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Completion of meiosis in *Drosophila* oocytes requires transcriptional control by Grauzone, a new zinc finger protein

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SUMMARY

Mutations in *grauzone* or *cortex* cause abnormal arrest in *Drosophila* female meiosis. We cloned *grauzone* and identified it as a C2H2-type zinc finger transcription factor. The *grauzone* transcript is present in ovaries and at later developmental stages. A Grauzone-GFP fusion protein is functional and localizes to nuclei of both nurse cells and follicle cells during oogenesis. Three lines of evidence indicate that *grauzone* and *cortex* interact: reducing *cortex* function enhanced the *grauzone* mutant phenotype; *cortex* transcript abundance is reduced in the absence of *grauzone* function and Grauzone protein binds to the *cortex* promoter. These results demonstrate that activation of *cortex* transcription by *grauzone* is necessary for the completion of meiosis in *Drosophila* oocytes, and establish a new pathway that specifically regulates the female meiotic cell cycle.

Key words: *grauzone*, *cortex*, Meiosis, *Drosophila*, Oocyte

INTRODUCTION

Meiosis is a specialized cell division that is essential for sexual reproduction. In this process, the germ cells produce haploid gametes, allowing the diploid number to be maintained after fertilization. Meiosis consists of a single round of DNA replication followed by two nuclear divisions. The first meiotic division results in pairing and segregation of chromosome homologs. A second division then ensues without DNA synthesis, in which the sister chromatids segregate producing haploid gametes. This second division, apart from occurring without DNA synthesis, thus has the hallmarks of a mitotic division.

In animals, meiosis takes place in both spermatocytes and oocytes. In many respects, the process is similar in both germ lines, since the fundamental goal is the same. However, there are some striking differences in the male and female processes. Meiosis is a continuous process in the male germ line. In the female germ line, meiosis is usually interrupted at prophase of the first meiotic division to allow for oocyte growth and the oocyte remains arrested at this stage until activated by an external signal. The mature egg then often arrests again, usually at metaphase I or metaphase II, until fertilization or some other signal triggers meiotic completion and further development.

Although certain signaling mechanisms that arrest and restart the female meiotic cell cycle during development are understood, other details of how the oocyte progresses through meiosis remain obscure. Functions have been uncovered in genetic screens that are required for the proper progression of meiosis. Two *Drosophila* female-sterile mutants, *grauzone* (*grau*) and *cortex* (*cort*), were identified that arrest the meiotic cell cycle inappropriately (Schüpbach and Wieschaus, 1989; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). A wild-type mature *Drosophila* oocyte arrests in metaphase I after an initial arrest in prophase I to allow for oocyte growth. The metaphase I arrest is released when the egg passes through the oviduct into the uterus and the activated egg completes two meiotic divisions without delay. In eggs from *grau* or *cort* homozygous females (referred to subsequently as *grau* or *cort* mutant eggs or embryos), early meiotic events up to metaphase I arrest appear to be normal. The first observable defect is unequal chromosome segregation in meiosis I and meiosis arrests erroneously at metaphase II. The chromatids on the meiosis II spindles can sometimes separate from each other, but the eggs fail to exit from meiosis II (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). The aberrant meiotic arrest cannot be rescued by a premature release of sister chromatid cohesion, suggesting that it is not caused by sustained cohesion (Page and Orr-Weaver, 1996). Since mitosis and male meiosis do not seem to be affected by these two mutants, *grau* and *cort* functions appear to be required specifically for female meiosis.

In addition to blocking the progression of the meiotic cell cycle, mutations in *grau* and *cort* cause other defects (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Bashirullah et al., 1999). Cortical microtubules in wild-type stage 14 oocytes exist in a long fibrillar state, which quickly disassemble at egg activation (Theurkauf and Hawley, 1992). In contrast, cortical microtubules in laid *grau* or *cort* eggs remain in a preactivated state. Bicoid protein, which is normally

produced during the first hour of embryogenesis, fails to be translated in the fertilized *grau* and *cort* embryos. The impaired translation is due to a defect in cytoplasmic polyadenylation of *bicoid* mRNA, a process required for the translation of Bicoid (Sallés et al., 1994; Lieberfarb et al., 1996). Finally, the stability of certain maternal mRNAs is enhanced in *grau* and *cort* eggs and embryos (Bashirullah et al., 1999).

Among the many cell cycle regulating genes that have been described, *grau* and *cort* are the only two genes known for which mutation causes an arrest in metaphase II. Therefore, the molecular characterization of *grau* and *cort* might elucidate new aspects of meiotic cell cycle progression specific to the female germ line. In this paper, we report the cloning of *grau*. *grau* encodes a new member of the C2H2-type zinc finger protein family and exerts its effect on meiosis by regulating the transcription of *cort*. These genes thus constitute two members of a pathway that control exit from the second meiotic division. Given the similarity between meiosis II and the mitotic cell division, analysis of this pathway might shed light not only on the metaphase-anaphase transition in meiosis, but also aspects of cell cycle regulation in mitosis.

MATERIALS AND METHODS

Fly stocks and mapping

The five *grau* alleles, *RM61*, *QF31*, *QE70*, *QQ36* and *RG1*, and two *cort* alleles, *QW55* and *RH65*, were generated by Schüpbach and Wieschaus (1989) in an EMS screen for female-sterile loci on the second chromosome and were obtained from T. Schüpbach. The deficiency chromosomes (used for mapping) were obtained as follows: *Df(2R)XE-916* from Todd Laverty; *Df(2R)l17s* from Elizabeth Underwood; *Df(2R)D4*, *Df(2R)C4* and *Df(2R)E2* from Rachel Kraut; *Df(2R)R1-8*, *Df(2R)X58-5*, *Df(2R)X58-7*, *Df(2R)X58-8*, *Df(2R)X58-9*, and *Df(2R)X58-11* from Terry Orr-Weaver. All the other fly stocks were obtained from the Bloomington stock center.

grau was mapped using the male-recombination method (Chen et al., 1998).

Analysis of genetic interaction between *grau* and *cort*

The $cort^{QW55}$ *cn* $grau^{RM61}$ *bw*, $cort^{QW55}$ *cn* $grau^{QF31}$ *bw*, $cort^{RH65}$ *cn* $grau^{RM61}$ *bw* and $cort^{RH65}$ *cn* $grau^{QF31}$ *bw* doubly mutant chromosomes were constructed from the chromosomes $cort^{QW55}$ *cn bw*, $cort^{RH65}$ *cn bw*, *cn* $grau^{RM61}$ *bw* and *cn* $grau^{QF31}$ *bw*. Putative recombinant lines were tested for the presence of *cort* and *grau* by complementation with the original *cort* chromosomes and *Df(2R)Pu-D17*, a deficiency that uncovers the *grau* locus.

