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A Novel Drosophila *Minute* Locus Encodes Ribosomal Protein S13

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ABSTRACT

Minutes comprise >50 phenotypically similar Drosophila mutations believed to affect ribosomal protein genes. Common traits of the Minute phenotype are short and thin bristles, slow development, and recessive lethality. To further investigate the proposed *Minute* to ribosomal protein correspondence, loss-of-function *Minute* mutations were induced by *P*-element mutagenesis. Here, we report a previously undescribed *Minute* locus that maps to 32A on chromosome *2L*; this *Minute* allele is named *P{lacW}M(2)32A*^{1} and the gene *M(2)32A*. Flies heterozygous for *P{lacW}M(2)32A*^{1} have a medium Minute phenotype. The gene interrupted by the *P*-element insertion was cloned. Sequence analyses revealed that it encodes the Drosophila homologue of eukaryotic ribosomal protein S13. It is a single-copy gene and the level of *RPS13* transcript is reduced to ~50% in *P{lacW}M(2)32A*^{1} heterozygotes. Both transcript level and phenotype are restored to wild type by remobilizing the *P* element, demonstrating that the mutation is caused by insertion of the *P*-element construct. These results further strengthen the notion that *Minutes* encode ribosomal proteins and demonstrate that *P*-element mutagenesis is a fruitful approach to use in these studies.

MINUTES are a group of ≥50 or more mutations scattered throughout the Drosophila genome and associated with similar dominant visible phenotypes, such as short slender bristles and delayed development, and with recessive lethality. Heterozygotes often display other secondary effects such as rough eyes, reduced viability, small body size, plexus venation of the wings, deformed or otherwise affected antennae, and low fertility, especially in females (Lindsley and Zimm 1992). In his pioneering study of *Minutes*, Schultz (1929) found that they are nonadditive in their phenotypic effect, *i.e.*, the phenotype of a *M1/+;M2/+* fly is not more severe than the phenotype of any of the single mutants. He concluded that the genes code for proteins with similar function(s) and suggested that *Minutes* are deficiencies. Unfortunately, the nonadditive property of the *Minute* mutations makes it impossible to determine if a deletion uncovers one or more closely linked *Minute* loci.

The phenotypic similarity between *Minutes* and *bobbed* mutations lead Ritossa *et al.* (1966) to suggest that *Minutes* might affect tRNA genes. Although initially attractive, this hypothesis has since been proven wrong (Sinclair *et al.* 1981). The clear demonstration that *M(3)99D* (Kongsuwan *et al.* 1985), *M(2)60E* (Hart *et al.* 1993) and *M(3)95A* (Andersson *et al.* 1994) encode RP49, RPL19 and RPS3, respectively, directly supports the recent proposal that *Minutes* correspond to mutations in ribosomal protein genes. Furthermore, the observation that most cloned ribosomal protein genes in *Drosophila melanogaster* have been cytologically mapped to polytene chromosomal regions at or near *Minute* loci (Andersson *et al.* 1994; Ntwasa *et al.* 1994) provides indirect support for this hypothesis.

Recent results indicate that ribosomal proteins are not simply required structural elements, but are also implicated in regulatory processes important in normal development. Thus, in Drosophila, *RpS6* behaves as a tumor suppressor gene (TSG) and is encoded by the *aberrant immune response 8* (*air8*) locus (Watson *et al.* 1992; Stewart and Denell 1993). Overexpression of the Drosophila ribosomal protein S15a suppresses a mutation in the *Saccharomyces cerevisiae cdc33* gene, which encodes the cap-binding subunit of eukaryotic initiation factor 4F (eIF-4F; Lavoie and Lasko 1993). Mutations of *cdc33* lead to arrest in the cell cycle at the G1 to S transition. *string of pearls* (*sop*) is a recessive female sterile mutation in *D. melanogaster* that arrests oogenesis at stage 6 (Crampton and Laski 1994). The *sop* gene product was identified as the Drosophila homologue to RPS2 of yeast and rat, the equivalent of prokaryotic r-protein S5. Drosophila ribosomal protein S3 is capable of cleaving DNA containing apurinic/apyrimidinic (A/P) sites (Wilson *et al.* 1994). Ribosomal proteins have also been implicated in regulatory processes that may be important in carcinogenesis (Henry *et al.* 1993 and references therein). Since the exact functions of the above ribosomal proteins are unknown, the link between phenotype and gene function remains to be determined.

It is also interesting to note that two genes on the human sex chromosomes, one on the *X* and one on

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the *Y*, encode isoforms of ribosomal protein S4 (FISHER *et al.* 1990). The proteins RPS4X and RPS4Y differ at 19 of 263 amino acids, and *RPS4X*, unlike most genes on the *X* chromosome, is not *X* inactivated. It has recently been argued that *RPS4* haploinsufficiency contributes to the Turner syndrome in humans (ZINN *et al.* 1994). Many other distinct phenotypes, mostly including growth retardation, have been associated with monosomy for particular portions of autosomes and haploinsufficiency of other ribosomal protein genes may contribute to these phenotypes (FISHER *et al.* 1990).

Many more *Minute* mutations need to be analyzed to determine the generality of the *Minute* to ribosomal protein correspondence, to enable studies of the mechanisms leading to the Minute phenotype, and to determine whether mutations in nonribosomal genes can lead to the Minute phenotype. The fact that most, if not all, *Minutes* are deficiency heterozygotes makes them useless in a molecular approach to address these questions. We therefore decided to induce and clone loss-of-function Minute mutations by using the *P{lacW}* mutagenesis procedure described by BIER *et al.* (1989). Here we describe a novel Minute mutation, *P{lacW}M(2)32A*[1], identified by its delayed larval development, short and thin bristles, and recessive lethality. We show that the gene, *M(2)32A*, which is interrupted by the *P* element insertion, encodes a Drosophila ribosomal protein.

