On the Selection and Evolution of Regulatory DNA Motifs

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Received: 10 September 2001 / Accepted: 2 April 2002

Abstract. The mutation and selection of regulatory DNA sequences are presented as an ideal model system of molecular evolution where genotype, phenotype, and fitness can be explicitly and independently characterized. In this theoretical study, we construct an explicit model for the evolution of regulatory sequences, making use of the known biophysics of the binding of regulatory proteins to DNA sequences, under the assumption that fitness of a sequence depends only on its binding affinity to the regulatory protein. The model is confined to the mean field (i.e., infinite population size) limit. Using realistic values for all parameters, we determine the minimum fitness advantage needed to maintain a binding sequence, demonstrating explicitly the “error threshold” below which a binding sequence cannot survive the accumulated effect of mutation over long time. The commonly observed “fuzziness” in binding motifs arises naturally as a consequence of the balance between selection and mutation in our model. In addition, we devise a simple model for the evolution of multiple binding sequences in a given regulatory region. We find the number of evolutionarily stable binding sequences to increase in a step-like fashion with increasing fitness advantage, if multiple regulatory proteins can synergistically enhance gene transcription. We discuss possible experimental approaches to resolve open questions raised by our study.

Key words: Transcription regulation — Mutation-selection model — Error threshold — Regulatory sequences

Introduction

The regulation of gene expression involves many different proteins known as transcription factors which bind passively to specific sites on the genomic DNA (see, e.g., Gerhart and Kirschner 1997). In bacteria, each such site (called an “operator”) typically consists of a contiguous sequence of 20–30 nucleotides which binds a specific transcription factor with a much higher affinity than would a random DNA sequence of comparable length (Stormo and Fields 1998; von Hippel 1979). Known examples of different operators for the same factor usually differ from the maximum affinity binding sequence in a number of positions, typically in as many as 20 to 30% of the significant positions that contribute most to the specificity of the interaction. The ensemble of viable binding sequences is collectively referred to as the binding “motif” for a factor; its “fuzziness” creates a difficult computational problem for the prediction of binding sites via informatic methods (see, e.g., Lawrence et al. 1993; Stormo and Hartzell 1989, and references therein). In many known cases, a single regulatory region contains multiple operators for the same factor, each of which deviates from the maximum affinity binding sequence.

Why are the motifs fuzzy? One possible scenario is that the binding affinity of each operator is tuned evolutionarily to maximize the function of each
regulated gene or operon. An alternative scenario is that the function is insensitive to the detail of the binding affinity as long as it is above some threshold. In the former case, fuzziness in the binding arises due to the particular distribution of functional requirement. In the latter case, binding sequences in different regulatory regions are deemed “equal,” and fuzziness results from maximizing the sequence “entropy.” While anecdotal examples of both cases are known, understanding whether either case dominates in biology is not only of interest for its own sake, but also very important for the choice of proper informatics tools for motif finding. In this paper, we describe a detailed theoretical study of the latter case from an evolutionary perspective, recognizing that as with any other portion of the genome, the binding sequences are subject to the opposing forces of mutation and selection over evolutionary time scales. In particular, we address the quantitative question of how large a selective advantage the presence of a binding motif needs to provide, to guarantee its survival against mutations, and how large an advantage before multiple motifs are justified. To make the study concrete and explicit, we confine the discussion mostly to gene regulation in bacteria or phages and focus on the binding of one specific transcription factor to its operator(s) in the regulatory region of one specific gene or operon. We do not treat the interactions among different factors, since in bacteria such as E. coli, the majority of genes are regulated by a single factor (Gralla and Collado-Vides 1996).

Another motivation for our study is that the evolution of transcription factor binding motifs seems to be a well-suited starting point for an attempt to establish a link between the microscopic molecular mechanisms in the cell and the “macroscopic” principles of evolution: In general, the most important ingredient in an evolutionary study is to relate the genotype on which mutation acts to the fitness of the organism on which selection acts through some quantifiable phenotype. This relation is particularly simple for the operator binding problem at hand, where a natural choice of the phenotype is the binding probability of the transcription factor to the operator. Regardless of whether the factor acts as an activator by attracting a polymerase to transcribe the gene, or as a repressor to block transcription, it can function only when it is bound to its operator. The fraction of time an operator is occupied in equilibrium is given by the binding probability. To regulate the transcription of the gene, e.g., in reaction to a change in the environmental conditions or to trigger a different phase of the cell cycle, the cell changes the factor–operator binding probability by varying the concentration of the (activated) factor inside the cell. The concentration may vary from practically zero in the “OFF state” to typically several hundred copies per cell in the “ON state.” We make the reasonable (but critical) assumption that the fitness gain an operator contributes to the organism depends solely on the binding probability \( P \) in the ON state, with the value of \( P \) itself determined by the actual sequence of the operator through the binding energetics.

