTTTGGGTTCACGCACATCACAGAAAGACAAACAAGCTGCTGGGGAAGCAAAGCCACTCCAG
D K E H S M T R K P L G S R T S Q K D K Q A A G E A K P L Q
1531 CATCCTTCTCTGCAGGAACTTAAAAAACAGTCTGCGATTCCGAATAGTCCGCGTTCCAATTGTCGCGAACCAATAGATTCGAGTGAAAAA
H P S L Q E L K K Q S A I P N S P R S N C R E P I D S S E K
1621 CAAGGTGGAGTGTGTGAGATCTGTAAATTGGAATACGATATCCTGAATATCCATTTGCAGAGCAAAGATCACGAGCTGTTTGCAAAGAAT
Q G G V C E I C K L E Y D I L N I H L Q S K D H E L F A K N
1711 TCGGATAATTTTCTGGCTCTAGACACTCTAATTCAAAGTTCGGCGGATGTGAATAGGTTCCTGGAGGAGGAGCCCGTAGAGAGTGAACTT
S D N F L A L D T L I Q S S A D V N R F L E E E P V E S E L
1801 GACATGGATGTCGATGAATCGTTGAGTAATGAGGAGTTGCAGAGTCCCCGTCAGAGGCCATCGCCAGCGTTGCGGGAGAAGTCCAAAAGG
D M D V D E S L S N E E L Q S P R Q R P S P A L R E K S K R
1891 ATAACCAAGGGAAAGCATTCGTCAGAAAAGTTTCAAGGAGTTGCAGTGGCATCACCTCAAACGCCATTCCCTGGTGCCAAGAAGGTTCAA
I T K G K H S S E K F Q G V A V A S P Q T P F P G A K K V Q
1981 GGAAATTCACCTGGTAGTCTTTCTGAACTTCAGCGTCAGGAACATCCAACAACAGCCGCAGCAACGCCAACAACGAATTCAGGACGAAGG
G N S P G S L S E L Q R Q E H P T T A A A T P T T N S G R R
2071 AAAACACAGAACTCCGGTCTGTCACCCCCTAAGAGAGCCATGTTGCCGCCTTCTTCTATTTATAAAGTAGTAGAAACTAGGGAGGAATGT
K T Q N S G L S P P K R A M L P P S S I Y K V V E T R E E C
2161 GCTACGCCTCCCAGAGGGCGAGGAAGACCTCCCAACCAAGTGGATTCGCCCTCGCTGATTGTAAAGTTCCAAAAGATCCGACAAACAGAG
A T P P R G R G R P P N Q V D S P S L I V K F Q K I R Q T E
2251 TTACAACGACTCAATGGAGAGGCAGAGAACTTTATGTTTCCCAGAACAGCGGTGCCAACTACAAGGAGCAGCAGTGAGCTGCCCACGGAT
L Q R L N G E A E N F M F P R T A V P T T R S S S E L P T D
2341 GTCGATCGACAAACCACCTCGGATGTAAGAGGCAGATACAGTATCTCCTCCGCCAGCCTAGACACTAGCACCAGCGAAGCAGAAACTAAG
V D R Q T T S D V R G R Y S I S S A S L D T S T S E A E T K
2431 GAATCTTCAGGATTACCTACTAGCATACGCAAGCGAGCTCAAGCAGTGGGAAGGCGAAGAAAAGTAGGAGGAGCAGCGGCGCAGGATGTT
E S S G L P T S I R K R A Q A V G R R R K V G G A A A Q D V
2521 TTCCAACGCCAATTATCAACGGGGAGTAGTAGCAGCAATAGTAACCAGCAGCGCTTTCCAAGCGCCCCCATCCAGCCAGAAGAAGGGCCT
F Q R Q L S T G S S S S N S N Q Q R F P S A P I Q P E E G P
2611 CAACCGCAGCCAAAACCGCAGCTAAAGATCAAGATAAAGCAGGAGCAATTGGTGGCCACCAGGAAAAGCAGTCGCACAGCTACGGCTATT
