Study of Dosage Compensation in Drosophila

Pei-Wen Chiang* and David M. Kurnit†,1

Manuscript received March 3, 2003
Accepted for publication June 27, 2003

**Human Medical Genetics Program, University of Colorado Health Sciences Center, Denver, Colorado 80220 and †Departments of Pediatrics and Human Genetics, University of Michigan, Ann Arbor, Michigan 48109-0650*

ABSTRACT

Using a sensitive RT-QPCR assay, we analyzed the regulatory effects of sex and different dosage compensation mutations in Drosophila. To validate the assay, we showed that regulation for several genes indeed varied with the number of functional copies of that gene. We then confirmed that dosage compensation occurred for most genes we examined in male and female flies. Finally, we examined the effects on regulation of several genes in the MSL pathway, presumed to be involved in sex-dependent determination of regulation. Rather than seeing global alterations of either X chromosomal or autosomal genes, regulation of genes on either the X chromosome or the autosomes could be elevated, depressed, or unaltered between sexes in unpredictable ways for the various MSL mutations. Relative dosage for a given gene between the sexes could vary at different developmental times. Autosomal genes often showed deranged regulatory levels, indicating they were in pathways perturbed by X chromosomal changes. As exemplified by the *BR-C* locus and its dependent *Sgs* genes, multiple genes in a given pathway could exhibit coordinate regulatory modulation. The variegated pattern shown for expression of both X chromosomal and autosomal loci underscores the complexity of gene expression so that the phenotype of MSL mutations does not reflect only simple perturbations of genes on the X chromosome.

FOR the X-linked genes, which differ in dose between male and female, there is a regulatory mechanism of dosage compensation operating such that the resulting expression of these X-linked genes is usually equivalent in the two sexes. In Drosophila, regulation is posited to occur by hypertranscription of loci on the single X chromosome in males (reviewed in Kelley and Kuroda 1995; Bashaw and Baker 1996; Cline and Meyer 1996; Lucchesi 1998; Stuckenholz *et al.* 1999). The current model for dosage compensation in Drosophila proposes that five MSL (male-specific-lethal) proteins, MSL1, MSL2, MSL3, MLE (encoded by maleless), and MOF (encoded by male-absent on the first), form a heteromeric complex on the male X chromosome. Binding of the MSL complex to the male X chromosome leads to the acetylation of histone H4 at lysine 16 (H4Ac16) and an increase of transcription (reviewed in Kelley and Kuroda 1995; Bashaw and Baker 1996; Lucchesi 1998; Stuckenholz *et al.* 1999). Further, the twofold upregulation is superimposed on the gene-specific transcription factors that control the amount, time, and tissue of expression. The finding that MOF is a homolog of human histone acetyltransferases TIP60 (HIV Tat-interacting protein of 60 kD) and MOZ (monocytic leukemia zinc finger protein) provides evidence for the role of histone acetyltransferase in dosage compensation (Hilfiker *et al.* 1997; Gu *et al.* 1998).

[1]*Corresponding author:* Department of Pediatrics, MSRB I, Room 3520, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0652. E-mail: sesame@umich.edu

Even though the current model of dosage compensation in Drosophila receives wide acceptance, direct experimental data in support of this model are still lacking. A twofold reduction in females of *Sgs4*, derived from the X chromosome, was reported (Belote and Lucchesi 1980; Breen and Lucchesi 1986). However, when more transcripts were analyzed using Northern blot analysis, a different conclusion was reached (Hiebert and Birchler 1994; Bhadra *et al.* 1999). In the alternative dosage compensation model proposed by Birchler (1996), the concentration of MSL complex on the X chromosome is not related to dosage compensation. Rather, the concentration of MSL complex on the X chromosome prevents nonspecific binding of the MSL complex to autosomes. The dosage compensation complex cannot accumulate on the male X chromosome in the mutants. Instead, the complex spreads on the autosomes. As a result, autosomal genes are hypertranscribed but the expression of X-linked genes is not changed.

The study of dosage compensation provides a unique opportunity to understand regulation at the chromosomal level. However, the conflicting results in the study of dosage compensation point to the desirability for a rapid and reliable assay to monitor the regulation of given genes. Recently, we developed a sensitive reverse transcriptase-quantitative PCR (RT-QPCR) assay to measure the relative expression of steady-state RNA in Drosophila. The success in the analysis of deficiency flies validated our approach (Chiang *et al.* 1999). The RT-QPCR assay has the following advantages compared with previous quantitative assays of gene expression: (1) Only

a small amount of total RNA is needed in the assay, (2) transcripts from any gene (abundant or not) can be analyzed because the assay works over six orders of magnitude, and (3) the reproducibility and resolution of the RT-QPCR assay was documented by studies of different systems in Drosophila (Chiang *et al.* 1999).

To assay the hypotheses that seek to explain gene expression from the X chromosome in the different sexes, a more sensitive and convenient method should be used to test actual gene expression. To this end, we used *TaqMan* RT-QPCR to measure the level of steady-state RNA for genes located on both the X chromosome and the autosomes in wild-type flies and in flies with dosage compensation mutations. We demonstrated the use of the technique to detect gene dosage in normal males and females. Our data demonstrate that different loci on both the X chromosome and the autosomes show different patterns of expression in the mutants. The variegated pattern shown for expression of both X chromosomal and autosomal loci underscores the lack of a simple explanation for the anomalies in the dosage compensation mutants. This study of mutants of dosage compensation illustrates the complexity of regulation that may best be comprehended by the elucidation of regulatory pathways where the variation of sets of genes occurs concomitantly.

MATERIALS AND METHODS

Research design: The steady-state RNA (total RNA) from a mix of at least 20 third instar larvae was analyzed for each sample. The standard curve method (user bulletin no. 2; ABI PRISM 7700 sequence detection system) was used for the measurement (Chiang *et al.* 1999). RNA derived from the mix of at least 20 third instar larvae from the Canton-S female fly was used as the standard. The quantity of RNA samples was estimated by spectrophotometry and was diluted to different concentrations. For the standards, four concentrations (5, 2.5, 1.25, and 0.6 ng/μl) were used in the analysis (although knowledge of the absolute amounts of RNA is necessarily approximate, the relative amounts used for the analysis are accurate within the limits of pipetting accuracy). To test the reproducibility of the experiments, two different concentrations of experimental samples (2.5 and 1.25 ng/μl) and three repeats of each dilution were used in the analysis. The experiment was repeated if reproducibility of the three repeats yielded variation >30%. Similarly, the experiment was repeated if a twofold difference (the cutoff was between 0.7 and 1.3) was not observed. During calculation, the numbers 5, 2.5, 1.25, and 0.6 were assigned to the standards and the quantity of specific RNA in the samples was calculated on the basis of relative amount of targets in the samples using the standard as a reference. Since absolute quantitation of RNA is difficult to achieve, a relative ratio approach was used in our calculation. After testing different markers in different wild-type and mutant flies, autosomal markers derived from either *RNA polymerase II* or the chromatin protein *Dspt4* were found to be the most reliable markers for the calculation of relative ratio (data not shown). Therefore, all the measurements in this study were done using a marker from *RNA polymerase II* as the reference for the calculation of relative ratio. As a result, 1.0 on the ordinate of the tables designates a relative amount of RNA product equal to the relative amount of RNA product obtained using a primer set for *RNA polymerase II*. The *Dspt4* marker was also included in all the measurements to ensure validity. The ratio between *Dspt4* and *RNA polymerase II* was always close to 1 in all the measurements, suggesting the consistency of our analyses. This normalization procedure enabled us to compare accurately the expression of a given gene in two flies.

The primer sets were designed following the rules recommended by the manufacturer (PE Biosystems, Foster City, CA) and are listed in Table 1. The *TaqMan* Gold RT-PCR reagents were used in our analysis (PE Biosystems). All primer sets were designed to amplify only RNA as they crossed exons. Only the *BR-C* primer set potentially reacted with both DNA and RNA, as the set was designed so that the first 6 nucleotides of the *BR-C* forward primer derived from the first exon and the remaining 14 nucleotides derived from the second exon. Using this strategy, the amplification from genomic DNA was still depressed by 100-fold compared with the cDNA reaction, enabling us to distinguish genomic DNA from cDNA.

Flies: *yw; mle^{l}/CyO, y^{+}* and the the four hypomorphic *mle* alleles (*mle-AAA*, *mle-GNT*, *mle-DQIH*, and *mle-GET*) were provided by Mitzi Kuroda. *yw; mle^{RK}/CyO, y^{+}* and *Df(1)l^{D34}* were provided by Barry Ganetzky. *y^{1} mof^{1}/Basc* was provided by John Lucchesi. *C(1)DX, y^{1}, f^{1}/y^{2}Y67g19.1* was provided by Cynthia Bayer. Deficiency flies *Df(1)S39/FM6* (deletion of *BR-C*), *Df(1)5D/FM6* (deletion of *Dspt6*), *Df(1)Pgd-kz/FM6* (deletion of *6-Pgd*), and *Df(1)N-264-105/FM1* (deletion of *Sgs4*) were ordered from the Bloomington Stock Center. *FM7i* and *C(1)DX, y^{1}, f^{1}/Y* were also obtained from the Bloomington Stock Center. The metafemale was produced by crossing an attached-X strain (*C(1)DX, y^{1}, f^{1}/Y*) marked with yellow (*y*) to a standard male strain and selected on the basis of gonad size and the color of the mouth hook (*y^{+}*). The flies were kept at 25° in this study. The difference of the size of gonads between male and female can be easily differentiated at the third instar larvae (the male has a gonad significantly bigger than that of the female). Therefore, in most of our analysis, RNA from third instar larvae was used.