For a few exemplary transcription factors, the variation in binding affinity upon mutation of the binding sequence has been studied in great detail experimentally (Fields et al. 1997; Oda et al. 1998; Sarai and Takeda 1989; Takeda et al. 1989). In particular, Fields, Stormo, and co-workers have shown for the case of the mnt repressor that its binding (free) energy is approximately a sum over independent contributions from each of the nucleotide positions in the binding sequence (Fields et al. 1997). Typically, only 10–15 positions in a binding site have a strong preference for specific nucleotides, while the other positions do not contribute significantly to the binding energy. Known binding sequences display a fuzziness of up to three or four mismatches in these significant positions. A useful simplified “two-state model” for transcription factor binding is obtained by taking only the significant bases into account and assigning to each of them the same binding energy \( \epsilon \), i.e., a match (to the optimal binding sequence), is favored by an energy difference \( \epsilon \) over a mismatch. This model, introduced long ago by von Hippel and Berg (1986), takes into account the effect of sequence-specific binding by a single parameter \( \epsilon \), without reference to detailed binding energies which have not yet been measured for most transcription factors.

Based on the two-state model and our assumption on the contribution of the binding of the factor toward fitness, we construct an explicit theory for the evolution of the binding sequences. Within the mean-field approach originally proposed by Eigen (Eigen 1971; Eigen et al. 1989) in the context of quasispecies evolution, we characterize the balance between the opposing forces of selection and mutation quantitatively. We determine the critical selection pressure needed to keep a motif from mutating away and show how the fuzziness in the motifs arises naturally above the selection threshold. We further apply the theory to investigate the frequently observed occurrence of multiple motifs in a given regulatory region and elaborate on various plausible causes. Toward the end, we provide extended discussions on experimental approaches to pursue the open questions suggested by this study.

**Model and Equations**

We focus on the operator sequence located in the regulatory region of a gene of interest. By assumption, this gene is regulated by a single transcription factor. Let \( \mathcal{S} = \{S_1, S_2, \ldots, S_L\} \) denote the \( L \) signifi-
Table 1. Fitness of the organism in two cellular states, with and without the binding of the transcription factor

|                      | State A | State B |
|----------------------|---------|---------|
| Factor unbound       | \( \phi_A \) | \( \phi_B \) |
| Factor bound         | \( \phi_A + \delta \phi_A \) | \( \phi_B - \delta \phi_B \) |

cant nucleotides of the operator which specify transcription factor binding. We keep the alphabet size, \( \mathcal{A} \), as a variable in our equations, since, as we will see below, this facilitates the intuitive understanding of certain dependencies; however, \( \mathcal{A} = 4 \) and \( S_j \in \{A, C, G, T\} \) is the only case of interest here. To describe the evolution of \( \hat{S} \) in a population of bacteria or phages, we need to specify the action of selection and mutation.

**Selection Mechanism**

It should be clear that gene regulation is needed only in the presence of a changing cellular state, triggered either internally, e.g., cell cycle, or externally through a change in the environment. Hence to study the fitness of a regulatory mechanism, we must invoke at least two states. Selection arises when the growth rate of an organism depends on the probability \( P_\hat{S} \) that the factor binds to the sequence \( \hat{S} \) in the state that prefers factor binding (the “ON state”). For the sake of concreteness, let us consider an environment that oscillates between two states. We assume that in State A (the ON state), the environment induces a certain concentration of activated factors, say, on average, \( N_{TF} \) factors per cell (either these may be produced upon entering State A or preexisting factors may be activated for binding by inducers that cause an allosteric transition). Let the growth rate or “fitness” of the organism in this state be \( \phi_A \) if the factor is never bound (binding motif not present), and \( \phi_A + \delta \phi_A \) if the factor is always bound (see Table 1). When the environment is in State B (the OFF state), let the fitness be \( \phi_B \) if the factor is never bound and \( \phi_B - \delta \phi_B \) if the factor is always bound. In the following we assume that the concentration of activated factors in State B is practically zero, so that the operator is never occupied (hence the parameter \( \delta \phi_B \) does not enter our model).

