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Effects of the *maleless* Mutation on *X* and Autosomal Gene Expression in *Drosophila melanogaster*

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ABSTRACT

The mutational effect of the *maleless* (*mle*) gene in *Drosophila* has been reexamined. Earlier work had suggested that *mle* along with other male-lethal genes was responsible for hypertranscription of the *X* chromosome in males to bring about dosage compensation. Prompted by studies on dosage sensitive regulatory genes, we tested for effects of mle^{ts} on the phenotypes of 16 *X* or autosomal mutations in adult escapers of lethality. In third instar larvae, prior to the major lethal phase of *mle*, we examined activities of 6 *X* or autosomally encoded enzymes, steady state mRNA levels of 15 *X*-linked or autosomal genes and transcripts from two large genomic segments derived from either the *X* or from chromosome *2* and present in yeast artificial chromosomes. In contrast to the previously hypothesized role, we detected pronounced effects of *mle* on the expression of both *X*-linked and autosomal loci such that a large proportion of the tested genes were increased in expression, while only two *X*-linked loci were reduced. The most prevalent consequence was an increase of autosomal gene expression, which can explain previously observed reduced *X*:autosome transcription ratios. These observations suggest that if *mle* plays a role in the discrimination of the *X* and the autosomes, it may do so by modification of the effects of dosage sensitive regulatory genes.

DOSAGE compensation in Drosophila is the equivalence of expression of genes on the *X* chromosome despite their unequal dosage in the two sexes (MULLER *et al.* 1931; MULLER 1950). It is achieved by a twofold increase in the transcription of *X*-linked genes in males (MUKHERJEE and BEERMANN 1965). Several studies have demonstrated that the ability of a gene to dosage compensate can depend on either or both of two broad parameters–*cis* regulatory control and genomic position (SPRADLING and RUBIN 1983; HAZELRIGG *et al.* 1984; MCNABB and BECKENDORF 1986; SASS and MESELSON 1991; HIEBERT and BIRCHLER 1992 and additional references therein); however, the mechanism for the doubling of transcriptional rates along the entire *X* chromosome is unknown.

A current model relating to dosage compensation proposes that a set of genes, identified in screens for male-specific lethal mutations, encode products that act to double the transcriptional rate of the *X* chromosome in males only (BELOTE and LUCCHESI 1980a). These are *male-specific lethal-1* (*msl-1*) [2-53.3], *msl-2* [2-9.0] (BELOTE and LUCCHESI 1980b), *msl-3* [3-25.8] (UCHIDA *et al.* 1981), and *maleless* (*mle*) [2-55.2] (GOLUBOVSKY and IVANOV 1972; FUKANAGA *et al.* 1975). They are all recessive male lethal in the third larval instar to prepupal stage, and their action depends directly or indirectly on the splicing mode of the sex determination gene, *Sex-lethal* (*Sxl*) (for reviews see BAKER 1989; HENIKOFF and MENEELY 1993). Of these, *mle* has served a central role in tests of the model. A reduction in the ratio of *X*-linked:autosomal gene expression in *mle* male larvae has been found and interpreted to reflect a lack of *X* chromosome hypertranscription. Such ratios have been calculated using the techniques of polytene chromosome transcriptional autoradiography (BELOTE and LUCCHESI 1980a; OKUNO *et al.* 1984), enzyme activity measurements of various *X*- and autosomally encoded enzymes (BELOTE and LUCCHESI 1980a), and steady state mRNA measurements of the *X*-linked *Sgs4* and autosomal *Sgs3* genes (BREEN and LUCCHESI 1986). Consistent with the sex-specific phenotype of *mle*, the MLE protein associates preferentially with the *X* chromosome relative to the autosomes in wild type males (KURODA *et al.* 1991). *X* chromosome binding has been used as an assay to demonstrate interactions among male-specific lethal loci (GORMAN *et al.* 1993).

The rationale for undertaking a reexamination of *mle* stemmed initially from the phenomenon of *trans*-acting regulatory dosage effects, which are observed in aneuploidy in a variety of higher eukaryotes (BIRCHLER 1979, 1981; BIRCHLER and NEWTON 1981; BIRCHLER *et al.* 1989, 1990). Such dosage effects occur when a change in copy number of a chromosomal segment affects the expression of a target gene elsewhere in the genome, usually by negative (or inverse) regulation but sometimes by positive (or direct) regulation. Only a subset of unlinked

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genes is affected in a given segmental aneuploid, and an individual target gene may be affected by more than one varied segment. Such effects have been observed in human (WHATLEY *et al.* 1984), mouse (REICHERT 1986), maize (BIRCHLER 1979, 1981; BIRCHLER and NEWTON 1981), and *Drosophila* (BIRCHLER 1984, 1992; BIRCHLER *et al.* 1989, 1990; RABINOW *et al.* 1991; SABL and BIRCHLER 1993). Single genes have been identified that appear to be responsible for such aneuploid effects, for example *Inr-a* (RABINOW *et al.* 1991). This gene elevates the expression of the *white* locus approximately twofold, when present at one-half of the normal diploid copy number and reduces the expression of *white* to two-thirds, when present in three halves the diploid level. Thus, there is an inverse correlation between the number of functional copies of *Inr-a* and the expression of *white*. We note that many regulatory genes identified in higher eukaryotes exhibit dosage effects, for example the *Drosophila* genes *bicoid* (DRIEVER and NUSSLEIN-VOLLHARD 1989; STRUHL *et al.* 1989), *Krüppel* (SAUER and JACKLE 1993), *dorsal* as well as other basic helix-loop-helix proteins (WARRIOR and LEVINE 1990; JIANG and LEVINE 1993) and the *Polycomb* group genes (MESSMER *et al.* 1992).

Dosage-sensitive inverse regulation has been invoked to explain the phenomenon of autosomal dosage compensation, which is the equivalent expression of genes linked to a varied segment of an autosome compared to the normal diploid. In Drosophila, it was originally described for trisomies of *2L* and *3L*, in which the majority of the monitored loci were dosage compensated (DEVLIN *et al.* 1982, 1988); such compensation occurs at the transcriptional level (DEVLIN *et al.* 1984). Autosomal dosage compensation was also found for the *alcohol dehydrogenase* (*Adh*) locus on chromosome *2* when a large chromosomal segment surrounding the gene was varied (BIRCHLER *et al.* 1990). By subdividing this segment, dosage compensation of *Adh* was shown to result from the opposing effects of increased copy number of the *Adh* structural gene and an inverse effect exerted by a specific neighboring region, that was also present on the original varied segment (BIRCHLER *et al.* 1990). Using a reporter construct, the inverse effect was shown to require the *Adh* promoter (BIRCHLER *et al.* 1990). A similar mechanism is responsible for *Adh* dosage compensation on the long arm of chromosome *1* in maize (BIRCHLER 1981).

It is reasonable to suggest that dosage compensation of the *X* chromosome in Drosophila could have arisen by a similar mechanism, whereby the difference in *X*-linked gene dosage between the sexes would be compensated by the action of dosage sensitive inverse regulator loci also present on the *X*. The inverse effect is of the appropriate magnitude–*i.e.*, approximately twofold, to account for such a change in male *X* chromosome activity. This hypothesis predicts dosage compensation of *X*-linked genes in metafemales (3*X*:2A) by reducing the expression of each of the three *X* chromosomes to the inverse level of the diploid–*i.e.*, to 2/3. Consistent with this hypothesis, dosage compensation of the *X* chromosome has been demonstrated in metafemales (LUCCHESI *et al.* 1974; STEWART and MERRIAM 1975; DEVLIN *et al.* 1985, 1988). In subsequent experiments, autosomal gene expression in metafemales was examined and found to be reduced by the inverse effect (BIRCHLER *et al.* 1989; BIRCHLER 1992), presumably accounting for their greatly reduced viability. This example of inverse regulation is similar to the effects of trisomies for *2L* and *3L* on the expression of unlinked genes, including some on the *X* chromosome (DEVLIN *et al.* 1988).

If the *X* carries dosage sensitive regulators that produce an inverse effect on a subset of autosomal genes, as do all autosomal arms tested, the question arises as to why autosomal transcription does not double in normal males. Some genes do indeed exhibit elevated expression in males, for example *glass* (SMITH and LUCCHESI 1969; BIRCHLER 1984), *brown* (RABINOW *et al.* 1991), *purple* (YIM *et al.* 1977), *pink* (BIRCHLER *et al.* 1989) and *light* (DEVLIN *et al.* 1990; J. A. BIRCHLER, unpublished data), but in general only a few approach the twofold level. Conceivably, a function exists in males to counteract the response of the autosomes to *X* chromosome inverse regulation, by altering a rate limiting step of gene expression for example. If such a function were lost by mutation, mutant males would exhibit an increase in transcription of certain autosomal genes, due to restoration of the rate limiting aspect of inverse regulation by the *X* chromosome.

