

Effects of Recombination on Complex Regulatory Circuits

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ABSTRACT

Mutation and recombination are the two main forces generating genetic variation. Most of this variation may be deleterious. Because recombination can reorganize entire genes and genetic circuits, it may have much greater consequences than point mutations. We here explore the effects of recombination on models of transcriptional regulation circuits that play important roles in embryonic development. We show that recombination has weaker deleterious effects on the expression phenotypes of these circuits than mutations. In addition, if a population of such circuits evolves under the influence of mutation and recombination, we find that three key properties emerge: (1) deleterious effects of mutations are reduced dramatically; (2) the diversity of genotypes in the population is greatly increased, a feature that may be important for phenotypic innovation; and (3) *cis*-regulatory complexes appear. These are combinations of regulatory interactions that influence the expression of one gene and that mitigate deleterious recombination effects.

MUTATION and recombination are the two main forces generating genetic variation, the raw material that natural selection feeds upon. Although a small fraction of the variation generated by mutation and recombination yields evolutionary innovations, the majority of this variation may be deleterious. Recombination can rearrange entire genes and even larger units of organization. It thus has potentially much greater effects on the phenotype than mutations, in particular point mutations of single nucleotides.

Its potentially large deleterious effects on well-adapted genotypes notwithstanding, recombination is clearly very successful evolutionarily, as the near ubiquity of sexual reproduction in eukaryotes attests (BIRKY 1996; JUDSON and NORMARK 1996; SCHON *et al.* 1998). The reasons for this ubiquity are less clear. (For reviews see BARTON and CHARLESWORTH 1998; OTTO and LENORMAND 2002; OTTO and GERSTEIN 2006.) It is undeniable that sexual reproduction and recombination have clear benefits to individuals or populations. For example, they can help a population avoid the consequences of Muller's ratchet, which is the accumulation of slightly deleterious mutations caused by genetic drift in finite populations (MULLER 1964). Second, recombination can help bring together beneficial mutations from different individuals that would

otherwise have to arise and go to fixation sequentially in an asexual population (FISHER 1930; MULLER 1932). It can thus help speed up adaptive evolution during periods of directional selection where a population is far from a fitness optimum (KEIGHTLEY and OTTO 2006). Additionally, it may cause the more rapid elimination of deleterious mutations (KONDRASHOV 1998). These and many other benefits of sex may depend on multiple details of how mutations and selection affect the fitness of individuals and the mean fitness of a population. For example, sex can be advantageous for the elimination of deleterious mutations when the combined negative effects of several such mutations on fitness are stronger than the sum or the product of their individual effects (OTTO and FELDMAN 1997; KONDRASHOV 1998). The question whether these conditions are often met has received considerable attention (BONHOEFFER *et al.* 2004; KOUYOS *et al.* 2006, 2007; SANJUAN and ELENA 2006; SANJUAN *et al.* 2006).

Against these and other potential advantages of sex stand two major disadvantages. The first is that populations of sexually reproducing and anisogamous organisms are vulnerable to the invasion of asexual variants where only females bear offspring. If an asexual female variant arose that reproduced at the same rate as sexually reproducing females, this variant would double in frequency every generation, because it produces only female offspring. It would thus have a reproductive advantage over sexually reproducing females (MAYNARD SMITH 1978). The second major disadvantage of recombination is that it breaks up *beneficial allele combinations*,

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reducing the mean fitness of the population and increasing the genetic load (MULLER 1950). (A population's genetic load designates a mean fitness lower than could be attained in the absence of some evolutionary force, such as mutation, migration, or recombination.) The simple and intuitive nature of the latter mechanism makes it likely that it is rather general. Not surprisingly then, a reduction in population mean fitness caused by recombination has also been demonstrated for multi-locus genetic systems involving quantitative traits influenced by large numbers of individual loci. For a summary of pertinent results see BURGER (1999).

Population genetic models of any evolutionary phenomenon must make simplifying assumptions about how genotypes relate to phenotypes or fitness, to render models tractable. Complex genetic systems—from proteins to genetic circuits—may defy some of these assumptions (WAGNER 2005; KANEKO 2008). Much of what is known empirically about the effects of recombination in systems where the genotype–phenotype relationship is complex stems from the gene and protein level. Although recombination is more frequent on levels of organization above the molecule (ZHANG *et al.* 2002), we know much less about its effects on these important levels. In particular, there have been relatively few studies of the effects of recombination on gene circuits, *i.e.*, groups of genes that jointly perform a biological task. Is the phenotype produced by a biological circuit (for example, a gene expression phenotype) more susceptible to mutation or to recombination? How does recombination affect population properties in such circuits? Building on previous work (WAGNER 1996; SIEGAL and BERGMAN 2002; AZEVEDO *et al.* 2006; GARDNER and KALINKA 2006; MISEVIC *et al.* 2006; HUERTA-SANCHEZ and DURRETT 2007; MACCARTHY and BERGMAN 2007), we consider these questions for a model of transcriptional regulation circuits known to be important in organismal development ([supporting information, Figure S1](#)). Despite being quite abstract, variants of this model have proven highly successful in explaining the regulatory dynamics of early developmental genes in the fruit fly *Drosophila*, as well as in predicting mutant phenotypes (MJOLSNESS *et al.* 1991; SHARP and REINITZ 1998; JAEGER *et al.* 2004). The model has also helped elucidate a number of fundamental evolutionary questions. Among them are the questions why mutants often show a release of genetic variation that is cryptic in the wild type (BERGMAN and SIEGAL 2003), how adaptive evolution of robustness occurs in genetic circuits (WAGNER 1996; SIEGAL and BERGMAN 2002; LECLERC 2008), and whether recombination can influence epistasis (AZEVEDO *et al.* 2006).