An example for the general situation described above could be the binding of the lac repressor to its operator in the lac operon of E. coli. In this case, the ON state would be the glucose-rich environment, and the OFF state would be the glucose-poor and lactose-rich environment. \( \phi_{A,B} \) would be the growth rate of E. coli in the two environments in the absence of the lac repressor. \( \delta \phi_A \) would be the increment in fitness when the unnecessary lac operon is turned off and \( \phi_B - \delta \phi_B = 0 \) is the deleterious situation when lactose is present as the main source of sugar, but the lac operon is not operative due to the undesirable binding of the repressor.

In this study, we discuss mainly the time-averaged effect over evolutionary time scales, which are much larger than the time scales of cellular or environmental fluctuations. We choose \( \tau/\ln 2 \) as our unit of time, with \( \tau \) denoting the average generation time in the absence of the factor, so that the time-averaged growth rate there can be set to 1. We assume that the cell can quickly adjust the cellular concentration of the factor\(^1\) so that the operator with sequence \( \hat{S} \) is occupied with probability \( P_\hat{S} \) in the ON state and never occupied in the OFF state. It is then plausible to assume that the time-averaged growth rate \( \Phi_\hat{S} \) depends linearly on \( P_\hat{S} \) (see also the discussion in the section on Selection Threshold and Fuzzy Motifs),

\[
\Phi_\hat{S} = 1 + \alpha \cdot P_\hat{S}
\]

Here \( \alpha \) is a dimensionless parameter which characterizes the selection pressure on the binding sequence \( \hat{S} \). In the limit \( \alpha \ll 1 \), there is hardly any selection pressure on the sequence at all; the opposite limit \( \alpha \to \infty \) corresponds to the case where the failure of the factor-operator binding is lethal to the organism. If the fraction of time, the bacteria population encounters environment A is \( \hat{f} \), the selection pressure becomes

\[
\alpha = \hat{f} \cdot \delta \phi_A
\]

In an experiment, \( \alpha \) can be adjusted according to Eq. (2) by changing the fraction of time \( \hat{f} \) the ON state is presented. Below, we investigate the statistics of the selected sequence \( \hat{S} \) for a wide range of \( \alpha \)’s.

**Mutation Process**

We consider only single-nucleotide substitutions and focus on mutations in the binding sequence \( \hat{S} \), assuming that the net result of mutation and selection on the rest of the genome gives the overall background fitness of 1 (with our time unit of \( \tau/\ln 2 \)). Furthermore, we neglect the difference between transversions and transitions and assume a constant rate \( v_0 \) at which a base mutates into any other base. The total mutation rate of a site of length \( L \) is then

\[^1\text{In the present article, we do not consider the “search problem” of how a transcription factor locates its operator among millions of other sites on the DNA [see Berg et al. (1981), Winter and von Hippel (1981), and Winter et al. (1981) for a thorough experimental and theoretical investigation of this problem and Gerland, Moroz, and Hwa (submitted for publication) for a bound on the protein-DNA interaction parameters that results from the requirement of reasonable search times]. Rather we treat protein–DNA binding as an equilibrium process characterized only by the binding probability. This is justified by the fact that the search time is typically of the order of 1 min, which is much smaller than the characteristic time scale of changes in gene expression.}\]
\( v = v_0 L \). For bacteria such as *E. coli*, \( v_0 \) is of the order of \( 10^{-9} \) under normal conditions and hence \( v \sim 10^{-8} \). The mutation rate is higher for RNA viruses, which rely on the less accurate reverse transcriptase for replication. For that case, \( v_0 \) is in the range \( 10^{-5} \) to \( 10^{-4} \) and hence \( v \sim 10^{-4} - 10^{-3} \).