Females of different genotypes (Table 1) were crossed to *Canton-S* male flies and maintained on apple juice plates for 3 days. Embryos were collected over the course of 2 hours and aged for 1-2 hours at room temperature. They were dechorionated in 50% Clorox bleach, devitellinized, fixed in methanol and rehydrated by standard methods (Theurkauf, 1994). The embryos were stained with 1 μg/ml DAPI (Sigma) in PBST (130 mM NaCl, 70 mM Na_2HPO_4, 35 mM NaH_2PO_4 and 0.1% Triton X-100) for 20 minutes, briefly washed in PBST, mounted in 70% glycerol, 30% PBS and examined immediately with a Nikon microscope with a DAPI filter. Embryos with mitotically dividing nuclei were scored as developing embryos.

P-element-mediated transformation and rescue

The rescue constructs were generated by subcloning fragments (Fig. 2A,B) from the P1 clone Ds02397 into the *CaSpeR* transformation vector. Injections were performed by using the helper plasmid *plChsπΔ2-3* (Spradling, 1986). Plasmid DNA at 0.4 mg/ml was coinjected with 0.1 mg/ml of helper plasmid into embryos from the *yw* strain. Up to ten independent lines were established for each construct. Transformed flies were crossed to the *grau* mutant flies. Homozygous *grau* female flies with or without the transgene were crossed to *Canton-S* male flies for the fertility test.

Molecular techniques

Poly(A) selected RNA was isolated using the poly(A)pure mRNA isolation kit, according to the manufacturer's instructions (Ambion). RNA samples were analyzed by electrophoresis, transferred and hybridized according to standard protocols using ^{32}P-labelled *grau* antisense RNA probe, *S-29* cDNA probe, *cort* genomic DNA and *cup* genomic DNA probe.

grau cDNA was isolated by 5′ RACE and 3′ RACE reactions according to Zhang and Frohman (1997).

Sequencing of the wild-type and the mutant DNA was done by automated sequencing (Applied Biosystems). Sequences were assembled and analyzed by use of the GCG programs (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI).

Grau-GFP construct

The *grau-GFP* fusion was generated from the 4.3 kb *CaSpeR* rescue construct. A *GFP* fragment was amplified from a *GFP-pKS(+)* clone (kindly provided by Thomas Kornberg, UCSF) by PCR using primers containing a *Sma*I site: 5′-CCCGGGATGAGTAAAG-GAGAAGAACTTTTCACT-3′ and 5′-CCCGGGCTATTTGTATAGT-TCATCCATGCCATG-3′. The PCR product was cloned into a TA cloning vector (Invitrogen, San Diego, CA). Plasmid DNA was prepared, digested with *Sma*I, and the *GFP* fragment was gel-isolated. A *grau* fragment was PCR amplified using primers: 5′-TTCCGCAAATCGATTTCCTTAAGCATTTCCCGGGCCTACCCG-ATTAG-3′ and 5′-TGCCAACATGCACTCCCACAAG-3′ and cloned into a TA cloning vector. This generates a *Sma*I site at the *grau* stop codon. The plasmid DNA was prepared, digested with *Sma*I enzyme, dephosphorylated and ligated to the *Sma*I-*GFP*-*Sma*I fragment. The ligation mixture was transformed into bacteria and plasmid DNA was isolated. The orientation of the *GFP* inserted into the *grau* fragment was confirmed using PCR with different combinations of primers. The correct clones contained a *GFP*-coding region fused in frame with *grau* at the original *grau* stop codon. The plasmid DNA was digested with *Cla*I and *Nsi*I, generating a *Nsi*I-*grau*-*GFP*-*Cla*I fragment. The *Xho*I-*Pst*I *grau* fragment from the 4.3 kb-*CaSpeR* construct was subcloned into a *pKS(+)* vector (Stratagene). The resulting plasmid was digested with *Cla*I and *Nsi*I, and ligated with *Nsi*I-*grau*-*GFP*-*Cla*I fragment. The selected *Xho*I-*Nsi*I-*grau*-*GFP*-*Cla*I-*Pst*I (referred to as *Xho*I-*grau*-*GFP*-*Pst*I) clones contained the C-terminal part of Grau protein fused with GFP and the *grau* 3′ regulatory sequences. The *Xho*I-*grau*-*GFP*-*Pst*I clones were digested with *Xho*I and *Pst*I. The *Xho*I-*grau*-*GFP*-*Pst*I fragment was gel purified and ligated to the 4.3 kb-*CaSpeR* DNA predigested with *Xho*I and *Pst*I. After transformation, the correct clones were selected that contained full-length *grau*-*GFP* fusion under the control of *grau* endogenous regulatory region in *CaSpeR* vector.

Microscopy

Imaging Grau-GFP in living egg chambers was done as described (Theurkauf and Hazelrigg, 1998).

Construction and purification of GST-Grau fusion protein:

The full-length *grau* coding region was amplified from a wild-type ovarian cDNA pool using the Expand Long Template PCR System (Boehringer Mannheim). The amplification was performed using the 5′ primer, 5′-CATGAATTGAATGGATATCTGCCGCCTCTG-3′, and the 3′ primer, 5′-CATGAATTCTTCCGGGCCTAACCCGAATAG-3′, both of which contain *Eco*RI restriction sites at their ends. The PCR product was digested with *Eco*RI, and inserted into *Eco*RI digested *pGEX-3X* vector (Amersham Pharmacia Biotech), forming a *GST-grau* fusion. The *GST-grau* fusion plasmid was sequenced by automated

sequencing (Applied Biosystems) to confirm that the fusion was in the correct reading frame and lacked PCR-induced mutations.

The GST-Grau fusion protein was expressed in *E. coli* BL21 cells according to manufacturer's instructions (Amersham Pharmacia Biotech).

GST-Grau was released from the insoluble fraction by treatment with 8 M urea, followed by dialysis into 0.2 M Tris-HCl, pH 8.0, 0.5 M NaCl, allowing the protein to renature. GST-Grau was then affinity purified on a glutathione sepharose 4B column (Amersham Pharmacia Biotech) according to manufacturer's instructions. Eluted GST-Grau was dialyzed overnight at 4°C against 10 mM PBS. Glycerol was then added to 10%, and the protein was aliquoted and stored at –80°C until use. Similar binding results were obtained with this denatured/renatured preparation and protein derived directly from the soluble fraction of the bacteria.