The model (WAGNER 1996) represents a regulatory circuit of N transcriptional regulators, which are represented by their expression patterns $\mathbf{S}(t) = (S_1(t), S_2(t), \dots, S_N(t))$ at some time t during a developmental or cell-biological process and in one cell or domain of

an embryo. These transcriptional regulators can influence each other's expression through cross-regulatory and autoregulatory interactions, which are encapsulated in a matrix $w = (w_{ij})$. The continuously valued elements w_{ij} of this matrix indicate the strength of the regulatory influence that gene j has on gene i ([Figure S1](#)). This influence can be activating ($w_{ij} > 0$), repressing ($w_{ij} < 0$), or absent ($w_{ij} = 0$). Put differently, the matrix w represents the (regulatory) genotype of this system, while the expression state is its phenotype. We model the change in the expression state of the circuit $\mathbf{S}(t)$ as time t progresses according to the difference equation $S_i(t + \tau) = \sigma[\sum_{j=1}^N w_{ij}S_j(t)]$, where τ is a constant, and $\sigma(\cdot)$ is a sigmoidal function whose values lie in the interval $(-1, +1)$. This equation reflects the regulation of gene i 's expression by other genes. We are here concerned with circuits whose expression dynamics start from a prespecified initial state $\mathbf{S}(0)$ at some time $t = 0$ during development and arrive at a prespecified stable equilibrium or “target” expression state $\mathbf{S}(\infty)$. We call such circuits *viable* and name the set of all viable circuits the “viable ensemble.” The initial state can be thought of as being determined by regulatory factors upstream of the circuit, which may represent signals from the cell's environment or from other domains of an embryo. Transcriptional regulators that are expressed in the stable equilibrium state $\mathbf{S}(\infty)$ may affect the expression of genes downstream of the circuit. We think of their expression as critical for the course of development, deviations from $\mathbf{S}(\infty)$ being highly deleterious; we thus consider that a circuit is lethal if it is not viable.

In the context of this model, we show that mutations that change the same number of regulatory interactions as a recombination event typically have much stronger deleterious effects. We also ask whether recombination is costly in terms of the genetic load it causes for a population undergoing recombination at each generation. We find that recombination may even *reduce* the genetic load in populations subject to stabilizing selection. Recombination also increases dramatically the genetic diversity in populations as shown by a “genotypic diversity index” that we define below. Finally, recombination in general changes alleles that remain on the same chromosome (haplotype) in a genome; in the context of our model, we find that this effect of recombination creates particular *cis*-regulatory complexes that mitigate recombination's effect on a regulatory network.

METHODS

Implementation of mutations: We here describe how a circuit genotype (specified by w) is allowed to change through mutations. We constrain mutational changes to modify only one regulatory interaction at a time. Furthermore, since biological circuits are sparse, we

want this to be reflected in the allowed $\{w_{ij}\}$. We do this by forcing the number M of (nonzero) regulatory interactions to be in a given range (M_-, M_+) . In practice, the value of M_- does not matter much (CILIBERTI *et al.* 2007) and so for simplicity it could be taken to be zero or any value substantially smaller than M_+ . For the maximum of the range, we set $M_+ = zN$; z is then the average number of interactions per gene. For most of our simulations, we set $z = 3$, but other values lead to the same qualitative behavior as long as they are not too small. Also, we call two circuits (viable or not) *nearest neighbors* if they differ by just one regulatory interaction.

A mutation may cause (i) an existing interaction to disappear, in which case the respective w_{ij} is set to zero, (ii) a new regulatory interaction to appear, in which case the new value is chosen as a Gaussian random variable with mean zero and variance one ($N(0, 1)$), and (iii) an existing interaction to change in magnitude. For such a mutation, we force the sign of the interaction to remain unchanged by choosing an $N(0, 1)$ random variable and multiplying it by (-1) if it is of the wrong sign. Each putative interaction w_{ij} to be mutated is chosen at random; if the interaction is already present, we take the mutations of cases i and iii to be equiprobable.

Implementation of recombination: We implement recombination akin to how it would occur in a sexually reproducing haploid organism. In our model, an entire row w_i of the matrix w represents the promoter and enhancer regions of gene i , through which other genes can exert their influence on this gene. Although an enhancer region can be large, the individual transcription factor binding sites on it are genetically closely linked, and recombination will occur only very rarely between them. In contrast, different genes of a gene regulatory circuit will often be unlinked (in particular they can occur on different chromosomes) and thus they will recombine freely. We here focus on free recombination between circuit genes and neglect recombination within genes (promoters/enhancers). Specifically, recombination occurs between two parent circuits $w^{(1)}$ and $w^{(2)}$. To form an offspring $w^{(o)}$ through recombination, we cycle over all pairs of rows i of the parent matrices and choose one of the two rows at random from the parents to form $w_i^{(o)}$. Because we constrain the number of nonzero interactions to be in the range (M_-, M_+) , it is possible that a putative offspring circuit $w^{(o)}$ will not contain the required number of interactions. If so, the recombination process is repeated until a $w^{(o)}$ is found that does fulfill the constraint.

Population dynamics to reach mutation–selection balance or recombination–mutation–selection balance: We work with nonoverlapping generations and fixed population sizes P . Given a list of P parents, we produce the next generation that will consist of P viable individuals. We do this by creating offspring sequentially. In the absence of recombination, a parent is chosen at random

(with replacement), and with probability μ a single regulatory interaction is mutated as described above to produce a child. In the presence of recombination, two of the P parents are chosen at random (with replacement). From this “mating pair” a recombined genotype is produced (see the procedures described above), and then again with probability μ a mutation is applied to produce the child.

Because either process (mutation and recombination/mutation) can produce an offspring that is nonviable, we repeat these processes until a population of P viable offspring has been reached. This population constitutes the next generation. We iterate for as many generations as needed to erase the memory of the initial conditions and to reach a population in the steady state. In finite populations, fluctuations from generation to generation occur in the steady state, but the process is stationary: averages over many populations evolving in parallel are the same as averages over a long time of just one population. We note that our procedure ensures that the number of offspring per mating pair is Poisson distributed in the absence of selection.

RESULTS

Recombination has much weaker effects than point mutations on gene expression phenotypes: In our first analysis, we compare the effects of mutation and recombination on a circuit’s expression phenotype ($\mathbf{S}(0) \rightarrow \mathbf{S}(\infty)$). We consider only free recombination; that is, we view the circuit genes we study as being unlinked. The reason is that in this case the effects of recombination are likely to be most visible. Recombination occurs between two parent circuits $w^{(1)}$ and $w^{(2)}$. In a recombination event, regulatory regions w_i (rows i of the matrix w representing regulatory genotypes) are exchanged with probability $\frac{1}{2}$ between $w^{(1)}$ and $w^{(2)}$. The result is one or more new “offspring” circuits w' whose phenotype we evaluate. Such a recombination event changes some number m of regulatory interactions w_{ij} when compared to a parent circuit. We are interested in the probability $R_R(m)$ that a recombination event changing m regulatory interactions of a viable circuit maintains viability. This probability is a measure of the circuits’ robustness to recombination. We compare this probability to $R_\mu(m)$, which is defined analogously but by using instead m independent mutations.

