Ribosomal Protein Insufficiency and the Minute Syndrome in Drosophila: A Dose-Response Relationship

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ABSTRACT

Minutes comprise >50 phenotypically similar mutations scattered throughout the genome of Drosophila, many of which are identified as mutations in ribosomal protein (rp) genes. Common traits of the Minute phenotype are short and thin bristles, slow development, and recessive lethality. By mobilizing a *P* element inserted in the 5′ UTR of *M(3)95A*, the gene encoding ribosomal protein S3 (RPS3), we have generated two homozygous viable heteroalleles that are partial revertants with respect to the Minute phenotype. Molecular characterization revealed both alleles to be imprecise excisions, leaving 40 and 110 bp, respectively, at the *P*-element insertion site. The weaker allele (40 bp insert) is associated with a ~15% decrease in *RPS3* mRNA abundance and displays a moderate Minute phenotype. In the stronger allele (110 bp insert) *RPS3* mRNA levels are reduced by ~60%, resulting in an extreme Minute phenotype that includes many morphological abnormalities as well as sterility in both males and females due to disruption of early gametogenesis. The results show that there is a correlation between reduced *RPS3* mRNA levels and the severity of the Minute phenotype, in which faulty differentiation of somatic tissues and arrest of gametogenesis represent the extreme case. That heteroalleles in *M(3)95A* can mimic the phenotypic variations that exist between different *Minute*/rp-gene mutations strongly suggests that all phenotypes primarily are caused by reductions in maximum protein synthesis rates, but that the sensitivity for reduced levels of the individual rp-gene products is different.

THE intriguing phenotypic syndromes of the *Minute* mutations in Drosophila have been studied in detail for more than 70 years and several hypotheses as to their origin have been postulated (Sinclair *et al.* 1981, and references therein). However, except for the suggestion that these mutations affect various components required for protein synthesis, none of these ideas has survived experimental scrutiny. Dividing cells require the normal complement of household genes and, therefore, should be particularly sensitive to a reduced rate of protein synthesis. In Drosophila, the imaginal discs are engaged in rapid growth during the second and third larval instar, with cell division occurring every 6–15 hr (Nöthiger 1972). In pupae, the abdominal histoblasts, which are mitotically dormant during the larval stages, undergo rapid cell division (Robertson 1936; Garcia-Bellido and Merriam 1971). Bristle formation during the pupal period (Howells 1972; Mitchell *et al.* 1977), and normal gametogenesis in both sexes, depends on rapid and flawless protein synthesis. Clearly, the panorama of striking phenotypes observed in *Minutes* (*e.g.*, prolonged development, short and thin bristles, missing and deformed antennae, notched or otherwise malformed wings, small body, rough eyes, reduced fertility and viability, and recessive lethality) is compatible with faulty protein synthesis.

Accumulating data now support the notion that the phenotypic characteristics of *Minute* mutants are attributable to mutations in ribosomal protein (rp) genes. This correlation has been confirmed for nine rp genes, including those encoding the r-proteins 49 (Kongsuwan *et al.* 1985), S2 (Cramton and Laski 1994), S3 (Andersson *et al.* 1994), S5 (McKim *et al.* 1996), S6 (Watson *et al.* 1992; K. Watson, personal communication), S13 (Sæbøe-Larssen and Lambertsson 1996), L9 (Schmidt *et al.* 1996), L14 (Sæbøe-Larssen *et al.* 1997) and L19 (Hart *et al.* 1993). In addition, a haploinsufficiency for the r-protein p40 gene (*sta*) results in the stubarista phenotype, which has Minute-like characteristics including shortened antennae, irregular aristae, short and sparse bristles, and female sterility (Melnick *et al.* 1993). All phenotypes, except the female sterility, could be rescued by transformation with a 4.4-kb genomic fragment harbouring the p40 wild-type gene. Whether or not the *sta* mutant is developmentally delayed was not mentioned.

While all characterized single-gene *Minute* mutants are mutations in rp genes, a reverse correlation is apparently not true. This is emphasized by studies of a chro-

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mosomal deletion that removes the two closely linked *RpS14* genes (Dorer *et al.* 1991). The mutation is recessive lethal but heterozygotes do not display any visible phenotype. The fact that there are two functional *RpS14* genes present per haploid genome (Brown *et al.* 1988) may explain the lack of phenotype in this haploinsufficient mutant. There is also the possibility that mutations in genes other than ribosomal protein genes may lead to a Minute phenotype. Complete or partial inactivation of genes involved in protein synthesis such as aminoacyl-tRNA synthetases or protein synthesis factors and mutations that affect ribosome synthesis and transport may lead to a Minute phenotype or a phenotype similar to Minute. The *bobbed* (ribosomal RNA genes; Ritossa 1976) and *mini* (5S RNA genes; Procunier and Dunn 1978) genes are two examples.

Unlike mutations generated by chemical mutagens or radiation, single *P*-element insertions allow new alleles of the gene to be generated rapidly by imprecisely excising the original element. Studying a range of mutant alleles that includes true nulls and partial revertants is frequently important for understanding gene function and regulation. Imprecise excisions can be selected that delete the gene's promoter and coding sequences or leave small insertions, revealing the true phenotype. *P{lac92}M(3)95A* is a recessive lethal *P*-element insertion in the 5′ untranslated region (UTR) of the gene encoding ribosomal protein S3 (RPS3) and produces a strong Minute phenotype in heterozygous mutants (Andersson *et al.* 1994). The present paper describes two *P*-element excision alleles of *P{lac92}M(3)95A*. The new alleles are homozygous viable and partial revertants with respect to the Minute phenotype and exhibit an additive phenotypic effect when combined with each other or with the original mutation. Molecular characterization revealed both alleles to be insertional mutations at the original *P*-element insertion site and to be associated with reduced *RPS3* mRNA levels and distinct phenotypes. Strikingly, a reduction of *RPS3* mRNA levels to ~40% of wild type is shown to cause a more severe Minute phenotype compared to *P{lac92}M(3)95A*, including serious morphological abnormalities and sterility due to arrest of early gametogenesis.

MATERIALS AND METHODS

Fly stocks and generation of *P{lac92}M(3)95A* excision alleles: Fly stocks were maintained on standard potatomash, yeast and agar substrate at 21°; all crosses were done at 25°. The original *P{lac92}M(3)95A* mutant was recovered from a mutagenesis screen and has been described earlier (Andersson *et al.* 1994). Partial revertants were obtained by crossing *P{lac92}M(3)95A ry/ry*^{506} *Sb P[ry*$^{+}$ *Δ2-3](99B)* males to *Df(3R)ry*^{81}*/MKRS, ry Sb* or to rosy ebony females, respectively, and the non-Stubble rosy progeny were selected and scored for the presence of a Minute phenotype.