MATERIALS AND METHODS

Drosophila stocks, *P*-element mutagenesis and genetic mapping: Fly stocks were maintained on standard potatomash, yeast and agar substrate at 21°; all crosses were done at 25°. For detailed description of genetic markers, mutations and their abbreviations, see LINDSLEY and ZIMM (1992). Stocks were kindly provided by the European Drosophila Stock Center in Umeå, Sweden, and by the Bloomington Drosophila Stock Center.

The *P{lacW}M(2)32A*[1] mutant was recovered from a small scale mutagenesis screen using the *P{lacZ white*$^{+mC}$ *ori Amp=lacW}* (Flybase "Genetic nomenclature for *Drosophila melanogaster*, March 23, 1995; PIROTTA 1988) enhancer trap procedure described by BIER *et al.* (1989). This *P* element, inserted into the *X* chromosome, contains the bacterial *lacZ* and Drosophila *white*$^{+mC}$ genes, and bacterial plasmid sequences for rapid cloning. Seventy individual vials were set up, each with two *yw*$^-$ *P{lacW};TMS, Sb* Δ*2-3(99B)/+* males crossed to five *y w*$^-$ females carrying the second chromosome balancers *CyO* and *Sp*, and the third chromosome balancers *Sb* and *D*. Two Minute white$^+$ males were obtained from ~10,000 progeny and balanced with *CyO* and *D*, respectively.

Revertants were obtained by crossing *yw*$^-$*;P{lacW}M(2)32A*[1]*/Sp;TMS, Sb* Δ*2-3(99B)/+* (nonwhite) males to *yw*$^-$*;CyO/Sp* females and the white non-Stubble progeny were selected and scored for the presence of a Minute$^+$ phenotype.

Cytogenetic mapping by *in situ* hybridization to salivary gland polytene chromosomes was performed essentially as described by PARDUE (1986). Deficiency and duplication mappings were done by crossing *yw*$^-$*;P{lacW}M(2)32A*[1]*/CyO* flies to strains carrying the abberations shown in Figure 2. For complementation test *y w*$^-$*;P{lacW}M(2)32A*[1]*/CyO* was crossed to the closely mapped *M(2)30A* and *M(2)36F*[2].

General nucleic acids techniques: Plasmid rescue of DNA sequences flanking the *P*-element insertion was done as described by PIROTTA (1986). High molecular weight genomic DNA and total RNA were prepared essentially as described by JOWETT (1986). Poly(A)$^+$ mRNA was isolated directly from crude lysates using magnetic oligo(dT) beads (Dynal AS; 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) were done using a TE80 Transvac vacuum blotter (Hoefer Scientific Instruments). Double stranded probes for hybridization to nucleic acid blots were labeled with the DIG DNA labeling kit (Boehringer Mannheim). Strand specific probes were generated using biotinylated single-stranded templates bound to streptavidin-coated magnetic beads (Dynal AS; ESPELUND *et al.* 1990) in a standard DIG labeling reaction (Boehringer Mannheim). DIG labeled probes were detected with the DIG luminescent detection kit (Boehringer Mannheim). Dideoxy sequencing (SANGER *et al.* 1977) were performed using the Sequenase 2.0 kit (USB) on biotinylated single-stranded templates bound to streptavidin-coated magnetic beads (Dynal AS; HULTMAN *et al.* 1989), and the sequencing reactions were carried out with dITP (substituted for dGTP) and addition of pyrophosphatase as recommended by the manufacturer to eliminate sequence artifacts. The primer extension analysis was carried out on poly(A)$^+$ mRNA prepared from adult females using AMV reverse transcriptase (Promega) and an end-labeled oligo (5'-ccaggagcgtgcatac-3') complementary to the first exon of the *M(2)32A* gene.

Screening genomic and cDNA λ libraries: The genomic λ-GEM11 library used here was made from an isofemale line of the wild-type stock Samarkand (A. LAMBERTSSON, unpublished dat) and was probed with the plasmid rescue clone (see Figure 3). The cDNA library used was an oligo(dT) primed λ ZAP II library from third instar larvae made by Dr. C. THUMMEL, University of Utah and kindly provided by Dr. K. WATSON, Harvard University.

Quantitative Northern analysis: Quantitation of signals from bands on Northern blots was done on Polaroid photos of exposed X-ray film with band intensities in the range 10–40% of saturation. The photos were digitized with a 600 DPI scanner and analyzed with the computer program IMAGEQUANT version 3.3 (Molecular Dynamics).

Magnetic solid phase inverse Northern (MSPIN) analysis: The magnetic solid phase inverse Northern technique was developed to identify coding regions in genomic DNA, and in this particular study, to point out the regions of interest surrounding the inserted *P* element at the *M(2)32A* locus. The procedure produces a single stranded template (first-strand cDNA) covalently attached to magnetic beads [Dynabeads Oligo(dT); Dynal AS] that is qualitatively and quantitatively characteristic of the mRNA population upon which it was synthesized. This template is reusable, and can also be applied to other procedures such as the generation of subtractive RNA populations.

Two micrograms poly(A)$^+$ mRNA was mixed with 0.2 mg Dynabeads Oligo(dT) (Dynal AS) in hybridization buffer (40 mM Tris-HCl pH 8.3; 0.2 M KCl) in a total volume of 40 μl and incubated on ice for 5 min to allow hybridization. The tube was gently agitated now and then to the keep beads suspended (applies to all further incubations as well). The beads were then washed once with hybridization buffer to remove excess of RNA. First strand cDNA was synthesized by adding 40 μl synthesis buffer [50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 1 U/μl AMV reverse transcriptase (Promega)] and incubating 5 min at room temperature, 5 min at 37° and 30 min at 42°. The beads were then washed once in 1× SSC, twice in denaturing solution (0.125 M NaOH; 0.1 M NaCl), twice in washing buffer

(0.1% SDS; 1× SSC), twice in 1× SSC and once in water. A DIG labeled probe was synthesized by including the beads/cDNA in a standard DIG labeling reaction (Boehringer Mannheim) on a rotary wheel at 37° overnight. After washing the beads with 1× SSC the single stranded probe was eluted in TE buffer by heat denaturation (care should be taken at this step not to let the beads dry out if they are to be reused). Comparison of this MSPIN probe to DIG labeled control DNA by luminescent detection estimated the probe to contain ~0.6 μg labeled DNA (data not shown). The MSPIN probe was then applied to Southern blots containing digestions of cloned genomic DNA to identify coding regions.

