study will be needed to determine whether this is the case.

Finally, there are at least three other potential impacts of a rise in anthropogenic H_2 emissions that are beyond the scope of this study but deserve further consideration: (i) Our model predicts that a rise in H_2 concentration at the surface will make the mean OH concentration in the troposphere decrease by about 7%, whereas that in the stratosphere will increase by 10%; these changes will affect the lifetimes of other trace gases that react with OH (such as CH_4 and CO). (ii) An increase in the mesosphere of H_2O derived from H_2 could lead to an increase in noctilucent clouds, with potential impact on Earth's albedo and mesospheric chemistry. (iii) H_2 is a microbial nutrient, and thus increased partial pressures of H_2 over natural soils might have unforeseen effects on microbial communities.

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Sex-Dependent Gene Expression and Evolution of the *Drosophila* Transcriptome

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Comparison of the gene-expression profiles between adults of *Drosophila melanogaster* and *Drosophila simulans* has uncovered the evolution of genes that exhibit sex-dependent regulation. Approximately half the genes showed differences in expression between the species, and among these, ~83% involved a gain, loss, increase, decrease, or reversal of sex-biased expression. Most of the interspecific differences in messenger RNA abundance affect male-biased genes. Genes that differ in expression between the species showed functional clustering only if they were sex-biased. Our results suggest that sex-dependent selection may drive changes in expression of many of the most rapidly evolving genes in the *Drosophila* transcriptome.

Sexual dimorphism is ubiquitous among higher eukaryotes. Differential selection pressure between the sexes has been postulated to explain the substantial between-sex differences observed in morphology, physiology, and behavior, indicating the existence of different optimal sex-dependent phenotypes (*1*). Studies of gene expression during the life cycle of *Drosophila melanogaster* have found that, for sexually mature males and females, a substantial fraction of the *Drosophila* transcriptome displays sex-dependent regulation (*2–4*). Increasing evidence suggests that molecular mechanisms associated with sex and reproduction change substantially faster between species than those more narrowly restricted to survival (*5*, *6*). New data also suggest that some of the interspecific changes that are driven by differential selection between the sexes have a regulatory origin (*7*, *8*). However, the evolutionary pattern of differences in gene expression between the sexes on a genomic scale is presently unknown.

We performed competitive hybridizations with cDNA microarrays (fig. S1) (*9*) to identify genome-wide regulatory differences in sex-biased genes between *D. melanogaster* and *D. simulans*. These morphologically nearly identical species belong to the *melanogaster* subgroup of the subgenus *Sophophora* and diverged ~2.5 million years ago (*10*, *11*). Our results are based on the 30 hybridizations outlined in fig. S1, which were performed with either cDNA to assay differences in transcript abundance or else genomic DNA as controls (*9*). The microarrays contained 4776 coding sequences amplified from cDNA clones (*9*). The hybridizations with genomic DNA were performed to detect coding sequences whose apparent transcript abundance might be affected by sequence divergence or by changes in gene-copy number. The species differ in an estimated 3.8% of nucleotides at the DNA sequence level (*12*) and in copy number of some transposable elements (*13*) and a few multicopy genes (*14*). Across the six interspecific DNA hybridizations, genomic DNA from *D. melanogaster* showed an average of 4.2% greater hybridization than genomic DNA from *D. simulans*, in good agreement with the estimated sequence divergence. The distribution of hybridization intensities across coding sequences was essentially gaussian (*15*) with only a few outliers identified, mostly as transposable elements such as the retrotransposon *springer* or multicopy genes such as *Stellate* (*14*, *16*). Apart from these exceptional sequences, the differences in genomic hybridization are well within the limit of detection of significant differences in gene expression with our level of replication. Accordingly, no correction for sequence divergence between the species was required for the estimates of transcript abundance.

The cDNA hybridization data were analyzed by a Bayesian method (*17*) that yielded an estimated mean and 95% credible interval of the relative level of expression of each gene in each sex of each species (table S1). Genes were classified as differentially expressed between sexes within species or for the same sex between species if their 95% credible intervals failed to overlap (Fig. 1). The main categories into which the 4776 coding sequences were classified are shown in Table 1. Comparison with the reported pattern of expression in *D. melanogaster* was used to validate our classification (*9*). Random permutations of the data provided an estimated false-positive rate of 0.03%; hence, no adjustment was made for multiple tests.

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More than half of the genes show a sex bias in expression; many of these genes are known or expected to be expressed in reproductive organs (*3*, *4*). Among the 2493 genes that show no detectable change in level of expression since the divergence of *D. melanogaster* and *D. simulans*, 57.5% show sex-biased expression (Fig. 1A). This is a somewhat greater fraction of sex-biased genes than reported previously (*2*, *3*), which we attribute largely to our level of replication.