Characterization of Minute phenotypes: Estimation of phenotypic characters (developmental time, fertility and viability) was performed by crossing *P{lac92}M(3)95A/+*, *P{lac92}M(3)95A*^{prv9}*/+*, *P{lac92}M(3)95A*^{prv11}*/+* and *+/+* females (maternally Canton-S wild type and isolated from non-crowded cultures) with Canton-S wild-type males in five parallels, and the cultures were monitored for deposition of eggs, hatching, pupation and eclosion. Estimations of developmental delay and viability (fraction of hatched eggs appearing as adults) were calculated by comparing mutants and wild type within each vial, and female fertility (egg production rate) to wild-type crosses. All experiments were carried out with non-crowded cultures at 25°.

General nucleic acid techniques: High molecular weight genomic DNA was prepared essentially as described by Jowett (1986). Poly(A)$^{+}$ mRNA was isolated directly from crude lysates using magnetic oligo(dT) beads (Dynal AS, Oslo; Jakobsen *et al.* 1990). Denaturing RNA gels and hybridizations were as described by Galau *et al.* (1986). Northern and Southern transfers of nucleic acids to Hybond-N nylon membranes (Amersham, Little Chalfont, UK) were done using a TE80 Transvac vacuum blotter (Hoefer, San Francisco). Probes were labelled with [^{32}P]dCTP using biotinylated single-stranded templates (antisense) bound to streptavidin-coated magnetic beads (Dynal AS) in a standard random priming reaction (Espelund *et al.* 1990). Exposure and quantitation of Northern blots was done with the Bio-Rad GS-250 phosphor imaging system, using area integration techniques for proper elimination of background. Dideoxy sequencing (Sanger *et al.* 1977) of *P*-element excision alleles was performed using the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) on biotinylated single-stranded templates bound to streptavidin-coated magnetic beads (Dynal AS; Hultman *et al.* 1989), and the templates used were PCR-products generated on genomic DNA using specific primers [5′-acgtgtctcgcgcgggcacact-3′ (upstream) and 5′-atggcggtcagctcccgaatgc-3′ (downstream)] spanning the *P*-element insertion site. The sequencing reactions were carried out with dITP (substituted for dGTP) and addition of pyrophosphatase as recommended by the manufacturer to eliminate sequence artefacts.

Light, scanning and transmission electron microscopy: For light microscopy, ovaries of homozygous *prv9* and wild-type animals were dissected, fixed for 0.5–2 hr in 4% glutaraldehyde/0.1 M cacodylate buffer, transferred to phosphate-buffered saline (PBS) and further dissected to reveal ovarioles and egg chambers. These preparations were then stained with DAPI (1 μg/ml in PBS) for 45–60 min, destained overnight in PBS and mounted in 50% glycerol.

For scanning electron microscopy (SEM) ovaries, testes, and adult flies were fixed in 3% glutaraldehyde/0.1 M cacodylate buffer, washed in PBS buffer, and dried in a series of ethanol baths. Samples were dried in a Balzers critical point drier, mounted on stubs and coated with Au/Pd in a Polaron SEM coating unit E5000. Scanning was performed in a JEOL JSM 6400 scanning electron microscope at 10 kV.

For transmission electron microscopy analysis ovaries and testes were dissected, fixed as described for SEM. Samples were transferred to propylene oxide, and put in Epon and then sectioned with a diamond knife in a LKB ultratom III. Sections were contrasted with lead citrate and uranyl acetate and analyzed at 80 kV in JEOL 100CX and 1200EX microscopes.

RESULTS

Generation of *P{lac92}M(3)95A* partial revertants: To mobilize the *P* element inserted in the *P{lac92}M(3)95A* mutant, a dysgenic cross was set up between *P{lac92}M(3)95A ry/TM6B, Tb* females and *ry*^{506} *Sb P[ry*$^{+}$ *Δ2-3](99B)/TM6B,Ubx* males, and *P{lac92}M(3)95A ry/ry*^{506} *Sb P[ry*$^{+}$ *Δ2-3](99B)* males were collected from the progeny. These

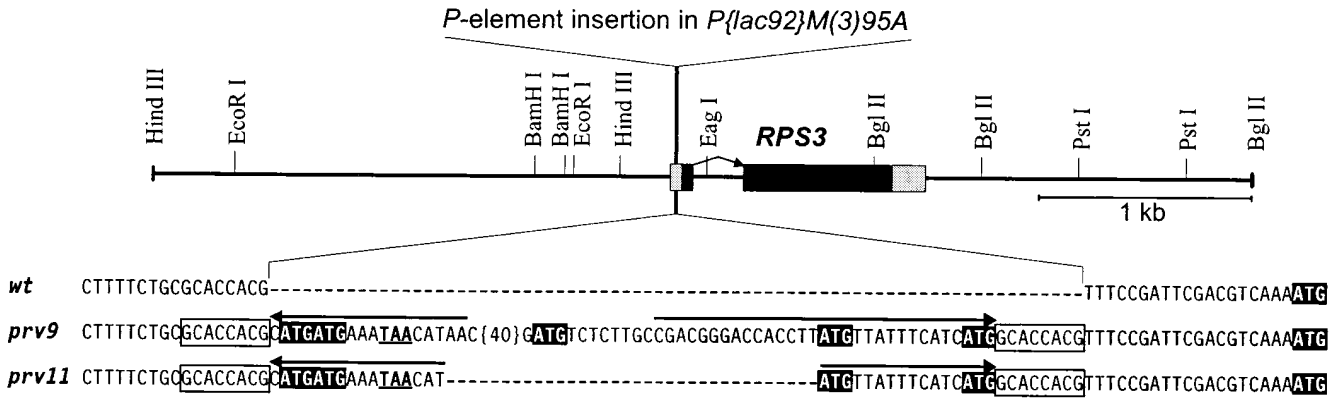

Figure 1.—Genomic organization of the *M(3)95A* gene in wild-type, *P{lac92}M(3)95A*, and partial revertant *prv9* and *prv11* alleles. The top part shows a schematic diagram of the wild-type *M(3)95A* gene. Black boxes represent protein coding regions while shaded boxes represent non-coding parts of the exons. The *P*-element insertion site in the *P{lac92}M(3)95A* mutant is indicated by a vertical line. The bottom part shows a sequence alignment of 5′UTRs from wild-type (wt), *prv9* and *prv11* alleles. Gaps are indicated by dashes, the 8-bp target-site duplications are boxed, ATG start codons have black background, in-frame stop codons are underlined, and sequences from the *P*-element inverted repeats are indicated by horizontal arrows.

males were crossed to *Df(3R)ry*^{81} */MKRS, ry Sb* females, and non-Stubble rosy males and females were selected and classified with respect to their bristle phenotype. While most of the progeny appeared to be either wild type (precise excision) or *M(3)95A* (large insertion or deletion), two partial revertants were found that display intermediate phenotypes. In heterozygous flies the two alleles, termed *P{lac92}M(3)95A*^{prv9} (*prv9*) and *P{lac92}M(3)95A*^{prv11} (*prv11*), have a moderate and weak/wild-type Minute bristle phenotype, respectively.