We first compared $R_R(m)$ and $R_\mu(m)$ for a random sample of circuits (that is, they were generated randomly from the viable ensemble; see File S1) with identical phenotype. Figure 1A shows the resulting data for circuits comprising 12 genes. There are significant differences between mutational and recombinational robustness even for events that affect only $m = 1$ regulatory interactions. Specifically, $>90\%$ of recombinant

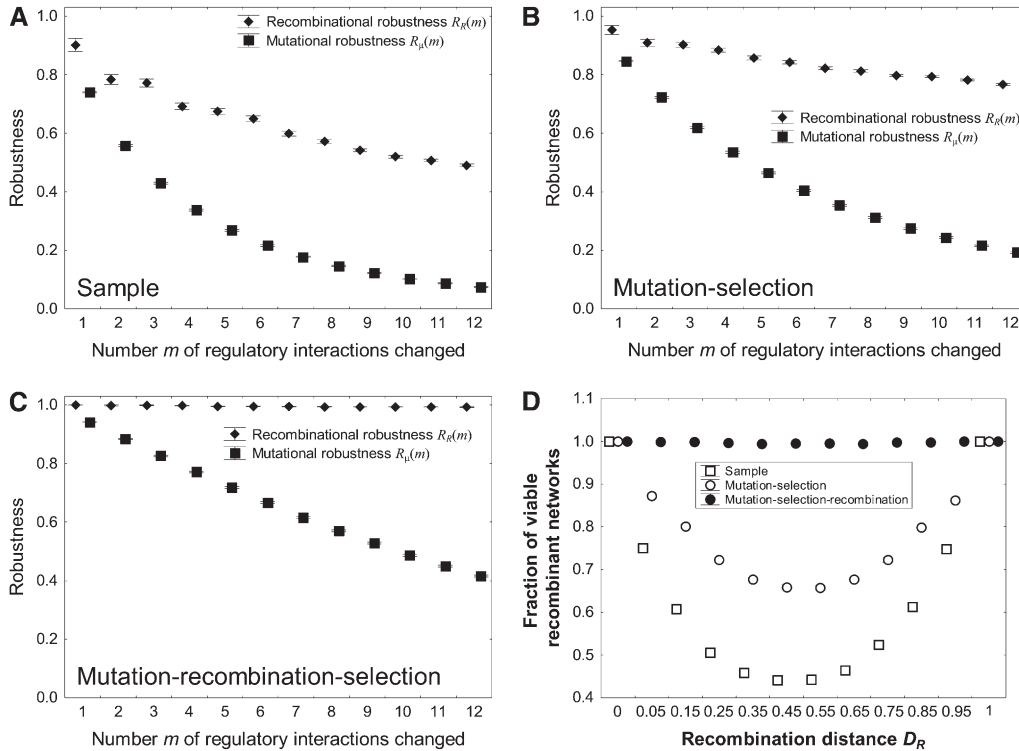


FIGURE 1.—Mutational and recombinational robustness. (A) Mutational robustness $R_\mu(m)$ and recombinational robustness $R_R(m)$ as a function of the number m of individual regulatory interactions that changed in a circuit as a result of mutation or recombination. The data shown were produced using 10^6 circuits randomly sampled from the viable ensemble (see File S1). (B) The same as in A, but for a population of circuits in mutation–selection balance, where selection confines the population to the set of circuits with the same phenotype. (C) The same as in B, but for a population of circuits in mutation–recombination–selection balance. (D) The fraction of *viable* recombinant offspring circuits (vertical axis) as a function of the normalized recombination distance D_R between parent and off-

spring. Data are shown for circuits in the viable ensemble (“Sample”), for circuits from a population in mutation–selection balance, and for circuits from a population in mutation–selection–recombination balance. Note the very high fraction of viable recombinants for the population in mutation–selection–recombination balance. All panels are based on circuits with $N = 12$ genes, number of regulatory interactions per circuit in the interval $(N, 3N)$, and orthogonal vectors $\mathbf{S}(0)$ and $\mathbf{S}(\infty)$. Note that all relevant circuit properties depend only on the angle between these vectors (CILIBERTI *et al.* 2007). B–D are based on populations of 1000 circuits and $\mu = 1$ mutation per circuit and generation. This corresponds to the large population size regime, approaching the infinite population limit. Lengths of error bars show one standard deviation.

offspring that differ from their most closely related parent by only one regulatory interaction are viable, whereas only 75% of circuits with one regulatory interaction changed due to mutations are viable. These differences become more extreme as the number m of changes increases. For example, when a recombination event changes $m = 12$ regulatory interactions, 50% of all offspring circuits are viable, whereas $<8\%$ of circuits with 12 random mutations remain viable. In sum, the data in Figure 1A show that if one or more regulatory interactions w_{ij} were already part of a viable circuit, then replacement of these interactions w_{ij} with those of another viable circuit via recombination greatly increases the likelihood that the recombinant circuit is viable compared to the case where the w_{ij} are changed by random mutations.

The circuits we studied thus far are randomly sampled from all circuits with the same phenotype and were not subject to any evolutionary process. However, we know that evolution can dramatically increase mutational robustness in such circuits (WAGNER 1996; SIEGAL and BERGMAN 2002; AZEVEDO *et al.* 2006). We thus next examined the difference between $R_R(m)$ and $R_\mu(m)$ for populations of circuits that are subject to random

mutations of individual regulatory interactions, as well as to selection to preserve phenotype. Figure 1B shows that in these populations $R_\mu(m)$ increased significantly compared to Figure 1A. This is consistent with previous observations (WAGNER 1996; AZEVEDO *et al.* 2006). In addition, recombinational robustness also increased substantially. For example, whereas in a random sample of circuits with the same phenotype 50% of recombination events changing $m = 12$ mutations did not affect the phenotype (Figure 1A), in a population in mutation–selection balance 75% of recombination events led to no change. Continual mutation pressure alone has thus decreased the death rate due to recombination by a factor 2.