Q P Q P K P Q L K I K I K Q E Q L V A T R K S S R T A T A I
2701 GTGACAGCAGCCACCGCCAGCAGTCATCAGCAGCAGCAGCTAAGGCAGACAACTTGCCGGAAAATGGCCAATAAACTGGAGGATAGGATG
V T A A T A S S H Q Q Q Q L R Q T T C R K M A N K L E D R M
2791 GGGGAATTAGTAAAGCCCAAAATAAAAATAAAGAAGGAAGTGATCGAAGAGCAGAAAGTGAAGGAGTTGGAAGATCTGGAAGAGATATTA
G E L V K P K I K I K K E V I E E Q K V K E L E D L E E I L
2881 GATAAAGAATTAGATGAGGAGGTGGACAGTAGTTGCAGTAGTGGTAGCGATGAAGACTACATTGCTGGTAGCCAACGAAGAATCACGGCA
D K E L D E E V D S S C S S G S D E D Y I A G S Q R R I T A
2971 GCTCCTCGTAAATCAACAGATACTCGTGAACAAAGAGCAGCTAGGCGGCTTTCCAGATTAACCATAAATCGTAGTGCCGGAGAGCTGGAG
A P R K S T D T R E Q R A A R R L S R L T I N R S A G E L E
3061 TTAACGGAGGTCAAAACGTCTCCTTCGAAAAGCCGCACTAAGATCCAGAAACCGTCAAGTCCCACGAAAAATAAAGTTAAACAAACGAAG
L T E V K T S P S K S R T K I Q K P S S P T K N K V K Q T K
3151 GCAGTGCCACCAGCGATAGATTTATTCTTTGATTGCAGTAAAAGCGAAAGATTTCGGGAAATGCAGTATACCTTCGAATCGTTGCCAAGT
A V P P A I D L F F D C S K S E R F R E M Q Y T F E S L P S
3241 GGGGAGCTTTGGAATAGAGTTTTTCTGCGACAGGATGCGGGTGAAGAGAACTATTACACGTATTATGGTAGCACTAATTATAGGAAACTG
G E L W N R V F L R Q D A G E E N Y Y T Y Y G S T N Y R K L
3331 CCCTATGAAATGGGTCCCATACCCATGGCCAAAACATTGCCAGCACACAGTTGTGCATTATGCCGCGAGGCAAGCGAAGTAAAGCAGGAT
P Y E M G P I P M A K T L P A H S C A L C R E A S E V K Q D
3421 AAGGGAGAGCAGATCAAGCTGGAAGATCAGAAGCCCGCGCCCAAGAAAGAAGTTAAGAAGGAGGAGGAAGTCCAGTCCTCTTCCTCATCC
K G E Q I K L E D Q K P A P K K E V K K E E E V Q S S S S S
3511 GCGACCTACAAGAACAAAAAGCTCCATCTGCTCCAACGTTATCAGCAGGAACAGGAGCAACTGCAGCAGTTGGAGGGAAATTCCTTGGCC
A T Y K N K K L H L L Q R Y Q Q E Q E Q L Q Q L E G N S L A
3601 ACTGCCGGAGCCAAATGTGATAGCAAGGCGAGCACACCAGAATTACTGGAAAGGGAGTTTGCCTCGGGTTCTATGGGGGATAGAGTGCAG
T A G A K C D S K A S T P E L L E R E F A S G S M G D R V Q
3691 CTGATAGAGAGAGTGAGGAGCACATCCAGTTCAAGCTGCAGTAACAGTCAACGGAGCGGCATAACCTGCAGGAATAAGCAGCTGGCCCGG
L I E R V R S T S S S S C S N S Q R S G I T C R N K Q L A R
3781 ATTGCCGAATTACCACCAAGAAAATCCCCTAGAGAACATGCTTCTACTCTGGCTTTGGTCAGTTGCATTATACGGCAGCGTCAGGACTCA
I A E L P P R K S P R E H A S T L A L V S C I I R Q R Q D S