RESULTS

Testing the resolution of quantitative PCR: We have shown that RT-QPCR analysis has the twofold resolution required to detect reductions of *Dspt4* and *Dspt6* transcripts in deficiency flies (Chiang *et al.* 1999). To further confirm this resolution of RT-QPCR, deficiency flies with a deletion of *BR-C*, *Sgs4*, *para*, or *6-Pgd* were analyzed by RT-QPCR (Figure 1). Each of the deficiency chromosomes was paired with the balancer, *FM7i*. The heterozygous deficiency/*FM7i* larvae were separated from homozygous *FM7i/FM7i* larvae on the basis of the color of the mouth hook. As expected, the expression of *Sgs4*, *para*, and *6-Pgd* was reduced in the deficiency flies *Df(1)N-264-105/FM7i*, *Df(1)l^{D34}/FM7i*, and *Df(1)Pgd-kz/FM7i*, respectively, compared with the expression in *FM7i/FM7i*. Surprisingly, the expression of *BR-C* was not reduced in the deficiency fly *Df(1)S39/FM7i* compared with the *FM7i/FM7i* female. Since the *BR-C* region was shown to be deleted in *Df(1)S39* (Belyaeva *et al.* 1980), a DNA QPCR analysis was performed to confirm the deletion of *BR-C* in *Df(1)S39*. The relative gene copy number ratio of *BR-C/Dspt6* was measured in *Df(1)S39/FM7i* and *FM7i/FM7i*. In accordance with the previous

TABLE 1

List of the PCR primers used in this study

6-Pgd	Forward	5′-TGCTGGTCAAGGCTGGAAGT-3′
	Reverse	5′-GAGATGTGTCCTGATACTCCGAGTT-3′
	Probe	5′-6-FAM-TCGACGACTTCATCCAGCAGCTGGT-TAMRA-3′
Dspt6	Forward	5′-GGAGAATCTGGGCGTCAAAGT-3′
	Reverse	5′-CGCTTTCGTTGTCGTGGAT-3′
	Probe	5′-6-FAM-AGACGCTTGAACCGCCGTCTCTCG-TAMRA-3′
BR-C	Forward	5′-CTCAAGAGCACACCCTGCAA-3′
	Reverse	5′-CGTGCAGGTCCATGAAGTTG-3′
	Probe	5′-6-FAM-CACCCGGTCATACTGCTGCAGGATG-TAMRA-3′
Sgs4	Forward	5′-GGTGGAGGAAGCGGATGTATT-3′
	Reverse	5′-TGAGACTTCGGTTTGGAATCG-3′
	Probe	5′-6-FAM-TGAAGGACCTGCTAACACCGAAGTGCC-TAMRA-3′
para	Forward	5′-GGACACCAATCACAAGCTCGAT-3′
	Reverse	5′-GATGTTTAATCTTGCCAGCTTCGT-3′
	Probe	5′-6-FAM-ACTCCAGGCCAATTTCGTAGTCGCGAT-TAMRA-3′
mof	Forward	5′-ACGTCCACTATGTTGGTCTCAATC-3′
	Reverse	5′-CGCATTGTCCGAGATCCTATG-3′
	Probe	5′-6-FAM-TGCCCACCCATCCGTCCAGG-TAMRA-3′
Lsp-1α	Forward	5′-TGGTCACTGCGACCGCTTA-3′
	Reverse	5′-GACGATCTCGAAGAGGAACTTCTG-3′
	Probe	5′-6-FAM-TCAGGAACGCCTTGTCAGCGACCTT-TAMRA-3′
Dspt4	Forward	5′-TTGACGCGATACCCAAGGAT-3′
	Reverse	5′-CTAGTGTGATCATAGACATTGTCCTTGTT-3′
	Probe	5′-6-FAM-CTCAAATTGATCAAAACTCTTCACTAGGGAGCAAA-TAMRA-3′
st	Forward	5′-GAAACCCGATCCGGACTCTAC-3′
	Reverse	5′-CCAGGCAGCAAAGCCAGTAT-3′
	Probe	5′-6-FAM-CCACGGGACAATATTATGCGGCCAA-TAMRA-3′
Sgs3	Forward	5′-CTACCGCCCTAGCGAGCAT-3′
	Reverse	5′-GCATCCACAATCGCAACAGT-3′
	Probe	5′-6-FAM-CTGCTTATTGGCTCCGCTAATGTTGCC-TAMRA-3′
Sgs5	Forward	5′-TTTGTGCCACCTGCTGAAATT-3′
	Reverse	5′-GAAGGGCCAACAATAGGAATAAGTC-3′
	Probe	5′-6-FAM-CAGCGGAGAACAGCAAGCCATTCC-TAMRA-3′
Sgs7	Forward	5′-TCGCTTGCATCCTGCTCAT-3′
	Reverse	5′-CACACGGTTGGCACTCACA-3′
	Probe	5′-6-FAM-CACCACCCAGGGCTAGATCGGAGAA-TAMRA-3′
Sgs8	Forward	5′-GTCATTGCGTGCATCATGCT-3′
	Reverse	5′-TCCACAAATCACGCATGAACA-3′
	Probe	5′-6-FAM-CGATCCTGCCTCGGGCTGCA-TAMRA-3′
mle	Forward	5′-CGACCAGGCTTCTGCTTCA-3′
	Reverse	5′-CCGGCGTAAGATTGTCCTCTAG-3′
	Probe	5′-6-FAM-CTGTTCGCGTGCCCGCTTCC-TAMRA-3′
Adh	Forward	5′-GGTCTGGACACCAGCAAGGA-3′
	Reverse	5′-TCAATGCGGTCGAGGATCA-3′
	Probe	5′-6-FAM-CTGCTCAAGCGCGATCTGAAGAACCT-TAMRA-3′
Pgi	Forward	5′-GGATGGAGCTCACTTCATGGA-3′
	Reverse	5′-GCCTTGAAGAAGTTGGAGTACCA-3′
	Probe	5′-6-FAM-ACACCATTCGAGAAGAATGCTCCTGTTATCCT-TAMRA-3′
her	Forward	5′-ACCATTAGCAACCCGCAGATT-3′
	Reverse	5′-ATTGACTGGAACCATCGCAACT-3′
	Probe	5′-6-FAM-TGATAAGCACGTCATCGCTGTCAACGTT-TAMRA-3′
da	Forward	5′-GTCAACACTCGCTGCAACAAA-3′
	Reverse	5′-CATACAAGTGCATCGGCTCATC-3′
	Probe	5′-6-FAM-AGCAATAGATCCTAATATCCACTGTTAAT-TAMRA-3′
Aldox-1	Forward	5′-AGAATGACTACGAAATTCTCAATCAATG-3′
	Reverse	5′-GCGGATAAAGGTGTTCAGAGTGA-3′
	Probe	5′-6-FAM-TGCCCTATGCAGTGAATCTAACTAACCTTCCG-TAMRA-3′
Ade2	Forward	5′-GCACCGCTGGCTACTGTGT-3′
	Reverse	5′-GTCGCAGGGTATTTAAAGTCCAA-3′
	Probe	5′-6-FAM-CTCTTCACATTCCAGGTTACAAACAGCCGTA-TAMRA-3′
RNA pol	Forward	5′-CCTTCAGGAGTACGGCTATCATCT-3′
	Reverse	5′-CCAGGAAGACCTGAGCATTAATCT-3′
	Probe	5′-6-FAM-TCCAGTGTGGCCATTGTACATGACCTCA-TAMRA-3′