**Binding Probability**

As mentioned above, the binding (free) energy \( E_S \) of the transcription factor to the binding sequence \( S \) is given, to a good approximation, by a sum over independent contributions from each nucleotide at the \( L \) significant positions (Fields et al. 1997; Stormo and Fields 1998), i.e.,

\[
E_S = \sum_{i=1}^{L} e_i(S_i).
\]

Each of these positions typically prefers a particular nucleotide by a binding energy of several \( k_B T \)’s (we exclude from \( S \) those positions which contribute only a fraction of \( k_B T \) toward the total binding energy). Furthermore, we adopt the “two-state model” (Berg and von Hippel 1987; von Hippel and Berg 1986), by assuming that each \( e_i(S_i) \) can take on only two possible values, 0 if \( S_i \) matches the preferred base \( S_i^* \) or if \( \varepsilon > 0 \) for a mismatch, i.e.,

\[
e_i(S_i) = \varepsilon \cdot (1 - \delta_{S_i, S_i^*}).
\]

The binding energy of a site \( S \) is then only a function of the number of mismatches, or Hamming distance \( r_S = |S - S^*| \), from the optimal binding sequence \( S^* \), i.e.,

\[
E_S = E(r_S) = r_S \varepsilon.
\]

(3)

Given its binding energy, the average occupancy of a site is determined by equilibrium thermodynamics. Since a binding site can only be occupied or unoccupied (but not multiply occupied), its binding probability \( P_S = P(r_S) \) is given by a Fermi function,

\[
P(r) = \frac{1}{1 + e^{(r-r_0)/k_B T}}
\]

(4)

which is also known as an Arrhenius function (see, e.g., Atkins 1998). Here, \( \mu = \varepsilon r_0 \) is the chemical potential for the transcription factors in the ON state [this function is plotted in Fig. 1 (right) with realistic parameter values]. Note that \( r_0 \) corresponds to the number of mismatches for which the probability of binding is 50%.

In total, we are left with three dimensionless parameters for the two-state model of protein–DNA binding: \( L \), \( \varepsilon/k_B T \) and \( r_0 \). As mentioned before, the number of significant positions in a binding site is typically in the range \( 10 \leq L \leq 15 \). By inspection of the known binding energies for exemplary transcription factors (Fields et al. 1997; Oda et al. 1998; Sarai and Takeda 1989; Takeda et al. 1989), we find the mean specificity of the significant sites to be typically \( \varepsilon = 1-3k_B T \). In (Gerland, Moroz, and Hwa submitted for publication), it is argued on rather general ground that this is actually the optimal range of \( \varepsilon \) for the transcription factors. The chemical potential \( \mu \) depends directly on the average number of factors \( N_{TF} \) in the cell; the work of Gerland, Moroz, and Hwa (submitted for publication) suggests that it can be approximated by \( \mu = \mu_0 + k_B T \ln[N_{TF}] \), where \( \mu_0 \) represents the binding free energy of a single factor to the rest of the genome. For those factors whose binding energies \( e_i(S_i) \) have been measured, we find that \( \mu_0 = -0.8k_B T \) (mnt) and \( \mu_0 = -1.9k_B T \) (\( \lambda \) repressor and cro). Hence, \( \mu = k_B T \ln[N_{TF}] \); see Fig. 1 for details. For \( \varepsilon = 2k_B T \) and \( N_{TF} = 50-5000 \), we get \( r_0 = \mu/\varepsilon = 2-4.3 \). Clearly, \( r_0 \) is the parameter that we have the least information about; but we see that it has a limited range, and in any case, most of our qualitative conclusions will be insensitive to the specific choice of \( r_0 \). [Note that the above analysis is for factors that have a binding site only in a single regulatory region. For those factors which are global regulators and have many operators located throughout the genome (e.g., the factor CRP in *E. coli*), the number \( N_{TF} \) above needs to be appropriately adjusted by the number of operators (Sengupta et al. 2002).]

**Evolution Equation**

In this study, we focus on the steady-state properties of the mutation/selection process defined above. For
a large population size and close to the steady state, we may consider only the dynamics of the average population and neglect fluctuations due to the discreteness of the individual organisms. We denote the average number of individuals at time \( t \) with binding sequence \( S \) by \( N_S(t) \). The time evolution of \( N_S(t) \) is described by

\[
\frac{\partial}{\partial t} N_S(t) = \frac{v_0}{\mathcal{A} - 1} \sum_S N_S(t) \delta_{S - \delta S\mid 1} - v N_S(t) + \Phi_S N_S(t)
\]

(5)

The first term on the right-hand side describes the mutational flow into \( N_S \) from all sequences \( S \) that are a single-nucleotide mutation away, while the second describes the reverse process. The third term represents the (time-averaged) selection/amplification process. Equation (5) is similar to the “para-muse model” considered in a different context by Baake et al. (1997).