This may be the case with mutants of *mle*. In both published accounts of polytene chromosome transcriptional autoradiography of *mle* males, involving three experiments, absolute transcription of autosomes was increased in *mle* males, and *X* chromosome levels were virtually unaffected (BELOTE and LUCCHESI 1980a; OKUNO *et al.* 1984). In these experiments it was assumed that autosomal transcription would be unaffected by *mle*, so it was measured as an internal control on *X* transcription. In the context of the time, it was deemed an appropriate assumption. Thus it was concluded that *X* chromosome transcription was reduced in *mle* homozygous males. The same assumption was made in a study of mRNA levels of *Sgs4* and *Sgs3* (BREEN and LUCCHESI 1986), in which the data were treated as an *X*:autosome expression ratio. Therefore, an alternative explanation for the reduced *X*:autosomal transcription ratio in *mle* males is an increase in autosomal transcription rather than a decrease from the *X*.

Moreover, the female lethality of Sxl^f was postulated to be due to a hyperactivation of the *X* chromosome expression by activation in females of the male-specific lethal loci (CLINE 1984). If the hyperactivation of the *X* chromosomes in Sxl^f homozygous females could be eliminated, one would predict a return to viability. This

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could be tested by examining the viability of the Sxl^f/Sxl^f; *mle/mle* genotype, which, by this hypothesis, would give viable *XX* males with a combined *X* chromosome expression equivalent to normal females. However, this genotype was constructed and no amelioration of the *Sxl* lethality was found (SKRIPSKY and LUCCHESI 1982).

To clarify the role of *mle*, we sought to determine in absolute terms its effect on *X*-linked and autosomal gene expression. Both genetic and molecular analyses were performed on the mle^{ts} allele in homozygous males compared to heterozygous male and female siblings. Initially, we tested for effects on the phenotypes of 16 *X* or autosomal mutations in adult escapers of lethality. The results of these observations prompted a molecular analysis in larvae, where the *mle* effects were originally studied. We examined enzyme activities of six *X*- or autosomally encoded enzymes, steady state mRNA levels of fifteen *X*-linked or autosomal genes, and transcripts from two large genomic segments derived from *X* and autosomal locations and present in yeast artificial chromosomes. We detected pronounced effects of *mle* on the expression of both *X* and autosomal loci. The majority of these changes were increases in expression, with a greater proportion of autosomal loci showing elevated expression than *X*-linked loci. Only two *X*-linked loci were reduced. These data are inconsistent with the hypothesis that *X* chromosome transcription is reduced to one-half of normal in homozygous *mle* males. In view of earlier transcriptional autoradiography data, and the experiments presented here, it is more likely that the canonical mutational effect of *mle* is an elevation of the expression of autosomal genes.

MATERIALS AND METHODS

Fly strains: Stocks were maintained on cornmeal dextrose media at 25°. The strain carrying the *Adh-w* construct was provided by JANICE FISHER and TOM MANIATIS (Harvard University), and it contains both of the *Adh* promoters. The stock described in Figure 2 was constructed by crossing mle^{ts} vg^{79d5} females to *T(2;3), CyO Tb/++* males, then backcrossing the *Cy* male progeny. The mutations used in the phenotypic analyses (see Figures 1 and 2) are described in LINDSLEY and ZIMM (1992). Those mutations located on chromosome *3* were made homozygous in a background of mle^{ts} $vg^{79d5}/SM6a$. The two located on chromosome *2*–*pr* and *If* were recombined onto the mle^{ts} vg^{79d5} chromosome. The *pr* allele was analyzed as a homozygote and *If* was analyzed as a heterozygote. Flies were aged 4 days before photographing.

Enzyme activity and protein measurements: Enzyme activity measurements of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), β-hydroxy acid dehydrogenase (β-HAD), alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH) and α-glycerophosphate dehydrogenase (αGPDH) were performed as described by BIRCHLER *et al.* (1989), with the following modifications. Ten sets of five wandering third instar larvae for each genotype were collected and stored at −80° in 250 μl of extraction buffer in 1.5-ml microcentrifuge tubes. Immediately before the assays they were homogenized using a Teflon pestle (Bel-Art) and kept on ice. All assays were performed within the same 16-hr period. After 5 min of centrifugation at 10,000 × *g*, 220 μl of supernatant was removed for enzyme activity and protein determinations. After incubations at 30°in the appropriate reaction conditions for each enzyme, reduction of NAD or NADP was measured by determining optical density at 340 nm with a Beckman DU-50 spectrophotometer.

Protein determinations were performed using 10 μl extract in 990 μl of Bio-Rad protein assay dye reagent concentrate (previously diluted with 4 volumes of distilled water). Amount of protein, (μg)/larva, was calculated using the linear OD_{595} response obtained from bovine serum albumin in 1.5-μg increments from 1.5 to 30 μg in 1 ml.

Total larval nucleic acid isolation: Total DNA and RNA were extracted using a modification of the technique of RAHA *et al.* (1990). Three samples of five wandering third instar larvae for each of the four genotypes shown in Figure 5 were collected and stored at −80°in STEL buffer (0.2% SDS, 10 mM Tris-Cl, pH 7.5, 10 mM EDTA, and 100 mM LiCl). Phenol and phenol-chloroform were equilibrated with STEL until the pH was above 7.0. Larvae were homogenized, extracted twice with phenol, once with phenol-chloroform, once with chloroform-isoamyl alcohol (24:1), and precipitated at −80° with 0.1 volume of 5 M LiCl and 2.5 volumes of cold ethanol. Nucleic acid was resuspended to an approximate concentration of 0.5 μg/μl as determined by spectrophotometric analysis. Separate DNase I and RNase A digestions confirmed that the upper and lower bands on ethidium stained gels corresponded exclusively to genomic DNA and rRNA, respectively.

Triplicate isolations of the four larval genotypes shown in Figure 3 were subjected to electrophoresis in 1% agarose. In the same gel, a dilution series of identically prepared nucleic acid was electrophoresed, and the gel was stained with ethidium bromide and destained. Two Polaroid negatives (type 55 Polaroid film) were prepared identically of the two sets of lanes. Lanes were scanned with the LKB 2202 Ultroscan laser scanning densitometer at the slowest scanning speed in order to obtain the most information from the bands, and analyzed with LKB GelScan interface and software package. The densitometer parameters, including absorbance range, speed, reference absorbance, and background curve were the same for each scan. The integration values of the peaks of the dilution series were plotted against concentration to obtain a standard curve. Relative quantification of DNA compared to RNA was performed on lanes containing 1.5 μg of total nucleic acid, an amount which fell within the linear response range.

RNA isolation: RNA was extracted by the guanidine-HCl method (COX 1968). Briefly, wandering third instar larvae were harvested and frozen at −80° in 8 M guanidine-HCl (Ultrapure, Schwarz/Mann) in 1.5-ml microcentrifuge tubes. Larvae were homogenized in 8 M guanidine-HCl using a powered tissue homogenizer (Tekmar); RNA was precipitated in 0.5 volume ethanol. Four more extractions with 4 M guanidine-HCl and ethanol precipitations followed. Finally the RNA was extracted from the pellet three times with sterile water, the second time at 56°. After ethanol precipitation from the pooled water extractions, the RNA was dissolved in sterile water at a concentration of 4.0 μg/μl and stored at −80°.

Northern analysis: Total RNA was separated on formaldehyde-agarose gels (1.5%) (LEHRACH *et al.* 1977) at 10 μg/lane. Gels were run at approximately 50 V for 18 h. Formaldehyde was present in the tank buffer at the same concentration as in the gel (6.7%). The RNA was capillary transferred to Biotrans nylon membrane overnight using 20 × SSC, then UV cross-linked to the filter (CHURCH and GILBERT 1984). Hybridizations were performed as described (BIRCHLER and HIEBERT 1989).

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Band intensities were determined by laser scanning densitometry. A dilution series of total RNA was prepared to test whether rRNA would provide an accurate control for loading differences between lanes containing 10 μg total RNA. A gel containing four replicas of a series of 5, 10 and 20 μg was blotted and hybridized with an antisense ^{32}P-labeled rRNA probe. The blot was exposed on Kodak X-AR film for a length of time that did not saturate the film. A densitometric scan of the autoradiogram gave linear increases in band density with amount of sample loaded. The data in Table 3 are from scans of autoradiograms with non-saturating exposures.

RNA probes: Northern blots were probed with ^{32}P-labeled antisense RNA probes made with T3, T7 or SP6 RNA polymerases from the linearized constructs described below. Clones obtained that lacked promoters for *in vitro* transcription were reinserted into appropriate vectors as described below. *In vitro* transcription was performed as described by BIRCHLER and HIEBERT (1989).