Approximately half of the genes (2283 out of 4776) have evolved a difference in the level of expression between the species. This is far greater than the number of genes that show significant differences in expression among strains within a species (*2*). Indeed, pairwise comparisons among eight strains of *D. melanogaster* analyzed with the same microarrays and methods reported here yielded an average of only 677 genes with significant differential expression (*18*).

Among the genes with differential expression between *D. melanogaster* and *D. simulans*, only a small number (380 out of 2283, or 16.6%) show parallel differences in expression in both sexes (Fig. 1B). Instead, the majority of genes (1903 out of 2283, or 83.4%) exhibit an evolutionary pattern that is sex-specific. In particular, 952 genes retain the same sex bias in *D. melanogaster* and *D. simulans* but have evolved different levels of expression between males or females (Fig. 1C). For the remaining 951 genes, the evolutionary change entails the gain, loss, or reversal of sex-biased expression (Fig. 1D). Among the 20 genes that show reversal of sex bias, six illustrate the most extreme discordance, in which the sex that shows the greatest level of expression in one species shows the smallest level of expression in the other.

For genes that show a significant difference in expression between the sexes in both species, male-biased and female-biased genes show different patterns in relation to the magnitude of the bias in expression (table S2). For sex-biased genes with mean expression between the sexes differing by a factor ≤ 2, no significant sex bias in gene expression was detected [G test, adjusted G value (G_{adj}) = 2.4, df = 1, not significant]; for genes differing by a factor >2 but ≤ 4, there is a tendency for female overexpression ($G_{adj} = 73.6$, df = 1, $P < 9.5 \times 10^{-18}$). However, for genes differing by a factor >4, there is a pronounced excess of genes with male overexpression ($G_{adj} = 49.1$, df = 1, $P < 2.4 \times 10^{-12}$). Although genes with male-biased expression are underrepresented in the cDNA library (*19*) that was used to construct our microarray, this seems unlikely to account for the pattern reported here.

Male-biased genes also show greater expression divergence between species than either female-biased genes or non–sex-biased genes. Fig. 2 compares the divergence of expression level for genes that are male-biased, female-biased, or non–sex-biased in their expression. Across all genes, the mean divergences of the distributions of all three classes are significantly different from each other, with the greatest mean divergence among the male-biased genes and the smallest among the female-biased genes (male- versus female-biased: Student's t test, $t = 8.1$, df = 1863, $P < 1.3 \times 10^{-15}$; male- versus non–sex-biased: $t = 3.6$, df = 1549, $P < 2.7 \times 10^{-4}$; female- versus non–sex-biased: $t = -6.1$, df = 2800, $P < 1.5 \times 10^{-9}$).

Because of their potentially opposite fitness effects in males and females, genes with sex-limited expression are candidates for the occurrence of sexually antagonistic mutations or mutations that have an epistatic interaction with antagonistic loci (*20*). Theory predicts an asymmetrical distribution of sex-biased genes between the X chromosome and auto-