Gel shift assay

Oligonucleotides corresponding to a 32 bp region of the *cort* promoter were annealed and radiolabelled with [^{32}P]γATP using T4 polynucleotide kinase (New England Biolabs). Nonspecific competitor was a double-stranded oligonucleotide (5′-GCCTGATTT-CCCCGAAATGACGG-3′) containing the IFN-γ-activated site (–129 to –107) from the interferon regulatory factor-1 gene (Gift of NC Reich, University at Stony Brook). Antibodies used were anti-GST antibody (Z-5, Santa Cruz Biotech) and control rabbit IgG (Santa Cruz Biotech).

The gel-shift experiment was performed as described (Kotanides and Reich, 1993). In a 20 μl reaction volume, 1 μg of GST or GST-Grau was incubated in gel shift buffer (12 mM Hepes, pH 7.9, 10% glycerol, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.06 mM EGTA, 0.5 mM DTT) with 2 μg poly(dI-dC) and 0.15 μg non-specific plasmid DNA. The protein was either incubated alone or with the addition of cold competitor DNA (100×) for 10 minutes at room temperature. Reactions to which antibodies were added were incubated at room temperature for 30 minutes. Radiolabelled probe (1 ng) was added, and incubation was continued for an additional 20 minutes at room temperature. Samples were then loaded onto a pre-electrophoresed 4.5% polyacrylamide gel (40:1 acrylamide: bisacrylamide) and electrophoresed in 0.25× TBE at 4°C. The gel was dried briefly and visualized by autoradiography.

RESULTS

Isolation of the *grau* gene

By meiotic mapping, *grau* was positioned at 2-97 on the second chromosome (Schüpbach and Wieschaus, 1989). Complementation tests, using various chromosomal deficiencies, showed that *grau* is uncovered by three deficiencies: *Df(2R)Pu-D17* (57B1-5; 58B), *Df(2R)C4* (57B1; 57E1) (Rachel Kraut, personal communication) and *Df(2R)E2* (57B1; 57B13-14) (Rachel Kraut, personal communication). These results located *grau* between 57B1-5 and 57B13-14 (Fig. 1A).

Using P-element-mediated male-recombination mapping (Chen et al., 1998), we further refined the location of *grau* between two P-elements: *l(2)07806* and *l(2)03050* (Fig. 1B). P1 clone Ds02397 containing the flanking sequences of these two P-elements has been sequenced by the Berkeley *Drosophila* Genome Project (BDGP). From the BDGP database, we determined that the region between these two P-elements is about 48 kb.

We made five rescue constructs that spanned ~85% of the DNA within this region (Fig. 2A). After P-element-mediated transformation (Spradling, 1986), we obtained transformed lines containing each of the constructs. The transformed lines, which had integrated the P-elements in the first or the third chromosome, were crossed into a *grau* mutant background. One of the constructs containing a 13 kb fragment fully rescued the female sterility conferred by the *grau* mutation.

The genomic DNA sequence within this 13 kb fragment was compared to the BDGP EST database. Five ESTs were identified within this fragment (Fig. 2B). Because the *grau* gene is required for the completion of meiosis in female flies, we reasoned that its transcript should be present in ovaries. Using RT-PCR and northern blot analysis, we found that each of these ESTs represents a different gene and transcripts for all five genes are present in the ovaries (data not shown). Six more constructs were made so that each construct contained the DNA for one or two of the genes (Fig. 2B). Again rescue experiments were performed using transgenic lines containing each construct. Three of the constructs were able to fully rescue the *grau* mutant phenotype. The common region shared by these constructs is a 4.3 kb fragment, which encodes a single transcription unit (Fig. 2B). The combined mapping and rescue results indicate that the 4.3 kb fragment corresponds to the *grau* gene.

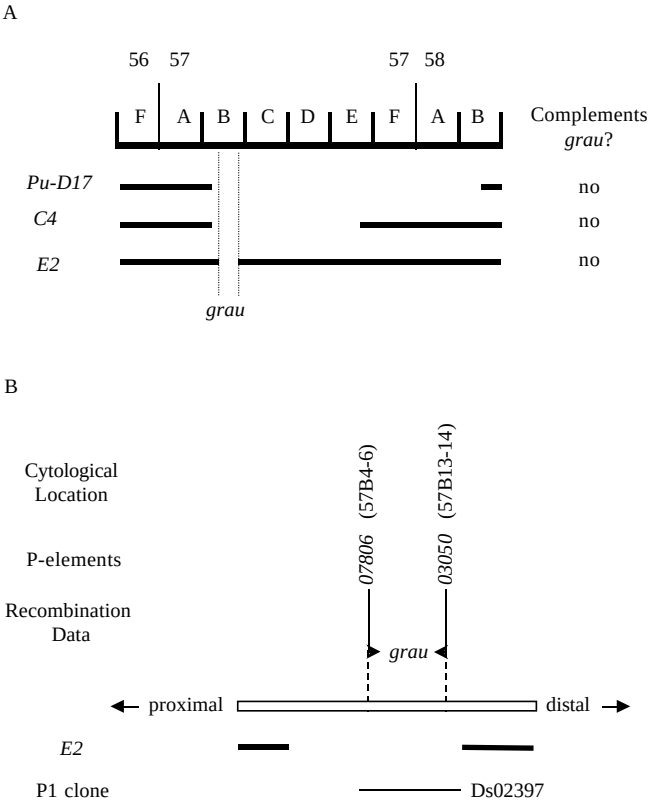

Fig. 1. Mapping of the *grau* gene. (A) Complementation test between *grau* and deficiencies. The *grau* gene is mapped between the proximal and the distal breakpoints of *Df(2R)E2:* 57B1-5; 57B13-14. The black line represents the region not deleted on the chromosome. Empty space between the lines represents the region deleted. (B) By male recombination mapping, the *grau* mutations were mapped between P-elements *l(2)07806* and *l(2)03050*. The insertion sites for these two P-elements are deleted in the deficiency *Df(2R)E2*. The flanking sequences from these two P-elements are contained within the P1 clone Ds02397 (Chen et al., 1998).