A construct of the *w* gene (GOLDBERG *et al.* 1982), pIBIw-cDNA, provided by R. LEVIS (Fred Hutchinson Cancer Research Center, Seattle), contains exons 3 through 6 inserted into pIBI76. A genomic clone of *Adh*, pSPZ1Adh3′, consists of a 2-kb *Bam*HI-*Eco*RI fragment from the 3′ end of the gene (GOLDBERG 1980), inserted into the pSPZ1 vector. A construct of the *β Tub56D* gene (BIALOGAN *et al.* 1985), provided by M. MORTIN (NIH, Bethesda, Maryland), consists of a 0.2-kb *Bam*HI fragment containing coding sequences inserted into pBluescript. The *Rp49* clone (KONGSUWAN *et al.* 1985), provided by L. RABINOW (Waksman Institute, Piscataway, New Jersey), contains coding sequences inserted into pBluescript. A construct of the *r* cDNA, pcrud5 (SEGRAVES *et al.* 1984), provided by W. ZERGES (Princeton University), contains a 5-kb *Eco*RI fragment in pGEM2. A construct of the *bw* cDNA (DREESEN *et al.* 1988), pVZ1bw+, was provided by S. HENIKOFF (Fred Hutchinson Cancer Research Center). The *st* construct, pG1stXB0.9 (TEARLE *et al.* 1989), provided by A. HOWELLS (The Australian National University), contains a 0.9-kb *Xho*I-*Bam*HI fragment inserted in pGEM1. The *v* construct, spv8.7 (SEARLES and VOELKER 1986), provided by L. SEARLES (University of North Carolina), consists of a 1.9-kb fragment containing most of the coding region inserted in pGEM1. A construct of the *y* cDNA (GEYER and CORCES 1987) consists of a 5.0-kb fragment containing the entire cDNA, provided by P. GEYER (University of Iowa), in the *Sal*I site of pBluescript IIKS(+). It is designated pBSIIKS(+)ycDNA. A construct of the *sis-b* gene (synonymous with the *ASC* T4 transcription unit of the *achaete-scute* complex) (CAMPUZANO *et al.* 1985), provided by J. MODOLLEL (University of Madrid), consists of a 1.5-kb *Eco*RI genomic fragment containing the entire coding region subcloned into pBluescript. A subclone of the *Sgs4* gene (MUSKAVITCH and HOGNESS 1982) was provided by S. BECKENDORF. It is designated pGEMSgs4 and consists of the pGEM1 vector (Promega) with a 0.7-kb insert of the *Sgs4* coding region. A construct containing the *Sgs3* gene, pBSIIKS(+)Sgs3, was made by subcloning a 1.6-kb *Sal*I-*Xho*I fragment from λcDm2008 (MEYEROWITZ and HOGNESS 1982), provided by S. BECKENDORF (University of California, Berkeley), into pBluescript IIKS(+). A construct, pBSIIKS(+)Zw, containing a genomic segment of the *Zw* gene, encoding G6PD (GANGULY *et al.* 1985) was made from a pUC9 construct provided by R. GANGULY (University of Tennessee, Knoxville). It consists of a 1.4-kb *Pst*I fragment containing most of exon 4 inserted in Bluescript IIKS+ (Stratagene). A genomic clone of *Gpdh*, encoding αGPDH (VON KALM *et al.* 1989), was provided by D. SULLIVAN (Syracuse University). It contains a 2-kb *Xho*I-*Hin*dIII fragment containing exons 3 and 4 inserted into pBluescript SK(+). It is designated pBSSK(+)αGPDH. A construct of the *Pgd* gene encoding 6PGD (SCOTT and LUCCHESI 1991), was provided by M. SCOTT and J. LUCCHESI (Emory University, Atlanta). It contains a 1.7-kb fragment of cDNA in pBluescript KS(−). It is designated pBSKS(−)1.7AS. The construct pSPZ2Dmry22c#1, consists of a 0.9-kb *Hin*dIII fragment of the 28S rRNA repeat (DAWID *et al.* 1978) inserted into the pSPZ2 vector.

Yeast artificial chromosomes: The two YACs, N23-10 and R14-41 (a gift of the laboratory of D. HARTL, Harvard University), were prepared from cultures in YCD/AHC selective media [per liter: 1.7 g yeast nitrogen base without amino acids and without $(NH_4)_2SO_4$ (Difco), 5 g $(NH_4)_2SO_4$, 10 g casein hydrolysate (U.S. Biochemical Corp.), 15 mg adenine hemisulfate (Sigma), 20 g glucose, pH adjusted to 5.8]. Cultures were grown for 2 days at 30°. Cells were pelleted at 1500 × *g* for 10 min, rinsed in distilled water, pelleted again, and resuspended in 5 ml spheroplasting solution [5 ml SCE (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7.0), 125 μl β-mercaptoethanol, 1 mg Zymolyase (ICN Immunobiologicals)], and mixed with an equal volume of 1% low melting point agarose in SCE. After gelling at 5°, plugs of cells were lysed for 36 hr at 55° in 12 ml of lysing solution (0.5 M EDTA, 1% Sarcosyl, 10 mM Tris-Cl, pH 8.0, 0.5 mg/ml Proteinase K).

Lysed plugs were fractionated on 1% agarose using OFAGE (PC 750 Pulse Controller, Hoeffer Scientific Instruments), with the following parameters: 200 V for 16 hr with 45-sec switch time, then 15-sec switch time for 10 hr. The CHEF system (Bio-Rad) was also used, with parameters as follows: switch time = 30 s, no ramp; start ratio = 1; voltage = 200 V; duration = 24 hr. Individual YAC bands were excised and DNA isolated by treatment with β-agarase (New England Biolabs) or Gene-Clean (Bio 101). DNA probes labeled with ^{32}P were prepared as described (FEINBERG and VOGELSTEIN 1983).

RESULTS

Effects of *mle^{ts}* on *X* and autosomal mutant phenotypes: A preliminary test of the effect of *mle* on gene expression was done by analyzing its ability to modify the phenotypes of various *X* and autosomal mutations. Stocks were constructed that would segregate for *mle^{ts}*, the same allele used in most previous studies, in a background of other mutations having visible adult phenotypes. The *mle^{ts}* allele produces homozygous adult male escapers of lethality at a rate of approximately 1/150 males at 18°. The mutations were chosen on the basis of the ability to detect changes in their expression levels by inspection of phenotypic severity–*i.e.*, hypomorphs and neomorphs. Phenotypes were examined in males and females of homozygous and heterozygous *mle^{ts}*, although in no case was a major effect observed in females. Stocks were screened until at least two escapers, but often more, were examined. Sixteen experiments were performed, nine involving *X*-linked mutations, and seven involving mutations on chromosomes *2* or *3* [see LINDSLEY and ZIMM (1992) for descriptions].

Figure 1 shows representatives of the effects of *mle^{ts}* on various *X*-linked mutant phenotypes. The *Bar* mutation is a dominant neomorph, causing a reduction in the number of ommatidia, and resulting in a bar-shaped eye. *Bar* is dosage compensated at the phenotypic level, as hemizygous males and homozygous females are both af-

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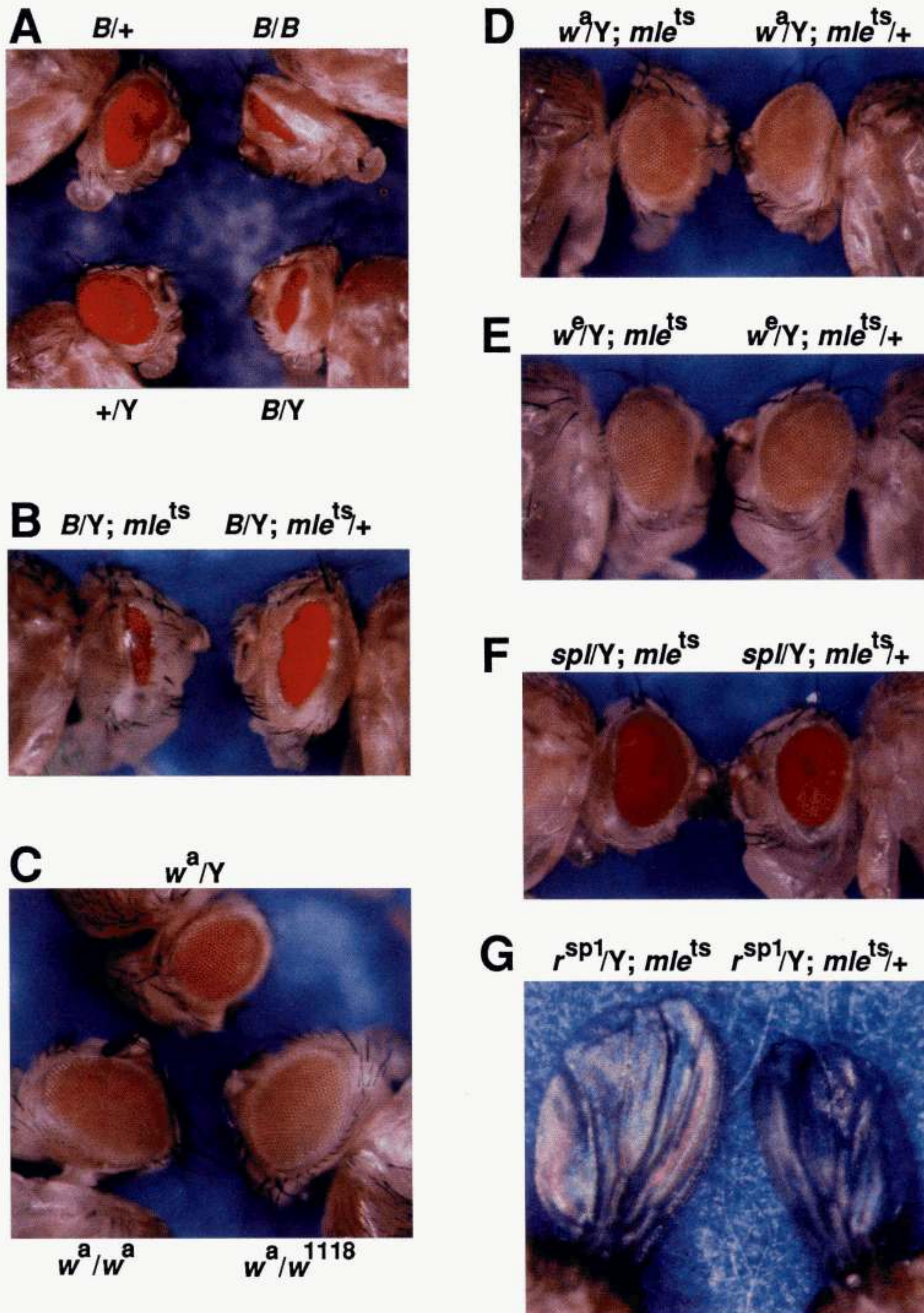