Genomic organization of partial revertant alleles: To determine the nature of the mutations generated in the excision events, a genomic fragment covering the *P*-element insertion site was used to probe a Southern blot containing *Bam*HI + *Bgl* II digested genomic DNA from wild type, *P{lac92}M(3)95A/TM2*, and partial revertant stocks (results not shown). The results showed that the *P* element had excised imprecisely and left a small insertion in both revertant alleles. To characterize these insertions at the nucleotide level, PCR products were generated from genomic DNA with biotinylated sequence-specific primers flanking the insertion site and sequenced using a direct approach on streptavidin-coated magnetic beads. The resulting sequences (Figure 1) showed that *prv9* and *prv11* has retained 110 and 40 nucleotides (nt) at the *P*-element insertion site, respectively, located within the *M(3)95A* 5′ UTR. In both alleles the insertion includes a 8-bp target site duplication and part of the *P*-element inverted repeats, and *prv9* contains an additional 53-bp fragment of internal *P*-element sequences. In both aberrant mRNAs the inverted repeats may form a hairpin structure that is associated with an energy release of 20.4 and 16.5 kcal/mol at 37° in *prv9* and *prv11*, respectively. While hairpin structures in this energy-range are easily dissolved by helicase activity (Pelletier and Sonenberg 1985a), their location 16 nt from the 5′ terminal end may result in interference with the binding of eIF-4B to the cap structure. It has been shown that a hairpin structure with a $\Delta G \approx -14$ kcal/mol located six nt downstream of the cap site abolishes this binding, whereas a more extensive structure located 37 nt downstream of the cap site does not (Pelletier and Sonenberg 1985b). Located within the 5′UTR these inserts also contain four and five upstream ATG start-codons (uATG). Two of these are in a suboptimal context and are in both alleles located in the most terminal ends of the insert [5′ end: CATGATG; 3′ end: CATCATG; Drosophila consensus: (C_{46},A_{27},T_{14},G_{13}) (A_{70},G_{19},C_6,T_5) (A_{51},C_{21},T_{16},G_{12}) (A_{43},C_{30},G_{18},T_9) ATG; Brown *et al.* 1994]. Translational initiation at the most upstream uATG would produce a three amino acid nonsense product, while at the most downstream uATG which is in frame with the wild type ORF, it would produce a RPS3 protein with a N-terminal extension of ten amino acids. Whether or not these initiations occur *in vivo* has not been investigated. The promoter region of rp genes in higher eukaryotes are in general known to reside both upstream and downstream of the transcription start site (Hariharan *et al.* 1989; Atchison *et al.* 1989). Thus, the insertion present in the 5′ UTR of *prv9* and *prv11* is likely to impair transcriptional initiation. Since the insert in *prv9* is considerably longer than in *prv11*, this effect should be most manifest in *prv9*, consistent with the differences observed in mRNA levels (see below).

Quantitative Northern analysis: To examine *RPS3* mRNA levels (transcriptional efficiency and/or mRNA stability) in the partial revertants, three Northern blots with separate poly(A)$^+$ mRNA extractions from wild type, *P{lac92}M(3)95A ry/TM6B, prv9/prv9*, and *prv11/prv11* adult females were hybridized with single-stranded *RPS3* and *RPL14* (Sæbøe-Larssen *et al.* 1997) cDNA probes; one of the Northern blots is shown in Figure 2. Quantitation was carried out by phosphor imaging

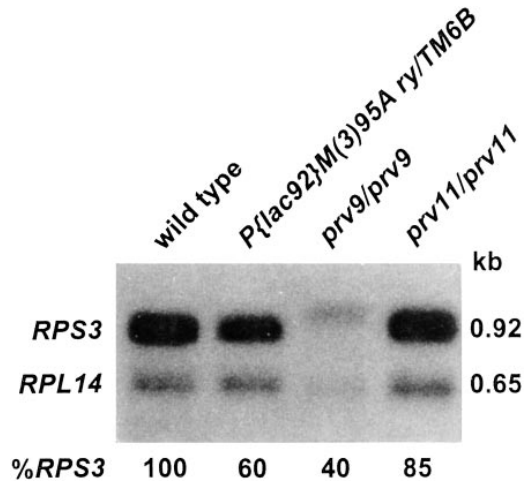

Figure 2.—Quantitative Northern analysis of poly(A)$^+$ mRNA from wild-type, *P{lac92}M(3)95A ry/TM6B, prv9/prv9,* and *prv11/prv11* adult female flies. The blot was hybridized with sense-specific *RPS3* and *RPL14* cDNA probes, the latter included for loading reference. Rounded means of percentage-wise reductions in *RPS3* mRNA levels are indicated in the lower part.

while the picture in Figure 2 is a Polaroid of exposed X-ray film. First, the Northern analysis showed that the *prv9* and *prv11* alleles produce *RPS3* mRNA that is increased in size by approximately the number of nucleotides inserted in their 5′ UTR. Thus, transcription is initiated at or near the wild-type initiation-site and is fixed with respect to the upstream region. In *prv11*, this choice of initiation site has been verified by primer extension analysis (results not shown). Second, quantitative analysis showed that *RPS3* mRNA abundance in *P{lac92}M(3)95A ry/TM6B* flies is reduced by 40 ± 7%. This is close to what would be expected for a haploinsufficient mutant and corroborates the data reported by Andersson *et al.* (1994). In homozygous *prv9* and *prv11* flies, the *RPS3* mRNA levels are 40 ± 5% and 85 ± 2% of wild type, respectively. Thus, an approximate correlation exists between the size of the fragment inserted in the *M(3)95A* promoter region and reduced mRNA levels. It is also possible that the difference in *RPS3* mRNA levels between *prv9* and *prv11* may be ascribed to the nature of the inserted sequence.