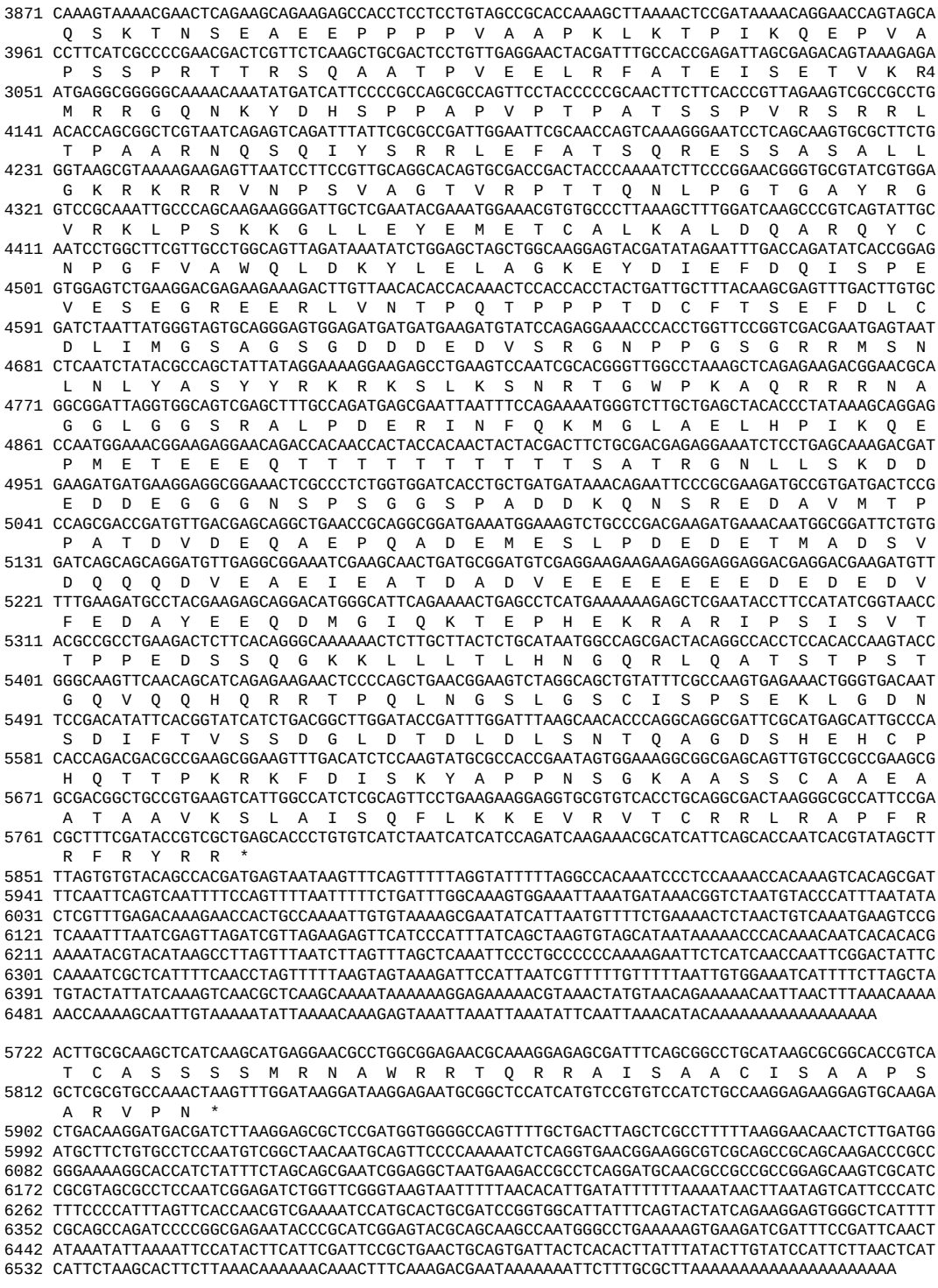


Fig. 7A. For legend see p. 4291.

this process specific to the follicle cells and to only the origins associated with the chorion gene clusters.

Twelve genes have been identified that have mutant alleles affecting amplification (Calvi and Spradling, 1998; Royzman et al., 1999). The first of these genes to be cloned, *k43*, was found to encode the *Drosophila* homolog of *S. cerevisiae* Origin Recognition Complex (ORC) subunit #2 (Orc2) (Gossen et al., 1995; Landis et al., 1997). In *S. cerevisiae* ORC is required for origin activity, however activation of the origin requires binding of additional proteins prior to S phase. Firing of the *S. cerevisiae* origin in S phase also requires the activity of the Cdc7-Dbf4 protein kinase. Cdc7 is a cdk-like serine/threonine protein kinase whose activity requires binding of the regulatory subunit Dbf4 (Jackson et al., 1993). Dbf4 binds to proteins associated with the origin, thus providing a likely mechanism for recruitment of Cdc7 to the origin (Dowell