Computer analysis: Homology searching and general analysis of nucleic acids and protein sequences were done with the program packages UWGCG (University of Wisconsin), SEQSEE (Wishart *et al.* 1994) and PC-GENE (Intelligenetics Inc. and Genofit SA). Restriction enzyme maps were constructed with the program COMAP (K. O. Hofmann, University of Cologne, Germany).

Nucleotide sequence accession numbers: The nucleotide sequence data reported in this paper have been deposited into the EMBL, GeneBank and DDBJ sequence data bases under the accession numbers X91853 [*M(2)32A* gene and its flanking regions] and X91854 (*RPS13* cDNA).

RESULTS

***P*-element mutagenesis:** To induce the Minute phenotype, a small-scale *P*-element mutagenesis screen was set up with 70 individual vials, each with two *y* w^- *P{lacW}; TMS, Sb* Δ*2-3(99B)/+* males crossed to five *y* w^- females carrying the second chromosome balancers *CyO* and *Sp*, and the third chromosome balancers *Sb* and *D* (Bier *et al.* 1989). Two Minute white$^+$ males were obtained from ~10,000 progeny; one was localized to the second chromosome and balanced with *CyO*, and the other to the third chromosome and balanced with *D* (S. Sæbøe-Larssen and A. Lambertsson, unpublished observations).

With respect to the two most striking characters of *Minutes*, retardation of larval development and defective bristle formation, we characterize the present mutation as having a medium Minute phenotype (larval development is prolonged 24–30 hr). To study fertility and viability, mutant females and males were crossed with wild-type males and females, respectively, and eggs were collected and counted every fourth hour. When eclosion began, flies were collected, classified and counted. These crosses also allowed us to record the total developmental time for siblings in the same vial. Under noncrowded culture conditions at 25°, viability of both sexes appears to be on a par with wild type, and female fertility, as measured by egg production rate, is reduced by ~40%. Of the many other characters found in *Minutes*, *e.g.*, rough eyes, plexus venation of the wings, small body size, missing or duplicated bristles, and reduced or malformed aristae, only rough eyes have been occasionally observed.

Chromosomal localization of the *P{lacW}M(2)32A^1* mutation: The DNA sequence flanking the *P*-element insertion was recovered by plasmid rescue (Pirotta 1986). This plasmid rescue clone (pRCM32A1), which

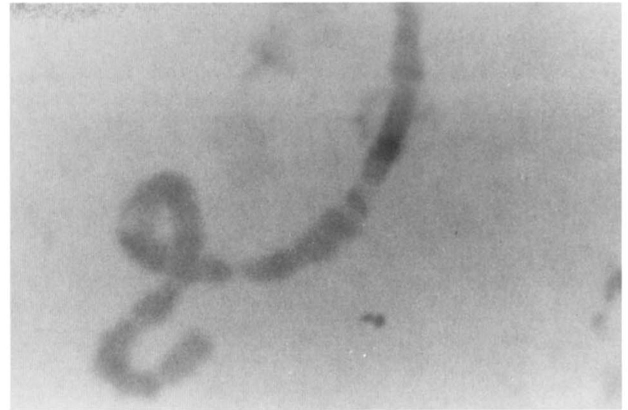

Figure 1.—Chromosomal localization of the *P*-element insertion. Oregon R wild-type salivary gland chromosomes hybridized with the plasmid rescue clone pRCM32A1 (*cf.* Figure 3A). The biotinylated probe only hybridizes to region 32A on chromosome 2L.

contains 2-kb genomic DNA and 1.9 kb from the 3′ end of the *P* element, was used as probe to map the cytological position of the *P*-element-induced *Minute* mutation by *in situ* hybridization to wild type and *y* w^-*; P{lacW} M(2)32A^1/ CyO* salivary gland polytene chromosomes. These *in situ* hybridizations showed that the element has inserted in region 32A on chromosome 2L (Figure 1), and that there is only one *P* element in the *y* w^-*; P{lacW} M(2)32A^1/ CyO* stock (results not shown but see Figure 4). Both the *P{lacW}* insert and the Minute phenotype were mapped by recombination (data not shown) to genetic map position II-42, which is in good agreement with the deficiency mapping of *P{lacW} M(2)32A^1* summarized in Figure 2 (see below). We also carried out a complementation analysis with the closely localized *Minutes* *M(2)30A* and *M(2)36F^2*, and complementation was found in both cases.

Deficiency mapping of *P{lacW}M(2)32A^1*: The cytogenetic position of the *P{lacW} M(2)32A^1* mutation is defined genetically by overlapping deficiencies on chromosome arm 2L and duplications (Figure 2). The transpositions *Tp(2;3)prd$^{2.27.3}$* and *Tp(2;3)dp^{h27}* consist of *Df(2L)prd$^{2.27.3}$* and *Dp(2;3)prd$^{2.27.3}$*, and *Df(2L)dp^{h27}* and *Dp(2;3)dp^{h27}*, respectively. Both *Dp(2;3)prd$^{2.27.3}$* and *Dp(2;3)dp^{h27}* produce Minute phenotype when combined with *P{lacW} M(2)32A^1/Df(2L)prd$^{2.27.3}$* and *P{lacW} M(2)32A^1/Df(2L)dp^{h27}*, respectively, and produce wild-type flies with *P{lacW} M(2)32A^1/+*. Deficiencies *Df(2L)Prl*, *Df(2L)J2* and *Df(2L)J27* all complement *P{lacW} M(2)32A^1*, producing Minute flies. These results confirm the *in situ* and recombination data, and we therefore conclude that *P{lacW} M(2)32A^1* is located in 32A (for exact breakpoints of the aberrations used here, see Lindsley and Zimm 1992). To our knowledge, no previously described *Minute* has been mapped to this region before (Lindsley and Zimm 1992), and the present *Minute* mutation is therefore named *P{lacW} M(2)32A^1*.