The *P{lac92}M(3)95A^1* phenotype: The phenotypic measurements obtained for *P{lac92}M(3)95A* in these studies supplement those reported by Andersson *et al.* (1994). All flies involved in these analyses were constructed to have identical genetic and maternal background (wild-type Canton-S) which produces the most accurate results. The P{lac92}M(3)95A phenotype features larval development prolonged by ~51 hr, a ~45% reduction of female fertility (egg production rate) and vitality (fraction of hatched eggs appearing as adults) reduced by ~10%. The scutellar bristles of *P{lac92}M(3)95A/+* are reduced in length and thickness by ~40%. These data describe a strong Minute phenotype that is the result of a ~40% reduction in *RPS3* mRNA abundance.

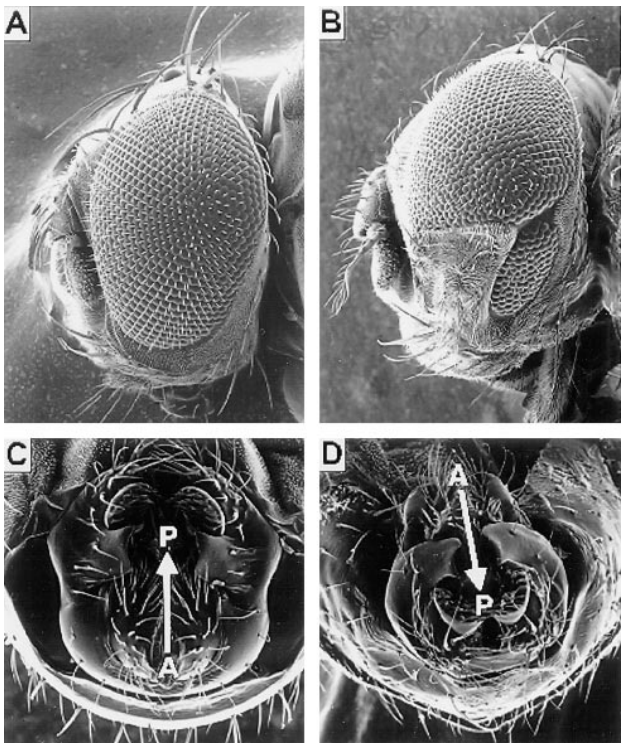

Figure 3.—Scanning electron micrographs showing eyes and male terminalia from wild-type and homozygous *prv9* flies. (A) Wild-type eye. (B) Homozygous *prv9* eye; the eye phenotype shows variable penetrance. (C) Wild-type genetalia and anal plate. (D) Homozygous *prv9* male terminalia showing uncompleted rotation of the genetalia and anal plate. *A*, anus; *P*, penis.

The *P{lac92}M(3)95A^{prv9}* phenotype: Heterozygous *prv9/+* flies exhibit larval development prolonged by ~16 hr and shortening of scutellar bristles by ~20% but no significant change in vitality or female fertility. The *prv9/+* heterozygote is classified as a moderate Minute.

In homozygous condition *prv9* has an extreme Minute phenotype, including ~60% shortening of scutellar bristles, larval developmental time prolonged by 70–80 hr, complete sterility, and small body size. Many flies also have morphological lesions indicating defective imaginal disc development. Frequently observed lesions are rough and malformed eyes (Figure 3, A and B), reduced and malformed aristae, and thin-textured wings. Another conspicuous effect observed is an incomplete rotation of the segment A9 in males (Figure 3, C and D), which bears the external genitalia. During normal male development the genitalia (segment A9) rotate 360° in the pupal stage so that the vas deference loops once about the intestine (Gleichauf 1936). In homozygous *prv9* males this rotation is incomplete in about 65% of the eclosed males. Several different degrees of incomplete rotation were observed, and it is notable that when rotation is incomplete the last tergite and analplate protrude markedly from the body. Little is known about the mechanisms behind this process.