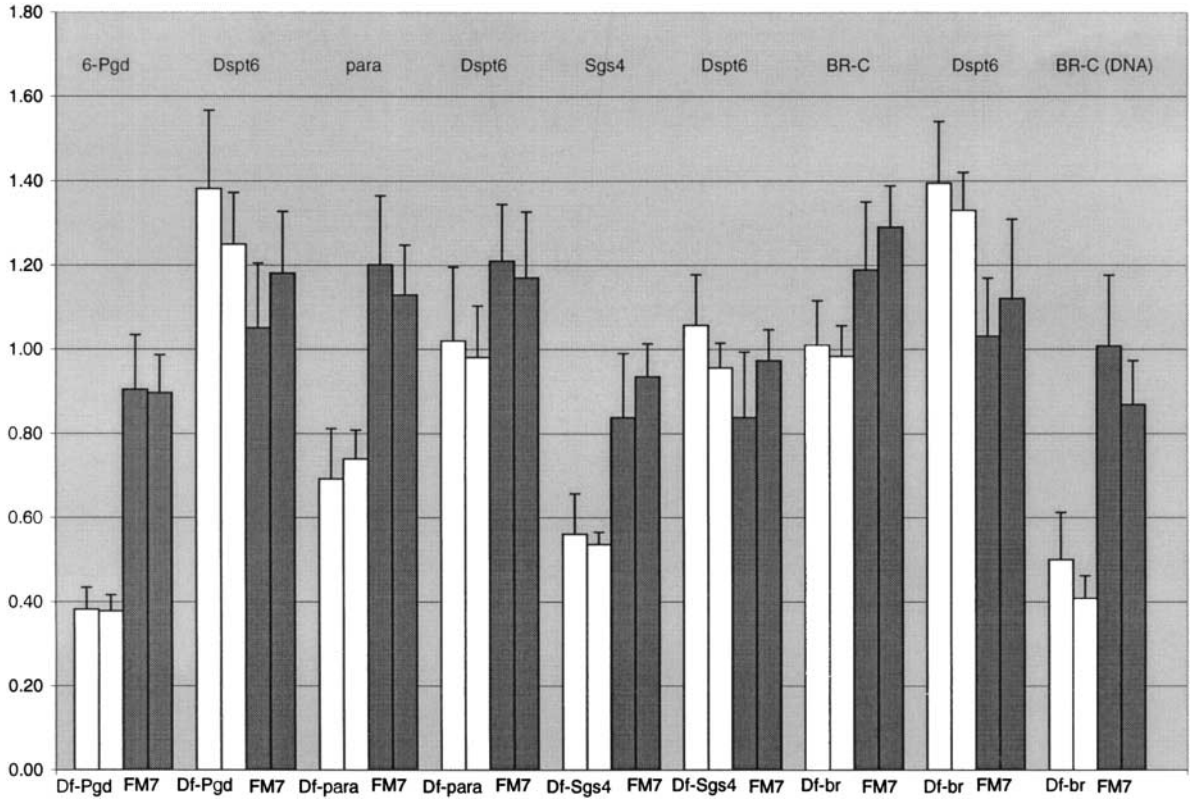

FIGURE 1.—Analysis of deficiency flies. Each analysis was done at two different dilutions, with a minimum of four separate analyses for each dilution. Both heterozygous deficiency and homozygous balancer flies were analyzed. The expression of *Dspt6* was also analyzed as a control. The order of each analysis is: (1) heterozygous deficiency (white); (2) 1:1 dilution (with an equal volume of water) of heterozygous deficiency (white); (3) homozygous balancer (gray); and (4) 1:1 dilution of homozygous balancer (gray). The copy number of *BR-C* (DNA) was also analyzed by DNA QPCR in heterozygous deficiency (white) and homozygous normal (gray) flies. In all figures, the standard deviation (95%) is indicated.

electron microscopic analysis (BELYAEVA *et al.* 1980), *BR-C* was indeed deleted in *Df(1)S39* (Figure 1).

To demonstrate gene dosage in Drosophila, 11 primer sets derived from 5 X-linked genes (*Sgs4*, *6-Pgd*, *Dspt6*, *para*, and *BR-C*) and 6 autosomal genes (*st*, *Dspt4*, *Sgs3*, *Sgs5*, *Sgs7*, and *Sgs8*) were analyzed. The expression of these 11 genes was dosage compensated in the male as shown by *TaqMan* RT-QPCR in wild-type male and female third instar larvae from Canton-S (Figure 2 for the X-linked genes and data not shown for the autosomal genes). Previously, the *Lsp-1*α protein was shown not to be dosage compensated in male flies (ROBERTS and EVANS-ROBERTS 1979). To provide more evidence of the resolution of RT-QPCR, the expression of *Lsp-1*α was analyzed in wild-type male and female third instar larvae from Canton-S. As shown in Figure 2, 30% less *Lsp-1*α transcript was detected in male flies compared with female flies.

A cross between *C(1)DX, y*^{1}*, f*^{1}*/Y* females and wild-type Canton-S males generates two females, *C(1)DX, y*^{1}*, f*^{1}*/Y* and *C(1)DX, y*^{1}*, f*^{1}*/X* (metafemale), with identical genetic backgrounds except for the sex chromosome. These two females can be separated by the color of the mouth hook. To confirm the genotypes of the third instar larvae from metafemale and female, we analyzed the relative DNA copy number of *Dspt6* (an X-linked gene) in metafemale, female, and male against an autosomal gene standard, *Dspt4*. As shown in Figure 3, the QPCR analysis clearly differentiated the DNA copy number differences of *Dspt6* among metafemale (three copies), female (two copies), and male (one copy).

Mutations of *mle* affect expression of a subset of genes on both the X chromosome and the autosomes: We analyzed one null allele of *mle* (*mle*^{1}; FUKUNAGA *et al.* 1975; Figure 4, A and B) and one missense mutation of *mle* (*mle*^{RK}; KERNAN *et al.* 1991; Figure 4, C and D). Both *mle* alleles are male lethal at the late third instar to the early pupal stage. Homozygous female larvae are viable for both *mle* alleles. Homozygous third instar larvae from male and female were separated for each allele and total RNA was isolated from the larvae. Since no significant difference was detected among the heterozygous male, heterozygous female, and homozygous female (data not shown), the analysis was performed using the homozygous male and homozygous female third instar larvae. Both homozygous male and homozygous female larvae have the same genetic background except for the sex chromosome. Therefore, an effect from the

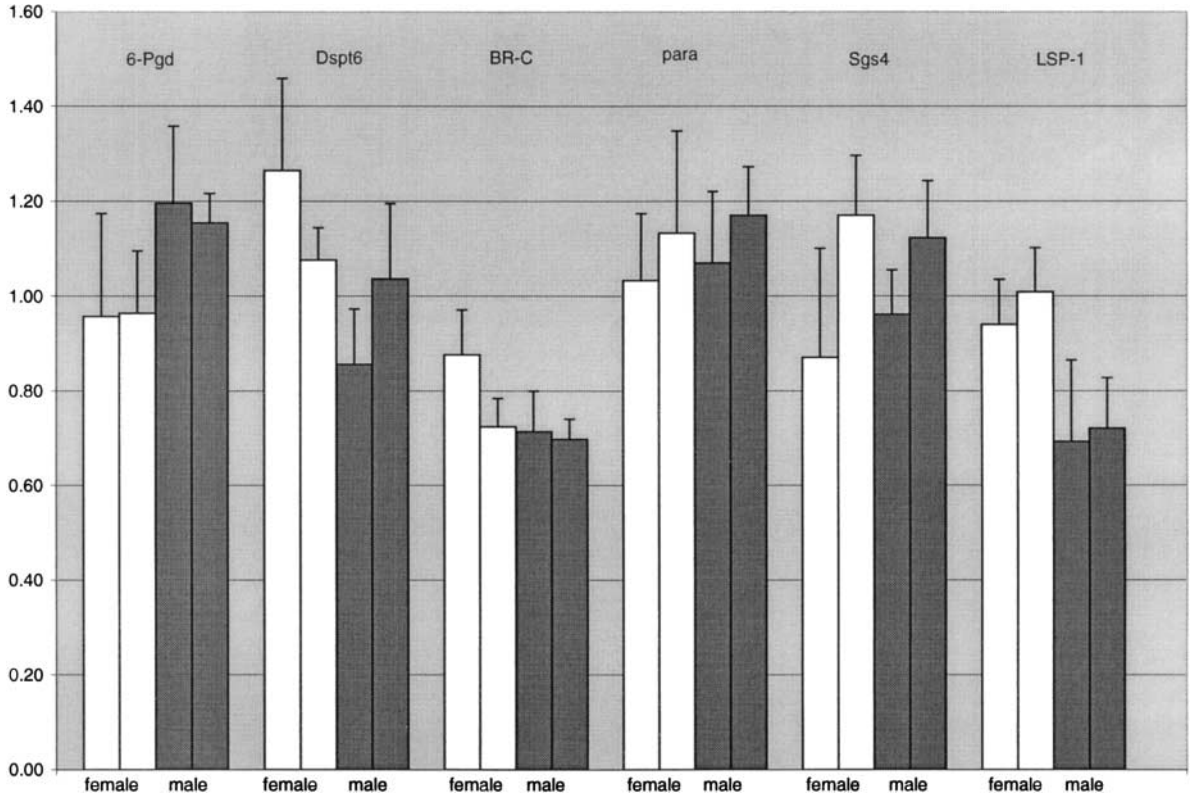

Figure 2.—RT-QPCR analysis of wild-type larvae. The order of each analysis is: (1) female (white); (2) 1:1 dilution of female (white); (3) male (gray); and (4) 1:1 dilution of male (gray).

mle mutations could be detected in our analysis. The amount of *Dspt6* transcript was reduced twofold for both *mle* alleles in the homozygous male compared with the homozygous female. In contrast, transcripts derived from *para* and *6-Pgd* were not significantly different in the homozygous male compared with the homozygous female. Three- to fourfold reductions of *BR-C* and *Sgs4* transcripts were observed in the homozygous male mle^{RK} larvae compared with the homozygous female mle^{RK} larvae. A twofold reduction was detected in homozygous mle^{1} male larvae for *BR-C* and a two- to threefold reduction was detected for *Sgs4* compared with homozygous mle^{1} female larvae. For the three autosomal (chromosome 3L) genes, *Sgs3*, *Sgs7*, and *Sgs8*, a five- to eightfold reduction and a two- to threefold reduction were seen in homozygous mle^{RK} and mle^{1} males, respectively. For the autosomal 3R gene *Sgs5*, the reduction was two- to threefold in mle^{1} and fivefold in mle^{RK}. In contrast, the autosomal 2R gene *Dspt4* was not altered in the homozygous male compared with the homozygous female in both mle^{RK} and mle^{1}. Finally, a four- to fivefold increase of the expression of autosomal 3L gene *st* was detected in both mle^{1} and mle^{RK} flies.

Analysis of *mle* hypomorphic alleles: We then analyzed four *mle* alleles generated through site-specific mutagenesis (Richter *et al.* 1996; Lee *et al.* 1997; Figure 5). All four alleles were analyzed in third instar larvae on an otherwise mle^{1} background. A total of seven primer sets (*6-Pgd*, *Dspt6*, *para*, *BR-C*, *Sgs4*, *Sgs5*, and *st*) were used in the analysis. As shown in Figure 5, A and B, none of the genes analyzed from the *AAA* and *GNT* alleles showed twofold variation compared with the homozygous female. The largest variation we observed was an approximate 40–50% reduction of *br* transcript in the homozygous *AAA* and *GNT* males.