Since the fitness function \( f \) depends on the sequence \( S \) only through the binding probability \( P_S \), which depends only on the number of mismatches \( r \) according to Eq. (4), it is advantageous to introduce a “radial distribution” \( N(r, t) \) in the (discrete) Hamming distance space (Nowak and Schuster 1989),

\[
N(r, t) = \sum_S N_S(t) \delta_{r - \delta S\mid 1}
\]

(6)

With \( \Phi_S \) denoting the “radial fitness” function, the evolution equation for \( N(r, t) \) becomes

\[
\frac{\partial}{\partial t} N(r, t) = \Phi(r) N(r, t) + \frac{v_0}{\mathcal{A} - 1} \Delta_r [(r + 1) N(r + 1, t)] - v_0 \Delta_r [(L - r) N(r, t)]
\]

(7)

where \( \Delta_r [f(r)] \equiv f(r) - f(r - 1) \) denotes the discrete derivative, and

\[
\Phi(r) = 1 + xP(r)
\]

(8)

is a mesa-shaped fitness landscape. Equation (7) is obtained by observing that there are \( (L - r)(\mathcal{A} - 1) \) ways to mutate a site with \( r \) mismatches into a site with \( r + 1 \) mismatches, \( r \) ways to mutate it into a site with \( r - 1 \) mismatches, and \( r(\mathcal{A} - 2) \) ways to mutate a site without changing the number of mismatches.

We characterize the predictions of our model by numerically integrating the discrete radial evolution equation (7) using the set of realistic parameters given above. However, to gain insight about the qualitative behavior of the model, we also analyze the continuum-space evolution equation obtained in the limit of large \( L \),

\[
\frac{\partial}{\partial t} n(r, t) = D(r) \frac{\partial}{\partial r} n(r, t) - v(n(r, t)) + \varphi(r)n(r, t)
\]

(9)

where we use \( n(r, t) \) and \( \varphi(r) \) to denote the continuum generalization of the functions \( N(r, t) \) and \( \Phi(r) \), respectively. Note that the mutational dynamics is locally conservative, with a local current \( j(r, t) = D(r) \delta n(r, t) = n(r, t) \). The appropriate boundary conditions are \( j(0, t) = 0 \) and \( j(L, t) = 0 \).

The continuous radial evolution equation (9) reduces the evolutionary dynamics to a simple one-dimensional drift-diffusion equation, where the “diffusion coefficient” \( D(r) \) and the “drift velocity” \( v(r) \) are explicitly given by

\[
D(r) = \frac{v}{2} \left( 1 - \frac{\mathcal{A} - r}{\mathcal{A} - L} \right)
\]

(10)

\[
v(r) = \frac{v}{2} \left( 1 - \frac{\mathcal{A} - r}{\mathcal{A} - L} \right)
\]

(11)

Note that both \( D \) and \( v \) are proportional to the overall mutation rate \( v = v_0 L \), with only weak dependence on \( r \) for \( r \ll L \). The drift velocity drives the distribution away from the optimal binding site at \( r = 0 \), simply because the number of sequences with a fixed number of mismatches \( r \) increases quickly with \( r \). This purely entropic bias changes sign at \( (\mathcal{A} - 1)L/\mathcal{A} \), which is the average number of mismatches in a random binding sequence. Also note that the \( r \) dependence of the diffusion coefficient disappears when \( \mathcal{A} = 2 \), because for a two-letter alphabet, every mutation implies a change in \( r \). For \( \mathcal{A} > 2 \), there are mutations which do not change the Hamming distance and hence do not affect the diffusion process. This effect is reflected in the reduction of \( D \) in Eq. (10).

Our continuous radial evolution equation (9) is somewhat reminiscent of the evolution equation in fitness space introduced by Tsimring et al. (1996) in a general population genetics context. However, with our concrete model for protein–DNA binding, we can work directly in genotype space, which will enable us to make explicit predictions on the behavior of the binding sites.