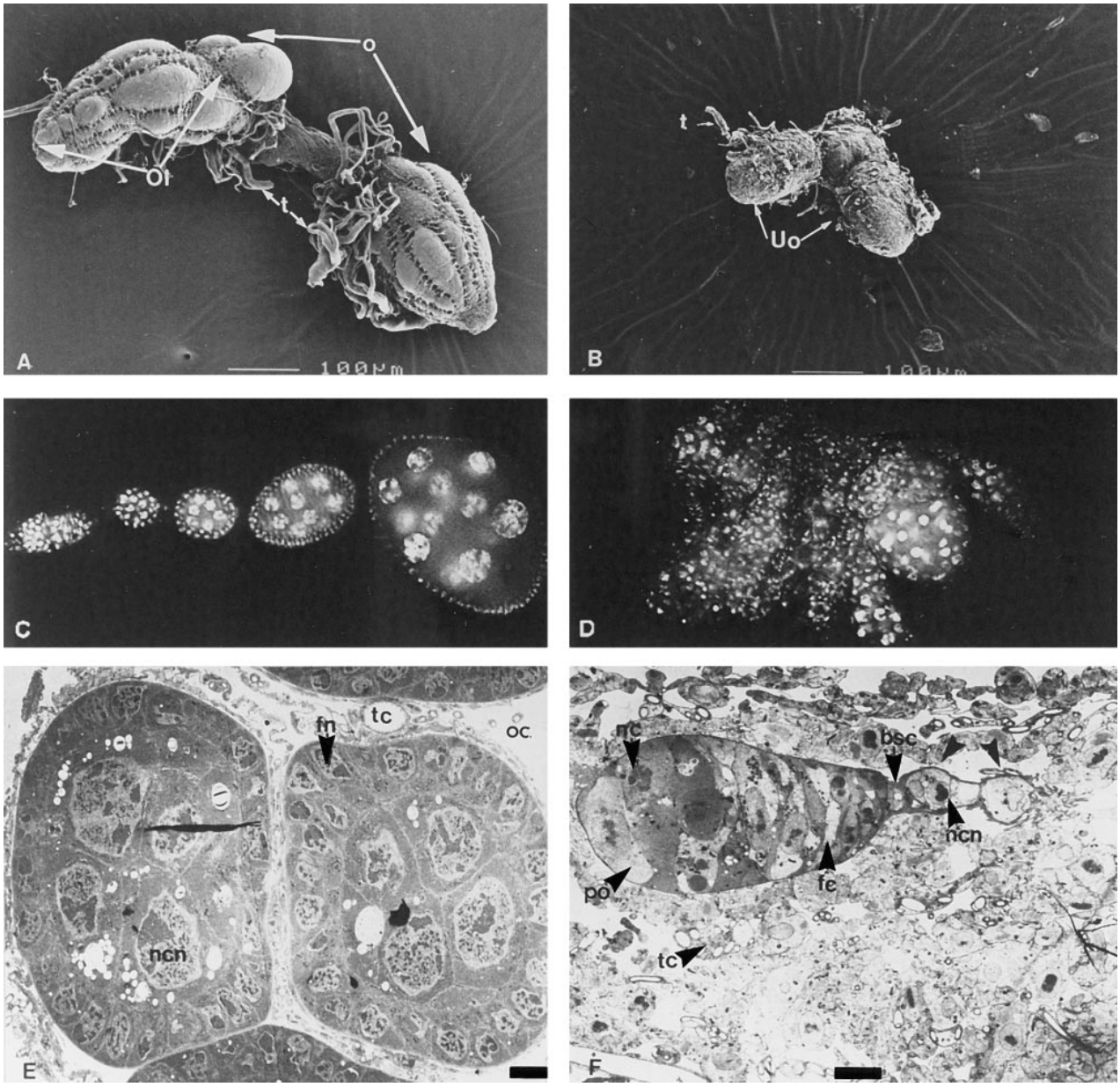

Figure 4.—The ovary phenotype of homozygous *prv9* female flies. (A–B) Scanning electron micrograph of ovaries from wild-type (A) and homozygous *prv9* (B) flies. *O*, ovary; *Ol*, ovariole (between arrows); *Uo*, undeveloped ovary; *t*, trachea. (C–D) Ovarioles from wild-type (C) and homozygous *prv9* (D) ovaries stained with DAPI which binds to DNA. The wild-type ovariole is seen to contain a germarium (left) and egg chambers of approximately stages 2, 3, 4, 5 and 7 in ascending order, while the homozygous *prv9* ovary contains scattered germaria with stalled stage 2 egg chambers with no enveloping follicle cells and many small nuclei in what appears to be a disorganized mass of cells. The mutant ovariole is enlarged three times compared to wild type. (E–F) Transmission electron micrograph of cross section from the apical part of wild-type ovary (E) with developing egg chambers and homozygous *prv9* ovary (F). The latter contains a germarium and stalled/degenerating stage 1 egg chambers (unmarked arrowheads). *bsc*, basal stalk cell; *fc*, follicle cell; *fn*, follicle cell nucleus; *nc*, nurse cell; *ncn*, nurse cell nucleus; *oc*, ovariolar cavity; *po*, pro-oocyte; *tc*, tracheal cell. Bar, 5 μm.

The effects on different body parts indicate that development of various imaginal discs is impaired as a secondary consequence of greatly reduced *RPS3* mRNA levels and reduced protein synthesis.

Both sexes of homozygous *prv9* flies are completely sterile (females lay no eggs and males are unable to fertilize wild type females), and dissection of the animals revealed undeveloped gonads to be the cause of this. A normal ovary consists of a cluster of about 16 parallel ovarioles held together by an enveloping peritoneal sheath which contains a network of anastomosing muscle fibres. In the adult female, each of the tubular ovarioles contains a germarium at its anterior end where the egg chambers are assembled and a vitellarium at its posterior end with seven to eight egg chambers in progressively older stages of oogenesis. Oogenesis starts

during the pupal stage and the oldest egg chambers at eclosion are in stage 7; it then takes more than 24 hr to produce the first mature egg. Scanning electron micrographs of ovaries from wild type (Figure 4A) and homozygous *prv9* (Figure 4B) animals clearly reveal the size differences and the absence of ovarioles in the mutant ovary. Ovaries were stained with DAPI, which binds to the DNA, and inspected with a fluorescence microscope. Whereas the different stages of oogenesis in normal ovaries are clearly and distinctly revealed by the nuclei of both nurse and follicle cells (Figure 4C), the undeveloped ovaries of homozygous *prv9* females (2–4 day old) are malformed and disorganized, and contain scattered germaria and stalled egg chambers that may correspond to stage 2 (Figure 4D). The nuclei of the enveloping follicle cells, which are seen at very early stages in a normal ovariole, are missing in the *prv9* ovaries. There are, however, numerous small nuclei present (Figure 4D) but whether these originate from nurse cells or follicle cells is not known at the moment. These findings were further confirmed by transmission electron microscopy studies. Whereas egg chambers with polyploid nurse cells and enveloping follicle cells are easily recognized in wild-type ovaries (Figure 4E), homozygous *prv9* ovaries contain occasional germaria with a germarial cyst and one or two egg chambers stalled at approximately stage 2 and lack enveloping follicle cells (Figure 4F). The *prv9* germaria are strikingly reminiscent of those present in developing ovaries in 48-hr-old pupae, in which follicle cells are easily observed (King *et al.* 1968). Since ovaries of newly hatched wild-type females contain ovarioles with stage 6 or stage 7 egg chambers, the results show that oogenesis in homozygous *prv9* is arrested at very early stages, which explains why the ovaries remain small.

Figure 5, A and B shows scanning micrographs of wild type and homozygous *prv9* testes from 3–5-day-old males. The *prv9* testes (Figure 5B) are considerably smaller than wild type (Figure 5A), and have small bulges spread along their length. Transmission electron microscopy of wild type and mutant testes revealed that, whereas the apical part of wild-type testes is filled with individualized spermatid bundles containing 64 spermatids (Figure 5C), there are neither spermatocytes nor spermatids nor sperms present in homozygous *prv9* testes (Figure 5D). There is, however, a disorganized mass of cells that may contain remnants of spermatid cysts. Both sections are in the apical part of the testis but, because the *prv9* testes are much smaller than wild type, the sections may not be fully comparable.

The *P{lac92}M(3)95A^{prv11}* phenotype: Homozygous *prv11*/*prv11* flies are characterized as moderate Minutes and feature larval development prolonged by ~22 hr and ~20% shortening of scutellar bristles. The egg production rate of females is reduced by ~40%, and viability is unaffected. In *prv11*/+ heterozygotes the only measurable phenotype is a ~5% reduction of scutellar bristle length, which can be recognized only after close examination of postalare bristles against the alula (wing flap).

Whether or not the ~15% reduction in *RPS3* mRNA abundance observed in homozygous *prv11* is the exclusive cause of the moderate Minute phenotype has not been addressed experimentally. However, the *prv11* phenotype is somewhat more severe than that of heterozygous *prv9*/+ flies, which have a ~30% reduction of *RPS3* mRNA abundance. Thus, it cannot be ruled out that the sequences inserted into the 5′ UTR of *prv11*, containing several uATGs and a putative hairpin structure, may have a negative effect on the translation of this aberrant mRNA and thereby contribute to the phenotype. In principle, the inserts present in *prv9* and *prv11* have the same basic features, but the *prv9* allele contains an additional 66-bp fragment that separates the inverted repeats. This separating fragment may impair the formation of a stem-loop structure, and thus, the two mRNAs may behave differently with respect to translational efficiency.