We examined the nearly lethal allele *GET* (Figure 5C) and the less severe sublethal allele *DQIH* (Figure 5D). A twofold reduction of *BR-C*, *Dspt6*, *Sgs4*, and *Sgs5* was observed in the homozygous *DQIH* male compared with the homozygous female. An approximate 30–40% reduction of the expression of *6-Pgd* was observed in the homozygous *DQIH* male and the expression of *para* was not changed in the homozygous *DQIH* male. In contrast with our finding of increased expression for *st* in mle^{1}, the expression of *st* did not vary in the homozygous *DQIH* male. A different picture emerged when the nearly lethal allele *GET* was analyzed. No significant variation was observed for the expression of *6-Pgd*, *para*, *Sgs4*, and *Sgs5* in the homozygous *GET* male. An approximate 30–50% reduction of the expression of *BR-C* and *Dspt6* was observed in the homozygous *GET* male. Finally, an approximate twofold increase of the expression of *st* was observed in the homozygous *GET* male.

Analysis of *mof* mutation: To further our study of MSL variants, mutation of a different MSL gene (*mof*; Hilfiker *et al.* 1997) was analyzed (Figure 6, A and B).

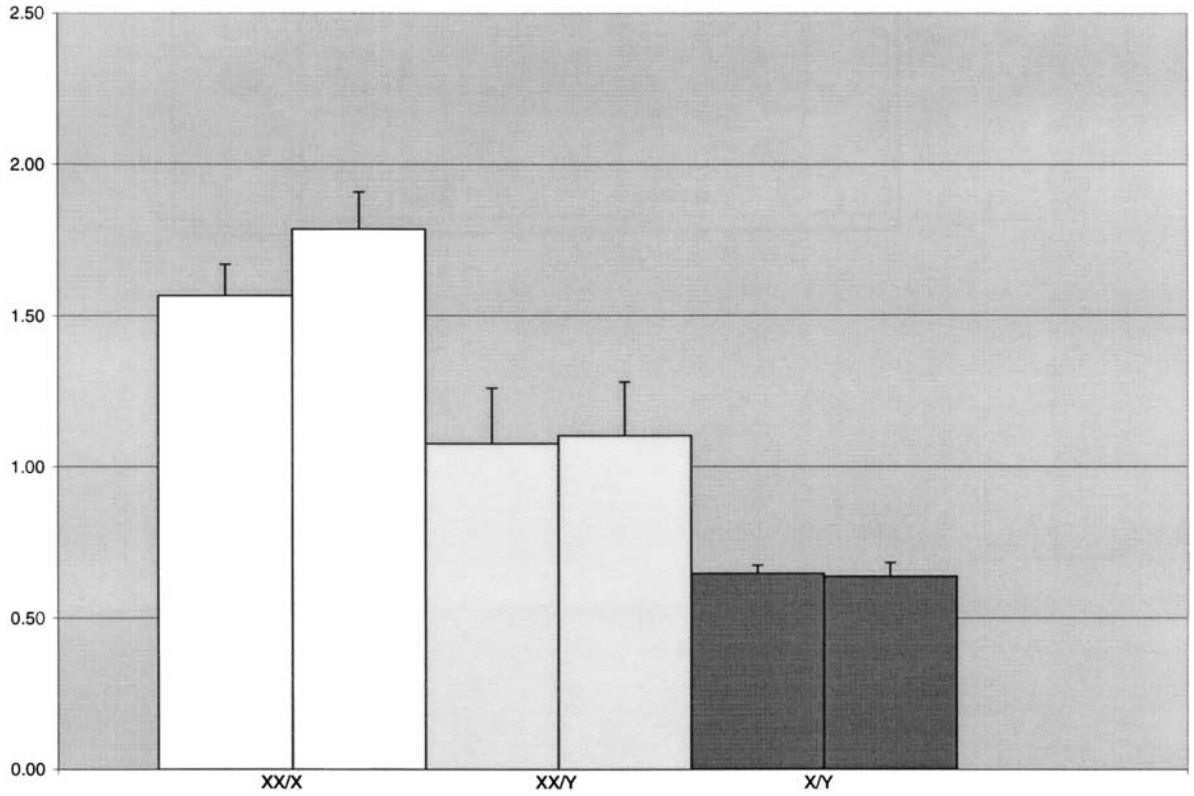

FIGURE 3.—DNA QPCR analysis of *C(1)DX* (XX/X; white); y^1, f^1/X, and *C(1)DX* (XX/Y; light gray); and y^1, f^1/Y, and *X/Y* (X/Y; dark gray). The relative DNA copy number of *Dspt6* (an X-linked gene) in metafemale, female, and male against an autosomal gene *Dspt4* was measured. For each genotype, no dilution and a twofold dilution of samples are measured.

Since *mof* is X linked, only three types of larvae could be isolated (*mof*^{1}*/balancer*, *balancer/Y*, and *mof*^{1}*/Y*). The expression of *Dspt6* in the *mof*^{1} hemizygous male was reduced 2-fold and the expression of *6-Pgd*, *para*, and *Dspt4* in the *mof*^{1} male did not vary significantly when compared to expression in the balancer male. However, a 5-fold reduction of *BR-C* was seen in the *mof*^{1} hemizygous male compared with the balancer male. Ten- to 30-fold reductions of *Sgs3*, *Sgs4*, *Sgs5*, *Sgs7*, and *Sgs8* were detected in the *mof*^{1} hemizygous male compared with the balancer male. Finally, a 2-fold increase of the expression of *st* was detected in the *mof*^{1} hemizygous male compared with the balancer male.

It was desirable to examine younger larvae to minimize any effects of dying that might confound analyses of older larvae. Younger y^1 *mof*^{1}*/Y* males (first and second instar) larvae can be separated from the mix of *Basc/Y* and y^1 *mof*^{1}*/Basc* larvae on the basis of the color of the mouth hook. Since the *BR-C* and *Sgs* genes are expressed later, beginning at the third instar, only *6-Pgd*, *para*, *Dspt6*, and *st* could be analyzed in these two groups of early larvae (Figure 6C). Similar to the analysis of third instar larvae, the expression of *Dspt6* was reduced twofold in younger y^1 *mof*^{1}*/Y* and the expression of *para* was not changed. However, the expression of *6-Pgd* was reduced twofold in younger y^1 *mof*^{1}*/Y* in contrast to the lack of significant change observed in the third instar larvae. Further, a less than twofold (40–50%) reduction of the expression of *st* was seen in the younger larvae in contrast to the twofold increase of expression in the third instar male. Since the expression of *6-Pgd*, *para*, *Dspt6*, and *st* was analyzed only in the third instar larvae from wild-type Canton-S (Figure 2), the expression pattern of these four genes at the younger developmental stage was also analyzed in wild type. The *FM7i* female was crossed to the wild-type Canton-S male. From this cross, the female offspring was y^+ and the male offspring was y^-. Therefore, the first and second instar male and female larvae could be separated from each other on the basis of the color of mouth hook. The RT-QPCR was performed on the younger wild-type samples and the expression of *6-Pgd*, *para*, *Dspt6*, and *st* was similar between male and female in all cases (Figure 6D). This control confirmed the suitability of these analyses in these young larvae.

DISCUSSION

Validity of the RT-QPCR methodology as used to investigate the dosage compensation model: It is postulated that the transcriptional product of the majority of genes, be they autosomal or X chromosomal, is equiva-

lent in males and females (reviewed in Kelley and Kuroda 1995; Cline and Meyer 1996). As shown in Figure 2, our sensitive RT-QPCR analysis agrees with this assertion, as dosage compensation between wild-type males and females can be seen at the regulatory level for five X-linked genes. The one case where dosage compensation was known not to exist (*Lsp-1α*; Roberts and Evans-Roberts 1979) did not show dosage compensation by RT-QPCR. These collective data are consistent with the hypothesis that RT-QPCR accurately quantitates transcriptional product, verifying the concept that the steady-state amount of product of a given gene is usually (but not always) equivalent in males and females. Taken together with our previous publication (Chiang *et al.* 1999), the work confirms the ability of the *TaqMan* QPCR and RT-QPCR methodologies to discern twofold dosage differences at both the DNA and RNA levels in Drosophila. These studies also substantiate the ability of the method to work with whole flies as opposed to using only selected tissues from a fly.

No simple rule for effect of MSL mutations on regulation: Our initial hypothesis was that application of the *TaqMan* QPCR and RT-QPCR methodologies would allow us to detect simple rules pertaining to the various entities comprising the MSL pathway. Surprisingly, our analyses demonstrated that there is no simple rule that can explain the effects of the MSL network on regulation. These analyses showed that both autosomal and X chromosomal genes are expressed in patterns that could not be predicted in a straightforward manner. There are genes on autosomes whose expression is altered and genes on the X chromosome that do not show dosage compensation, and the quantitative anomalies are often not consistent with the chromosomal dosage and/or dosage compensation.

Relative effects on gene expression for the various *mle* and *mof* mutants: Since males carrying homozygous *mle* lethal alleles and mof^1 hemizygous males can survive through the late third instar to early pupal stage, the amount of steady-state mRNA could be analyzed in third instar larvae. If lethality reflected gene expression in the homozygous males, one would expect the effect on the male X chromosome to be as follows: $mle^1 = mof^1 \cong GET > DQIH > GNT > AAA$. (The effect from mle^{RK} is more difficult to predict since a dominant negative effect could occur.) Instead, we observed a different order of negative effects on the expression of X-linked genes: $mof^1 > mle^{RK} > mle^1 \cong DQIH > GET > GNT = AAA$. The limited numbers of X-linked genes analyzed and the ultimate resolution of RT-QPCR could be responsible for the similar patterns observed in *GNT* and *AAA* alleles in our analysis. Even so, it is clear from our analysis that the expression patterns we observed are not simply related to the lethal effects caused by the *mle* or *mof* mutations.