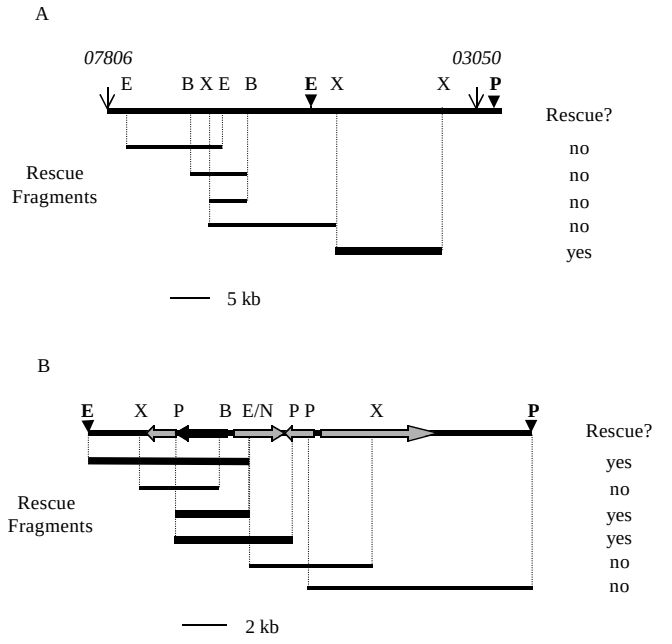

Fig. 2. Identification of the *grau* gene by rescue analysis. (A) Constructs containing different portions of the region between the two P-elements were generated and used to create transgenic flies. One fragment (thicker line) (13 kb) was able to rescue the *grau* mutant phenotype. The location of the two P-elements and the restriction sites used to clone each fragment are shown. The arrowheads indicate the region between the *Eco*RI site and the *Pst*I site that was used to generate more transgenic lines in panel B. Restriction sites are represented as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; X, *Xba*I. (B) The 13 kb *Xba*I-*Xba*I rescuing construct contains five maternally expressed genes (arrows). Constructs containing one or two gene(s) were used to generate transgenic flies. Three fragments were able to fully rescue the *grau* mutant phenotype (thicker lines). The black arrow represents the *grau* transcription unit. The arrowheads indicate the *Eco*RI and *Pst*I sites in A. Restriction sites are represented as follows: B, *Bam*HI; E, *Eco*RI; N, *Nhe*I; P, *Pst*I; X, *Xba*I.

The Grau protein

Part of the *grau* cDNA sequence was available from the BDGP EST project. We cloned the full-length cDNA by performing 5′RACE and 3′RACE (Zhang and Frohman, 1997). The full-length *grau* cDNA contains 1876 nucleotides. The structure of the *grau* gene was determined by comparing the *grau* cDNA sequence with the sequence of genomic clone Ds02397 (Fig. 3A).

The *grau* cDNA contains an open reading frame of 1713 nucleotides encoding a 570 amino acid polypeptide (Fig. 3B), with a predicted molecular mass of 65.9 kDa and a pI of 7.3. The first methionine shown is most likely to be the true N terminus of the protein because there are stop codons in all three open reading frames upstream. Using the GCG program (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI.), we found that the C-terminal half of the Grau

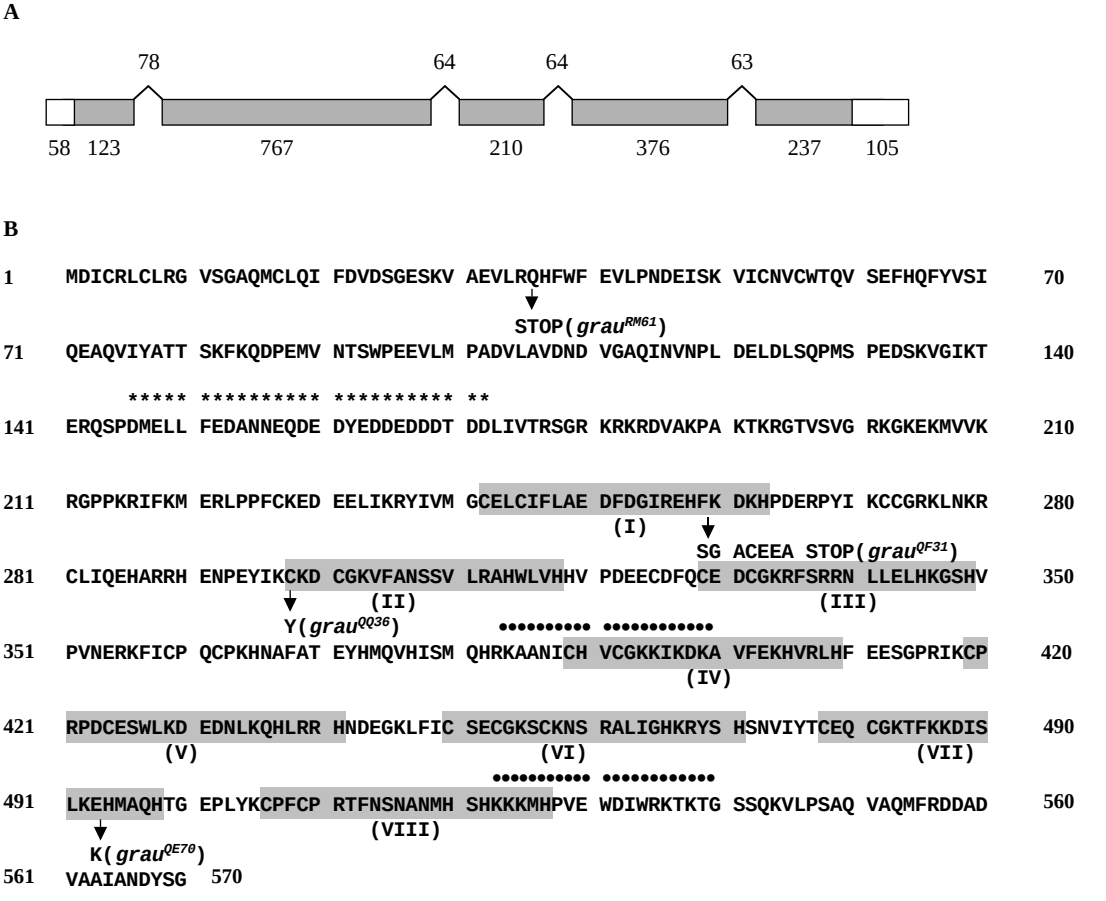

Fig. 3. The *grau* gene. (A) In the structure of the *grau* gene, there are four small introns. The white bars represent the 5′ and 3′ UTR (untranslated region). The gray bars represent the exons, and the lines represent the introns. The numbers indicate the sizes of the UTRs, exons and introns in base pairs. The sequence data will appear in the Genbank database with the accession number AF208016. (B) The deduced Grau protein sequence and the identified mutations. The eight C2H2-type zinc finger motifs are in shaded boxes. The two putative nuclear localization signals are labeled by dots above the amino acids, and the acidic amino acid patch is labeled with asterisks above the amino acids.