FIGURE 1.—The effect of mle^{ts} in males with *X*-linked hypomorphic and neomorphic mutations. Genotypes are given beside each mutant. See text for descriptions. In (B), the chromosome containing *Bar* is the balancer, *FM7*, y^{31d} sc^8 *v B*. In (F), the *spl* chromosome contains y^2 w^{i+A} *spl*. The heterozygous mle^{ts} genotypes are all $mle^{ts}/SM6a$.

fected to a similar degree, and heterozygous *Bar* females are less affected than hemizygous males (Figure 1A). Therefore, if the mutant effect of mle^{ts} were a twofold reduction in *Bar* gene expression, then homozygous mle^{ts} males would have larger, more normal eye morphology than their heterozygous brothers. In fact, the opposite was found. Homozygous mle^{ts} males (n = 6, where n equals the number of homozygous *mle* males observed) that were also hemizygous for *Bar* had even narrower eyes (Figure 1B), a more severe phenotype that resembles that of flies carrying three copies of the *Bar* mutation, and presumably derives from a greater level of expression. The *X*-linked mutation, *white-apricot* (w^a) is hypomorphic, resulting in an intermediate eye color between the wild-type and null alleles of the *white* (w) locus. The w^a allele is dosage compensated, with hemizygous males and homozygous females being equally pigmented, whereas hemizygous females are one-half as pigmented (Figure 1C). There was very little effect of mle^{ts} on the dosage compensation of w^a. The homozygous males were slightly darker than their heterozygous brothers (Figure 1D). More than 50 homozygous males have been observed. Another allele of w, *w-eosin* (w^e), is hypomorphic and lacks dosage com-

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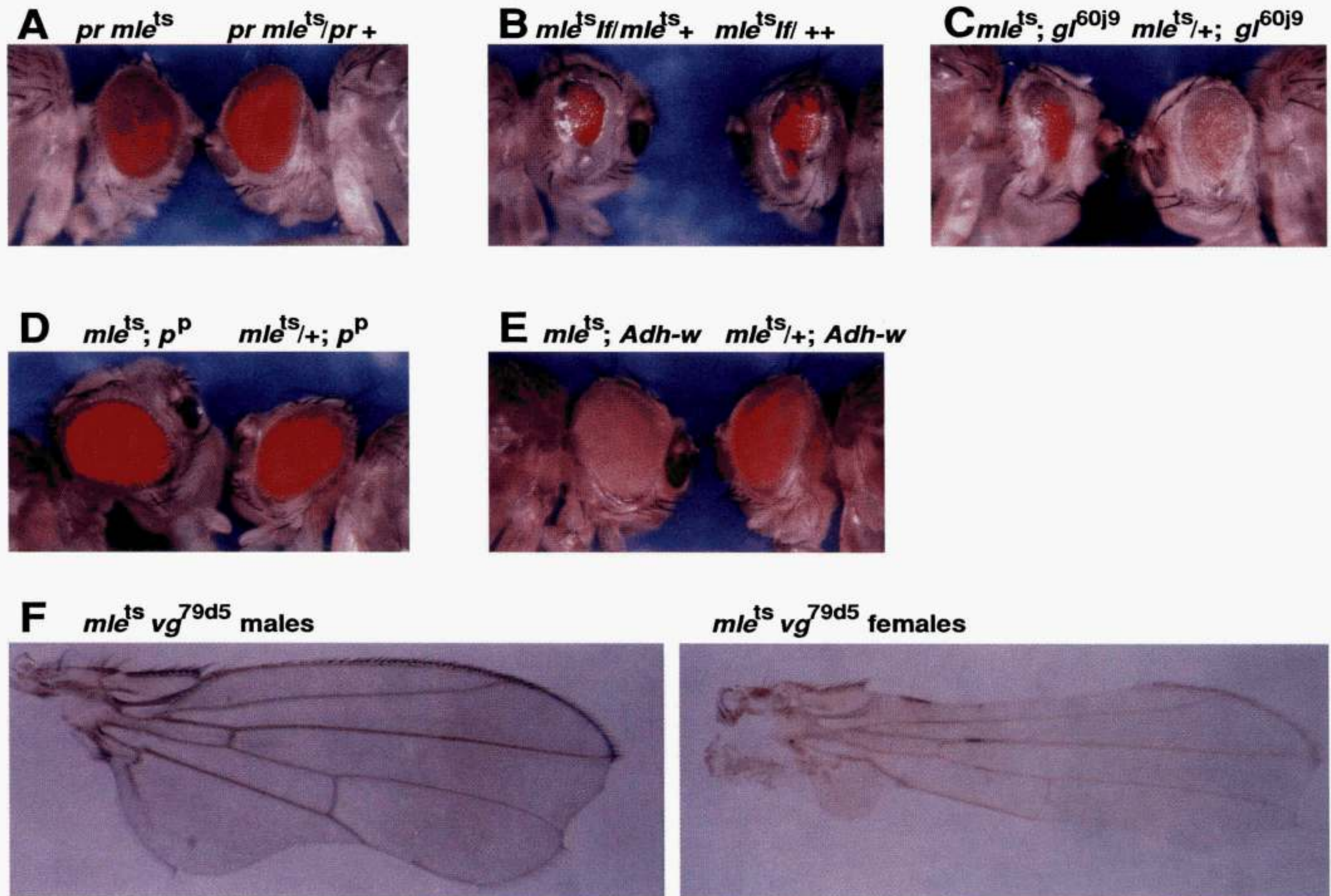

FIGURE 2.—The effect of mle^{ts} in males with autosomal hypomorphic and neomorphic mutations. Genotypes are given beside each mutant. See text for descriptions. In (A), the homozygous genotype is *Tft pr* mle^{ts} vg^{79d5}/*pr* mle^{ts} vg^{79d5} and the heterozygous genotype is *Tft pr* mle^{ts} vg^{79d5}/*pr cn*. In (B–E), the heterozygous mle^{ts} constitution is mle^{ts}/*SM6a*.

pensation. Thus, females have twice the eye pigmentation of males. Because of this, it was tested whether mle^{ts} would affect w^{e} differently than other *w* alleles. There was no effect of mle^{ts} on eye pigmentation in the presence of this allele ($n = 2$) (Figure 1E). The *split* locus is an *X*-linked hypomorph and causes the eye to appear rough and small. The effect of mle^{ts} is apparently an increase in the expression of *split* because eye size was increased in mle^{ts} males ($n = 2$) as compared to heterozygotes (Figure 1F). The recessive *X*-linked hypomorphic *rudimentary-sp1* mutation (r^{sp1}), has a truncated wing phenotype. In mle^{ts} males ($n = 3$), r^{sp1} is increased in expression as evidenced by a less severe rudimentary phenotype compared to heterozygous brothers (Figure 1G).