Finally, we carried out an analogous comparison for circuits in recombination–mutation–selection balance (Figure 1C). The results show that in such populations recombinational robustness increases dramatically: $>99\%$ of “offspring” circuits that differ from their parents in up to $m = 12$ regulatory interactions are viable. Mutational robustness has also increased further from the mutation–selection balance, but not quite as dramatically. For example, only 40% of circuits subject to 12 mutations are viable.

Recombination leads to populations with high genetic diversity: We next asked whether the extreme recombinational robustness we observe is caused by a population that is very impoverished in genetic variation, such that only a small number of genotypes continually (re)create each other through recombination.

Figure S2, a, shows that the number of different genotypes in a population in mutation–recombination–selection balance is greater than that in a population in mutation–selection balance, even though the average genotypic distance (defined via a Hamming distance of the matrices w , as detailed in METHODS) between individuals is smaller in the recombining population (Figure S2, b). In addition, Figure S2, c, shows the values of a genotypic diversity index I that takes into account both the number and the frequency of different genotypes, for the cases of recombining and nonrecombining populations. This index is defined as follows. Let a population have P individuals and k different genotypes; if p_i individuals have genotype i ($P = p_1 + \dots + p_k$), then I is given by

$$I = \frac{(\sum_i p_i)^2}{\sum_i p_i^2}.$$

I increases from a value of one if all genotypes in the population are identical ($p_i = P$ for some i) to a value of P if all individuals have different genotypes. We find that I is consistently higher in recombining populations (Figure S2, c). Taken together, these observations show that recombination in a population leads to a greater number of genotypes, while simultaneously increasing the similarity between these genotypes.

Recombination with mutation leads to high robustness and can produce less genetic load than mutation alone: Simple intuition suggests that recombination typically increases the genetic load of a population under stabilizing selection. That is, it should cause a reduction in mean fitness, because it breaks up favorable allele combinations. However, this is not what happens in some quantitative genetics models (BURGER 2000); furthermore, previous work (AZEVEDO *et al.* 2006; HUERTA-SANCHEZ and DURRETT 2007; MACCARTHY and BERGMAN 2007) has shown that for the complex genotype–phenotype map embodied by the circuits we study, recombination enhances robustness and modifies epistasis. Figure 2A shows the genetic load (related to the survival rate of offspring, see METHODS) for two kinds of populations. One of the populations is subject to mutation and selection, and its load is caused by mutations only. We denote this load as L_μ . The other population is additionally subject to recombination, and its load, $L_{\mu,r}$, is caused by both recombination and mutation. Both populations have achieved a balance of the evolutionary forces affecting them. Figure 2A shows the genetic load as a function of population size. For small population sizes, the genetic loads of the two populations are not distin-

guishable. However, as population sizes increase, the recombining population attains a significantly lower load.

To understand the reasons for this phenomenon, several observations are germane. The first is that the genetic loads of both populations depend linearly on the mutation rate μ (Figure 2B) and with a slope that is similar for the two kinds of populations. What this suggests is that mutation and not recombination is the prime cause of the load in both populations. To help understand this observation, it is useful to recall the analysis of Figure 1, which showed that the recombinational robustness in populations subject to recombination becomes extremely high. For example, in populations with recombination, the probability that a recombination event changing one or two regulatory interactions leaves the circuit's phenotype intact is $R_R(1) = 1.00$ and $R_R(2) = 0.998$ (based on $>10^5$ recombination events), whereas the probability that one or two mutational changes leave it intact is $R_\mu(1) = 0.94$ and $R_\mu(2) = 0.88$. This means that in a recombining population of the circuits that we study, the deleterious effects of recombination are negligible compared to those of mutations and so $L_{\mu,r} \approx L_\mu = \mu(1 - \langle R_\mu \rangle)$, where $\langle R_\mu \rangle$ is the average mutational robustness in the population.

The second germane observation regards the relationship between the product of population size P and mutation rate μ on one hand and genetic load on the other hand. The product $P\mu$ is a key parameter of population genetics. It determines the amount of polymorphism in populations. Populations with $P\mu \ll 1$ (respectively $P\mu \gg 1$) are monomorphic (polymorphic) most of the time. Note that recombination will not affect a population's composition if the population is monomorphic, because all possible mating pairs of individuals are identical. It has been shown earlier for RNA molecules and for regulatory circuits (VAN NIMWEGEN *et al.* 1999; CILIBERTI *et al.* 2007) that populations subject to mutation and stabilizing selection on a given phenotype will evolve increased mutational robustness, but only if $P\mu \gg 1$. Briefly, the reason is that for robustness to increase, a population needs to show variation for robustness and thus be polymorphic. Figure 2C shows the relationship between $P\mu$ and the relative advantage of recombination experienced by a population. This advantage is expressed as the ratio of the genetic loads $L_{\mu,r}/L_\mu$. If this ratio is smaller than one, then the genetic load of the recombining population is smaller, and recombination is advantageous. It is clear from Figure 2C that the greater $P\mu$ is, the greater also the advantage of recombination.

Taken together, these observations provide the following explanation for the effect of recombination on the genetic load. In sufficiently large populations ($P\mu \gg 1$), recombination can increase both mutational robustness (Figure 1 and AZEVEDO *et al.* 2006) and robustness