Selection Threshold and Fuzzy Motifs

In this section, we use the evolutionary model (7) described under Model and Equations to address the following questions: How large a selection pressure is needed for the maintenance of binding motifs? and Can the fuzziness of the motifs be accounted for by the balance between mutation and selection? We first provide an analytical solution to a simplified continuum model and then show by numerical simulation that the qualitative features of the solution hold even for a small system such as \( L = 10 \). We compare these results to available data and discuss experimental ramifications.
Analytical Results

Various properties of the continuum model specified by Eqs. (9)–(11) can be obtained exactly. Here we present the results and discuss various qualitative features of the solution, in particular, the existence of a critical selection pressure for the maintenance of the binding motifs. Even though the continuum model is meaningful only for $L \gg r_0 \gg 1$, we will see from numerical simulation that the qualitative features are valid even for the more realistic parameter range where $r_0$ is not much larger than one, and $L \sim 10$.

For the analytical study, we neglect the $r$ dependence of the diffusion coefficient (10) and the drift velocity (11) and use $D = \gamma/2$, $v = \gamma$. This is justified as long as $r_0 \ll L$, since as we will see, most of the interesting “action” of this system occurs around $r = r_0$. Equation (9) then reduces to the asymmetric “quantum well” problem well studied in the context of various statistical mechanics problems (Hatano and Nelson 1997). [It differs from the DNA unzipping problem studied by Lubensky and Nelson (2000) only by an (unimportant) boundary condition at $r = 0$.] An explicit solution can be obtained by further approximating the Fermi function (4) by the Heavyside step function $\theta(r)$, such that the fitness landscape becomes

$$\tilde{\Phi}(r) = 1 + \alpha \theta(r_0 - r)$$

This idealized form of the fitness function is known as truncation selection (Kondrashov 1988).

The solution of this simplified continuum problem is of the form $n(r,t) = n_0(r) = e^{\gamma t}$, where $n_0(r)$ is the stationary distribution associated with the largest growth rate $\gamma$. It is controlled by one dimensionless parameter, the effective selection pressure

$$\tilde{\epsilon} = \frac{2\alpha}{\gamma}$$

We have $\gamma = 1$ if $\tilde{\epsilon}$ is below the critical value

$$\tilde{\epsilon}_c = 1 + \frac{\eta_c^2}{r_0}$$

where $\eta_c$ is of order one and depends only weakly $r_0$. In this regime, $n_0(r)$ is given by the continuum version of the (skewed) binomial distribution

$$\Omega(r) = (\alpha - 1)^r \binom{L}{r}$$

as if the fitness plateau at $r < r_0$ is not present. For $\tilde{\epsilon} > \tilde{\epsilon}_c$, the solution is given in terms of the eigenvalue problem

$$\gamma''(r) + \tilde{\epsilon} \theta(r_0 - r) \gamma(r) = \tilde{\lambda} \gamma(r)$$

with the boundary condition $\gamma(0) = \gamma'(0)$, where $\gamma(r)$ is the eigenfunction corresponding to the largest eigenvalue, $\tilde{\lambda}(\tilde{\epsilon})$, which must exceed 1. [Thus, the precise definition of $\tilde{\epsilon}_c$ is $\lambda(\tilde{\epsilon}_c) = 1$.] In this regime, the growth rate becomes $\gamma = 1 + (\tilde{\epsilon} - 1)\tilde{\lambda}/2 > \tilde{\lambda}_0$, and the stationary distribution is $n_0(r) = \gamma(r) e^{-\tilde{\lambda} r}$. The form of the latter can be straightforwardly analyzed for large $\tilde{\epsilon}$’s (such that $\tilde{\lambda} > 1$). It is strongly peaked at $r^* \approx r_0$, indicating that the motifs are marginally conserved or maximally fuzzy above the selection threshold.