Several autosomal mutations were tested for interaction with mle^{ts} (Figure 2). The hypomorphic *purple* (*pr*) mutation is on chromosome *2* and causes a reduction in the amount of pteridines, or red pigments in the eyes of homozygotes. This mutation is sexually dimorphic such that males exhibit more red pigmentation than females. In mle^{ts} males ($n = 12$), expression of *pr* is decreased, resulting in a darker brown eye color (Figure 2A). A dominant neomorphic mutation on chromosome *2*, *Irregular facets* (*If*), gives an eye phenotype characterized by reduction of area to one half of normal, and fused or absent facets. The effect of mle^{ts} is to increase the expression of *If*, as evidenced by a more severe phenotype ($n = 4$) (Figure 2B). The hypomorphic *glass-60j9* (gl^{60j9}) mutation on chromosome *3* causes smaller eye area, reduced pigmentation, and fused facets. The eye area phenotype was more variable than eye color, so the latter was used as the gauge of expression level. It was found that in mle^{ts} males ($n = 5$) eye pigmentation was darker than in heterozygotes, indicating a greater expression level (Figure 2C). The hypomorphic *pink-peach* (p^{p}) mutation on chromosome *3* gives a pink eye color. Expression of *pink* is increased in mle^{ts} males ($n = 7$) as evidenced by their darker eye pigmentation (Figure 2D). A transformed fusion construct, *Adh-w* on chromosome *3*, links the autosomal *Adh* promoter with the *w* structural gene. This insertion produces an intermediate level of the *w* gene product in a background that is null for *w*. Therefore, any modulation in phenotype is attributable to an effect on the *Adh* promoter. The effect of mle^{ts} was a reduction of expression ($n = 2$) (Figure 2E). The autosomal *vestigial-79d5* (vg^{79d5}) allele, which produces an abnormal wing

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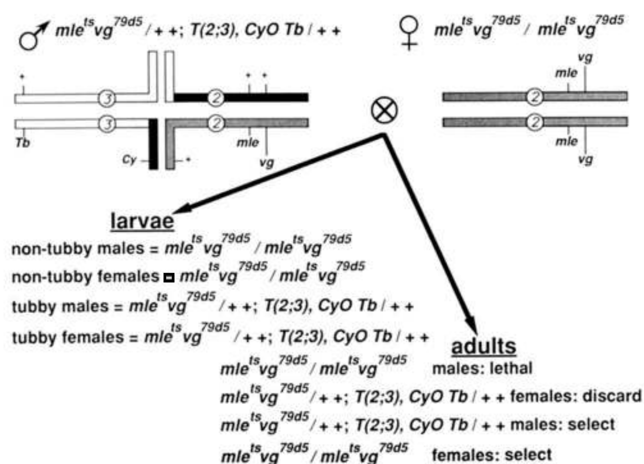

FIGURE 3.—Stock description of mle^{ts}. To use the larval marker, *Tb*, on chromosome *3*, the translocation heterozygote shown was used as the male parent. Centromeres are designated with numbers according to their chromosome of origin. Filled bars represent the *CyO* balancer chromosome; shaded bars represent the mle^{ts} vg^{79d5} chromosome. The stock is maintained by transferring all males and mle^{ts} vg^{79d5} females, but discarding the translocation heterozygous females to avoid recombination between *Tb* and *Cy*.

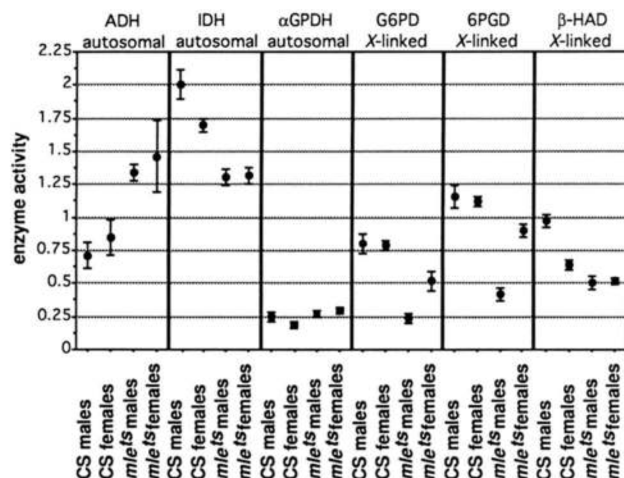

FIGURE 4.—Effects of mle^{ts} on larval enzyme activities. Mean values ($n = 10$) are given in OD_{340} spectrophotometric units from enzyme activity assay divided by OD_{595} units from protein determination assay. Bars represent 95% confidence intervals. mle^{ts} homozygotes were of the genotype, mle^{ts} vg^{79d5} (see Figure 2 for stock description). CS refers to Canton S strain.

margin phenotype, served as a marker for mle^{ts} homozygotes in this study, and provided another autosomal locus to observe for interaction with mle^{ts}. Comparing male and female homozygotes, it was found that vg^{79d5} had a less severe phenotype in males, potentially due to a higher expression level (Figure 2F).

Five other mutations were tested for their response to mle^{ts} (data not shown). Two were non-dosage compensating *w* alleles: *w-apricot-like* (w^{apl}) ($n = 3$) and *w-ivory* (w^{i}) ($n = 2$). There was no effect of mle^{ts} on the phenotypes of these. The other three included a *w* gene construct (F4-2) truncated 360 bp upstream of the start site and present in a *P* element vector on chromosome *2* (LEVIS *et al.* 1985) ($n = 2$), the hypomorphic *w-apricot-2* (w^{a2}) ($n = 2$), which is a point mutation in the coding sequence of *w*, and a duplication of w^{+} sequences, *Dp(1;1)*, w^{61e19} (GREEN 1963) ($n = 2$) in combination with *zeste-1* which reduces expression from w^{+} when the latter is in a paired or duplicated configuration. None of these genotypes showed an eye pigmentation response to mle^{ts}.

In total, the phenotypic analyses revealed three *X*-linked mutations were elevated in expression in mle^{ts} homozygotes, and six different *w* alleles were unchanged. Regarding autosomal mutations, four were elevated, two were reduced, and one was unchanged. While such assays are more qualitative than quantitative, they have the advantage of being an absolute, as opposed to a relative, measure of expression of specific genes. The collective results served as an impetus for the following molecular study.

Effects of mle^{ts} on enzyme activities: The phenotypic observations were of adult escapers of lethality. Earlier work on *mle* had tested for effects prior to the major lethal phase in late larvae to early pre-pupae. Accordingly, we repeated the earlier studies on enzyme activities. A stock was constructed for this purpose that includes a dominant larval marker mutation, *Tubby* (*Tb*) on chromosome *3*, linked to the chromosome *2 CyO* balancer by a reciprocal translocation (Figure 3). One-half of the larvae from this stock are heterozygous for the translocation that carries mle^{ts} and the *Tb* marker, while the other half are mle^{ts} homozygotes. The translocation was used in order to include the *Tb* marker which gives a readily apparent larval phenotype. All data on enzyme activities and RNA, described below, derive from this stock.

A previous report of the effects of mle^{ts} on the activities of several enzymes in larvae showed that mle^{ts} causes an activity reduction of *X*-encoded enzymes and has little effect on autosomally encoded ones (BELOTE and LUCCHESI 1980a). To examine the enzyme expression in absolute terms, we repeated six of eight of these enzyme activity measurements relative to total protein on mle^{ts} males and females compared to Canton S wild-type males and females (Figure 4), and also determined total protein per larva (see below). Three autosomally encoded enzymes were tested–alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH), and α-glycerophosphate dehydrogenase (αGPDH). Each showed statistically indistinguishable activity levels between mle^{ts} males and females; however, there were deviations from the Canton S values. For ADH, the mle^{ts} values were higher than Canton S in both sexes, and for IDH the values for both sexes were lower, but changed from sexually dimorphic expression in Canton S to sexual equivalence in mle^{ts}.

Three *X*-encoded enzymes were tested: G6PD, 6PGD and β-HAD. In two cases, G6PD and 6PGD, mle^{ts} male

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TABLE 1

Total protein comparisons between Canton S and homozygous *mle*^{ts} males and females

Strain	Sex	*n*	Mean	SE	M/F ratio
Canton S	M	10	159.4	2.3	0.745
	F	10	214.0	2.2	
mle^{ts}	M	10	148.7	2.0	0.789
	F	10	188.5	2.5	

Mean values are in micrograms of total protein/larva. Calculations were made from OD_{595} spectrophotometric units of *n* number of protein extracts of five larvae each. Homozygous *mle*^{ts} were of the genotype *mle*^{ts} *vg*^{79d5}. Protein determinations were made from a standard curve, generated under identical conditions using bovine serum albumin.