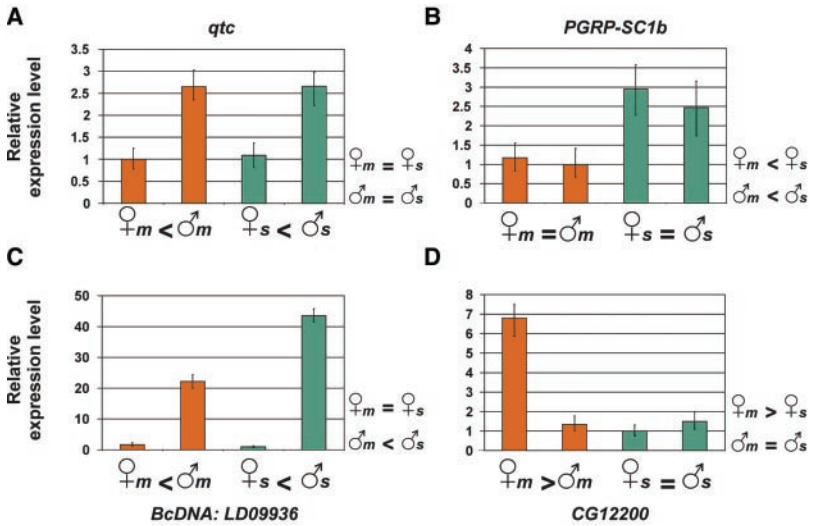

Fig. 1. Illustrative examples of gene classification according to the relative levels of expression for the four sex-by-species combinations compared. (**A**) *qtc*, a male-biased gene expressed to the same extent in both species. (**B**) *PGRP-SC1b*, a non–sex-biased gene with greater expression in *D. simulans*. (**C**) *BcDNA:LD09936*, a male-biased gene expressed with a greater bias in *D. simulans*. (**D**) *CG12200*, a female-biased gene overexpressed in *D. melanogaster* relative to *D. simulans*. The 95% credible intervals associated with the estimated mean expression levels that were obtained by a Bayesian approach (*17*) are compared between sexes within *D. melanogaster* and *D. simulans* (below the graphs) and for the same sex between species (to the right). Inequality signs denote statistically significant differences. Expression levels appear as a fold-change relative to the node(s), with the lowest level of expression set to 1 (*17*). m, *D. melanogaster*; s, *D. simulans*.

Table 1. Classification of genes by their pattern of evolution and sex bias. A Bayesian method was used to determine the 95% credible intervals of the mean expression level for all genes in the hybridizations in fig. S1 (*17*). Those intervals were then compared between sexes within species and for the same sex between species. A difference in gene expression was regarded as significant if the 95% credible intervals failed to overlap. In Bayesian analysis, the 95% credible interval is the analog of the 95% confidence interval in conventional frequentist statistics. Sixty-seven different categories of gene expression are possible among the four comparisons (fig. S2 and table S1).

Class	Number	% of total
By pattern of evolution		
No difference in expression in either sex between species and no difference in sex bias between species	2493	52.2
Sex-independent difference in expression between species	380	8.0
Increase or decrease in sex-biased expression between species	952	19.9
Gain, loss, or reversal of sex-biased expression between species	951	19.9
Total	4776	
By sex bias		
Total female-enriched	2031	42.5
Female-biased in both species	1507	31.6
Female-biased in *D. melanogaster* only	327	6.8
Female-biased in *D. simulans* only	197	4.1
Total male-enriched	1318	27.6
Male-biased in both species	911	19.1
Male-biased in *D. melanogaster* only	174	3.6
Male-biased in *D. simulans* only	233	4.9

somes (*21*). Among genes with a significant male bias in expression, we found a 32% deficit of genes on the X chromosome of *D. melanogaster* and *D. simulans* as compared to the autosomes (G_{adj} = 19.9, df = 1; $P <$ 0.0001). A deficiency of male-biased genes on the *D. melanogaster* X chromosome has been reported previously (*4*). The data presented here confirm the generality of this pattern in other *Drosophila* species and also highlight a significant overrepresentation of female-biased genes in expression on the X chromosome. We find a 22% excess of female-biased genes on the X chromosome (G_{adj} = 13.5, df = 1, $P <$ 0.001), a pattern not previously detected except in microarray experiments that directly compare testes and ovaries (*4*).

We looked for biologically coherent patterns of functional divergence between *D. melanogaster* and *D. simulans* to assess nonrandom changes in gene expression during the evolution of the *Drosophila* transcriptome. For this purpose, we searched for significant overrepresentation of particular molecular functions, biological processes, cellular components, or regulatory pathways in the subsets of genes differentially expressed between species (*9*, *22*). Genes that show differential expression between these species present a pattern of functional clustering that is primarily sex-by-species–specific but not necessarily related to germline function. Among genes that show no sex bias in expression in either species, functional clustering is undetectable under statistically conservative criteria that correct for multiple tests (tables S3 and S4). In contrast, sex-biased genes that are differentially expressed between *D. melanogaster* and *D. simulans* exhibit diverse patterns of functional and pathway enrichment (tables S5 to S8). Especially notable is the presence of genes that are potentially involved in mating behavior (*23*). Males of *D. simulans* show overexpression of genes involved in the phototransduction cascade (P = 0.009), whose products are all localized to the rhabdomere ($P = 5.7 \times 10^{-4}$). Greater male-biased expression of genes involved in phototransduction in *D. simulans* may reflect the fact that visual stimuli are more important for mating in *D. simulans* than in *D. melanogaster* (*24*), as *D. simulans* does not mate efficiently in the dark (*25*). In *D. melanogaster* males, two genes related to olfaction are upregulated (uncorrected P = 0.019). These newly evolved sex-interspecific expression differences closely mirror the epicuticular hydrocarbons that play a key role in the chemical communication during courtship. *D. melanogaster*, unlike *D. simulans*, displays a hydrocarbon profile that varies markedly between the sexes (*26*).