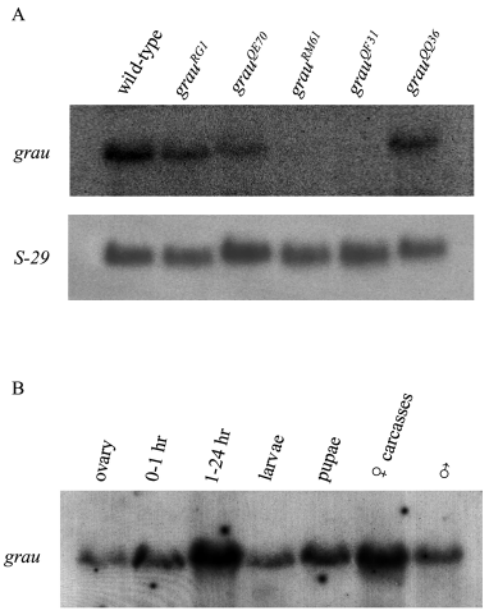

Fig. 4. Expression of *grau* mRNA. (A) Northern analysis of *grau* transcript in wild-type ovaries and in *grau* mutant ovaries. Poly(A)+ RNA from ovaries of wild-type flies and flies hemizygous for different *grau* alleles over *Df(2R)Pu-D17* was probed with 32P-labelled *grau* anti-sense RNA. Only one form of *grau* transcript was detected in the wild-type ovaries. *grau* transcript was undetectable in $grau^{RM61}$ and $grau^{QF31}$, and moderately decreased by $grau^{RG1}$, $grau^{QE70}$ and $grau^{QQ36}$. The proteosome gene *S-29*, which is also deleted by *Df(2R)Pu-D17*, was used as a standard for the amount of mRNA loaded on the gel. (B) Developmental expression of *grau* RNA. Poly(A)$^+$ RNA was isolated from each of the developmental stages indicated, a northern blot was prepared, and probed with ^{32}P-labelled *grau* anti-sense RNA probe (♀ carcasses: adult female bodies with the ovaries dissected out). The *grau* transcript is present during all these stages.

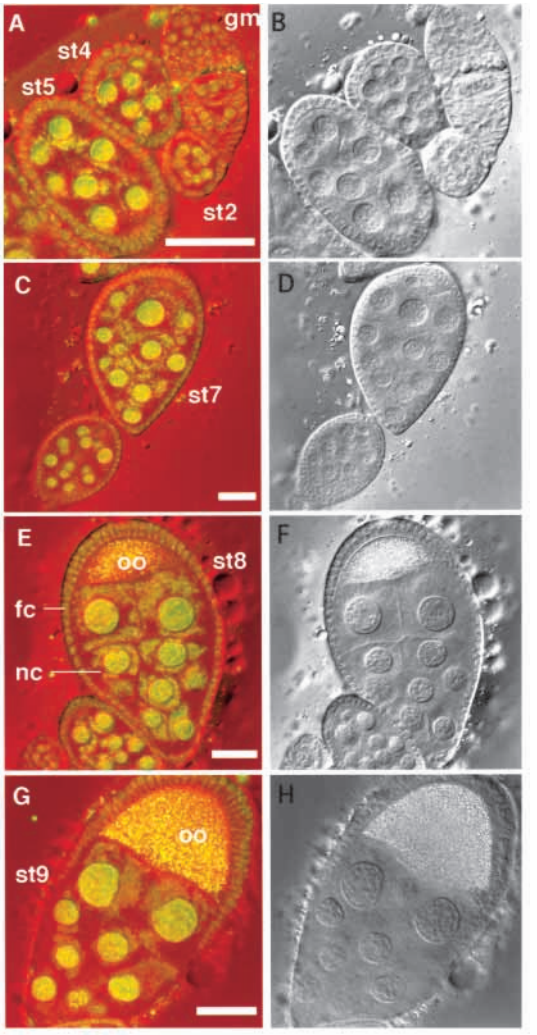

Fig. 5. Grau-GFP localization during oogenesis. Ovaries were isolated from transgenic flies carrying two copies of a *grau-GFP* transgene, teased apart and visualized by confocal-microscopy. Grau-GFP is shown in green and background in red. (A,C,E,G) Fluorescent images; (B,D,F,H) phase-contrast images. (C-H) The posterior end of the egg chamber is up. Grau-GFP was localized to nuclei in germarium cells and in early stage egg chambers (A), in stage 7 (C), stage 8 (E) and stage 9 (G) egg chambers. Expression was observed in both follicle cells (fc) and nurse cells (nc). Very little or no Grau-GFP signal was detected in the oocyte nucleus (data not shown). Bars, 40 μm.

protein consists of eight conserved C2H2-type zinc finger motifs, identifying it as a new member of the C2H2-type zinc finger protein family (Fig. 3B).

Other than the zinc finger motifs, there are several notable features in the Grau protein. First, there is a cluster of acidic residues extending from Asp146 to Asp172 (17 of 27 residues are Asp or Glu). Similar acidic domains have been found in many transcription factors, for example Gal4, and function as transcription activating regions (Ptashne, 1988). Second, there are two predicted bipartite nuclear localization signals within the Grau protein. The first is located from residue Arg382 to Lys399, and the second is from Lys522 to Thr539. Using the PSORT program (Nakai and Kanehisa, 1992), the Grau protein is predicted to be nuclear.

Nature of the *grau* mutant alleles

All five *grau* alleles, $grau^{RM61}$, $grau^{QF31}$, $grau^{QE70}$, $grau^{QQ36}$ and $grau^{RG1}$, were generated by Schüpbach and Wieschaus (1989) in an EMS screen for female-sterile loci on the second chromosome. Eggs from mothers for the five alleles had similar phenotypes when the females were homozygous or hemizygous over a deficiency that deletes *grau*, suggesting that all are null alleles (Page and Orr-Weaver, 1996). To gain additional information regarding the functional domains of the protein, we sequenced all the *grau* mutant alleles. PCR-amplified cDNA and genomic DNA were prepared from flies hemizygous for each *grau* allele, i.e., a *grau* mutant allele in *trans* to *Df(2R)Pu-D17*, a deficiency that deletes the *grau* locus. The PCR products were cloned into the TA cloning vector and sequenced.