The *P* element causes the Minute phenotype: To de-

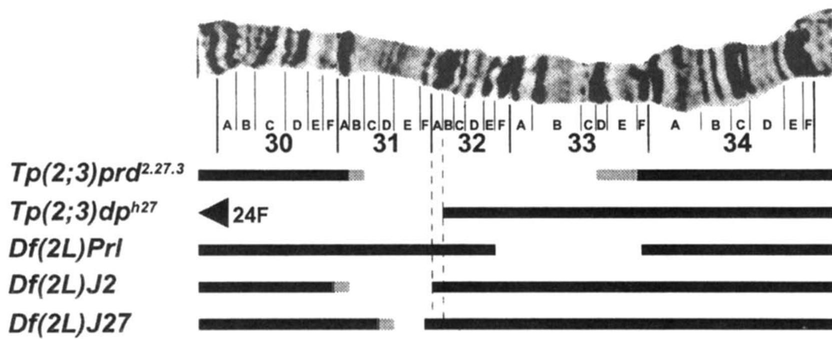

FIGURE 2.—Cytogenetic map of the 30–34 chromosome region. Only the reciprocal deficiency halves of the transpositions *Tp(2;3)prd*$^{2.27.3}$ and *Tp(2;3)dp*^{h27} are shown. The dashed vertical lines indicate the cytological position of *P{lacW}M(2)32A*^{1}. Uncertainty of breakpoints is indicated by hatched boxes. The arrowhead indicates that the distal breakpoint of *Df(2L)Prl* is at 24F. For exact breakpoints, see LINDSLEY and ZIMM (1992).

termine whether the Minute phenotype of *y w*$^{-}$;*P{lacW}M(2)32A*^{1}/*CyO* flies is caused by the *P*-element insert, a dysgenic cross was set up to remobilize the *P* element and thereby revert the phenotype to wild type. This was achieved by crossing *y w*$^{-}$;*P{lacW}M(2)32A*^{1}/*CyO* males to *y w*$^{-}$;*CyO/Sp;TMS, Sb* Δ*2-3(99B)/D* females, and *y w*$^{-}$;*P{lacW}M(2)32A*^{1}/*Sp;TMS, Sb* Δ*2-3(99B)/+* males were collected from the progeny. These males were crossed to *y w*$^{-}$; *CyO/Sp* females, and white non-Stubble males and females were collected and scored for Minute or Minute^{+} phenotype. Several revertants displaying a Minute^{+} phenotype were found, thus showing that the Minute phenotype can be reverted by mobilization of the *P* element. Since premeiotic *P* loss results in clusters of identical revertant progeny, only one revertant was retained from each father for further study. Revertant stocks were established from single males and one such revertant line was used for Southern, Northern and primer extension analyses (see below).

Isolation and identification of the *M(2)32A* gene: The gene interrupted by the *P*-element insertion was isolated by first recovering the DNA sequence 3′ of the *P*-element insert by plasmid rescue (PIROTTA 1986). This clone (pRCM32A1) was then used to probe a genomic Drosophila library, and overlapping clones covering ~28 kb were isolated and restriction enzyme mapped. Based on an alignment of the genomic clones and the plasmid rescue clone (Figure 3A), the *P{lacW}* insertion was localized to the region between *Eco*RI at position −0.5 kb and *Bam*HI at position +0.6 kb (positions are according to the map in Figure 3A). To verify this, the 1.1-kb *Eco*RI-*Bam*HI fragment was used to probe a whole-genomic Southern blot with *Eco*RI digested DNA from wild-type, mutant and revertant flies (Figure 4). As expected, the fragment hybridizes to a 2.5-kb *Eco*RI fragment in wild-type and revertant flies whereas the *P{lacW}M(2)32A*^{1}/*CyO* flies yield three hybridizing fragments: the 2.5-kb *Eco*RI fragment originating from the balancer chromosome, and two ~4.0 kb fragments that contain 3.7 and 1.9 kb of the *P* element 5′ and 3′ ends, respectively. This Southern analysis also shows that the sequence flanking the *P*-element insertion is a single copy sequence.

Magnetic solid phase inverse Northern (MSPIN) analysis of the 28-kb region (Figure 3A) detected several genes distant to the *P*-element insertion site that produce low abundant mRNAs, and one highly transcribed gene in the 2.5-kb *Eco*RI fragment flanking the *P*-element insertion site. To narrow down the region of interest the MSPIN analysis was applied to smaller subclones (Figure 3B). The schematic representation of these results (Figure 3C) shows that the *P* element has inserted in the left-most portion of the highly transcribed gene. No other genes were detected by this method within the region from −2.6 kb to +3.2 kb relative to the *P*-element insertion site.