Overall, our results highlight profound changes in sex-dependent gene expression that have taken place in the *Drosophila* transcriptome over 2 to 3 million years. The more rapid divergence in gene expression found here for males parallels the observation that male-specific morphological features evolve more rapidly than those of females (*27*, *28*). Random genetic drift and/or relaxed selection for sex-dependent gene expression could result in large, yet nearly neutral, interspecific differences in gene expression. However, the finding that reversals of sex-dependent expression between species are rare, whereas other kinds of changes are common, argues against this hypothesis. A purely neutral model of transcriptome divergence is also difficult to reconcile with the substantial excess of sex-biased genes among those that have evolved differences in expression, as well as with the underlying nonrandom pattern of functional divergence between *D. melanogaster* and *D. simulans* in sex-biased genes. The patterns of change are more consistent with the action of sex-dependent selection both within and between species. Sex-dependent selection may result from antagonistic fitness effects between the sexes, by sexual selection (*1*), or both together (*29*). Divergent, sex-dependent selection patterns between species could account for the interspecific differences reported here.

The nonrandom chromosomal distribution of sex-biased genes in *Drosophila* contrasts sharply with that found in mammals, where the X chromosome shows an excess of genes expressed in the male germ line (*30*). The *Drosophila* pattern is also at odds with that expected from the theory of sexually antagonistic alleles. In theory, X-linked mutations that favor males at the expense of females will be more likely to fix if they are recessive, whereas X-linked mutations that favor females at the expense of males are more likely to fix if partially dominant (*21*). Part of the discrepancy may reflect a transposition bias, for example, the preferential movement of reverse-transcribed copies of genes expressed late in spermatogenesis from the X chromosome to the autosomes (*31*). Alternatively, in contrast with loss-of-function mutations that tend to be nearly recessive (*32*), sex-limited and sexually antagonistic mutations might consist largely of gain-of-function mutations with partial dominance (*33*), in which case the pattern reported here would be expected.

Since the divergence between *D. melanogaster* and *D. simulans*, significant changes in adult gene expression have evolved in approximately half of the transcriptome, whereas the other half has retained the ancestral pattern of expression. The observations that 83% of the interspecific changes in gene expression are sex-dependent and that divergence in expression levels is greater in males suggest that sex-dependent selection is a major force driving the recent evolution of the *Drosophila* expression profile. The rapid accumulation of sex-related changes in biologically coherent functional categories and the contrasting chromosomal locations of male-biased and female-biased genes lend additional support to this view. Further experiments should help clarify whether a substantial fraction of the interspecific changes in gene expression are coordinately regulated by a limited number of genes or have evolved those differences largely independently from one another.

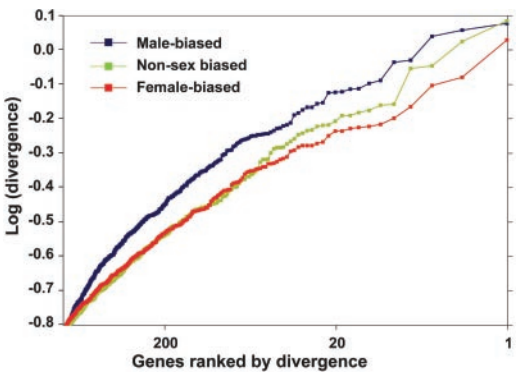

Fig. 2. Differential divergence in the level of expression among male-biased, female-biased, and non–sex-biased genes. Genes were ranked on a logarithmic scale by their difference in expression level between *D. melanogaster* and *D. simulans*. Blue, male-biased genes; red, female-biased genes; green, non–sex-biased genes. The plot includes 425 genes of each class, chosen so that the origin of the plot would correspond to genes whose divergence is essentially equal. Divergence is calculated as the coefficient of variation of the mean expression values for the sex that shows the expression bias in *D. melanogaster* and *D. simulans*. For non–sex-biased genes, the divergence is calculated as the average of the male and female coefficients of variation. Male-biased genes show a larger difference between *D. melanogaster* and *D. simulans* than female-biased genes or non–sex-biased genes.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/300/5626/1742/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S10
References and Notes

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Fusion of Cells by Flipped SNAREs

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The SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) hypothesis suggests that pairs of proteins known as vesicle (v-) SNAREs and target membrane (t-) SNAREs interact specifically to control and mediate intracellular membrane fusion events. Here, cells expressing the interacting domains of v- and t-SNAREs on the cell surface were found to fuse spontaneously, demonstrating that SNAREs are sufficient to fuse biological membranes.