Additivity of phenotypes: A complementation analysis was carried out at 25° by crossing wild type, *P{lac92}M(3)95A*, and partial revertants in all possible combinations (Table 1). These tests revealed that (1) *prv9* is lethal in combination with *P{lac92}M(3)95A*, (2) *prv9*/*prv11* heterozygotes have a strong Minute phenotype comparable with that of *P{lac92}M(3)95A*/+, and (3) *prv11*/*P{lac92}M(3)95A* heterozygotes have an extreme/semi-lethal Minute phenotype with a vitality (fraction of hatched eggs appearing as adults) of only 3–5%. (Most die as pupae or are too weak to break out of the pupal case.) Hatched *prv11*/*P{lac92}M(3)95A* flies are also sterile, have severe morphological lesions similar to those of *prv9* homozygotes, and usually live for one or two days only. The results show that there is an approximate correlation between reduced RPS3 mRNA levels and the severity of the Minute phenotype, in which disruption of gametogenesis and imaginal disc development represents the extreme consequence prior to lethality.

DISCUSSION

In the *P*-element excision experiment described in this paper we recovered both complete and partial revertants of *P{lac92}M(3)95A*. We have shown that different degrees of *RPS3* insufficiency produce distinct phenotypes, in which the penultimate effect prior to lethality constitutes arrest of gametogenesis and many morphological defects.

The insert present in *prv9* and *prv11* is located within a region generally known to constitute the rp-gene promoter in higher eukaryotes (Hariharan *et al.* 1989; Atchison *et al.* 1989), and, therefore, it seems likely that the reduced *RPS3* mRNA abundance in these mutants is the result of impaired transcription. This may explain the low levels observed in *prv9* (110 bp insert) compared to *prv11* (40 bp insert). An alternative explanation is that

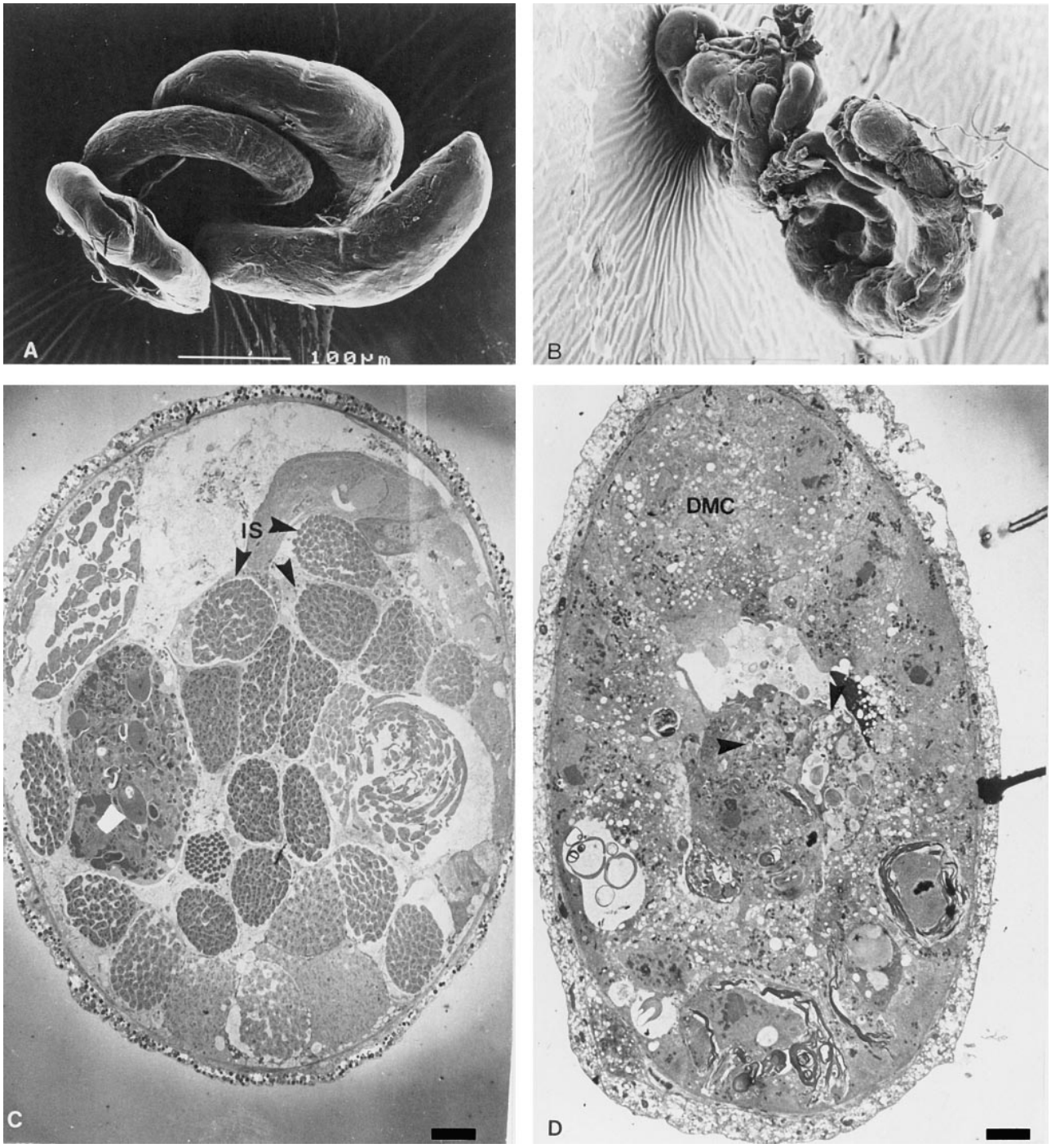

Figure 5.—Testes from wild-type and homozygous *prv9* male flies. (A–B) Scanning electron micrograph of half of a wild type testis (A) and a whole homozygous *prv9* testis (B). (C–D) Transmission electron micrographs of apical sections from wild-type (C) and mutant (D) testis. *IS*, individualized spermatid cysts; *DMC*, disorganized mass of cells. Small arrows indicate possible degenerating spermatid cysts. Bar, 5 μm.

translational initiation at uAUGs followed by premature termination could result in nonsense-mediated mRNA degradation (Theodorakis and Cleveland 1996, and references therein). Both *prv9* and *prv11* have an uAUG in a sub-optimal context close to the 5′ terminus that is followed by a stop codon after one triplet. However, since there is a four-fold difference in the reduction of *RPS3* mRNA between the two partial revertants this cannot be a likely explanation.