A phase transition occurs at $\tilde{\epsilon} = \tilde{\epsilon}_c$ where the stationary distribution switches from being mostly confined in the region $r < r_0$ (localized phase) to the binomial distribution (delocalized phase) when $\tilde{\lambda}$ approaches 1. This phase transition belongs to the same class of transitions as the one described by Eigen in the context of quasi-species evolution (Eigen 1971; Eigen et al. 1989; Higgs 1994). The critical selection pressure $\tilde{\epsilon}_c \sim \eta_0 L$ is recognized as the well-known form of the “error threshold.” Note also the dependence of $\tilde{\epsilon}_c$ on $r_0$ as given in Eq. (14): $\tilde{\epsilon}_c$ decreases upon increasing $r_0$, and since $\lambda$ is a monotonously increasing function of $\tilde{\epsilon} - \tilde{\epsilon}_c$, the effective growth rate $\gamma$ will also increase. This implies that a wider fitness landscape has a smaller mutational load and a larger effective fitness, which is a known result (see, e.g., Schuster and Swetina 1988).

The “order parameter” of the phase transition is the average number of mismatches in the stationary state, $\langle r \rangle = \int_0^L n_0(r)/\int_0^L n_0(r)$. In the localized phase, $\langle r \rangle \approx r_0$, while $\langle r \rangle = (\alpha - 1)L/\beta \rightarrow \infty$ in the delocalized phase. When $\tilde{\epsilon}$ approaches $\tilde{\epsilon}_c$ from above, $\langle r \rangle$ diverges as

$$\langle r \rangle \propto (\tilde{\epsilon} - \tilde{\epsilon}_c)^{-1}$$

indicating that this is a second-order phase transition.6

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6In the context of protein folding, it has been pointed out by R. Goldstein (2001) that the balance of mutation and selection may lead to maximal fuzziness in the space of amino acid sequences. Our results are similar in spirit, but more explicit due to the simplicity of the protein-DNA binding.

6It should be noted that both the critical value $\tilde{\epsilon}_c$ and the divergence of $\langle r \rangle$ near $\tilde{\epsilon}_c$ are modified if one explicitly includes the time dependence of the fitness landscape. In particular, if we take the fitness to be $\Phi(t) = \int_0^T f(t) dt$ (1 in the ON state and $f(t) = 0$ in the OFF state), with a stochastic $f(t)$, then the evolution dynamics becomes equivalent to the class of time-dependent depinning problems studied by Lubensky and Nelson (2000), with the critical behavior $\langle r \rangle \propto (\tilde{\epsilon} - \tilde{\epsilon}_c)^{-2}$. 

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3Inspired by the present system, solution of the mean-field evolution model for a general mesa-like fitness landscape has recently been developed by Peliti (2002).

4For $1 \ll r_0 \ll L$, $\eta_c$ is given to a good approximation by the solution of the equation $\eta_c = -r_0\tan(\eta_c)$ and hence $\eta_c \in [\pi/2, \pi]$. 

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6In the context of protein folding, it has been pointed out by R. Goldstein (2001) that the balance of mutation and selection may lead to maximal fuzziness in the space of amino acid sequences. Our results are similar in spirit, but more explicit due to the simplicity of the protein-DNA binding.
Numerical Results

To test whether the behavior derived above for the simplified continuum model holds approximately also for the discrete model (7) with realistic parameters, we performed a number of numerical studies. We determined the steady-state distribution $N_0(r)$ of Eq. (7) over a range of values of $\bar{z}$ for two sets of parameters: (a) a nearly continuum model, with $L = 1000$, $r_0 = 30$, and the step function landscape (12); and (b) the discrete model with $L = 10$, $r_0 = 3$, and the Fermi function landscape (8) with $\epsilon = 2k_B T$. Figure 2 shows the stationary distribution $N_0(r)$ for the discrete model in the delocalized regime ($\bar{z} = 0.5$), in the localized regime ($\bar{z} = 3.0$), and in the crossover region in between ($\bar{z} = 1.4$). We see that $N_0(r)$ is peaked slightly below $r_0 = 3$ in the localized regime and becomes indistinguishable from the binomial distribution (15) in the delocalized phase. Note that the distribution is broad in the crossover regime, which is consistent with the finding of a continuous second-order transition in the continuum model (see the last section).

To make the comparison more quantitative, we next examine the order parameter $\langle r \rangle$. Figure 3a shows $r_0/\langle r \rangle$ plotted as a function of $\bar{z}$, for the discrete model (triangles) and the nearly continuum model (diamonds). The nearly continuum model displays a sharp transition at $\bar{z} \approx 1.6$. The sharp transition becomes a pronounced crossover for the discrete model but still with a relatively well-defined threshold $\bar{z}_c$. The $r_0$ dependence of $\bar{z}_c$ is plotted in Fig. 3b over the relevant interval $1 < r_0 < 5$ [here, we have defined the threshold $\bar{z}_c$ as the value of $\bar{z}$, where the derivative of $r_0/\langle r \rangle(\bar{z})$ is maximal]. We see that it is relatively insensitive to the precise value of $r_0$, with $\bar{z}_c \approx 1$ as given qualitatively by the formula (14).