B

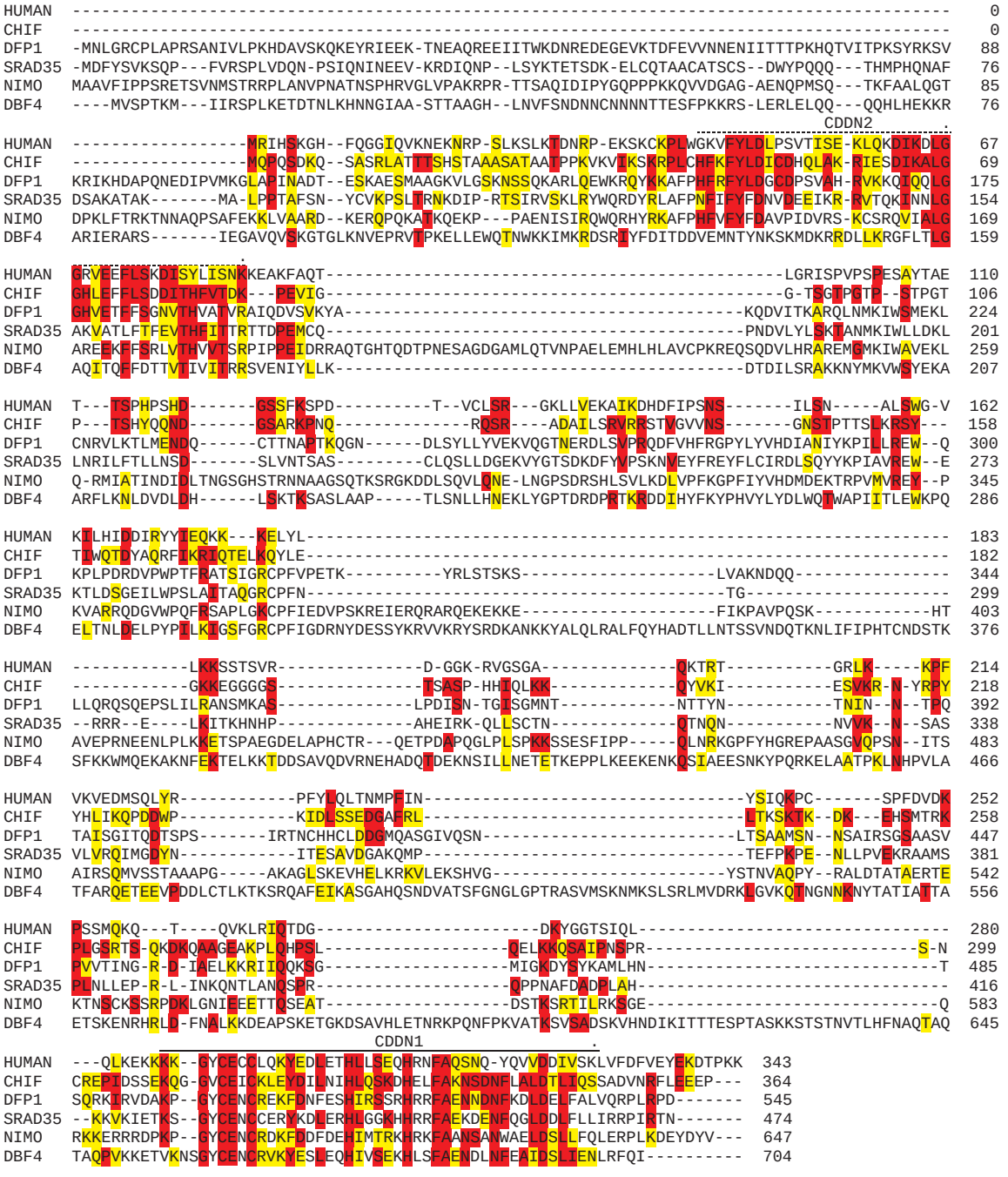


Fig. 7B. For legend see opposite page.

et al., 1994). The Mcm2-7 proteins are a family of six highly conserved proteins that associate with the ORC origin complex prior to S phase and which are required for initiation of DNA synthesis. Cdc7-Dbf4 phosphorylates a subset of the Mcm proteins in vivo and in vitro suggesting that this may be part of the mechanism by which Cdc7-Dbf4 activates the origin (Lei et al., 1997).