To analyze the transcription unit identified by the MSPIN method, a third instar larval cDNA library was screened with the 2.5-kb *Eco*RI fragment as double-stranded probe. Ten cDNA clones were isolated and found to have identical restriction enzyme patterns although differences in length at their 5′ ends were observed. Northern analysis revealed the transcript identified by this cDNA to be abundantly expressed in all developmental stages (data not shown). Figure 5 shows the analysis of male and female poly(A)$^{+}$ RNA from wild-type, *P{lacW}M(2)32A*^{1}/*CyO* and homozygous *P{lacW}M(2)32A*^{1rv1} flies. The blot was hybridized with a single-stranded cDNA probe (antisense) and with a Drosophila α-tubulin probe as loading control. First, the cDNA probe detects a message of ~0.6 kb that is ~3.5 times more abundant in females than in males when compared with total poly(A)$^{+}$ content. Second, quantitative Northern analysis shows that the abundance of this transcript is reduced by ~50% in *P{lacW}M(2)32A*^{1}/*CyO* flies compared with wild type, showing that the *P*-element insertion disrupts the locus encoding this message. The amount of transcript is restored to wild-type level (or more) in homozygous *P{lacW}M(2)32A*^{1rv1} male and female revertants. No other transcripts were observed on the same stripped blot or on identical Northern blots when hybridized with a double-stranded probe from the region −0.8 kb to +0.7 kb relative to the P{*lacW*} insertion site (data not shown). Also, no other messages were detected on Northern blots of total RNA hybridized with the same probe (data not shown). Taken together, these results show that *P{lacW}M(2)32A*^{1} is a mutation in the gene represented by our cloned cDNA. We will refer to this gene as *M(2)32A*.

Sequence and primer extension analyses: To identify the gene disrupted by the *P*-element insertion, one of

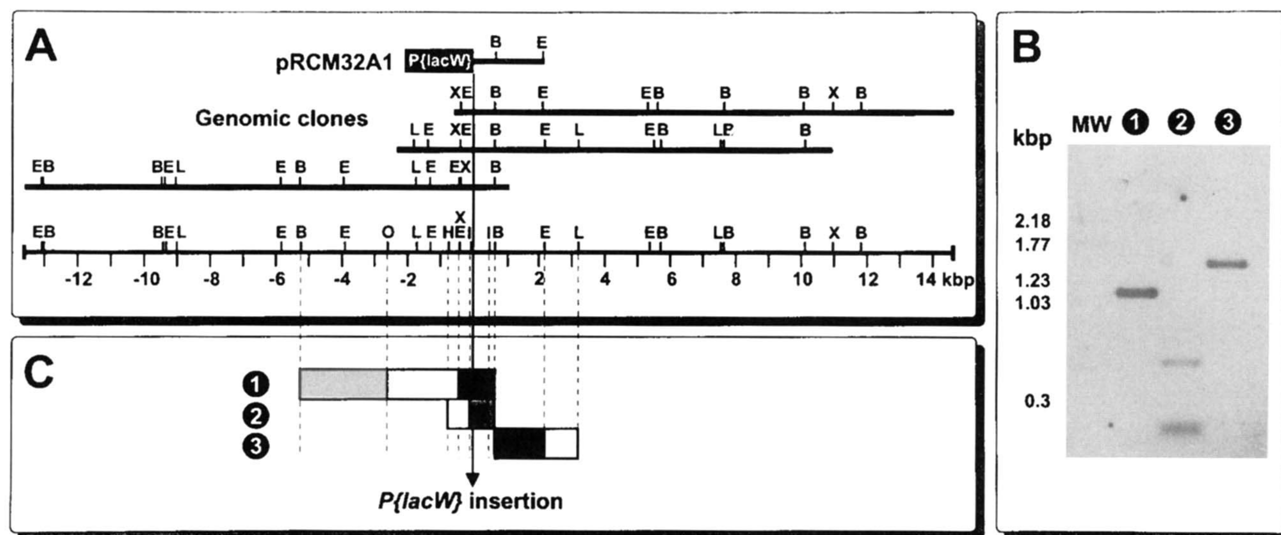

FIGURE 3.—Genomic mapping and inverse Northern analysis. (A) Restriction enzyme maps and alignment of the plasmid rescue clone pRCM32A1 and three genomic λ-clones (arms are not shown). The point of *P*-element insertion is indicated by a vertical arrow (extending into Figure 3C). Map positions (shown in the lower part) are numbered in kilobase pairs relative to the *P*-element insertion site. Restriction enzyme sites are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; I, *Hin*cII; L, *Sal*I; O, *Xho*I; and X, *Xba*I. (B) Magnetic solid phase inverse Northern (MSPIN) analysis of genomic plasmid clones from the region surrounding the point of *P*-element insertion. Lane 1 is *Bam*HI (−5.3) − *Bam*HI (+0.6) fragment digested with *Bam*HI + *Xho*I + *Xba*I, lane 2 is *Hin*dIII (−0.8) − *Bam*HI (+0.6) fragment digested with *Bam*HI + *Hin*dIII + *Hin*cII, and lane 3 is *Bam*HI (+0.6) − *Sal*I (+3.2) fragment digested with *Bam*HI + *Eco*RI + *Sal*I (positions are according to the map in Figure 3A). The blot was hybridized with an MSPIN probe (see MATERIALS AND METHODS) generated on a mixture of poly(A)$^+$ mRNA from third instar larvae and adult flies. (C) Schematic representation of the MSPIN analysis shown in Figure 3B (lanes have the same labels). Boxes represent the fragments produced in the respective digestions, and shading is applied to reflect the quantitative aspects. The *P*-element insertion site is indicated by a vertical arrow.

the isolated cDNA clones and 1.9-kb genomic DNA was sequenced. The sequence of the cDNA clone was then compared with the sequences in the EMBL and GeneBank data libraries. This search revealed a very high level of identity (98.9% in a 564 bp overlap) to the *D. melanogaster RP17* cDNA (McNabb and Ashburner 1993), and to *RPS13* cDNA sequences from *Musca domestica* (76%; Z. H. Zhou and M. Syvanan, unpublished results), *Rattus rattus* (73%; Suzuki *et al.* 1990), *Homo*