Due to the presence of uATGs and inverted repeats in the 5′ UTR of the *prv9* and *prv11* alleles, some uncertainty exists regarding the efficiency with which the mRNAs are translated to yield functional protein. A tendency of translational initiation at an uAUG would

TABLE 1

Phenotypic characteristics associated with *M(3)95A* allele combinations

Genotype	*RPS3* mRNA level[a]	Scutellar bristle length[a]	Developmental delay (hr)	Egg production rate[a]	Survival rate to eclosion[a]	Denoted Minute phenotype
prv9/M(3)95A	25[b]	NA	NA	NA	0	
prv11/M(3)95A	48[b]	35	NI	0	4	Extreme[c,d]
prv9/prv9	40	40	75	0	50	Extreme[d]
M(3)95A/+	60	60	51	55	90	Strong
prv9/prv11	63[b]	60	NI	NI	NI	Strong
prv11/prv11	85	80	22	60	NI	Moderate
prv9/+	70	80	16	ND	ND	Moderate
prv11/+	93[b]	95	ND	ND	ND	Weak/wild-type

NA, not applicable; ND, no detectable deviation from wild type; NI, not investigated.
[a] Percent of wild type.
[b] Putative values calculated from combination of alleles.
[c] Semi-lethal.
[d] Both males and females are sterile and have morphological defects.

diminish initiation at those further downstream (Jackson 1996, and references therein), and a secondary structure located close to the 5′ terminus may lessen initiation by interfering with the binding of eIF-4B to the cap structure (Pelletier and Sonenberg 1985b). In particular this applies to *prv11* in which a ~15% reduction in *RPS3* mRNA abundance is seen to cause a phenotype more severe than that of *prv9/+* (~30% reduction of *RPS3*). Obviously, the *prv11* mRNA is not translated with normal efficiency. The major difference between *prv9* and *prv11* is that the insert present in the *prv9* allele contains an additional 66-bp fragment that separates the inverted repeats. Supposing that this separating fragment has a negative effect on the formation of a stem-loop structure, the *prv9* mRNA may be translated with higher efficiency. This interpretation is consistent with the observation that *prv9/+* has a Minute phenotype less severe than that of *P{lac92}M(3)95A/+* (~40% reduction of *RPS3*).

A rp-gene mutation with a phenotype similar to *prv9* has been described previously. *string of pearls* (*sop*^{P}) is a *P*-element insertion in the promoter region of the gene encoding RPS2, and was reported to result in a recessive Minute phenotype (Cramton and Laski 1994). The *sop*^{P} mutation causes an incomplete inactivation of transcription, and 10–15% of homozygous *sop*^{P} embryos manage to reach the adult stage. Surviving homozygous *sop*^{P}/*sop*^{P} flies have a 60–70% reduction in *RPS2* mRNA levels and display an extreme/semi-lethal Minute phenotype as well as female sterility due to arrest of oogenesis at stage 5. The stage 5 cysts are normal in that they have 15 nurse cells and one oocyte positioned properly at the posterior end. Major differences between *prv9* and *sop*^{P} are the stages reached during oogenesis (2 and 5, respectively). Also, *prv9* males are sterile, many *prv9* flies also have morphological defects, and *sop*^{P}/+ flies show no Minute phenotype. One interpretation of these differences could be that the impairing of protein synthesis is more severe in *prv9* than in *sop*^{P}. Alternatively, RPS2 and RPS3 may have specific, but different, bifunctional roles during gametogenesis.

All cells involved in gametogenesis require the normal supplement of household genes to maintain a balance between the levels of soluble proteins, various membranes and ribosomes in order to optimize conditions for this process. The gonad primordium is established during embryogenesis when the migrating germ cells become enfolded by somatic cells of mesodermal origin (Sonnenblick 1941; Campos-Ortega and Hartenstein 1985). During the larval stages these groups of cells divide continuously; they begin differentiation during the pupal stages (King 1970). The absence of developing germ cells and the early stall of gametogenesis in homozygous *prv9* may be a secondary effect of a faulty differentiation of the somatic parts of the gonads. It is equally possible, however, that the growth and division of the germ-line stem cells are more sensitive to a reduction in protein synthesis than are the somatic portions of the gonads. The similarity between the defects in both females and males suggests that RPS3 plays a role common to the early stages of gametogenesis in both sexes. A flawless protein synthesis is a prerequisite in both oogenesis and embryogenesis where each cell is dependent on receiving a sufficient supply of proteins and organelles. Thus, a reduction in the maximum protein synthesis rate, caused by strongly reduced *RPS3* mRNA abundance, appears to be the most logical explanation for the arrested gametogenesis in homozygous *prv9* flies. This reduction appears to be deleterious at critical stages in gametogenesis and development, and different degrees of reduction in r-protein levels or other ribosomal components may arrest gametogenesis at specific stages.

There is evidence that some ribosomal proteins have extraribosomal function (Wool *et al.* 1996, and references therein), and it has been suggested that the blocking of

oogenesis in homozygous sop^P females (60–70% reduction in *RPS2* mRNA abundance) is the result of such a non-translational role played by this protein (Cramton and Laski 1994). However, the observation that an equivalent percentage-wise reduction in *RPS2* and *RPS3* mRNA abundance produces similar phenotypes indicates that arrest of oogenesis may be a general effect of strongly reduced protein synthesis. It is also possible that the many morphological defects frequently observed in homozygous *prv9* flies are a consequence of strongly reduced protein synthesis through incomplete growth/development of imaginal discs and somatic tissues.

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LITERATURE CITED

Andersson, S., S. Sæbøe-Larssen, A. Lambertsson, J. Merriam and M. Jacobs-Lorena, 1994 A Drosophila third chromosome *Minute* locus encodes a ribosomal protein. Genetics **137:** 513–520.

Atchison, M. L., O. Meyuhas and R. P. Perry, 1989 Localization of transcriptional regulatory elements and nuclear factor binding sites in mouse ribosomal protein gene *rpL32*. Mol. Cell. Biol. **9:** 2067–2074.

Brown, S. J., D. D. Rhoads, M. J. Stewart, B. Van Slyke, I. T. Chen *et al.*, 1988 Ribosomal protein S14 is encoded by a pair of highly conserved adjacent genes on the X chromosome of *Drosophila melanogaster*. Mol. Cell. Biol. **8:** 4314–4321.

Brown, C. M., P. A. Stockwell, M. E. Dalphin and W. A. Tate, 1994 The translational signal database (TransTerm) now also includes initiation context. Nucleic Acids Res. **22:** 3620–3621.

Campos-Ortega, J. A., and V. Hartenstein, 1985 *The Embryonic Development of Drosophila melanogaster*. Springer-Verlag, Berlin.