Cdc7 homologs have now been characterized and cloned from *S. pombe*, *Xenopus laevis*, mouse and human (Brown and Kelly, 1998; Hess et al., 1998; Jiang and Hunter, 1997; Kim et al., 1998; Sato et al., 1997). In addition, we have recently cloned a Cdc7-homologous gene from *Drosophila melanogaster* (unpublished data). This conservation suggests that regulation of DNA replication origin firing by Cdc7-family protein kinases is conserved through eukaryotic evolution. The cloning of the *chiffon* gene suggests that function of the regulatory subunit of the Cdc7 protein kinase may also be conserved.

The predicted chiffon protein was found to contain two domains, designated CDDN1 and CDDN2, which were also found in *S. cerevisiae* Dbf4. The CDDN domains were also found in *S. pombe* Dfp1 protein, which is the *S. pombe* homolog of Dbf4 (Brown and Kelly, 1998), as well as in an *S. pombe* gene highly related to Dfp1 (called ‘similar to

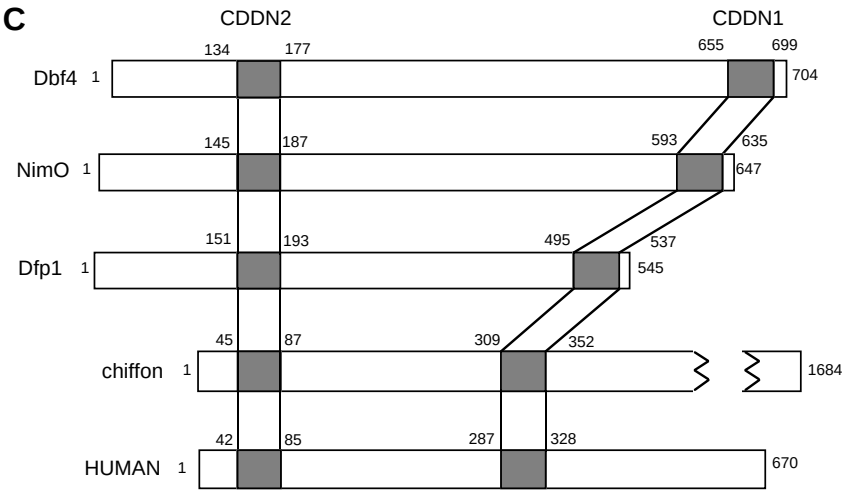


Fig. 7. *chiffon* cDNA sequence and predicted protein domains. (A) The cDNA sequence of *chiffon*, compiled from several overlapping cDNAs, as diagrammed in Fig. 5B. The composite sequence includes two alternatively spliced transcripts, as indicated. The putative start codon is at position 692-694, and the termination codons are indicated by asterisks. The regions of greatest similarity to yeast Dbf4 protein and other predicted proteins, designated CDDN1 and CDDN2 are indicated, by solid and dashed lines, respectively. *chiffon* potentially encodes two alternate open reading frames, one 5.085 kb and the other 5.133 kb, encoding proteins of 1695 and 1711 amino acid residues, respectively. The donor splice site g at position 5772 is outlined and underlined. (B) Protein sequence alignment of chiffon and related proteins. Residues identical to chiffon are outlined in red, conservative residues in yellow. The conserved CDDN1 and CDDN2 domains are indicated by solid and dashed lines, respectively. (C) Schematic representation of the Dbf4, NimO, Dfp1, chiffon and human proteins, with regions of similarity (CDDN1 and CDDN2) between chiffon and the other proteins indicated. Only regions of similarity with chiffon are diagrammed. Dbf4 sequence, NCBI accession #X60279 (gene sequence) and #g3643 (translated ORF). Dfp1 sequence, GenBank accession #AF110398 (Brown and Kelley, 1998). nimO sequence, GenBank accession #AF014812 (James et al., 1999). The human sequence was generated by combining two GenBank sequences. First, BAC clone 'RG135C18', GenBank accession #AC005164 (J. Kellen and J. Burkhart, Direct submission, unpublished). Our analysis identified an additional exon within the 3′ region of the 'RG135C18' BAC clone encoding a region homologous to the chiffon CDDN2 domain, as shown. Second, the 5′ end of the human sequence was obtained from an overlapping 'EST41568', accession #AA336887 (Adams et al., 1995). Finally, query of NCBI databases with the predicted human protein sequence identifies numerous mouse cDNA and EST clones, all apparently derived from a single gene, which encode a predicted protein containing the conserved CDDN1 and CDDN2 domains (data not shown).