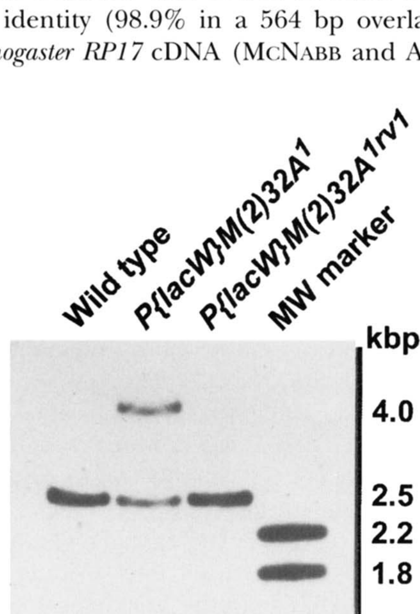

FIGURE 4.—Southern blot analysis of whole-genomic DNA from wild-type, mutant *y w*$^-$*;P{lacW}M(2)32A*^{1}*/CyO* and revertant *y w*$^-$*;P{lacW}M(2)32A*^{1rv1} flies digested with *Eco*RI. The blot was probed with a 1.1-kb *Eco*RI-*Bam*HI fragment containing sequences surrounding the point of *P*-element insertion.

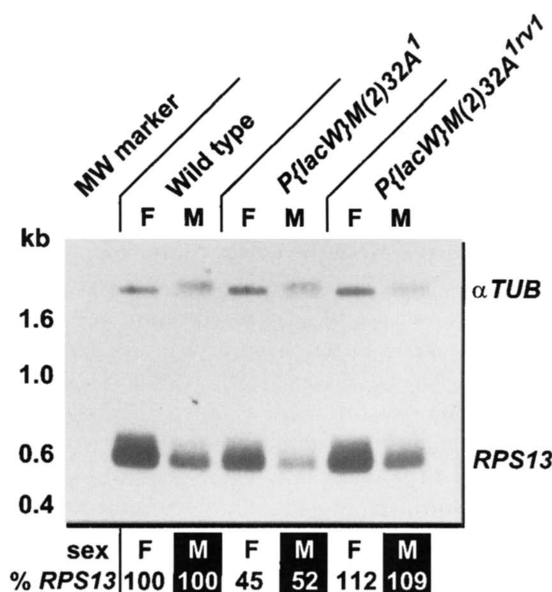

FIGURE 5.—Quantitative Northern analysis of poly(A)$^+$ mRNA from female (F) and male (M) wild-type, mutant *y w*$^-$*;P{lacW}M(2)32A*^{1}*/CyO* and revertant *y w*$^-$*;P{lacW}M(2)-32A*^{1rv1} flies. The blot was hybridized with *RPS13* cDNA and α-tubulin as probes. Quantitation of *RPS13* abundance (bottom) is calculated as percentage of wild-type levels when α-tubulin is considered as loading control.

sapiens (71%; CHADENEAU *et al.* 1993), *Zea mays* (68%; JOANIN *et al.* 1993) and *Brugia pahangi* (67%; ELLENBERGER *et al.* 1989), among others. The very high level of identity between the *RP17* cDNA and the one reported here, and the observations that both derive from single copy genes (see above; MCNABB and ASHBURNER 1993), suggest that these cDNAs originated from the same gene. When comparing the two cDNAs, we detected a 68-bp-long sequence at the 5′ end of *RP17* that is absent from our cDNA and that has no counterpart in our genomic sequence (data not shown; accession numbers: *Rp17* Z19052 and *RPS13* X91854). A closer look at the *RP17* cDNA identified this 68-bp sequence as an inverted repeat of a sequence near the 3′ end. The remaining differences are caused by five single nucleotide alterations. Two of these are found in the coding region and cause an alternative reading frame involving 10 amino acids. While our amino acid sequence in this region matches the other RPS13 sequences, the RP17 is dissimilar. It appears that the *RP17* cDNA contains sequence artifacts, especially the 68-bp 5′ end. Also, *Rp17* was cytologically mapped to 29A on the polytene chromosome map, which is inconsistent with the genetic data we present here. Based on the high level of identity to *RPS13* cDNAs from various species, we conclude that our cDNA corresponds to the eukaryotic *RpS13* gene. This cDNA will be referred to as *RPS13*. Given the sequence identity between *RPS13* and *RP17*, we believe that *RP17* also corresponds to this locus.

The Drosophila *RPS13* cDNA is 629-bp long [poly(A) tail included] and contains 17 nucleotides of untranslated leader, a 453-bp open reading frame encoding a protein of 151 amino acids, and a 123-bp untranslated trailer (poly(A) tail not included). The latter contains two polyadenylation signals (AATAAA) at positions 545 and 558, which are 49 and 36 nucleotides upstream of the polyadenylation site, respectively. As shown by primer extension analysis (see below), this cDNA is 13 nucleotides from being full length. Comparison of the *RPS13* cDNA sequence with the corresponding genomic sequence, *M(2)32A*, reveals two introns of 226 and 62 bp, respectively (Figure 6A). The splice donor and acceptor sequences and the branchpoint tetranucleotide of both introns match very well the Drosophila consensus splice sequences (MOUNT *et al.* 1992).

Primer extension analysis (Figure 6B) shows that there is one major transcription start site at position −30 relative to the open reading frame. Additional minor transcription start sites are found at positions −31, −33 and −34. Transcription is initiated in a pyrimidine rich tract, typical for mammalian and several Drosophila ribosomal protein gene cap sites (MAGER 1988; STEWART and DENELL 1993; ANDERSSON *et al.* 1994). The presence of a polypyrimidine tract has been found to be important for both promoter function and translational regulation (CHUNG and PERRY 1989; MOURA-NETO *et al.* 1989; HARIHARAN and PERRY 1990; LEVY *et al.* 1991). However, not all Drosophila ribosomal protein genes have this motif (MAGER 1988). The promoter of *M(2)32A* has no apparent TATA or CAAT motifs typically found in promoters of genes transcribed by RNA polymerase II.