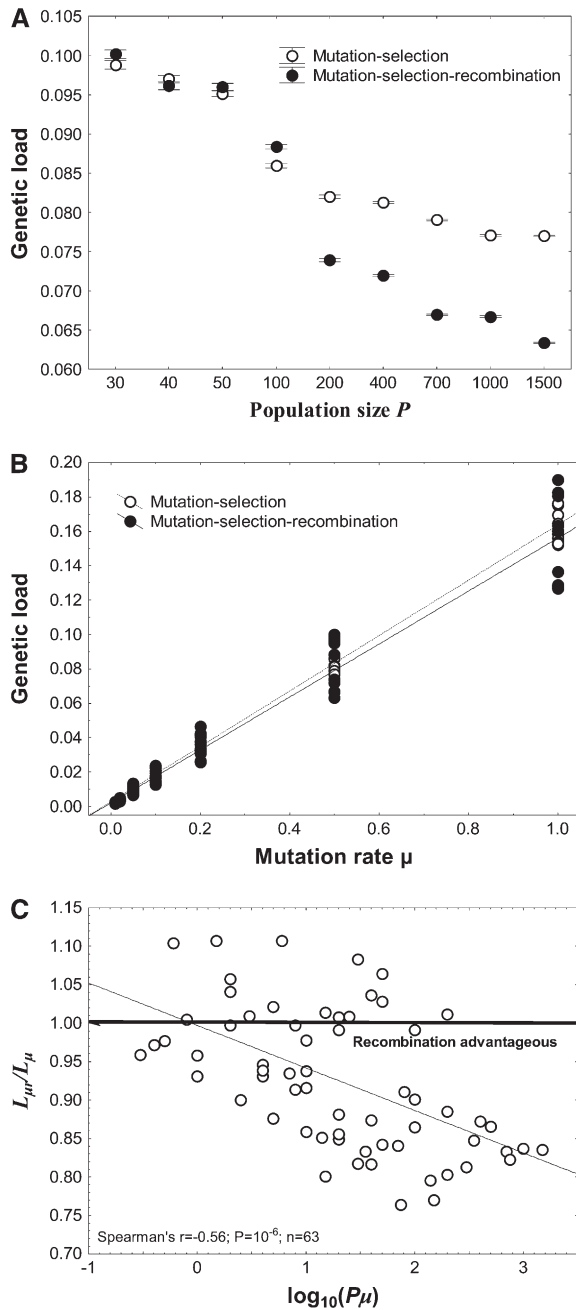


FIGURE 2.—Recombination can lead to reduced genetic load. (A) Population size (horizontal axis) *vs.* genetic load (vertical axis) for populations in mutation–selection(–recombination) balance. The mutation rate per circuit and generation is $\mu = 0.5$. Lengths of error bars correspond to one standard deviation. (B) Mutation rate (horizontal axis) *vs.* genetic load (vertical axis) for populations in mutation–selection (–recombination) balance. At each mutation rate and for each of the two kinds of population, the load is shown for populations of size $P = 30, 40, 50, 100, 200, 400, 700, 1000,$ and 1500 . Error bars are omitted for clarity, and many points are invisible, because they are congruent or nearly so. (C) Logarithmically transformed product of population size and circuitwide mutation rate (horizontal axis) is plotted against the ratio $L_{\mu r}/L_r$ (vertical axis) of the genetic loads of a population in mutation–selection (–recombination) balance ($L_{\mu r}$) and the load of a population in mutation–selection balance (L_r). If this ratio is smaller than one (thick horizontal line), then the load of the recombining

to recombination (Figure 1). This increase has two effects. First, recombination as a source of deleterious change becomes small compared to mutations. Second, in recombining populations, fewer mutations have deleterious effects (because of the increased mutational robustness), and thus, overall, the genetic load of such populations is lower. Put differently, the reduced load is a by-product of the increased robustness caused by recombination, and this increased robustness can manifest itself only in sufficiently polymorphic populations, because recombination is otherwise ineffective.

These observations describe the causes for an advantage of recombination qualitatively. However, circuit sizes, numbers of regulatory interactions, population sizes, and mutation rate may interact in their effects on the genetic load. For example, we have observed that when varying P and μ while holding $P\mu$ constant, the genetic load essentially does not change for the populations in mutation–selection balance, while in the case of recombining populations the load continues to decrease as P increases (results not shown). That $P\mu$ is not the sole determinant of the equilibrium genetic load is also clear from Figure 2C, because there is much unexplained variance in the statistical association shown. We leave a more quantitative analysis to future contributions.

Cooperative effects and *cis*-regulatory complexes: A complementary way of analyzing the effects of recombination derives from the following observations. If the parent circuits differ in H regulatory interactions, then one of the recombinant offspring will differ from one parent by m regulatory interactions, whereas the other offspring will differ by $(H - m)$ regulatory interactions. We can then express the distance of the offspring from either parent as a fraction of H , *i.e.*, as a recombination distance $D_R = m/H$. This recombination distance varies between 0 and 1. A value of D_R close to zero means that the offspring is close to the reference parent, whereas a value of D_R close to one means that the offspring is very distant from the reference parent, but very close to the other parent. Intermediate values of D_R mean that the offspring is roughly equally distant from either parent. Figure 1D shows that offspring with values of D_R that are very close to zero or one are very likely to be viable, whereas offspring with intermediate values of D_R have a lower likelihood to be viable. In other words, the likelihood that a recombination event is deleterious shows a U-shaped distribution, whose trough occurs at

population is lower, and recombination provides an advantage. Ranges of mutation rates and population sizes used are identical to those in B. The diagonal line is a linear regression line. All panels are based on circuits with $N = 12$ genes, number of regulatory interactions per circuit in the interval $(N, 3N)$, and orthogonal vectors $\mathbf{S}(0)$ and $\mathbf{S}(\infty)$. B and C are based on populations of 1000 circuits and $\mu = 1$ mutation per circuit and generation.