Our RT-QPCR analysis indicated that the interpretation of regulation of the dosage compensation mutants is complex. The twofold reductions of *BR-C*, *Sgs4*, and *Dspt6* in mle^1 and *DQIH* mutants supported the current model. However, we did not observe the expected twofold reduction for the X-linked loci, *para* and *6-Pgd*, both of which remain dosage compensated in mutant males [*para* may be a special case since *mle* may participate in its splicing and editing (Hanrahan *et al.* 2000)]. Analysis of younger mof^1 mutants (that are not sick) confirmed the analysis on *Dspt6* and *para* observed at the later third instar stage. Another unexpected finding from our analysis was that the reduction of transcripts from both *BR-C* and its downstream *Sgs* genes (including the *Sgs4* gene on the X chromosome) was greater than twofold in both the mof^1 and mle^{RK} male flies. The greater than twofold reduction suggested that the regulation of *BR-C* on the single X chromosome is influenced by these mutations.

Since the younger $y^1\ mof^1/Y$ larvae were still healthy and all four genes analyzed were dosage compensated at the younger stage of wild-type flies, the effects we observed in younger $y^1\ mof^1/Y$ males reflect the effect of the *mof* mutation on gene expression.

The similarity of the *mle* and *mof* data on the expression patterns of the genes we examined (*cf.* Figures 4 and 6) favors the hypothesis that the functions of these genes are alike. This provides evidence for the upregulation model (in which MLE and MOF act together to upregulate the male X; reviewed in Kelley and Kuroda 1995; Bashaw and Baker 1996; Cline and Meyer 1996; Lucchesi 1998; Stuckenholz *et al.* 1999), rather than the inverse model (in which the absence of MOF should differ from the absence of MLE that sequesters active MOF; Birchler 1996).

Interconnected regulation networks: We postulate that some of the effects we observed resulted from interconnected regulation networks. In particular, expression of the *Sgs* genes (on both the autosomes and the sex chromosome) correlated with the amount of *BR-C* transcript in the mutants. This correlation was seen in a variety of mutants. The most severe reduction was observed in the mof^1 mutant. A 5-fold reduction of *BR-C*, and 10- to 30-fold reductions of *Sgs3*, *Sgs4*, *Sgs5*, *Sgs7*, and *Sgs8* were detected in the mof^1 hemizygous male compared with the balancer male. A 3- to 4-fold reduction of *BR-C* and 3- to 8-fold reductions of *Sgs3*, *Sgs4*, *Sgs5*, *Sgs7*, and *Sgs8* were detected in mle^{RK} homozygous males compared with homozygous females. The reduction of expression of autosomal *Sgs* genes is ~2- to 3-fold in the mle^1 and *DQIH* homozygous males, where the reduction of *BR-C* is 2- to 3-fold. Thus, our analysis delineates a strong correlation between *BR-C* expression and expression of the *Sgs* regulation units in a variety of flies. Presumably this reflects the regulation network documented previously, in which *BR-C* mutations were shown to decrease the induction of the intermolt genes *Sgs3*, *Sgs4*, and *Sgs5* (Guay and Guild 1991; Karim *et al.*

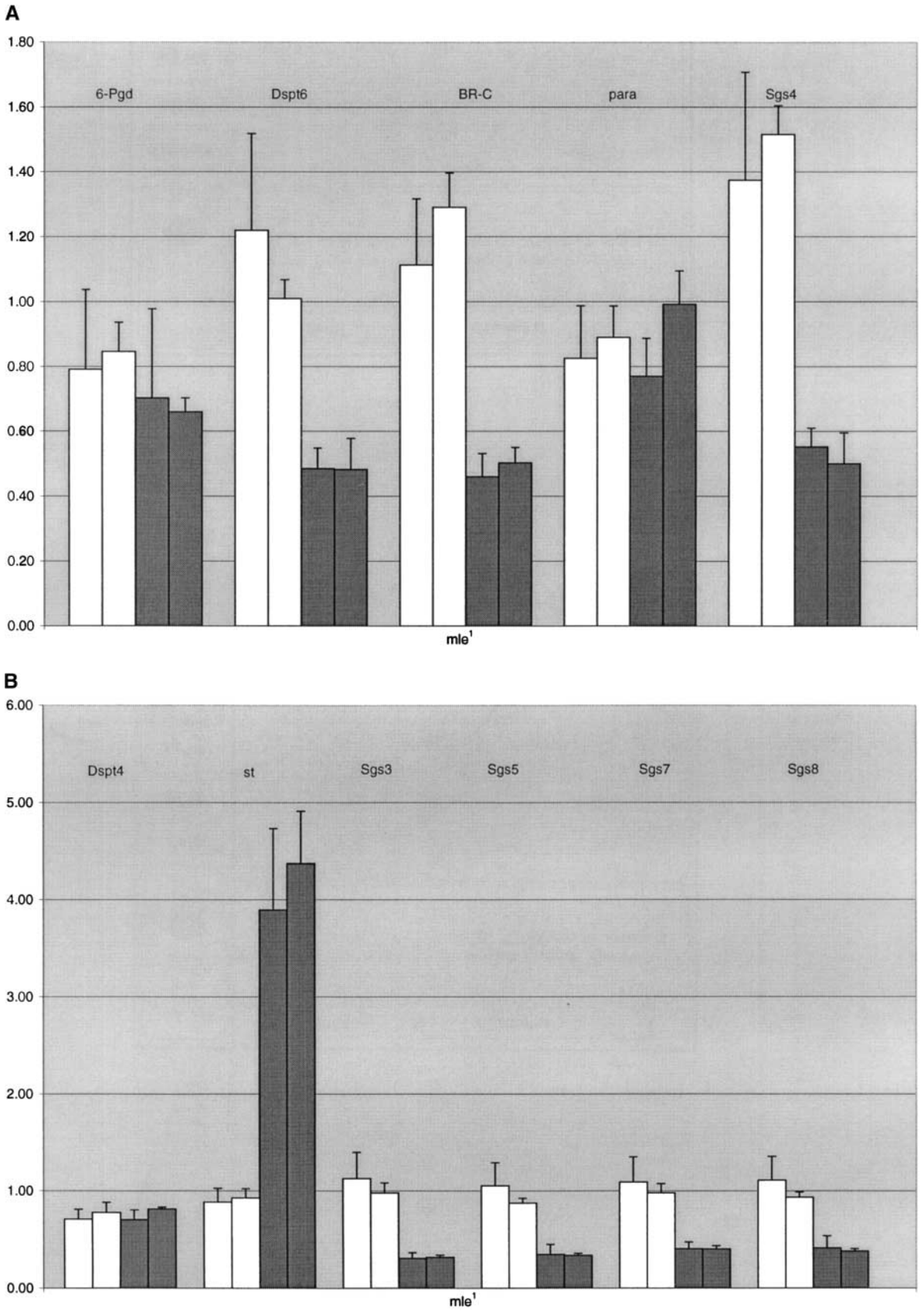

FIGURE 4.—RT-QPCR analysis of *mle*^{1} (A and B) and *mle*^{RK} (C and D). The order of each analysis is: (1) homozygous female (white); (2) 1:1 dilution of homozygous female (white); (3) homozygous male (gray); and (4) 1:1 dilution of homozygous male (gray). Analysis of the X chromosome genes are shown in A and C and analysis of the autosomal genes are shown in B and D.