$grau^{RM61}$ contained a point mutation (C to T) at base 158, generating a stop codon after 35 amino acids (Fig. 3B). Thus, this mutant allele would generate a severely truncated protein. The $grau^{QF31}$ allele contained a 74 bp deletion between base 823 and base 898. This deletion causes a frame shift and introduces a stop codon within the first zinc finger motif. Therefore, this allele would produce only half of the protein,

fused to seven amino acids of an aberrant sequence (Fig. 3B). The *grau*^{QQ36} allele contained a single base mutation (G to A) at base 945, resulting in a change of the first conserved Cys to Tyr in the second zinc finger motif (Fig. 3B). The *grau*^{QE70} allele also contained a single mis-sense mutation (G to A) at base 1529, resulting in the conversion of Glu493 to Lys. This residue is located in the seventh zinc finger motif. Even though it is not one of the conserved amino acids within this motif, it is adjacent to the conserved His494. Thus the mis-sense mutations in both *grau*^{QE70} and *grau*^{QQ36} were located within the zinc finger motifs. The coding region of the *grau*^{RG1} allele has also been sequenced, but no mutation was identified.

Northern blot analysis was performed to study *grau* expression in the ovaries of flies hemizygous for different *grau* alleles (Fig. 4A). A single *grau* transcript was present in the ovaries. In two out of the five *grau* mutant alleles, *grau*^{RM61} and *grau*^{QF31}, the transcript was undetectable. Mutations in both *grau*^{RM61} and *grau*^{QF31} result in premature stop codons (Fig. 3B), consistent with previous observations that non-sense mutations destabilize mRNAs (Hentze and Klulozik, 1999). The transcript was detected in the other three alleles: *grau*^{RG1}, *grau*^{QE70} and *grau*^{QQ36}. No size change of the transcript was observed in any of the mutant alleles.

Expression of the *grau* transcript during development

grau mutants are female sterile. The mutation has no known effect on homozygous flies, but oocytes and embryos derived from homozygous female flies fail to develop normally (Schüpbach and Wieschaus, 1989; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Bashirullah et al., 1999). To investigate *grau* expression more closely, we examined the *grau* transcript in various developmental stages by northern blot analysis (Fig. 4B). The *grau* transcript was present in ovaries, throughout embryogenesis, and in larvae, pupae and adult males. It was also present in female carcasses lacking ovaries (Fig. 4B). Therefore, *grau* may have functions after embryogenesis that have not yet been uncovered.

The Grau-GFP fusion protein is localized to the nucleus of both nurse cells and follicle cells

The phenotype of *grau* mutations suggested that its product acts during oogenesis and early embryogenesis (Schüpbach and Wieschaus, 1989; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Bashirullah et al., 1999). The nature of the Grau protein indicated that it might function as a transcription factor. Thus, it was important to determine whether the Grau protein localized to the cell nucleus during oogenesis.

We localized Grau by fusing its open reading frame to that of the *Aequorea victoria* green fluorescent protein (GFP) (Wang and Hazelrigg, 1994). To express Grau-GFP under the normal *grau* regulatory sequences, the GFP sequences were inserted immediately after the last amino acid of Grau in the 4.3 kb rescuing construct (Fig. 2B), and the construct was used to produce transformed *Drosophila* lines. Transformants carrying the *grau-GFP* fusion were crossed into a *grau* background to determine whether the fusion protein was functional. A single copy of the *grau-GFP* fusion was able to fully restore fertility to *grau* mutant females.

We examined the localization of Grau-GFP in ovaries from lines containing two copies of the transgene. Fluorescence was observed in the nucleus of both follicle cells and nurse cells. The expression of the fusion protein begins in the germarium, and was still evident at stage 9 (Fig. 5). Expression continued until at least stage 10B, with very little if any signal in the oocyte nucleus (B. C., unpublished data).

Table 1. Genetic interaction between *grau* and *cort*

Genotype*	Number of developing embryos	Total number of embryos	% of embryos developing
grau^{RM61}/*PuD-17*‡	18	637	2.8
grau^{QF31}/*PuD-17*	83	1461	5.7
cort^{QW55} *grau*^{RM61}/++	540	577	93.6
cort^{QW55} *grau*^{QF31}/++	830	849	97.8
cort^{RH65} *grau*^{RM61}/++	936	967	96.8
cort^{RH65} *grau*^{QF31}/++	988	1034	95.6
cort^{QW55} *grau*^{RM61}/+ *PuD-17*	0	1134	0
cort^{QW55} *grau*^{QF31}/+ *PuD-17*	0	1207	0
cort^{RH65} *grau*^{RM61}/+ *PuD-17*	0	1345	0
cort^{RH65} *grau*^{QF31}/+ *PuD-17*	0	1032	0

*Embryos from females heterozygous for a *grau* or *cort* mutation or heterozygous for the deficiency *Df(2R)PuD-17*, showed >92% development.
‡This deficiency deletes the *grau* gene.

Interaction between *grau* and *cort*

Embryos from female flies homozygous for *grau* or *cort* mutations show very similar defects, suggesting that the two gene products participate in the same developmental pathway. A more careful investigation allowed us to identify a difference between *grau* and *cort* mutants. While no embryos laid by *cort* homozygous females hatched into larvae, a small percentage of *grau* embryos developed into adult flies (Table 1). This limited fertility allowed us to test for interaction between the two genes. The small percentage of developing embryos derived from *grau* homozygous females (2~5%) was abolished when the females were also heterozygous for a *cort* mutation (0%, Table 1).

Recently, we have identified the *cort* transcription unit (T. C. et al., unpublished observations), allowing us to study the effect of mutation in one gene on the expression of the other. To examine whether *grau* RNA expression is affected by *cort*, we analyzed *grau* expression in ovaries from *cort* mutant females. The expression of *grau* RNA was not affected (data not shown). On the contrary, *grau* homozygous ovaries had severely reduced levels of *cort* transcript (Fig. 6A). We also analyzed RNAs isolated from ovaries containing different dosages of *grau* function: wild-type, heterozygous and null (Fig. 6B). With decreasing dosage of *grau* function, decreasing amounts of *cort* transcript were detected. These results indicate that *grau* acts upstream of *cort*, probably by regulating *cort* transcription. The presence of small amounts of *cort* transcript in *grau* null ovaries may account for the leaky phenotype of the *grau* mutants (Table 1).