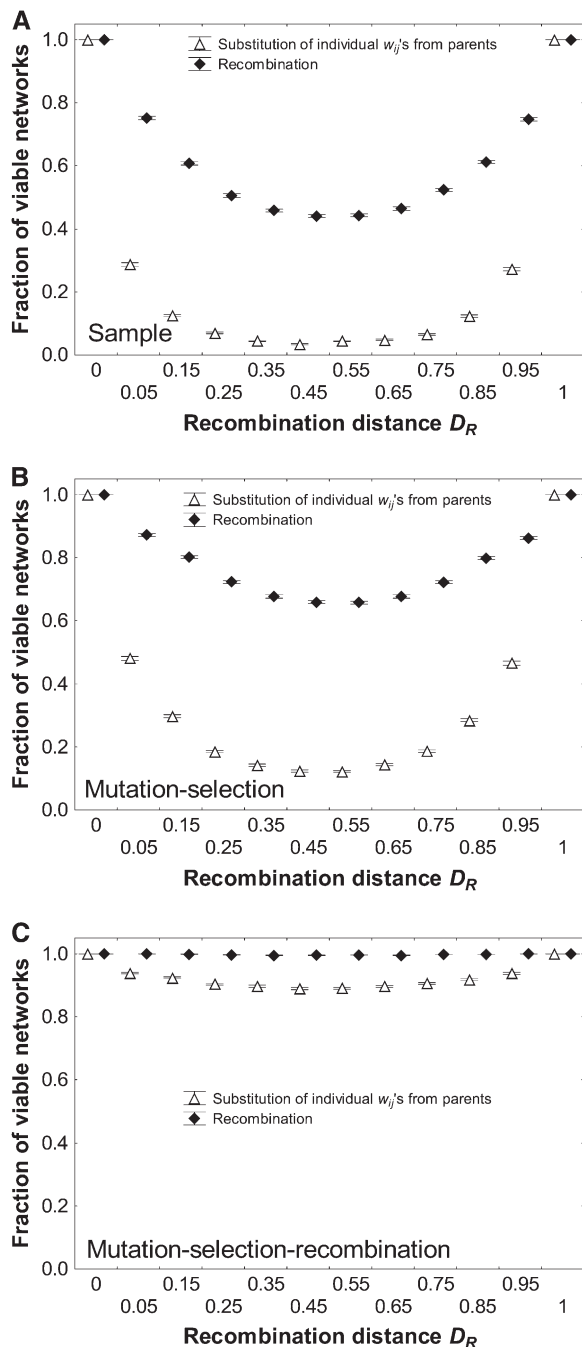


FIGURE 3.—The *cis*-regulatory interactions (w_{ij}) in the regulatory region of any one gene i affect the gene expression phenotype synergistically. We show here that recombination changing m regulatory interactions affects viability to a lesser extent than m independent substitutions of regulatory interactions between one and the other parent. Data shown are for (A) a random sample of circuits with the same phenotype (viable ensemble), (B) a population of circuits in mutation–selection balance, and (C) a population of circuits in mutation–recombination–selection balance. If the genotypic distance between parents is H and if m is the genotypic distance of the offspring to a reference parent (one of the two parents), then the normalized distance is $D_R = m/H$, which is shown on the horizontal axes of all panels. The diamonds denote the fraction of viable recombinant offspring circuits. We obtained the data for the triangles as follows. For two random parent circuits in the ensemble of interest, we first generated an off-

spring by recombination; let D_R be its normalized distance to the reference parent. We then took one of the offspring’s parents and changed (“substituted”) one at a time a randomly chosen interaction to that of the other parent; we repeated these substitutions until the circuit obtained had the normalized distance D_R . We then repeated this entire process for 10^4 mating pairs to estimate the fraction of viable offspring as a function of D_R . All panels are based on circuits with $N = 12$ genes, number of regulatory interactions per circuit in the interval $(N, 3N)$, and orthogonal vectors $\mathbf{S}(0)$ and $\mathbf{S}(\infty)$. Note that all relevant circuit properties depend only on the angle between these vectors (CILIBERTI *et al.* 2007). B and C are based on populations of 1000 circuits, and $\mu = 1$ mutation per circuit and generation. Lengths of error bars correspond to one standard deviation.

the maximum ($D_R = 0.5$) number of changes. This trough is deepest for parents taken at random from the viable ensemble (*cf.* METHODS), is flatter for populations subject to mutation and selection, and is even flatter for populations that are, in addition, subject to mutation, recombination, and selection balance (Figure 1D). These observations further underscore our earlier observation that both mutation and recombination can dramatically increase recombinational robustness. Figure 3 shows results of an additional analysis, which highlights that it is not only the change of individual w_{ij} ’s but also how they act together in regulating the expression of any one gene that is critical for preserving circuit viability. In this analysis we began with parent circuits that differ in H interactions and offspring at distance $D_R = m/H$ from one of the parents. We then changed m randomly chosen interactions in one of the parents to those of the other parent (regardless of whether they occurred in the regulatory region of the same gene, *i.e.*, the same row of w) and estimated the likelihood that the resulting changed circuit was viable. We found that this likelihood is generally lower than when the same number m of interactions is changed through recombination. This means that the joint change of regulatory interactions that *cooperate in the regulation of one gene*, as occurs with recombination, impairs circuits less. Breaking up these combinations of interactions is more disruptive. Also, in File S1, we show that when the offspring are constructed as just described and are exposed to additional mutations, then the likelihood to remain viable decreases in ways similar to those caused by mutations in the parents. Furthermore, robustness to these additional mutations also increases in populations subject to selection, mutation, and/or recombination (Figure S3).

To address the issue of how synergistic (cooperative) action emerges when populations are subject to recombination, we first investigated statistical properties of the strengths of regulatory interactions in the interaction matrix w . Figure 4A shows the distribution of the nonzero weights w_{ij} . In a random network (regardless of viability) these weights follow a Gaussian distribution in our model. Remarkably, this distribution is