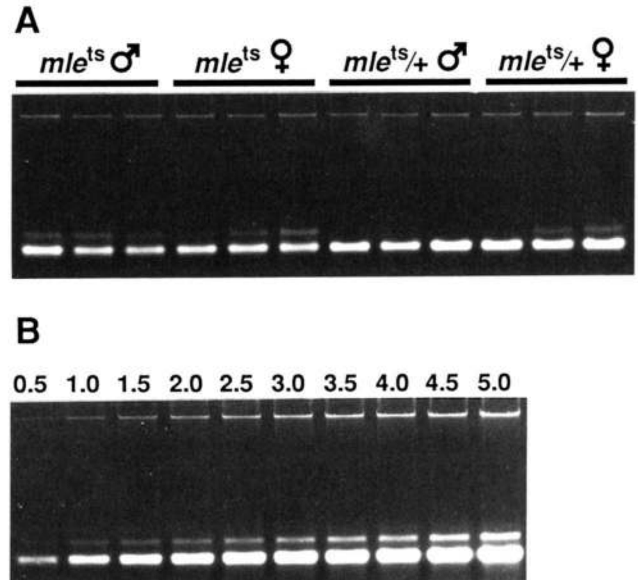

FIGURE 5.— Determination of DNA/RNA ratios in *mle*^{ts} *vg*^{79d5} males and females, and in *mle*^{ts} *vg*^{79d5}/++; *T(2;3), CyO Tb*/++ males and females. (A) Photograph of the ethidium bromide-stained agarose gel used to generate the data in Table 2. Three replicate total nucleic acid extractions are shown for each genotype. The amount loaded in each lane is 1.5 µg. The top band is genomic DNA, the lower bands are 28S rRNA, as confirmed by DNase I and RNase A digestions. Relative quantification was made by laser scanning densitometry of a photographic negative at low scan speed. Only the most predominant rRNA band was measured. (B) Linear dilution series of total nucleic acid, from 0.5 to 5 µg in increments of 0.5 µg. Densitometric scanning established a linear response range in which 1.5 µg occupied a central position. These samples in both (A) and (B) were electrophoresed in the same gel.

levels were reduced to approximately one-half the value in females. In the case of β-HAD, *mle*^{ts} male levels were reduced relative to the elevated, overcompensated Canton S level which is usually the case for this enzyme (BIRCHLER *et al.* 1989), thus bringing its activity down to the level of dosage compensation between *mle*^{ts} males and females. These enzyme data, both *X* and autosomal, are in basic agreement with the previous enzyme analysis of *mle*^{ts}.

In these enzyme activity determinations, an aliquot of each sample was diverted to an assay for total protein, to be used as a standard in individual samples. The same data were pooled to determine whether *mle*^{ts} male/female total protein ratios were changed relative to those of the wild-type strain Canton S. Between strains, total protein per male or female larvae was significantly different at the 95% level, as were the male/female ratios (Table 1). These differences were not of a magnitude sufficient to account for the observed effects of *mle*^{ts} on enzyme activities.

Effects of *mle*^{ts} *on X* and autosomal mRNA levels: To take the analysis of *mle*^{ts} closer to the level of transcription, steady state RNA levels were measured on northern blots of total RNA from larvae, using arbitrarily selected probes for 15 *X*-linked or autosomal loci. To control for differences in RNA hybridizations and preparations, each probe was used on multiple blots, and with RNA from at least three separate preparations.

In these experiments, the central question was whether the abundance of specific transcripts per unit DNA template was altered in the *mle*^{ts} mutant larvae. Pertinent to this objective was the choice of ribosomal RNA as a control for loading differences between lanes. Because the vast majority of RNA species in a total preparation is rRNA, it was possible to address the question of whether equivalent amounts of total RNA from different genotypes reflect the contribution of equivalent amounts of DNA. We answered this by measuring total DNA/rRNA ratios of the four genotypes using a technique for the simultaneous recovery of DNA and RNA (see MATERIALS AND METHODS). A photographic negative of an ethidium stained gel containing triplicate preparations (Figure 5A) was analyzed by laser scanning densitometry, as was a linear dilution series in the same gel (Figure 5B) to establish the standard curve. The results of this analysis are shown in Table 2. Between sexes of either genotype there were only slight differences in DNA/RNA ratios; however, between genotypes (*Tb*/+ *vs.* +/+) the differences were greater. Comparing homozygous males to heterozygous males, it was found that heterozygotes had 12.2% more RNA per unit DNA than homozygotes (significant at the 95% level), presumably the effect of the *Tb* marker. This factor was used to convert band densities, after adjusting for loading, in the analysis of northern data (see below).

Figure 6 shows representative northern blot autoradiograms of 8 *X*-linked and 7 autosomal probe hybridizations. The *X*-linked *white* gene showed no difference between homozygous and heterozygous males (Figure 6A), a result consistent with phenotypic data (Figure 1D), while on the same blot the autosomal *Adh* gene was markedly increased in homozygous males (Figure 6A). The autosomal *β1-tubulin* (β *Tub56D*) and *Rp49* gene transcripts were not significantly affected between heterozygous and homozygous males (Figure 6B). The

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TABLE 2

DNA/RNA ratios in homozygous and heterozygous mle^{ts} males and females

Genotype	Sex	n	Mean ratio	SE
mle^{ts}/mle^{ts}	M	3	0.402	0.008
	F	3	0.412	0.014
$mle^{ts}/+$	M	3	0.358	0.030
	F	3	0.371	0.004

Ratios were calculated from values obtained by scanning laser densitometry of a negative of the ethidium stained gel shown in Figure 5A, based on the linear response obtained by scanning the gel shown in Figure 5B. Homozygous mle^{ts} were of the genotype mle^{ts} vg^{79d5}. Heterozygous mle^{ts} were of the genotype mle^{ts} $vg^{79d5}/++$; $T(2;3)$, CyO $Tb/++$.

X-linked *rudimentary* (*r*) gene transcript was apparently increased in homozygous males relative to heterozygotes (Figure 6C), but differences in band densities between these genotypes were not significant at the 95% level (Table 3), although at the phenotypic level *r* expression showed an increase (Figure 1G). On the same blot, the autosomal *brown* (*bw*) transcript was markedly reduced in homozygous males relative to heterozygotes. The sexual dimorphism of *bw*, evident in the heterozygotes, in which the male level of transcript is approximately twice that of females, has been noted previously (RABINOW *et al.* 1991). The transcripts of the autosomal *scarlet* (*st*) gene and the *X*-linked *vermilion* (*v*) gene were both greatly increased in abundance in homozygous males (Figure 6D). The *X*-linked *yellow* (*y*) gene was virtually unaffected in homozygous males compared to heterozygotes, whereas the transcript of the *X*-linked *sisterless-b* (*sis-b*) gene was reduced (Figure 6E). Probes of the *X*-linked *Sgs4* and the autosomal *Sgs3* genes served to repeat an earlier experiment on the effect of mle^{ts} in which it was found that the former was reduced relative to the latter (BREEN and LUCCHESI 1986). Here a similar result was found; while *Sgs3* was not affected in homozygotes, *Sgs4* was reduced (Figure 6F).

The genes encoding the dehydrogenases, G6PD, αGPDH and 6PGD, are designated *Zw*, *Gpdh* and *Pgd*, respectively. Together with *Adh*, they correspond to four of the six enzyme activity measurements presented above. The steady state RNA responses of these enzyme loci to mle^{ts} did not correlate with the responses of their respective activity levels (Figure 4). In the case of *Adh*, its RNA level was greatly increased in homozygous males (Figure 6A), whereas at the enzyme level there was no significant difference. The *Gpdh* probe also gave a result different from that of its enzyme product; instead of equivalence between homozygous males and females, there was an increase in homozygous males and a decrease in homozygous females, while the heterozygotes were equivalent (Figure 6G). In the case of 6PGD, the *Pgd* probe did not detect a reduction in RNA (Figure 6G), as observed at the enzyme level. Similarly, enzyme activity of G6PD was reduced in mle^{ts} males relative to