Grau protein binds to the *cort* promoter

To determine if Grau protein can regulate the transcription of *cort* directly, we tested the ability of Grau to bind to a region of the *cort* promoter in vitro. For this purpose, Grau was expressed in bacteria as a GST-tagged fusion protein, purified and used in a DNA gel shift assay (Fig. 7). While GST alone showed no DNA-binding activity (Fig. 7, lane 2), recombinant GST-Grau protein bound to a 32 bp region of the *cort* promoter

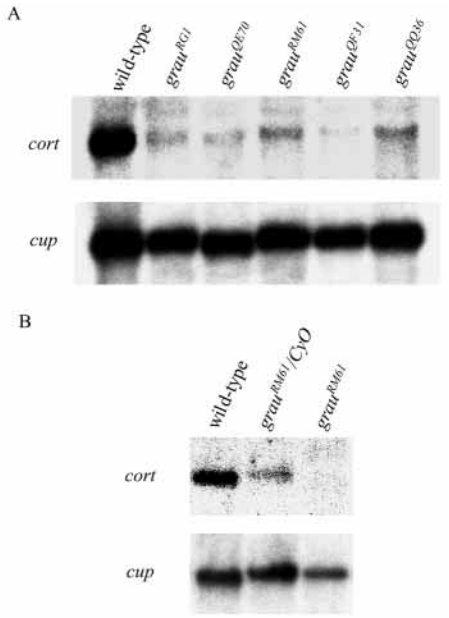

Fig. 6. Expression of *cort* transcript in wild-type and in *grau* mutant ovaries. (A) Poly(A)$^+$ RNA from wild-type ovaries and from ovaries of hemizygotes for different *grau* alleles over *Df(2R)Pu-D17* was probed with ^{32}P-labeled *cort* genomic DNA. Abundance of *cort* transcript was severely reduced by all the *grau* mutant alleles. The *cup* gene was used as a standard for the amount of mRNA loaded on the gel. (B) Poly(A)$^+$ RNA was analyzed from wild-type ovaries, from $grau^{RM61}/CyO$ and from $grau^{RM61}/Df(2R)Pu\text{-}D17$ ovaries. With reduced *grau* function, reduced amount of *cort* transcript was observed.

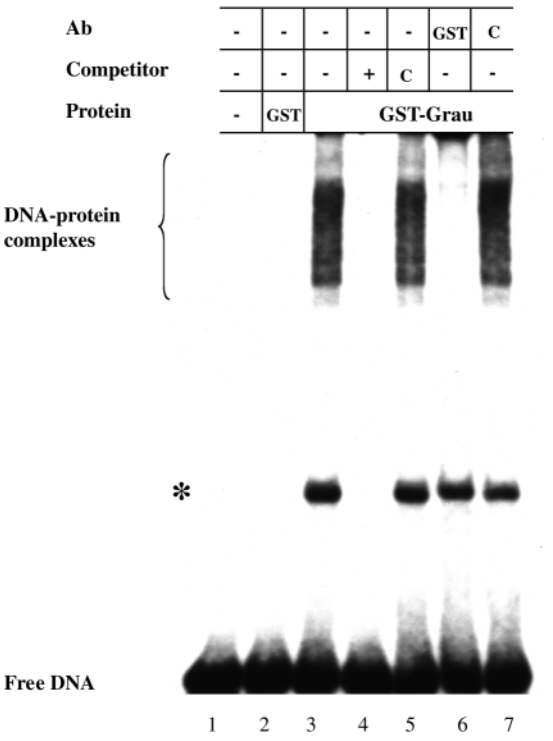

Fig. 7. GST-Grau protein binds to the *cort* promoter. Bacterially expressed and purified GST-Grau protein was analyzed for its ability to bind to a 32 bp region of the *cort* promoter by gel shift assay. DNA-binding reactions included either GST (lane 2) or GST-Grau protein (lane 3-7). The DNA-protein complexes were resolved by electrophoresis in a nondenaturing polyacrylamide gel, and visualized by autoradiography. Competition reactions were incubated with 100× cold specific competitor (+, lane 4) or 100× cold nonspecific competitor (C, lane 5). Super-shift reactions were performed with a specific antibody (anti-GST, lane 6) and a control nonspecific antibody (C, lane 7). A DNA-protein complex (asterisk) containing a degradation product of the fusion protein which lacks the GST tag is not supershifted by anti-GST antibody (lane 6).

(Fig. 7, lane 3). The shift included high molecular weight complexes and a smaller complex; the latter appears to be a complex between the DNA and a degradation product of the recombinant protein lacking the GST domain (see lane 6). To determine if the binding was sequence specific, competition reactions were performed. Excess cold *cort* probe abolished the mobility shift (Fig. 7, lane 4). The shift could not be competed, however, by excess cold competitor corresponding to the binding site of an unrelated transcription factor (Fig. 7, lane 5). GST-Grau also did not bind to other regions of the *cort* promoter or several other unrelated DNA targets (data not shown). To determine if the mobility shift was GST-Grau protein specific, antibody directed against the GST-tag was included in the gel shift reaction. Antibody directed against the GST-tag super-shifted the high molecular weight DNA-protein complex (Fig. 7, lane 6), whereas a control antibody had no effect (Fig. 7, lane 7). Thus, Grau binds to a 32 bp region of the *cort* promoter in a sequence-specific manner.

DISCUSSION

Mutations in the *grau* alleles affect the coding region of the protein

Two of the mutant alleles, $grau^{RM61}$ and $grau^{QF31}$, would generate proteins that are severely truncated. They appear to be null alleles since the *grau* transcript is almost undetectable in the mutant ovaries. The $grau^{QQ36}$ allele contains a mis-sense mutation, resulting in the conserved amino acid Cys to Tyr change in the second C2H2 zinc finger motif. The two Cys and two His residues serve as zinc ligands, bringing together the three conserved hydrophobic residues also within the zinc finger motif to form a stable structural domain (El-Baradi and Pieler, 1991). Substitution of the conserved Cys residue with Tyr would destabilize the structure. $grau^{QE70}$ also contains a single mis-sense mutation, resulting in the conversion of Glu493 to Lys. This residue is not one of the seven conserved amino acids within the zinc finger motif, but it is one of the residues that is directly involved in sequence-specific DNA contacts (Bernstein et al., 1994). It is possible that the Glu to Lys change prevents this finger from recognizing the DNA target.

Eggs from homozygous mothers of all the five *grau* alleles, including the two that had no *grau* RNA, had similar phenotypes. These results and genetic evidence (Page and Orr-Weaver, 1996) indicate that all are null alleles. Therefore, although Grau protein contains eight zinc finger motifs, mutations in a single finger totally abolish the protein function. It will be interesting to determine what role individual zinc

fingers play and whether these fingers are functionally distinguished from each other.