To determine the exact location of the inserted *P* element in *M(2)32A*, the original plasmid rescue clone, pRCM32A1, was partially sequenced and compared with the wild-type genomic sequence. The *P* element was found to be inserted in the middle of the 5′ untranslated leader between nucleotides −15 and −16 relative to the translation initiation codon (see Figure 6). No other rearrangements or deletions were detected.

Conceptual translation of the open reading frame predicts a protein of ~17 kD, which is well conserved between distantly related species (Figure 7). The Drosophila RPS13 protein shows 87% identity to both human and rat RPS13 (SUZUKI *et al.* 1990; CHADENEAU *et al.* 1993), 78% to the parasitic nematode *B. pahangi* RPS13 (ELLENBERGER *et al.* 1989), 74% to the imperfect yeast *Candida maltosa* RPS13 (SASNAUSKAS *et al.* 1992), 69% to the fission yeast *Schizosaccharomyces pombe* RPS13 (MARKS and SIMANIS 1992), 71% to the monocot plant *Z. mays* RPS13 (JOANIN *et al.* 1993), and 32% identity to the archaebacterium *Haloarcula marismortui* RPS15 (SCHOLZEN and ARNDT 1992). A search for sequence motives using the computer program SEQSITE (WISHART *et al.* 1994) identified a bipartite nuclear targeting signal (DINGWALL and LASKEY 1991), IKK{11}RKDKD in position 92-110, consistent with the need for transportation to the nucleolus.

DISCUSSION

The genetic and molecular data presented here show that *P{lacW}M(2)32A*^{1} is a novel *Minute* mutation and that the gene, *M(2)32A*, encodes ribosomal protein S13. Our result supports the hypothesis that *Minutes* are mutations in ribosomal protein genes. We have demonstrated that insertion of a *P* element within the untranslated 5′ leader sequence of the *M(2)32A* gene results in a ~50% reduction of *RPS13* mRNA abundance. We have also shown that both *RPS13* mRNA abundance and phenotype are restored to wild type in revertant flies. Therefore, we conclude that a *P* element inserted in the 5′ end of the *M(2)32A* gene causes a loss-of-function mutation, which results in the Minute phenotype. The present report also demonstrates that *P*-element mutagenesis is a fruitful method to clone loss-of-function *Minute* mutations.

In Drosophila, the larva increases in size by growth and polytenization of the cells, and dividing cells are found in the imaginal discs, the precursors of the adult system. Cells increasing in size (larval system) or dividing (imaginal system) must maintain a balance between the levels of soluble proteins, various membranes and ribosomes to optimize conditions for these processes. It has been shown that *Minute* cells divide more slowly than do non-*Minute* cells, and when present in the same