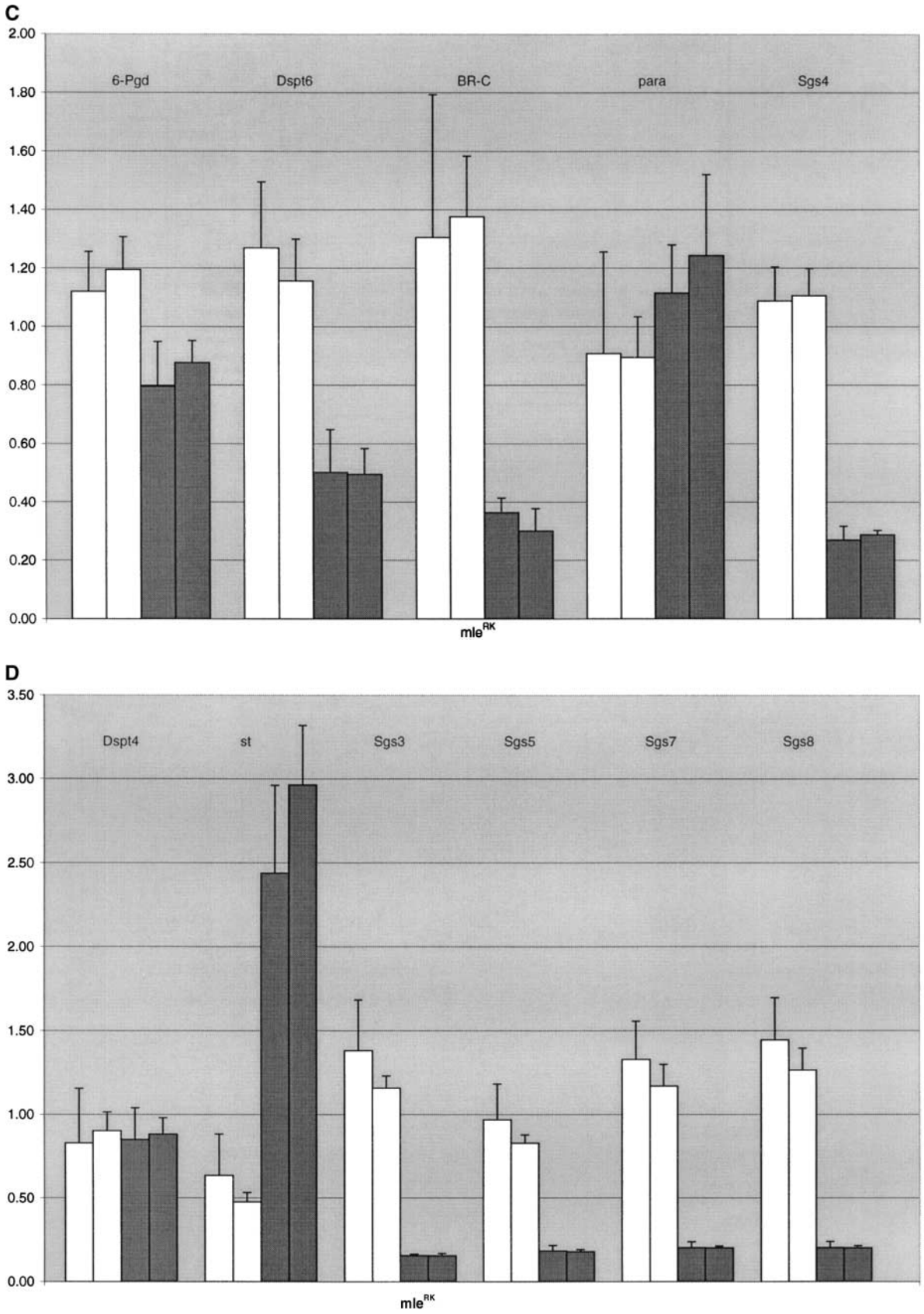

FIGURE 4.—*Continued.*

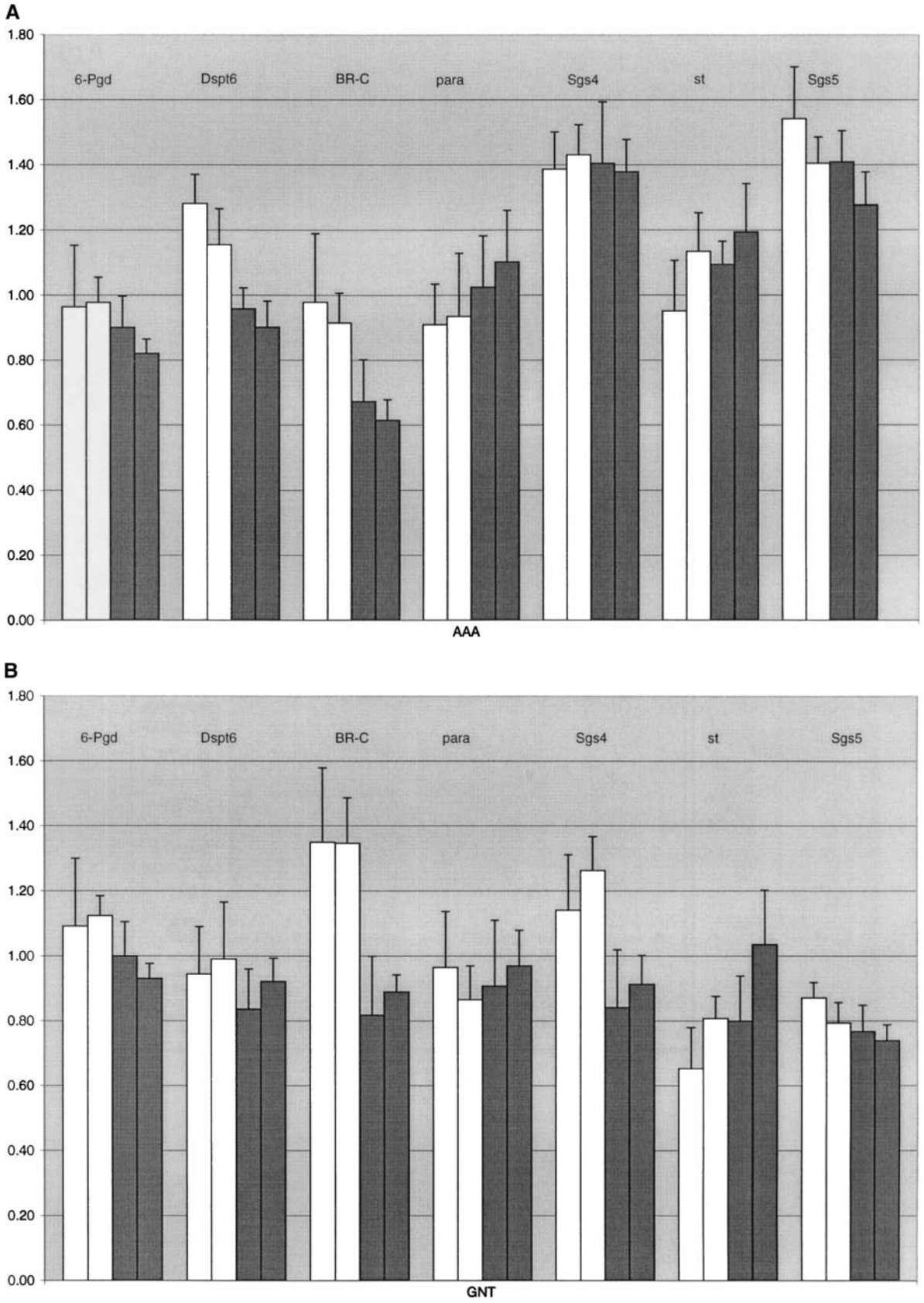

FIGURE 5.—RT-QPCR analysis of *AAA* (A), *GNT* (B), *GET* (C), and *DQIH* (D) on an otherwise deficient (mle^{l}) background. The order of each analysis is: (1) female (white); (2) 1:1 dilution of female (white); (3) male (gray); and (4) 1:1 dilution of male (gray).