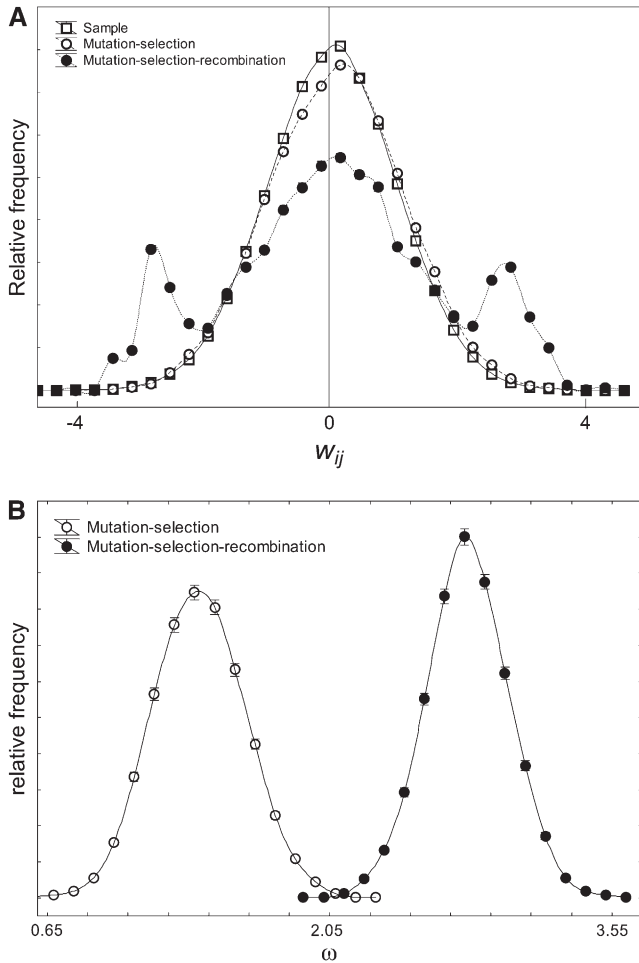


FIGURE 4.—Regulatory regions operate differently in populations subject to recombination. (A) Networks in nonrecombining populations (open circles) have essentially the same distribution of nonzero weights w_{ij} as networks sampled from the viable ensemble of networks (open squares) or as networks regardless of their viability (not shown). In contrast, populations subject to recombination (solid circles) give rise to a distribution that is broader and no longer bell shaped. (B) Populations with and without recombination can be distinguished by considering the distribution of the statistic ω , averaged over the different genes (regulatory regions) of a network (see text for details). Data in A are based on 10^5 circuits with $N = 12$ genes, and data in B are based on 10^4 circuits with $N = 20$ genes. The number of regulatory interactions per circuit lies in the interval $(N, 3N)$, and we use orthogonal vectors $\mathbf{S}(0)$ and $\mathbf{S}(\infty)$. Whiskers indicate one standard error and are too small to be visible in many cases. Curves were fitted to data using quadratic spline functions.

hardly changed at all for networks drawn at random from the ensemble of viable networks and for populations of viable networks subject to mutation and selection (Figure 4A). In striking contrast, populations undergoing recombination exhibit a distribution for the w_{ij} that is very non-Gaussian (Figure 4A). This distribution is not even bell shaped and has pronounced “shoulders” corresponding to a greater use of strong regulatory interactions (large values of w_{ij}). The larger

values must persist in the population only because they are advantageous under recombination and selection and point to a different usage of regulatory interactions in the two kinds of populations.

To examine this observation further, it is useful to remark that by shuffling the regulatory regions of different genes (as represented by different rows of the matrix w), recombination destroys potential statistical associations between *cis*-regulatory elements (as represented by w_{ij} 's) in the regulatory regions of *different* genes. However, this effect could be compensated by greater cooperativity between the *cis*-regulatory element w_{ij} 's *within* any one regulatory region. To assess whether this is the case we introduce a statistic ω_i to distinguish properties of the regulatory interaction w_{ij} 's influencing the expression of a gene i between populations undergoing recombination or just mutation. We define ω_i as

$$\omega_i = \sum_{j/S_j(0)=S_j(\infty)} |w_{ij}|.$$

For each network, we compute ω_i for each gene i and define ω as the average of ω_i over all genes. How can one interpret ω ? Note that ω_i sums the strengths $|w_{ij}|$ of the interactions in a given row i for all “incoming” regulatory interactions from genes j for which the expression level S_j is the same in the initial and target states. Such a gene's expression level $S_j(t)$ is less likely to change as the system approaches the target gene expression state (because it is already at the “correct” expression level) than the expression levels of genes that do not have the same expression in the initial and the target state. The expression pattern of these genes j is thus less variable or less “noisy,” which is why they are used in defining ω_i . If these genes are allowed to have a disproportionately large influence on the regulation of other genes, one would expect more robust gene expression dynamics and thus greater robustness for the target expression state. In Figure 4B we show the distribution of ω . Specifically, Figure 4B shows the distribution of ω for the genotypes in populations under mutation–selection balance and under mutation–recombination–selection balance. Clearly, populations undergoing recombination have much larger values of ω . Our previous observations showed that recombination leads to higher robustness. The right-shifted distribution of ω (Figure 4B) suggests that the mechanistic cause of this higher robustness is precisely the principle just outlined.

A second pertinent observation is that there are far more potential epistatic interactions between w_{ij} in different rows than within rows; however, among all these interactions, the intrarow interactions are more likely to be relevant for a robust gene expression pattern in recombining populations, especially if they are based on the more steadily expressed genes just discussed. The reason is that recombination constantly separates

regulatory regions of different genes. With this observation in mind, one would expect a difference in the distribution of ω_i that appears in populations subject to recombination, compared to nonrecombining populations, because recombining populations can take advantage of the most relevant epistatic interactions (those arising within a regulatory region) to ensure robustness of a gene expression pattern. This difference in the distribution of ω_i is exactly what we observe (Figure 4B).

In sum, a gene's regulatory region can be thought of as forming a *cis*-regulatory complex, whose allelic combinations are not disrupted by recombination in our model.

This perspective is in line with our earlier observations (Figure 3) that exchanging a regulatory complex (row i) in one individual with its counterpart in another individual is rarely deleterious, while exchanging sub-components of such a complex (individual w_{ij} 's) is generally far more deleterious.

DISCUSSION

We have made several key observations. First, if random mutations change a number m of interactions in a regulatory circuit, then it is much more likely that the change is deleterious than if the same number m of interactions is changed through recombination. This holds regardless of whether circuits are sampled randomly from an ensemble of circuits with the same phenotype or from populations subject to selection, mutation, or recombination. The pertinent observations from Figure 1 are strikingly similar to those made recently by Drummond and collaborators (DRUMMOND *et al.* 2005) for a completely different kind of model system, lattice proteins. The similarity suggests that recombination may affect dissimilar systems on different levels of organization similarly. While work on regulatory circuits is largely theoretical, for proteins there is experimental evidence that recombination does actually have weak effects on protein functions. For example, the products of recombination between two distantly related β -lactamases retained function with significantly higher probability than β -lactamase variants that involved the same number of amino acid changes as introduced by recombination, but where these changes were introduced through random point mutation (DRUMMOND *et al.* 2005). More anecdotally, a DNA shuffling experiment involving divergent interleukin-12 genes from seven mammalian species showed that between 40 and 90% of chimeric molecules retained interleukin-12 activity (LEONG *et al.* 2003). A similar study creating four random recombinant α -interferons from 20 different human α -interferon genes that differ, on average, at 17 amino acid positions, found that all four recombinant proteins generated were as

active as their "parents." The authors estimate that mutating the same number of positions as are changed in a recombination event among these proteins would abolish protein function (CHANG *et al.* 1999). Such weak deleterious effects may contribute to the promise recombination shows in engineering new proteins (STEMMER 1994; CRAMERI *et al.* 1997, 1998; ZHANG *et al.* 1997; CHANG *et al.* 1999; KOLKMAN and STEMMER 2001; LEONG *et al.* 2003; RAILLARD *et al.* 2001a,b). Because recombination can reorganize genetic systems on a much larger scale than point mutations, these weak effects on different levels of organization are intriguing. The question of whether they are general warrants further exploration.