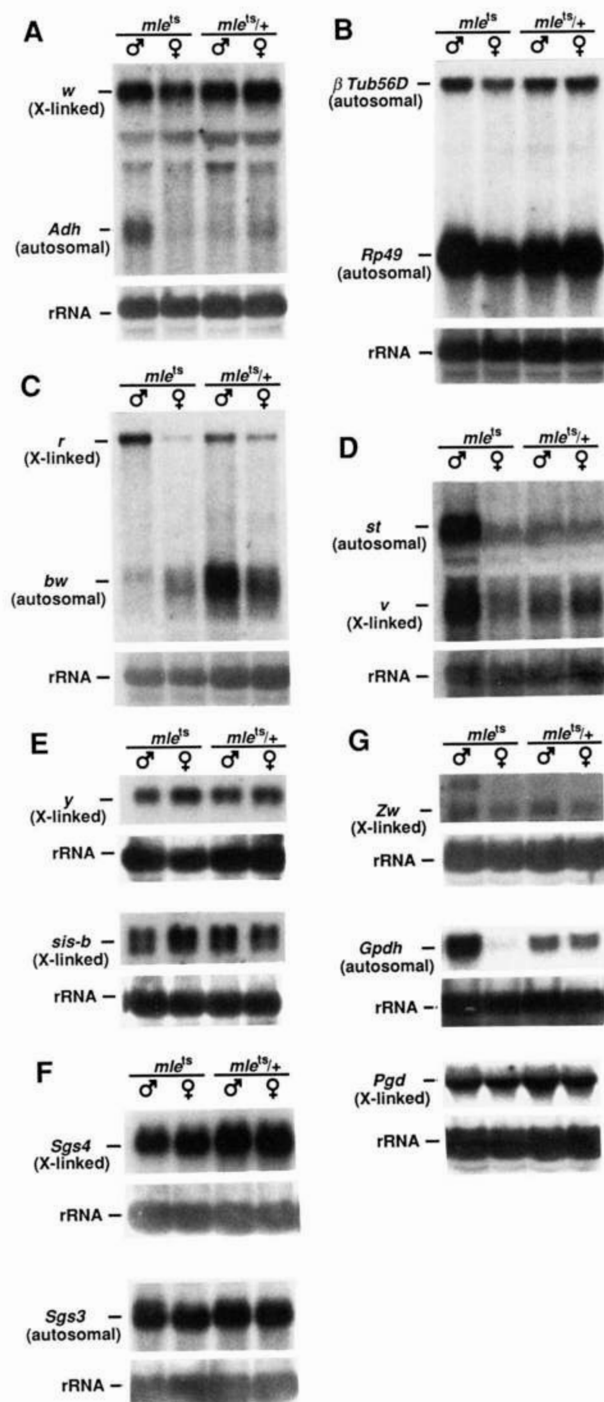

FIGURE 6.—Northern analysis of *X* and autosomal loci in mle^{ts} vg^{79d5} males and females, and in mle^{ts} $vg^{79d5}/++$; $T(2;3)$, CyO $Tb/++$ males and females. See text for descriptions. The locus from which the probe was derived is indicated along with its genomic position, either *X*-linked or autosomal. Below each panel is the corresponding rRNA loading control. Blots were hybridized with antisense RNA probes and each lane contains 10 μg total RNA.

females, but its mRNA was unaffected as shown by the *Zw* probe, and a higher molecular weight RNA species was greatly increased in abundance (Figure 6G). Therefore, for these loci there are incongruities between enzyme and mRNA levels for the same gene. The larvae for

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TABLE 3

Transcript abundance ratios of male *mle*^{ts} homozygotes/heterozygotes

Locus	Chromosome	*n*	Ratio	SE
white	*X*	5	0.98	0.05
rudimentary	*X*	3	1.53	0.37
vermilion	*X*	3	6.05*	0.32
Sgs4	*X*	5	0.54*	0.03
Zw (G6PD) [a]	*X*	3	0.98	0.13
Pgd (6PGD) [a]	*X*	6	0.82	0.06
sis-b	*X*	4	0.61*	0.03
yellow	*X*	3	0.90	0.21
Adh	*2L*	4	4.52*	0.39
Gpdh (αGPDH) [a]	*2L*	4	2.25*	0.23
brown	*2R*	5	0.05*	0.02
βTub56D	*2R*	3	1.01	0.08
scarlet	*3L*	3	7.31*	0.40
Sgs3	*3L*	4	0.84	0.06
Rp49	*3R*	5	1.24	0.16

Band densities were measured by scanning laser densitometry. Homozygote/heterozygote ratios are means of *n* number of ratios, obtained by scanning multiple northern blot autoradiograms of the type shown in Figure 6. Prior to determining ratios, band density values were adjusted for loading differences, and for different DNA/RNA ratios in *mle*^{ts} homozygotes relative to heterozygotes (Table 2). To quantitate RNA data, densitometry was performed using autoradiograms with non-saturating exposure levels. The suitability of rRNA as a control for loading differences was determined by scanning rRNA bands in serial dilutions, which established a linear response range for amount of RNA loaded per lane, in which 10 μg occupied a central position (see MATERIALS AND METHODS). Homozygous *mle*^{ts} were of the genotype *mle*^{ts} *vg*^{79d5}. Heterozygous *mle*^{ts} were of the genotype *mle*^{ts} *vg*^{79d5}/++; *T(2;3), CyO Tb*/++.

[a] The corresponding enzyme name is given in parentheses following its structural gene name.

* The indicated ratios were determined to differ from 1.0 with greater than 95% confidence.

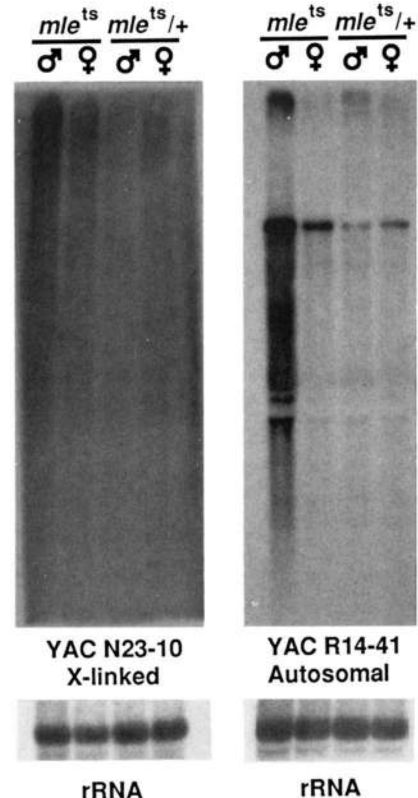

FIGURE 7.—Northern analysis using DNA probes from 210-kb *X*-linked and autosomal yeast artificial chromosomes (YACs). (Left) The probe was N23-10, an *X*-linked YAC at cytological position 8E3-9B12. (Right) The probe was R14-41, an autosomal YAC on chromosome *2* at position 47A1-16.

both the enzyme and RNA analyses were collected in an identical manner, so these differences cannot be attributed to collection procedures. This situation is discussed further below.

Male homozygote/heterozygote ratios of mRNA accumulation are presented in Table 3. Of the eight *X*-linked loci tested, two showed significant reduction below 1.0–*Sgs4* and *sis-b*. One *X*-linked locus, *v*, was significantly increased. Of the seven autosomal loci tested, three were significantly increased–*Adh*, *Gpdh* and *st*. One autosomal locus, *bw*, was significantly decreased.

Another test of the effect of *mle*^{ts} on steady state RNA levels was done by probing northern blots with two large genomic segments present in yeast artificial chromosome (YAC) constructs (GARZA *et al.* 1989). These were used to test for a generalized effect of *mle*^{ts} on many autosomal or *X*-linked loci by assaying large segments of the respective chromosomes. One of these, N23-10, contains *X* chromosome sequences covering the cytological position 8E3-9B12 (210-kb insert). The other, R14-41, contains chromosome *2* sequences covering the position 47A1-16 (210-kb insert). Figure 7 (left panel) shows that the N23-10 probe hybridized more strongly to the homozygous male lane than any of the others. This hybridization apparently derives from a single high molecular weight RNA that is present in high abundance and that obscures the detection of lesser species. The R14-41 probe showed a similar, but more dramatic difference in signal, with homozygous males showing a much greater level of hybridization than the other genotypes for two different high molecular weight RNAs (Figure 7, right panel). In neither of these experiments is generalized RNA degradation responsible for the diffuse hybridization within lanes; re-probings with rRNA (see Figure 7) and other single copy probes (data not shown) demonstrated that the RNA in the lanes was intact. In addition to the strongly hybridizing bands detected by R14-41, there were at least five other faintly hybridizing RNAs, four of which appeared to be increased in *mle*^{ts} homozygous males. The YACs undoubtedly contain sequences homologous to many transcripts, but it was not possible to determine from these autoradiograms exactly how many transcripts contributed to the overall signal.

DISCUSSION

We have analyzed the expression of *X* and autosomal loci at three levels–phenotypic, protein and steady state mRNA, showing that *mle*^{ts} has effects at each. Phenotypically, three of four *X*-linked loci were slightly in-

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creased in expression while one was unchanged; expression of autosomal mutations was elevated in four cases, reduced in two, and unchanged in one. Of the eight *X*-linked loci tested at the RNA level, two showed significant reduction, one was significantly increased, and five were unchanged; of the seven autosomal loci tested, three were significantly increased in expression, one was significantly decreased, and three were unchanged. The autosomal YAC gave a larger increase than the one derived from the *X*. Also, in some cases, an effect in homozygous females was detected. Overall, these data are inconsistent with the hypothesis that the effect of the mle^{ts} mutation is to eliminate dosage compensation.

Previously, the lethal effect of *mle* was attributed to a reduction of *X* chromosome expression to one-half the normal level in males. The positive and negative effects, on both *X*-linked and autosomal gene expression reported here, were largely restricted to homozygous males. Thus, the male lethality of mle^{ts} is likely caused by male-limited effects related to those described here.