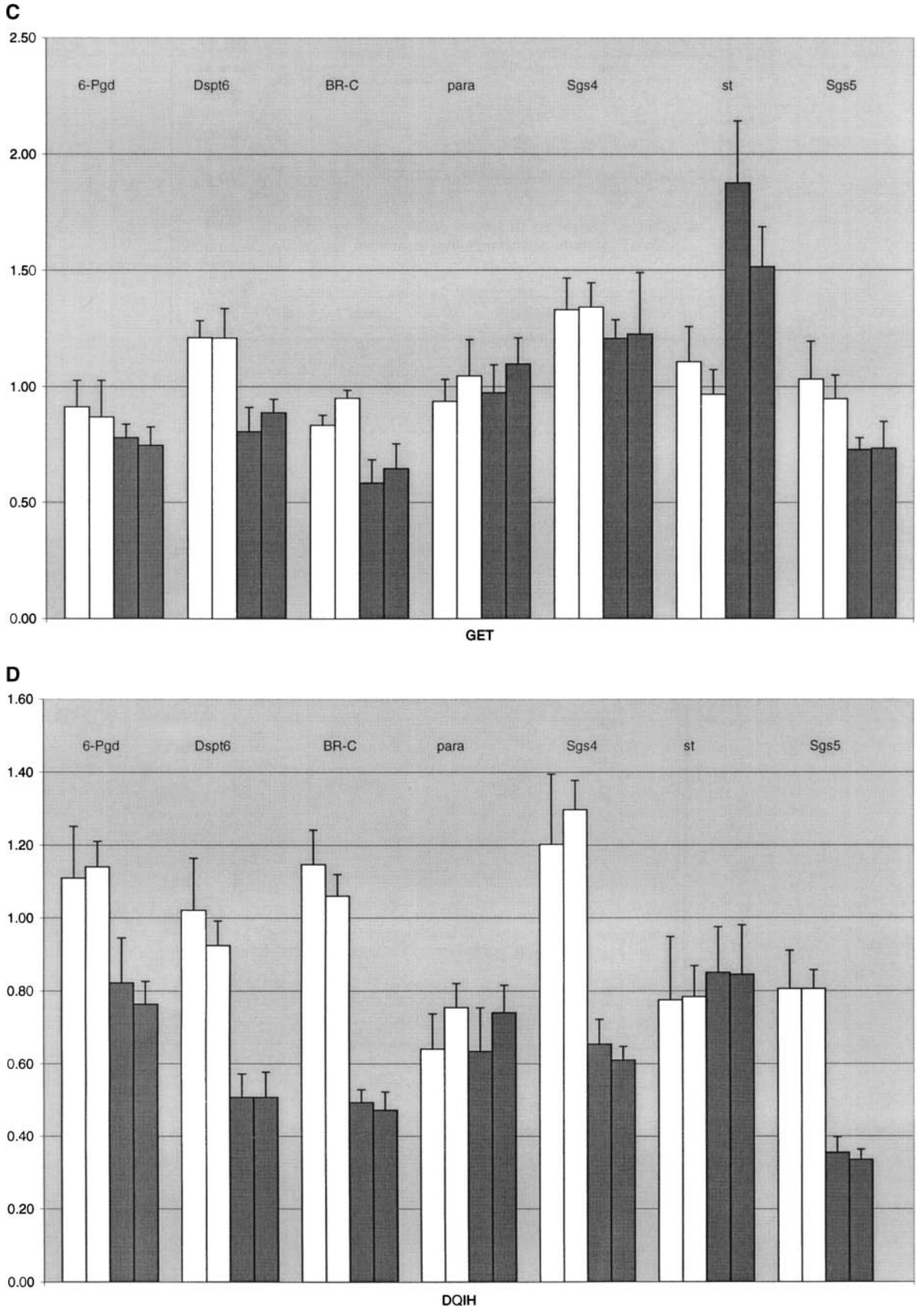

FIGURE 5.—*Continued.*

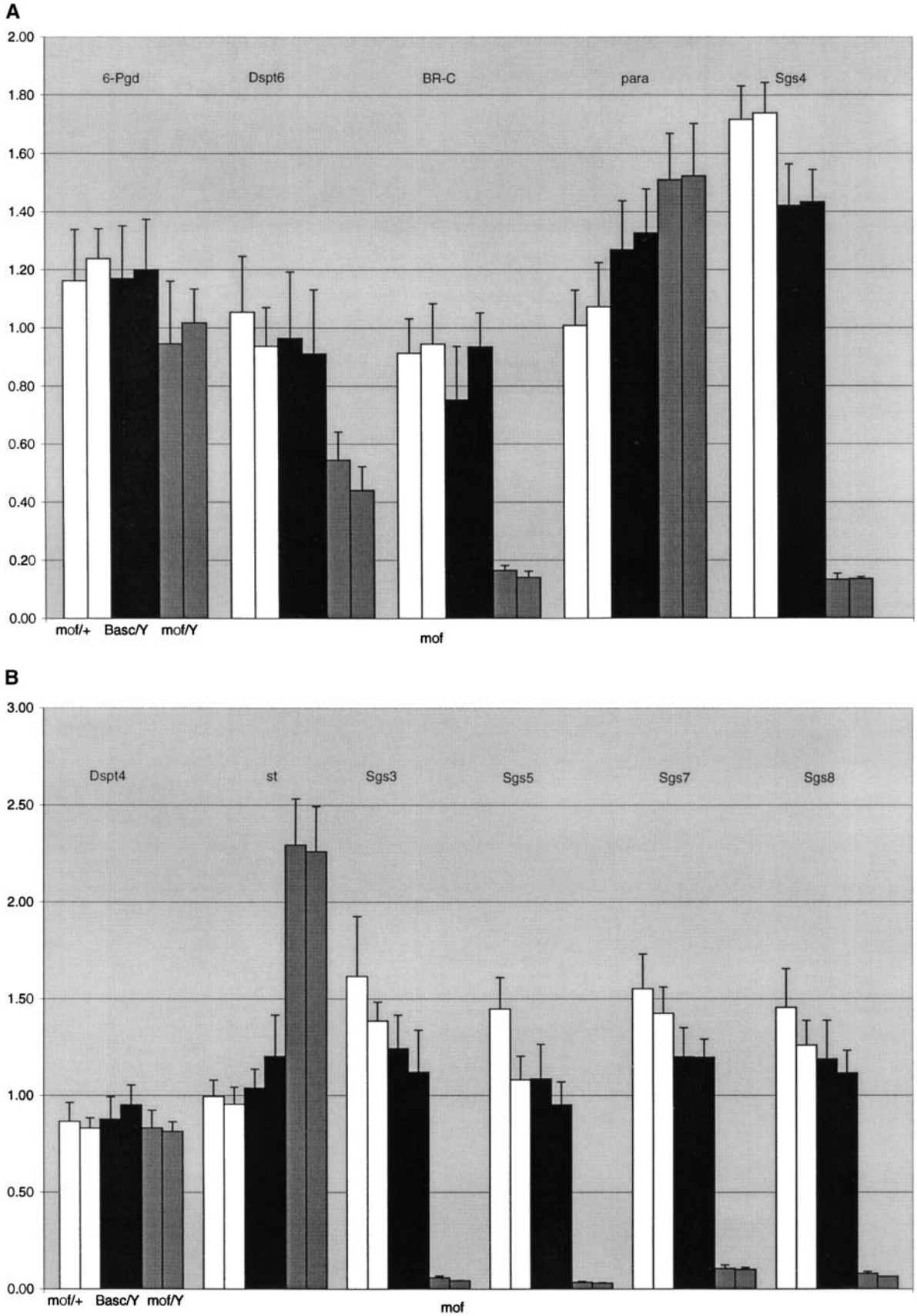

FIGURE 6.—RT-QPCR analysis of *mof*^{1}. (A and B) Third instar larvae. (C) Young *mof*^{1} larvae. (D) Young larvae from wild type. (A and B) The order of each analysis is: (1) *mof*^{1}*/Basc* (indicated as *mof/+*; white); (2) 1:1 dilution of *mof*^{1}*/Basc* (white); (3) *Basc/Y* (black); (4) 1:1 dilution of *Basc/Y* (black); (5) *mof*^{1}*/Y* (gray); and (6) 1:1 dilution of *mof*^{1}*/Y* (gray). *Basc* indicates the presence of a balancer chromosome.

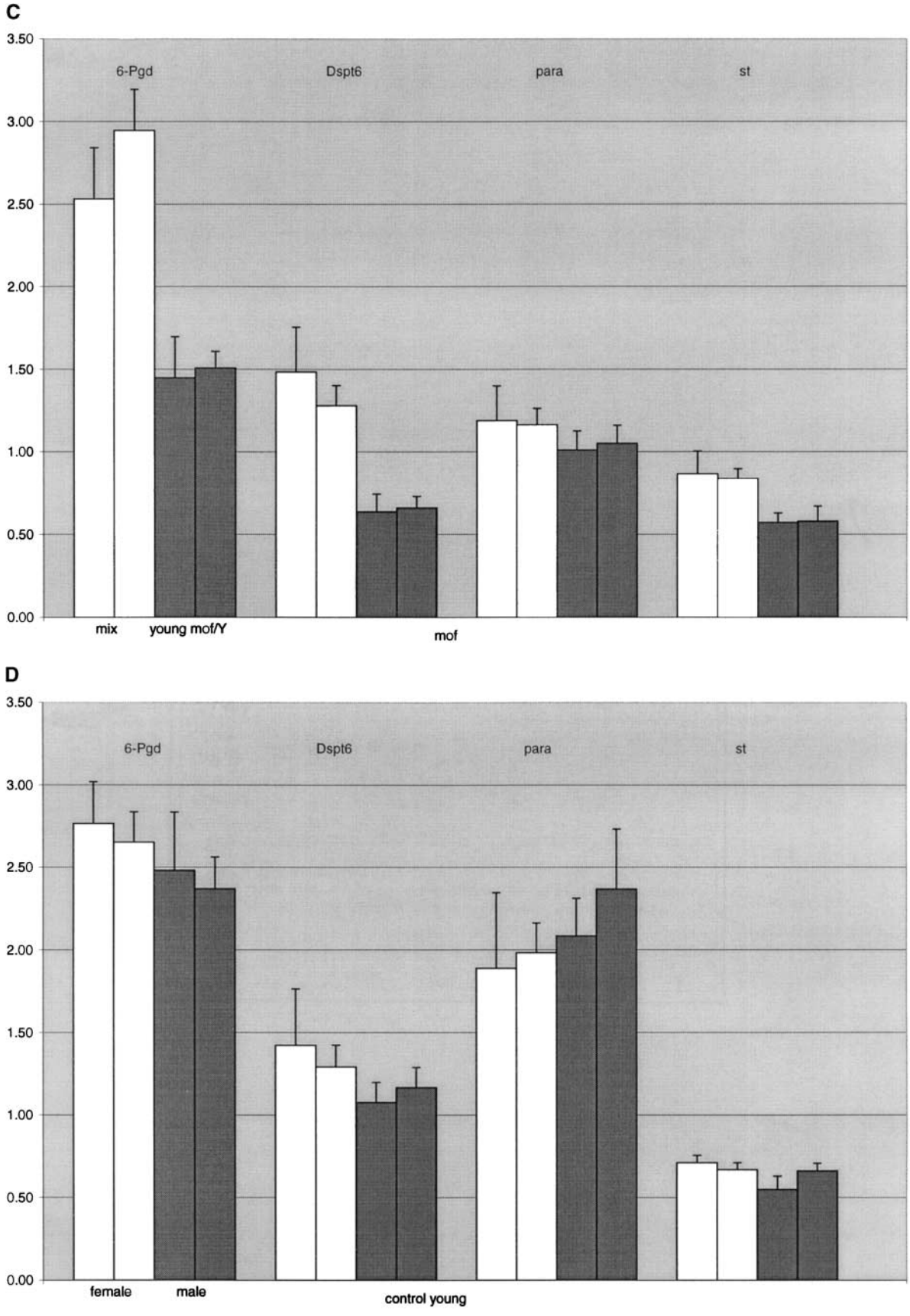

FIGURE 6.—*Continued.*

1993). Thus, the data is compatible with *BR-C* transcript levels controlling the transcript levels of the *Sgs* genes.

Developmental effects of MSL mutations: The effects on regulation of a gene can vary at different developmental stages. The expression of the autosomal gene *st* provides a different picture of altered regulation than the *BR-C* and *Sgs* genes do in that *st* is upregulated in general, rather than downregulated, in the MSL mutants. An approximately fourfold increase of expression relative to the wild type was observed in the mle^{1} and mle^{RK} homozygous male mutants. An approximately twofold increase was observed in the mof^{1} hemizygous mutant. In contrast to several other genes analyzed, the expression of *st* was changed in *GET* mutants but not in *DQIH* mutants. When younger mof^{1} larvae were analyzed, a small (40–50%) reduction of the expression of *st* was seen in younger male larvae in contrast to the twofold increase of expression in third instar males. Therefore, altered regulation of *st* in these mutants is developmentally regulated, manifesting different regulation asymmetries between the sexes at different developmental stages. This underscores the complexity of effects caused by these mutations.