A second central observation we made is that recombination may decrease the genetic load of a population under stabilizing selection. The explanation involves several factors. First, the joint action of selection and recombination dramatically increases robustness to recombination itself. That is, in a population in recombination–mutation–selection balance, recombinational robustness can be very high, in which case most of the genetic load of the population is caused by mutations. Second, recombination increases a population's *mutational* robustness to a much greater degree than mutations alone do (Figure 1). Combined, these two factors can make the load reach lower values in recombining populations than in nonrecombining populations, provided that the product of population size and mutation rate is not too small.

Third, we found that recombination allowed the emergence of cooperative effects among *cis*-regulatory sites (as represented by individual entries of the matrix w_{ij}); the genotypes in populations undergoing recombination build up *cis*-regulatory complexes within individual genes' regulatory regions that are not broken up by recombination; without recombination there is no emergence of such complexes.

Last but not least, we also found that recombination affects the number of different genotypes sustained in a population: recombination led to populations with significantly larger genotypic diversity, as measured either by the number of different genotypes or by an index of genotypic diversity.

Past work on the architecture of "genotype networks" (often called neutral networks), defined as the sets of genotypes that have the same phenotype (SCHUSTER *et al.* 1994), can help us understand some of these observations. Such genotype networks occur in multiple kinds of biological systems (WAGNER 2005). For the circuits we study, a genotype network can be represented as a graph whose nodes—regulatory circuits—correspond to genotypes, and where two genotypes are neighbors if they have the same phenotype and if they differ by one mutational change (in our case, their genotypes are at distance 1). The *degree* of a node in this genotype network is the number of neighboring nodes

it has; it is also a measure of the mutational robustness of the corresponding circuits. For our regulatory circuits, these genotype networks are vast, connected, and very heterogeneous in degree (CILIBERTI *et al.* 2007). Earlier work has shown that in populations evolving on such a network, mutation and selection alone will drive a population toward nodes of high mutational robustness (VAN NIMWEGEN *et al.* 1999). It is thus the architecture of genotype networks that allows populations in mutation–recombination–selection balance to localize in network regions with the highest robustness and thus have a very small genetic load.

Our system is very different from simple two- or three-locus models often considered when investigating the effects of recombination; the intuition derived from such simplified models is that recombination typically has deleterious effects because it breaks up coadapted gene complexes. This may be true for regulatory regions of different genes, but we also found that recombination allowed for greater cooperativity in the *same* regulatory region. Furthermore, simple models of few loci do not capture the fact that mutational robustness becomes higher as a result of mutation and even more so through recombination. To be sure, one could conceive simple two-locus models that incorporate a changeable mutational robustness. However, in more complex genotype–phenotype maps, this feature is a natural consequence of the organization of a genotype network.

For the gene regulatory circuits we studied, Azevedo and collaborators have studied the effects of recombination in the absence of mutations, and they found that populations subject to recombination and selection saw both their mutational and their recombinational robustness increase significantly (AZEVEDO *et al.* 2006). These same authors also showed that recombination can lead to synergistic epistasis (AZEVEDO *et al.* 2006), a phenomenon where multiple mutations have stronger effects on fitness than expected from the effects of single mutations. Synergistic epistasis seems to be necessary *if* one accepts that sexual reproduction is favored because recombination promotes the elimination of deleterious mutations from a population (KONDRASHOV 1998). This means that recombination may create the conditions for its own maintenance in a population (but see LECLERC 2008). While the evolution of negative epistasis is an intriguing observation, it does not address the question how recombination can become established in a population in the first place. The same holds for our observations. We show that recombination can provide a population-level advantage, but we do not claim that our results provide an explanation for the *origin* of recombination. Similarly, recombination may or may not provide an advantage under directional selection. Various work in population genetic theory and experimental observations demonstrate such an advantage (FISHER 1930; MULLER 1932; FELSENSTEIN

1974; DE VISSER *et al.* 1999; WILKE 2004; KEIGHTLEY and OTTO 2006; COOPER 2007), although this advantage may not be universal (DE VISSER *et al.* 2008). In a model of gene regulatory circuits closely related to the one considered here, recombination modifiers that increase the recombination rate do not readily become established in populations (MACCARTHY and BERGMAN 2007).

The effects of recombination on robustness and genetic load may be more important than those of mutations for a genetic circuit and thus deserve increased attention. For example, if the genes of a gene circuit are dispersed among chromosomes, as is often the case, their alleles are reshuffled in every single generation of sexual reproduction, yielding a 1000-fold greater rate of recombination than mutation, given typical recombination and mutation rates, as well as typical gene sizes of 100 kbp (DRAKE *et al.* 1998; MYERS *et al.* 2006). These observations notwithstanding, we note that the variation recombination acts upon is ultimately generated by mutation. Any effects of recombination on a population can manifest themselves only in polymorphic populations, because in monomorphic populations all recombinant offspring are identical to their parents. In the populations we consider, many individuals have an optimal phenotype, and the populations are in mutation–selection balance on a genotype network. In such populations only deleterious and neutral mutations can occur, and the neutral theory of molecular evolution (KIMURA 1983) shows that such populations will be polymorphic most of the time if the product $P\mu$ of population size P and mutation rate μ is much greater than one. It is thus not surprising that we see an effect of recombination on the genetic load only if $P\mu$ is much greater than one. The threshold at which recombination begins to have a beneficial effect may depend on several model details, including how mutation rates are defined. For instance, our mutation rate is a circuitwide mutation rate and not a rate of mutation per gene (regulatory region) or per single regulatory interaction. For genic mutation rates, recombination should begin to affect genetic loads at much smaller values of $P\mu$.

Recombination is commonly thought to have two major disadvantages, namely the twofold cost of sex and the increase in genetic load it causes (MULLER 1950; MAYNARD SMITH 1978). If our observations hold generally, then one of these disadvantages may disappear in complex genetic systems, while other potential advantages such as increased genetic diversity and modular regulatory control may emerge. The effects of recombination on systems with complex genotype–phenotype maps can be rather counterintuitive. However, because all real biological complex systems have complex genotype–phenotype maps, it is well worth exploring them further, both with abstract models and in suitable experimental systems.

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