Regulation of *grau* function during oogenesis and at later developmental stages

The *grau* transcript is present throughout different developmental stages as well as in male adult flies. However, all the defects that have been identified in the *grau* mutants occur during oogenesis in *grau* mutant females and in the embryos laid by them (Schüpbach and Wieschaus, 1989; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Bashirullah et al., 1999). Viability is not affected by lack of *grau* function, mutant male flies are fertile and no growth defects have been observed. It is not clear what function *grau* plays during later developmental stages and in adult male flies. It is possible that *grau* function is not required or, in the absence of *grau* function, another gene(s) may compensate for *grau* function at these stages. The *grau* gene is required to activate *cort* transcription. While *grau* is expressed through all developmental stages (Fig. 4B), *cort* transcript is present only during oogenesis (T. C. et al., unpublished data). This can be explained by several possibilities: the Grau protein may need special modification to be activated, or it may need a co-factor to be functional. If the Grau modification only occurs or the co-factor is only available during oogenesis, it is expected that *cort* transcription would be limited to oogenesis. An alternate possibility is that there may be an inhibitor of *cort* transcription present in stages except for oogenesis. Only in the absence of this inhibitor, would *cort* be transcribed in a *grau*-dependent manner. Experiments to study how Grau protein regulates *cort* transcription are underway.

Control of meiotic divisions

Drosophila metaphase I arrest is released at egg activation. Eggs laid by *grau* and *cort* mutant female flies are defective in proper chromosome segregation at meiosis I and fail to exit from metaphase-anaphase transition at meiosis II (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). It is possible that the unequal chromosome segregation at meiosis I may also be due to a defect in the metaphase-anaphase transition triggered by egg activation.

The *grau* and *cort* genes are the only two mutations identified so far that cause female meiotic arrest (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). The chromosome configuration in the mutant eggs suggests a block at meiotic metaphase-anaphase transition. Very little is known about the mechanisms that regulate this transition in meiosis. Much progress has been made, however, in elucidating the similar process during mitosis. Such studies have shown that the metaphase-anaphase transition in mitosis requires the destruction of cell cycle regulators, such as cyclin B and the anaphase inhibitor Pds1, through ubiquitin-mediated proteolysis (Glotzer et al., 1991; King et al., 1995; Murray, 1995; Zachariae and Nasmyth, 1996; Juang et al., 1997).

Since the degradation of anaphase inhibitors and mitotic cyclins allows progression through mitosis, similar inhibitors may also be present during meiosis. One possible role for *grau* and *cort* may be in the regulated degradation of these meiotic anaphase inhibitors and cyclins. In this paper, we identify Grau as a new member of the C2H2-type zinc finger family of transcription factors. Our recent identification of the *cort* transcription unit suggests it may be a cell cycle regulator (T. C. et al., unpublished result). The fact that Grau protein regulates *cort* transcription provides a possible explanation for the *grau* mutant phenotype. In the absence of Grau protein function, *cort* transcription is severely reduced. This may result in the production of much less Cort protein than is required for the metaphase-anaphase transition. Consistent with this hypothesis, the small amount of *cort* transcript present in the *grau* mutant ovaries may account for the small percentage of *grau* embryos developing into later stages (Fig. 6A; Table 1). Since no mutant phenotype has been observed in mitosis or male meiosis (Page and Orr-Weaver, 1996), *grau* appears to be required specifically during female meiosis.

The hypothesis that *grau* and *cort* may be necessary for the degradation of certain cell cycle regulators may also account for the persistent cortical microtubules in the mutant eggs. The inappropriate presence of meiotic Cyclin B results in persistent MPF activity. Since MPF activity has been shown to affect microtubules (Belmont et al., 1990), the cortical microtubule phenotype could be a result of MPF persistence at metaphase II.

Translational control, meiosis and early development

Gene regulation at the beginning of embryogenesis relies on post-transcriptional control of maternal mRNAs, since the oocyte and the early embryonic genomes are transcriptionally silent. This control includes activation as well as silencing of mRNAs at precise times and locations in development. One way to activate silent maternal mRNA translation is through cytoplasmic polyadenylation (Sallés et al., 1994; Wickens et al., 1996).

Translational activation of maternal mRNAs has to be coordinated with oocyte maturation and completion of meiosis. In vertebrates, the proto-oncogene *c-mos* (MAP kinase kinase kinase) provides a conceptual link between the regulation of the meiotic cell cycle and translational activation of maternal mRNAs: (1) c-Mos protein is only produced upon egg activation, (2) it activates MPF, and is thus critical for the resumption of meiosis, (3) it stabilizes MPF to maintain a metaphase arrest prior to fertilization, and (4) it is required for cytoplasmic polyadenylation and thus translation of other maternal mRNAs (Wickens et al., 1996; De Moor and Richter, 1997; Gebauer and Richter, 1997; Sagata, 1997; Murray, 1998; Barkoff et al., 1998).

It is intriguing that no *c-mos* homolog has been identified in *Drosophila*, since meiosis is a fundamental component of sexual reproduction and translational activation of maternal mRNAs is a very conserved process that regulates gene activity. Thus, analysis of this aspect in *Drosophila* development provides an opportunity to identify new molecules that function in regulating the meiotic cell cycle as well as translational control of maternal mRNAs.

The *grau* and *cort* mutants were targeted simultaneously in two genetic screens: a screen for mutants that arrest in meiosis (Page and Orr-Weaver, 1996) and a screen for genes that regulate translation of *bicoid* maternal mRNAs (Lieberfarb et al., 1996). Mutations in *grau* or *cort* impair translational activation of *bicoid* mRNA because of insufficient polyadenylation (Lieberfarb et al., 1996). Therefore, *grau* and *cort* are functionally parallel to *c-mos* in providing a link

between the regulation of the meiotic cell cycle and translational activation of maternal mRNAs.

How does Grau, a transcription factor, and its downstream target Cort, affect cytoplasmic polyadenylation and translational activation of *bicoid* mRNA? Translation of stored maternal mRNAs has to be coordinated with the act of egg activation or fertilization. Many maternal mRNAs that remain silent in arrested oocytes do not become cytoplasmic polyadenylated and translationally activated until egg activation or fertilization. It is conceivable that an inhibitor(s) of cytoplasmic polyadenylation and translation may be present or the cytoplasmic polyadenylation machinery in the arrested egg needs to be activated. In these cases, fertilization or egg activation would generate a signal(s) to remove the inhibitor or to activate the cytoplasmic polyadenylation machinery. With Cort being a potential meiotic cell cycle regulator, it is possible that the pathway to which *grau* and *cort* belong is required to remove the inhibitory factor of *bicoid* mRNA polyadenylation or to activate the cytoplasmic polyadenylation machinery upon egg activation. The final answer to this question awaits further investigation of translational control during early development as well as the identification of its relationship to the meiotic cell cycle.

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