The fusion of intracellular membranes is mediated by cognate SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) proteins (*1*) that assemble between lipid bilayers as SNAREpins (*2*). SNAREpins consist of a bundle of four helices (*3*). For fusion to occur, three of these (contributed by the target membrane or t-SNARE), must emanate from one of the membrane partners. The fourth helix, contributed by the cognate vesicle or v-SNARE, must be rooted in the opposing membrane (*4*). Energy made available from protein folding—the spontaneous assembly of the SNARE complex—is coupled by the SNAREpins to do work on the lipid bilayers, promoting fusion (*5*). SNARE-dependent fusion is exquisitely specific, so much so that the pattern of membrane fusion in the cell is predicted from the pattern of fusion of artificial liposomes by isolated SNAREs with an accuracy exceeding 99% (*4*, *6*–*8*).

Can SNAREpins also fuse entire cells? Viral-encoded fusion proteins use helical hairpin bundles that are analogous to SNAREpins (*9*). SNARE proteins normally face the cytoplasm, within which their helical domains can pair to link membranes for fusion. Thus, to ascertain whether SNAREs can fuse cells, their orientation must be "flipped," and cognate cells must be engineered that express either the v- or the t-SNAREs.

For this purpose (Fig. 1A), we inserted a signal sequence that specifies translocation across the endoplasmic reticulum (ER) ahead of the coding sequence of each of the three subunits of a plasma membrane exocytic SNARE complex: VAMP2, functionally defined as the v-SNARE, and Syntaxin 1A (Syntaxin) and SNAP-25, which together compose the t-SNARE (*10*). SNAP-25 contributes two helices to the bundle and Syntaxin contributes one. The membrane-anchoring sequence at the C-terminus of Syntaxin and VAMP is expected to terminate translocation initiated by the signal sequence and to anchor the flipped SNAREs to the plasma membrane after expression and intracellular transport. SNAP-25 is normally anchored to the cytoplasmic side of the plasma membrane by palmitoylation of a Cys-rich loop. To prevent disulfide bonds that could interfere with folding in the ER lumen (and which would not form in the reducing environment of the cytosol), we used a fully functional SNAP-25 mutant with all four Cys residues substituted by Ser to generate flipped SNAP-25 (*5*). Translocated SNAP-25 is expected to assemble with flipped Syntaxin on the lumenal side of the ER when they are coexpressed to form a flipped t-SNARE. The extracellular domains of the flipped v- and t-SNAREs do not contain any Cys residues.

SNAREs are unfolded or incompletely folded except when they are in the four-helix bundle. In particular, v-SNAREs like VAMP2, with their single coil motif, are intrinsically unfolded (*11*), and the t-SNARE (Syntaxin and SNAP-25) exists in a partially folded state in which the membrane-distal portion of the t-SNARE forms a stable three-stranded helical bundle, but the membrane-proximal portion is unzipped (*12*–*14*). Because the quality control system operative in the lumen of the ER serves to retain incompletely folded proteins (*15*), only a small fraction of the flipped SNAREs would be expected to reach the cell surface. In particular, the most unfolded SNARE, VAMP2, would be expected to be least efficiently transported. However, with a high enough level of expression, at least some of each SNARE should reach the cell surface.

In preliminary experiments, cell fusion only occurred when the cells that expressed the flipped v- and t-SNAREs were treated with tunicamycin, an antibiotic that inhibits Asn-linked glycosylation (*16*). This modification occurs in the lumen of the ER but not in the cytosol. SNAREs, which are not physiologically N-glycosylated, could become artifactually glycosylated when they are flipped. There are Asn-linked glycosylation sites (Asn-X-Ser/Thr) predicted within VAMP2 (Asn^{25}), SNAP-25 (Asn^{77} and Asn^{188}) and Syntaxin (Asn^{107} and Asn^{135}).

Flipped VAMP2 (Fig. 1B) and flipped SNAP-25 (Fig. 1C) were indeed Asn-glycosylated when expressed in COS cells. The artifactual glycosylation was prevented by glycosylation-site mutations that generated flipped VAMP2/T27A and flipped SNAP-25/T79AN188A, which were used in all further experiments. Two artifactual glycosylation sites are predicted in Syntaxin, both within its N-terminal regulatory domain, Habc (*17*, *18*), which folds back on the helical bundling domain to negatively regulate fusion by blocking access to the v-SNARE (*19*). Artifactual glycosylation, as well as substantial autoinhibition, were simultaneously eliminated by the removal of this domain in flipped Syntaxin.

COS cells transfected with flipped SNAREs expressed these proteins on the cell

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