The MLE and MSL-1 proteins bind to the *X* chromosome in males (KURODA *et al.* 1991; PALMER *et al.* 1993). In the case of MLE, this process depends on the functions of the *msl-1*, *msl-2* and *msl-3* loci (GORMAN *et al.* 1993). Because of these interactions, it is possible that the products of all of these loci, as well as the male *X* chromosome-specific acetylated form of histone H4 (TURNER *et al.* 1992), act together in producing the effects reported here.

Besides the present experiments, one other study has tested for an effect of *mle* on an *X*-linked mutant phenotype. GERGEN (1987) showed that dosage compensation is operative at the blastoderm stage of embryogenesis, and demonstrated that *mle*, *msl-1* and *msl-2* each had no effect on the *X*-linked *runt* mutant phenotype. The results were interpreted to suggest that at this developmental stage a separate mechanism operates to achieve dosage compensation. In the present study, later developmental stages have been tested, during which several *X*-linked genes were found to be similarly unaffected by *mle*.

Previous studies of gene expression in *mle* larvae employed polytene chromosome transcriptional autoradiography, enzyme activity measurements, and mRNA analysis of two loci (see Introduction). The conclusion in each of these was that *mle* caused a decrease of *X*-linked gene expression to one-half of normal, and therefore represented a function directly involved in the hyperactivation of the male *X* chromosome. In the transcriptional autoradiography studies involving the alleles, mle^{1} or mle^{ts} (BELOTE and LUCCHESI 1980a; OKUNO *et al.* 1984), however, inspection of the absolute data indicates that the reduced *X*:autosome transcription ratio in each of three experiments derived not from reductions in *X* activity values but from elevations of autosomal values. The same is true in a separate experiment involving a heteroallelic combination at the *msl-3* locus (OKUNO *et al.* 1984). Since only relative *X*:autosome ratios were sought in those experiments, controls on absolute grain count variation between males and females or between heterozygotes and homozygotes were not imposed. Nevertheless, a unanimous trend is apparent that autosomal transcriptional activity is increased in males homozygous for male-specific-lethal mutations. Because our results show several cases of increased expression of autosomal genes, it is formally possible that the reduced *X*:autosome ratios reported previously resulted from overall elevations in autosomal gene expression. Although effects in both directions were found on both the *X* and the autosomes, a greater proportion of autosomal loci showed elevations as compared to *X*-linked loci showing reductions. Thus, the cumulative effect, as measured by polytene chromosome autoradiography, could be increased autosomal transcription.

Significant discrepancies were observed between the enzyme activity data of ADH, G6PD, 6PGD and αGPDH, and the mRNA levels of their respective structural genes–*Adh*, *Zw*, *Pgd* and *Gpdh*. The enzyme activity data agreed generally with that from four male-specific lethal loci analyzed previously–mle^{ts}, *msl-1*, *msl-2* and *msl-3* (BELOTE and LUCCHESI 1980a) in that *X*-encoded enzymes were reduced; however, the mRNA levels gave a different response. As alluded to above, this difference was also found between enzyme activities and absolute autoradiography data in the original study (BELOTE and LUCCHESI 1980a). These discrepancies are not attributable to any lack of sensitivity of the enzyme activity measurements, because the assays have been optimized for linear responsiveness under the conditions used in these experiments (BIRCHLER *et al.* 1989). Unlike the situation with larval expression of *X*-encoded enzymes, the adult phenotypic responses of certain *X*-linked loci to mle^{ts} showed a direct correlation with their respective mRNA responses. These loci include *w* (Figures 1D, 1E and 6A) and *r* (Figures 1G and 6C). These phenotypic effects would be determined at the pupal stage. Although in general a correlation between enzyme activity and phenotypic expression would be expected as well, the different types of effect observed might depend on the developmental stage. The activity levels of the *X*-encoded enzymes, G6PD, 6PGD, and β-HAD were examined in adult males and found to be unaffected by mle^{ts} compared to heterozygous controls (data not shown). Thus the enzyme reductions are specific to the larval stage. We have shown that total protein level between males and females is changed only slightly by mle^{ts} (Table 1), and that the total RNA/DNA ratio is unchanged (Table 2), permitting speculation that because an apparent increase in mRNA does not result in increased protein, factors required for translation could be limiting in mle^{ts} larvae. If autosomal gene expression is generally increased in mle^{ts} males and *X* expression more or less

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unchanged (see above), then competition for translation would result in an overall reduction of *X*-encoded enzyme activities per total protein in homozygous males relative to the other genotypes, whereas mRNA levels of *X*-linked genes relative to rRNA would be nearly equal. Since the bulk of the data implicating the four male-specific lethal mutations in hyperactivation of the male *X* chromosome comes from enzyme studies, our results suggest a reinterpretation of their mutational effects. Further study will be required to understand this situation.

Male-specific lethal genes and the evolution of sex chromosomes: Dosage compensation may be viewed from the standpoint of the evolution of sex chromosomes and the effects of aneuploidy on gene expression. The evolution of heteromorphic sex chromosomes from originally homologous pairs is considered to have occurred repeatedly in higher eukaryotes, and the existence of numerous species that display intermediate stages suggests that the transition to heterogamy is gradual (CHARLESWORTH 1991). The transition to dosage compensation should likewise take place in a stepwise manner, especially in Drosophila for which whole arm monosomy, a condition equivalent in chromosome constitution to males, is an embryonic lethal condition (FITZ-EARLE and HOLM 1979). Consistent with this idea, incomplete dosage compensation has been observed for the evolving X^2 sex chromosome of *Drosophila miranda* (STROBEL *et al.* 1978).

The effects of monosomy on gene expression are more complicated than a simple twofold reduction of linked gene expression in light of studies on whole arm trisomy and segmental aneuploidy. In trisomies of *2L* and *3L*, most genes monitored on the varied arms were found to be dosage compensated, with others showing dosage effects (DEVLIN *et al.* 1988). Most unlinked genes that were monitored were reduced in expression, exhibiting an inverse response to the trisomics. Other experiments on segmental aneuploids have demonstrated inverse and direct effects on the expression of genes unlinked to the varied segments (O'BRIEN and GETHMAN 1973; RAWLS and LUCCHESI 1974; HODGETTS 1975; HALL and KANKEL 1976; PIPKIN *et al.* 1977; DETWILER and MACINTYRE 1978; MOORE and SULLIVAN 1978; OLIVER *et al.* 1978; KENNISON and RUSSELL 1987; SABL and BIRCHLER 1993). Thus, the lethal effect of monosomy is likely due to changes in expression levels of genes both linked and unlinked to the varied chromosome.

As the chromosome destined to form the *Y* becomes more degenerate, *trans*-acting dosage effects on *X* and autosomal gene expression would become more prevalent in males due to an effective change in *X* dosage. During the evolution of sex chromosomes, we hypothesize a tendency toward suppression of these effects given the usual near equality of both *X*-linked and autosomal gene expression between males and females. While inverse regulation could have been recruited as a mechanism to compensate the expression of *X*-linked genes by *X*-linked regulatory loci, inverse and direct effects of the *X* chromosome upon the autosomes would need to be ameliorated. Such refinement would require a function to distinguish between the *X* and autosomes. The product of *mle* along with other male-specific lethal loci may serve such a function as evidenced by results showing its preferential localization to the male *X* chromosome (KURODA *et al.* 1991), the mechanism for distinction being as yet unknown.

It perhaps could be argued that the MLE protein, having homology with nucleic acid helicases (KURODA *et al.* 1991), might interact directly with the loci examined in these experiments at the level of transcript elongation or pre-mRNA processing. This is suggested by the fact that many of its effects are greater than twofold and dosage-sensitive effects are usually within the twofold range. Also, the *mle* mutational effect does not equal that of "monosomy" for the *X* chromosome, because *mle* mutants die during the third larval instar or early pupal stages and not during embryogenesis as do autosomal whole arm monosomics. Therefore, *mle* mutants do not mimic completely the simple predictions of loss of dosage compensation or loss of modification of dosage effects.

Nevertheless, there are several observations that argue that the MLE protein is involved in *X* and autosomal distinction. First, the autoradiographic data repeatedly exhibit an altered *X* to autosomal ratio with the autosomal levels showing an absolute elevation in the homozygous *mle* males. Also the enzyme analyses show a differential effect of *X* and autosomally encoded products with the *X* products being reduced per total protein in larvae. This difference, however, appears to be due to a generalized increase in autosomal expression with a limitation on translation, as evidenced by the northern analysis of the RNA from the same genes with absolute standardization, via ribosomal RNA, to the DNA content. Overall, on the RNA level, few *X*-encoded products are reduced, but several of the autosomal ones are elevated. Last, there is a preferential association of the MLE protein with the *X* chromosome in wild-type males (KURODA *et al.* 1991), suggesting a chromosome specific basis for its effects, either by its presence on the *X*, its absence from the autosomes, or both. The group of male- specific lethal genes might have been recruited during evolution for a chromosome-specific modification of inverse and direct effects produced by the reduced dosage of the *X* chromosome in males.

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