A structural gene on the X chromosome could have positive and/or negative regulators on the same chromosome. Similarly, a structural gene on an autosome could also have positive and/or negative regulators on the X chromosome. Any specific effect on the X chromosome could then trigger a chain reaction for the expression of other genes on the X chromosome or on autosomes. The regulation of the *Sgs* genes by *BR-C* was shown previously (Guay and Guild 1991; Renault *et al.* 2001) and could explain the interrelationships we observed among these genes. The factor(s) directly or indirectly involved in the regulation of *st* is not known. For example, at least one of the factors could be on the X chromosome and this unknown factor could be regulated developmentally. The presence of developmentally regulated factors was further suggested by our analysis of *6-Pgd*. No significant variation of *6-Pgd* levels was observed in third instar larvae of the mof^{1} mutant but a twofold reduction was observed in the younger larvae of the mof^{1} mutant. The point that ensues from this analysis is that it is necessary to ascertain the behavior of a gene (and when applicable, its interconnected regulation network pathway) throughout development to describe the effects of that gene (and when applicable, its network). For example, the more than twofold reduction of *BR-C* in some dosage compensation mutants might result from the effect of an interconnected regulation network of *BR-C* and its positive regulator(s).

Implications for understanding development: The importance of the interconnected regulation networks, with its resultant complexities, was overlooked in the past:

1. Understanding the role of aneusomic genomes in tumor cells or in human genetic diseases (*e.g.*, Down syndrome) will involve the complexity of interconnected regulation networks. In other words, the etiology of some features of Down syndrome may not be caused simply by the expected 1.5-fold increase of steady-state transcriptional product from genes on chromosome 21 (Kurnit 1979). The disturbance of regulation of genes on other chromosomes may be involved as well.
2. A more detailed molecular analysis of different functional domains of the dosage compensation complex could be initiated on the basis of our analysis. The expected twofold reduction of *Dspt6*, *BR-C*, and *Sgs4* transcripts provides an opportunity to study the functional domains of MLE in the mle^{1} background. As illustrated in our study, the function of helicase activity in MLE could be separated from the lethal effect caused by a mutation of *mle*. Without the helicase activity, the hypertranscription activity of the MSL complex is still intact. Similar analyses may be extended to other *msl* genes.
3. No two genes share a common expression pattern in all the mutants we analyzed. Therefore, regulation happens at the level of each individual gene. Indeed, our analyses show that it is not possible to predict the dosage of a gene in a given MSL mutant. The situation is further complicated by the finding that dosage varies at different developmental stages. The fact that young mutant larvae can give different results than older mutant larvae is additional evidence for the regulation network idea, as indirect effects may be amplified with time and/or proximity to death.

Desirability of global analyses: With the demonstration that the MSL pathway yields more complex regulation of gene expression than was previously thought, this requires more complicated analyses to identify pathways affected by the MSL genes. In turn, indirect effects of the MSL mutants can be expected, potentially extending to unexpected effects affecting entire pathways. Thus, a more global comparison such as microarray analysis (Schena *et al.* 1995; Churchill 2002) is indicated to outline the genes that belong to potential pathways (*e.g.*, the *Sgs* genes regulated by *BR-C* gene) influenced by the MSL genes. Indeed, this technology has been used to study sex determination in Drosophila (Churchill and Oliver 2001; Jin *et al.* 2001). Greater detail for the members of an indicated pathway can then be obtained by analyses using methodologies such as those presented herein that can potentially detect small differences. Although our data demonstrated that regulation of a gene cannot be deduced *a priori*, it can be measured using RT-QPCR analysis in a given case. With the success of such analyses, more MSL alleles could be analyzed and the effect of the genes constituting the MSL pathway on expression of a given gene can be unraveled.

We thank James Birchler for his support of the initial phase of the work. Mitzi Kuroda provided the different *mle* flies used in this study. Kathy Welch aided our statistical analyses. This study was supported by a grant from the state of Michigan (MEDC-410).

LITERATURE CITED

Bashaw, G. J., and B. S. Baker, 1996 Dosage compensation and chromatin structure in Drosophila. Curr. Opin. Genet. Dev. **6:** 496–501.

Belote, J. M., and J. C. Lucchesi, 1980 Control of X chromosome transcription by the maleless gene in *Drosophila*. Nature **285:** 573–575.

Belyaeva, E. S., M. G. Aizenzon, V. F. Semeshin, I. I. Kiss, K. Koczka *et al.*, 1980 Cytogenetic analysis of the 2B3-4-2B11 region of the X-chromosome of *Drosophila melanogaster*. Chromosoma **81:** 281–306.

Bhadra, U., M. Pal-Bhadra and J. A. Birchler, 1999 Role of the *male specific lethal* (*msl*) genes in modifying the effects of sex chromosome dosage in Drosophila. Genetics **152:** 249–268.

Birchler, J. A., 1996 X chromosome dosage compensation in *Drosophila*. Science **272:** 1190.

Breen, T. R., and J. C. Lucchesi, 1986 Analysis of the dosage compensation of a specific transcript in *Drosophila melanogaster*. Genetics **112:** 483–491.

Chiang, P. W., W. L. Wei, K. Gibson, R. Bodmer and D. M. Kurnit, 1999 A fluorescent quantitative PCR approach to map gene deletions in the Drosophila genome. Genetics **153:** 1313–1316.

Churchill, G. A., 2002 Fundamentals of experimental design for cDNA microarrays. Nat. Genet. **32** (Suppl.): 490–495.

Churchill, G. A., and B. Oliver, 2001 Sex, flies and microarrays. Nat. Genet. **29:** 355–356.

Cline, T. W., and B. J. Meyer, 1996 Vive la difference: male vs. females in flies vs. worms. Annu. Rev. Genet. **30:** 637–702.

Fukunaga, A., A. Tanaka and K. Oishi, 1975 Maleless, a recessive autosomal mutant of *Drosophila melanogaster* that specifically kills male zygotes. Genetics **81:** 135–141.

Gu, W., P. Szauter and J. C. Lucchesi, 1998 Targeting of MOF, a putative histone acetyl transferase, to the X chromosome of *Drosophila melanogaster*. Dev. Genet. **22:** 56–64.

Guay, P. S., and G. M. Guild, 1991 The ecdysone-induced puffing cascade in Drosophila salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. Genetics **129:** 169–175.

Hanrahan, C. J., M. J. Palladino, B. Ganetzky and R. A. Reenan, 2000 RNA editing of the Drosophila para Na(+) channel transcript: evolutionary conservation and developmental regulation. Genetics **155:** 1149–1160.

Hiebert, J. C., and J. A. Birchler, 1994 Effects of the *maleless* mutation on X and autosomal gene expression in *Drosophila melanogaster*. Genetics **136:** 913–926.

Hilfiker, A., D. Hilfiker-Kleiner, A. Pannuti and J. C. Lucchesi, 1997 *mof*, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in *Drosophila*. EMBO J. **16:** 2054–2060.

Jin, W., R. M. Riley, R. D. Wolfinger, K. P. White, G. Passador-Gurgel *et al.*, 2001 The contributions of sex, genotype and age to transcriptional variance in Drosophila melanogaster. Nat. Genet. **29:** 389–395.

Karim, F. D., G. M. Guild and C. S. Thummel, 1993 The *Drosophila Broad-Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development **118:** 977–988.

Kelley, R. L., and M. I. Kuroda, 1995 Equality for X chromosomes. Science **270:** 1607–1610.

Kernan, M. J., M. I. Kuroda, R. Kreber, B. S. Baker and B. Ganetzky, 1991 nap^{ts}, a mutation affecting sodium channel activity in *Drosophila*, is an allele of *mle*, a regulator of X chromosome transcription. Cell **66:** 949–959.

Kurnit, D. M., 1979 Down syndrome: gene dosage at the transcriptional level in skin fibroblasts. Proc. Natl. Acad. Sci. USA **76:** 2372–2375.

Lee, C. G., K. A. Chang, M. I. Kuroda and J. Hurwitz, 1997 The NTPase/helicase activities of *Drosophila* maleless, an essential factor in dosage compensation. EMBO J. **16:** 2671–2681.

Lucchesi, J. C., 1998 Dosage compensation in flies and worms: the ups and downs of X chromosome regulation. Curr. Opin. Genet. Dev. **8:** 179–184.

Renault, N., K. King-Jones and M. Lehmann, 2001 Downregulation of the tissue-specific transcription factor Fork head by Broad-Complex mediates a stage-specific hormone response. Development **128:** 3729–3737.

Richter, L., J. R. Bone and M. I. Kuroda, 1996 RNA-dependent association of the *Drosophila* maleless protein with the male X chromosome. Genes Cells **1:** 325–336.

Roberts, D. B., and S. Evans-Roberts, 1979 The X-linked α-chain gene of Drosophila LSP-1 does not show dosage compensation. Nature **280:** 691–692.

Schena, M., D. Shalon, R. W. Davis and P. O. Brown, 1995 Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467–470.

Stuckenholz, C., Y. Kageyama and M. I. Kuroda, 1999 Guilt by association: non-coding RNAs, chromosome-specific proteins and dosage compensation in *Drosophila*. Trends Genet. **15:** 454–458.

Communicating editor: K. Golic