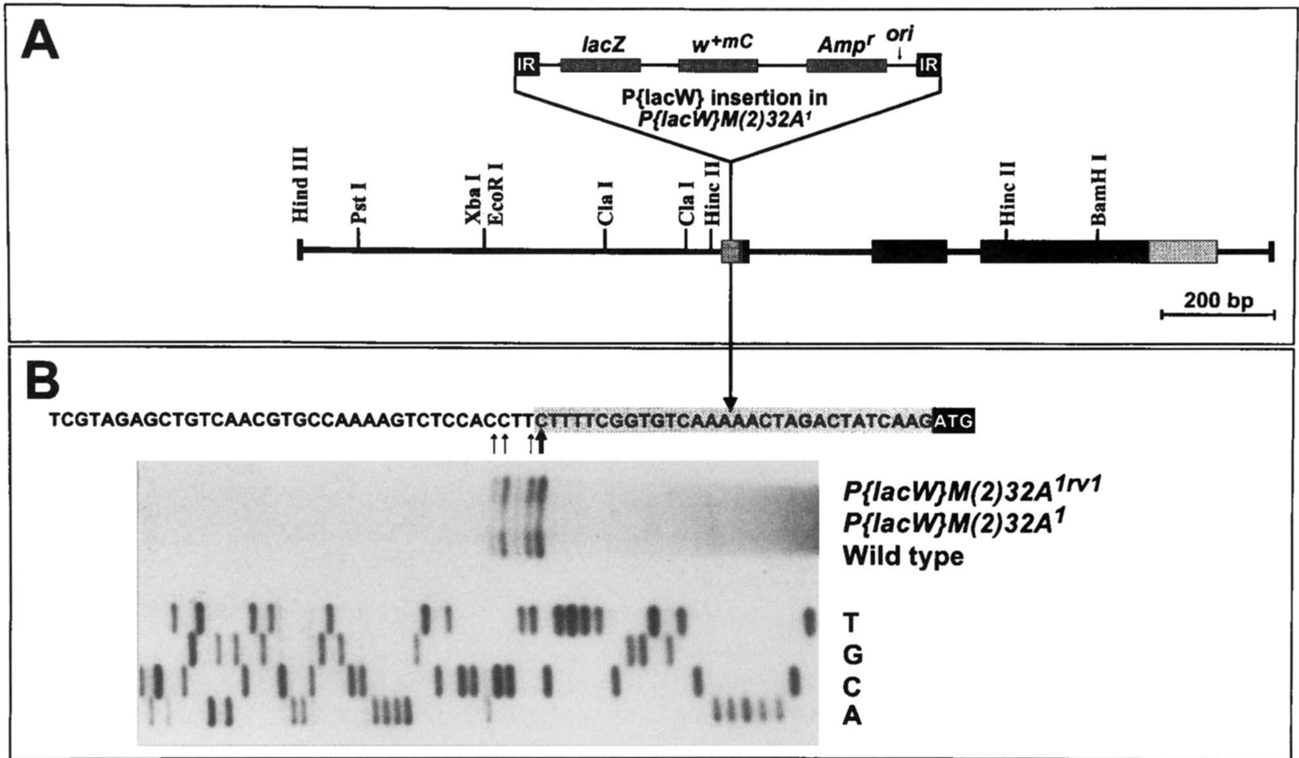

FIGURE 6.—Organization of the Drosophila *M(2)32A* gene. (A) Diagram representation of the genomic sequence (EMBL: X91853) harboring the *M(2)32A* gene. Exons are represented by boxes; black color indicate protein-coding regions while gray color indicate noncoding parts of the exons. The *P*-element (not shown to scale) insertion site in the mutant *y* w^-; *P{lacW}M(2)32A*^{1}/*CyO* stock is indicated. (B) Primer extension analysis with poly(A)$^+$ mRNA from wild-type, mutant and revertant adult flies extended from the primer described in MATERIALS AND METHODS. The sequence was generated using the same primer with a genomic clone as template and is shown at the top with background colors corresponding to the boxes in A. Transcription is seen to initiate in a pyrimidine rich tract, with cytosine in position −30 (from the translation initiation codon) being the most frequently used starting point. The vertical arrow shows the position of the *P*-element insertion in the mutant *y* w^-; *P{lacW}M(2)32A*^{1}/*CyO* stock.

tissue, the latter divide at the wild-type rate, implying that this effect is cell autonomous (MORATA and RIPOLL 1975). Since the discs are devoid of functions necessary for normal larval development (SHEARN *et al.* 1971), the possibility that larval delay is a consequence of delayed imaginal development would require additional complicated assumptions. It is more likely that mutations in ribosomal protein genes affect both larval and imaginal cells at the level of protein synthesis, resulting in lowered rates of both larval development and disc cell division.

The Minute phenotype is both distinct and complex but there are conspicuous differences between *Minutes* (LINDSLEY and ZIMM 1992). There may be at least two explanations to these differences. First, in eukaryotes most ribosomal protein genes appear to be transcribed in excess and regulated at the level of translation (JACOBS-LORENA and FRIED 1987). In Drosophila, transla-

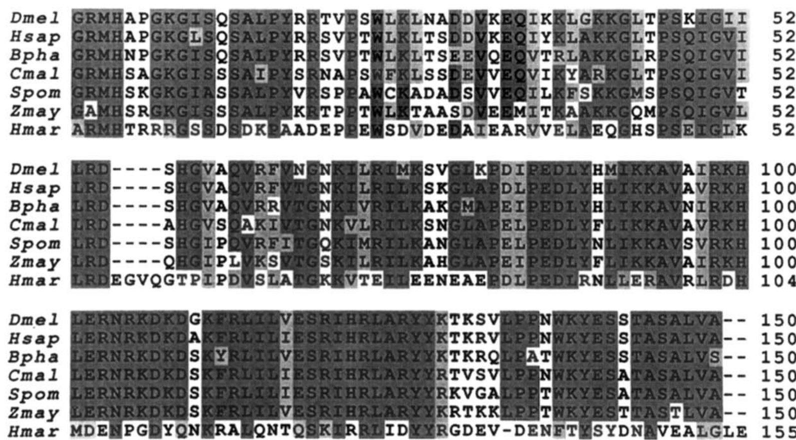

FIGURE 7.—RPS13 amino acid alignment. Comparison of the predicted RPS13 amino acid sequences from *D. melanogaster* (Dmel), *H. sapiens* (Hsap), *B. pahangi* (Bpha), *Candida maltosa* (Cmal), *S. pombe* (Spom), *Z. mays* (Zmay) and *Haloarcula marismortui* (Hmar) (see text for references). Dark gray shaded regions represent sequence identity while the light shaded regions represent conservative amino acid changes using a cut-off at 0.7 in the Dayhoff matrix values used by GRIBSKOV and BURGESS (1986). Shading is applied where at least five sequences have identical or similar amino acids. Amino acid substitutions that do not fit the consensus are not shaded and gaps are indicated by dashes.

tion of ribosomal protein mRNAs is selectively down-regulated during early embryogenesis and under heat-shock conditions, up-regulated in paragonial cells after copulation, and apparently unaltered when imbalanced synthesis (overexpression) of rRNA or ribosomal protein mRNA occurs (JACOBS-LORENA and FRIED 1987, and references therein). Thus, it is possible that loss of one allele of a ribosomal protein gene may result in increased translation of the mRNA produced by the remaining allele. The translation of some ribosomal protein mRNAs may, however, be more efficiently up-regulated than others, resulting in the quantitative differences in phenotype observed between *Minutes*. Other explanations could be that various ribosomal protein genes are expressed at different levels and/or that the threshold requirement is different for each component needed for ribosome assembly. In either case, haploinsufficiency of different ribosomal proteins should have a distinct effect on the Minute phenotype.

All *Minutes* described to date have a dominant phenotypic expression, which makes them easy to detect. Recessive *Minute* mutations, on the other hand, are difficult to detect or easily overlooked. A precedent now exists for a recessive ribosomal protein mutation to cause a Minute phenotype. Females homozygous for *string of pearls*, which encodes ribosomal protein S2, are sterile and show a mutant ovary phenotype (CRAMPTON and LASKI 1994). They also have typical *Minute* traits like short, thin bristles and delayed development. Homozygous males are fertile but show mutant bristle phenotype. There is also the possibility that mutations in genes other than ribosomal protein genes may lead to 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) and *mini* (5S RNA genes; KAY and JACOBS-LORENA 1987) genes are two examples.

Cells in multicellular organisms are distinctly different from each other as a consequence of the unique processes of cell differentiation, organogenesis and morphogenesis. As a result, not only the pattern of proteins synthesized is different in different cells, but in addition, the rates of protein synthesis are also differentially modulated during growth and development. A systematic genetic and molecular analysis, therefore, using an experimental system such as targeted disruption of genes involved in protein synthesis in Drosophila, will add to our understanding of the phenotypic consequences of haploinsufficiency for these gene products. As the same basic processes seem to work in all multicellular organisms, there is good reason to believe that such studies will illuminate all creatures, including ourselves.

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