rad35/dfp1'; K. Oliver et al., direct submission to GenBank, accession #AL049489.1). The CDDN domains were also found in the predicted translation product of the *Aspergillus nimO* gene. While this manuscript was in review, the *nimO* gene was reported to be required for DNA synthesis and mitotic checkpoint control in *Aspergillus* (James et al., 1999). That study points out the homology between nimO protein and Dbf4 in the CDDN1 region, and makes the interesting observation that the structure of the CDDN1 region is consistent with a single Cys_2-His_2 zinc finger-like motif with a short central loop of 9 bp. Finally, the CDDN domains were also found in a predicted human protein of unknown function and in several mouse cDNAs of unknown function. The data suggest a family of eukaryotic proteins related to Dbf4 and involved in initiation of DNA replication.

The similarity between chiffon and Dbf4 and Dfp1 suggests a model for chiffon's role in amplification: chiffon may function in the activation of the chorion gene origins as the regulatory subunit of a kinase involved in origin firing, most likely the *Drosophila* homolog of Cdc7. *S. cerevisiae* Dbf4 contacts Cdc7 through the carboxyl terminus where the CDDN1 domain is located. Thus we hypothesize that, analogous to Dbf4 function in *S. cerevisiae*, chiffon may contact the *Drosophila* Cdc7 homolog through the conserved CDDN1 domain and recruit it to the ORC via conserved ORC contact sites in the chiffon amino terminus, perhaps the CDDN2 domain. chiffon could also be hypothesized to recruit other, as yet unidentified proteins to the origin. Finally, we cannot at this time rule out alternative and less direct models for chiffon function during amplification.

In addition to the defect in chorion gene amplification, the *chiffon* null phenotype also includes rough eyes and thin thoracic bristles. While there are several possibilities for how *chiffon* might be required for normal eye and bristle development, these phenotypes are consistent with a defect in DNA replication and/or S phase control in the cells forming these structures. For example, *roughex* regulates cyclin levels and entry into S phase, and *roughex* mutants are viable with rough eyes similar to *chiffon* nulls (Thomas et al., 1997). *morula* is a regulator of mitotic and endo- cell cycles and hypomorphic *morula* mutants have rough eyes and thin thoracic bristles similar to *chiffon* null mutants (Reed and Orr-Weaver, 1997). Finally, specific hypomorphic mutations in either the *dDP* or *dE2F* subunits of the *Drosophila* cell cycle regulator E2F cause rough eyes, thin thoracic bristles and defective chorion gene amplification nearly identical to *chiffon* nulls (Royzman et al., 1997, 1999). Thus, *Drosophila* chorion gene amplification, eye development and thoracic bristle development appear to be processes that are particularly sensitive to defects in the cell cycle/DNA replication machinery.

There are a number of possibilities as to how *chiffon* might function in S phase regulatory pathways with the above-mentioned and/or other cell cycle and S phase regulators. Identification of proteins that interact with *chiffon* in vivo will begin to test these models and should facilitate the dissection of the mechanism(s) regulating chorion gene amplification. Identification of proteins that interact with the evolutionarily conserved CDDN domains of chiffon, using techniques such as yeast two-hybrid system, should be particularly informative.

The *chiffon* null phenotype demonstrates that *chiffon* is required for chorion gene amplification and normal eye and bristle development. *chiffon* might therefore be a tissue-specific regulator of DNA replication and/or cell cycle. However, it remains possible that *chiffon* might be required in additional tissues, or even in every cell for DNA replication and/or cell

cycle control. Because wild-type *chiffon* RNA is maternally supplied to *chiffon* mutant embryos, wild-type chiffon protein might persist through embryonic and larval development and mask a more general requirement for *chiffon* function. Additional experiments, such as analysis of *chiffon* mutant germline clones will be required to address these questions.

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