Crampton, S. E., and F. A. Laski, 1994 *string of pearls* encodes Drosophila ribosomal protein S2, has *Minute*-like characteristics, and is required during oogenesis. Genetics **137:** 1039–1048.

Dorer, D. R., A. Anane-Firempong and A. C. Christensen, 1991 Ribosomal protein S14 is not responsible for the Minute phenotype associated with the *M(1)7C* locus in *Drosophila melanogaster*. Mol. Gen. Genet. **230:** 8–11.

Espelund, M., R. A. P. Stacy and K. S. Jakobsen, 1990 A simple method for generating single-stranded DNA probes labeled to high activities. Nucleic Acids Res. **18:** 6157–6158.

Galau, G. A., D. W. Hughes and L. Dure, 1986 Abscisic acid induction of cloned cotton late embryogenesis-abundant (*Lea*) mRNAs. Plant. Mol. Biol. **7:** 155–170.

Garcia-Bellido, A., and J. R. Merriam, 1971 Clonal parameters of tergite development in Drosophila. Dev. Biol. **26:** 264–276.

Gleichauf, R., 1936 Anatomie und Variabilitat des Geschlechtsapparates von *Drosophila melanogaster* (Meigen). Z. Wiss. Zool. **148:** 1–66.

Hariharan, N., D. E. Kelley and R. P. Perry, 1989 Equipotent mouse ribosomal protein promoters have a similar architecture that includes internal sequence elements. Genes Dev. **3:** 1789–1800.

Hart, K., T. Klein and M. Wilcox, 1993 A *Minute* encoding a ribosomal protein enhances wing morphogenesis mutants. Mech. Dev. **43:** 101–110.

Howells, A. J., 1972 Levels of RNA and DNA in *Drosophila melanogaster* at different stages of development: a comparison between one bobbed and two phenotypically non-bobbed stocks. Biochem. Genet. **6:** 217–230.

Hultman, T., S. Ståhl, E. Hornes and M. Uhlén, 1989 Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. Nucleic Acids Res. **17:** 4937–4946.

Jackson, R. J. 1996 A comparative view of initiation site selection mechanisms, pp. 71–112 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Jakobsen, K. S., E. Breivold and E. Hornes, 1990 Purification of mRNA directly from crude plant tissue in 15 minutes using magnetic oligo dT microspheres. Nucleic Acids Res. **18:** 3669.

Jowett, T., 1986 Preparation of nucleic acids, pp. 275–286 in *Drosophila, A Practical Approach*, edited by D.B. Roberts. IRL Press, Oxford.

King, R. C., 1970 Ovarian Development in *Drosophila melanogaster*. Academic Press, New York.

King, R. C., S. K. Aggarwal and U. Aggarwal, 1968 The development of the female Drosophila reproductive system. J. Morph. **124:** 143–166.

Kongsuwan, K., Y. Qiang, A. Vincent, M. C. Frisardi, M. Rosbash *et al.*, 1985 A Drosophila *Minute* gene encodes a ribosomal protein. Nature **317:** 555–558.

McKim, K. S., J. B. Dahmus and R. S. Hawley, 1996 Cloning of the *Drosophila melanogaster* meiotic recombination gene *mei-218*: A genetic and molecular analysis of interval 15E. Genetics **144:** 215–228.

Melnick, M. B., E. Noll and N. Perrimon, 1993 The Drosophila *stubarista* phenotype is associated with a dosage effect of the putative ribosome-associated protein D-p40 on *spineless*. Genetics **135:** 553–564.

Mitchell, H. K., L. S. Lipps and U. M. Tracey, 1977 Transcriptional changes in pupal hypoderm in *Drosophila melanogaster*. Biochem. Genet. **15:** 575–587.

Nöthiger, R., 1972 The larval development of imaginal disks, pp. 1–34 in *Results and Problems in Cell Differentiation: The Biology of Imaginal Disks*, Vol. 5, edited by H. Ursprung and R. Nöthiger. Springer-Verlag, New York, Heidelberg and Berlin.

Pelletier, J., and N. Sonenberg, 1985a Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of a eukaryotic mRNA reduces translational efficiency. Cell **40:** 515–526.

Pelletier, J., and N. Sonenberg, 1985b Photochemical cross-linking of cap binding proteins to eukaryotic mRNAs: effect of mRNA 5′ secondary structure. Mol. Cell. Biol. **5:** 3222–3230.

Procunier, J. D., and R. J. Dunn, 1978 Genetic and molecular organization of the 5S locus and mutants in *D. melanogaster*. Cell **15:** 1087–1093.

Ritossa, F., 1976 The *bobbed* locus, pp. 801–846 in *The Genetics and Biology of Drosophila*, Vol. 1B, edited by M. Ashburner and E. Novitski. Academic Press, London.

Robertson, C. W., 1936 The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the prinicipal morphological changes. J. Morph. **59:** 351–398.

Sanger, F., S. Nicklen and A. R. Coulson, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **77:** 5463–5467.

Schmidt, A., M. Hollmann and U. Schäfer, 1996 A newly identified *Minute* locus, *M(2)32D*, encodes the ribosomal protein L9 in *Drosophila melanogaster*. Mol. Gen. Genet. **251:** 381–387.

Sinclair, D. A. R., D. T. Suzuki and T. A. Grigliatti, 1981 Genetic and developmental analysis of a temperature-sensitive *Minute* mutation of *Drosophila melanogaster*. Genetics **97:** 581–606.

Sonnenblick, B. P., 1941 Germ movements and sex differentiation of the gonads in the Drosophila embryo. Proc. Natl. Acad. Sci. USA **27:** 484–489.

Sæbøe-Larssen, S., and A. Lambertsson, 1996 A novel Drosophila *Minute* locus encodes ribosomal protein S13. Genetics **143:** 877–885.

Sæbøe-Larssen, S., B. Urbanczyk Mohebi and A. Lambertsson, 1997 The Drosophila ribosomal protein L14-encoding gene, identified by a novel *Minute* mutation in a dense cluster of previously undescribed genes in cytogenetic region 66D. Mol. Gen. Genet. **255:** 141–151.

Theodorakis, N. G., and D. W. Cleveland, 1996 Translationally coupled degradation of mRNA in eukaryotes, pp. 631–652 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Watson, K. L., K. D. Konrad, D. F. Woods and P. J. Bryant, 1992 Drosophila homolog of the human S6 ribosomal protein is re-

quired for tumor suppression in the hematopoitic system. Proc. Natl. Acad. Sci. USA **89:** 11302–11306.

Wool, I. G., Y. L. Chan and A. Glück, 1996 Mammalian ribosomes: The structure and the evolution of the proteins, pp. 685–732 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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