Viral and Bacterial Evolution

We expect the selection threshold described above to be detectable in evolution experiments with RNA viruses. The total mutation rate $v$ for the binding site for RNA viruses is of the order $10^{-3}–10^{-4}$ for a binding sequence of length $L = 10$. Assuming that the fitness gain of the virus in the ON state [i.e., the factor $\delta \phi_A$ in Eq. (2)] is of the order of 1–10%, then the effective selection pressure $\tilde{z} = 2\delta \phi_A/v$ on the discrete model.
viral regulatory sequence becomes of the order \( \tilde{z} \sim O(1) \) if the fractional exposure \( \tilde{f} \) to the ON state is set at a few percent level. By varying \( \tilde{f} \) over the range of several percent, we expect that the phase transition should be observable. Moreover, the anomalous dependence (see footnote 4) of the selection threshold on the temporal variation \( f(t) \) should also be observable by applying controlled temporal changes to the environment. The stationary distribution \( N_0(r) \) itself can be monitored in principle by sequencing a reasonable number (say 100) viral regulatory sequences after stationarity is reached.

A very different situation is expected for the evolution of bacteria or even DNA viruses. The total mutation rate \( v \) is of the order \( 10^{-8} \) for bacteria and \( 10^{-16} \) for DNA viruses. Consequently, \( \tilde{z} \) is expected to be four orders of magnitude larger than \( \tilde{z} \) for bacteria and two orders larger for DNA viruses. What is the behavior of the discrete model at such large values of \( \tilde{z} \)? In Fig. 4, we show the position of the peak \( r^* \) of the distribution \( N_0(r) \) obtained numerically as a function of \( \tilde{z} \) on a logarithmic scale. For values of \( \tilde{z} \) exceeding \( \sim 140 \), we find that the peak is pushed down to \( r^* = 0 \), contrary to the fuzziness depicted in Fig. 3.

This behavior is obviously an artifact of the specific feature of the Fermi function landscape used in (8): for very large \( \tilde{z} \)'s, there is an incentive for the distribution to move toward small \( r \)'s due to the very slight increase in the value of \( P(r) \) for smaller \( r \)'s. But it is unreasonable to expect that the simple relation between the binding probability \( P(r) \) and the fitness function \( \tilde{\phi}(r) \) assumed in this study to hold down to very small differences in \( P(r) \). Aside from various kinetic effects of binding and temporal variations of the environment that we have neglected, fluctuations due to finite population size (e.g., genetic drift) simply do not allow for the population to resolve the very small differences in fitness due to the small differences in \( P(r) \); see the theory of nearly neutral evolution (Ohta 1992). Thus, \( \tilde{\phi}(r) \) should be effectively \( r \) independent for small \( r \)'s. This can be implemented by replacing \( \tilde{\phi}(r) \) by a constant value \( \tilde{\phi}(r_0) \) when \( \tilde{\phi}(r_0) - 1 - \tilde{\phi}(r_0) \) is below some resolution limit (set by the effective population size of the organism). For low mutation rates (or large \( \tilde{z} \)'s), this amounts to replacing the fitness function by an infinite square well:

\[
\tilde{\phi}(r) = \begin{cases} 
\infty & \text{if } r \leq r_0 \\
0 & \text{if } r > r_0
\end{cases}
\]

(18)

The stationary distribution obtained in this case depends only on the width of the well \( r_0 \) and is shown in Fig. 5 for \( r_0 = 3 \). Note that it is highly peaked at \( r_0 \) as expected. Hence the binding sequence is fuzzy even as \( \tilde{z} \rightarrow \infty \). However, it is different from simply truncating the binomial distribution (15) for \( r > r_0 \) due to the mutational load, i.e., a fraction of the population with \( r = r_0 \) will receive an additional deleterious mutation and not make it to the next generation.

**Comparison to Known Sites**

It is useful to compare the above solution to biological data. Unfortunately, polymorphisms in the same binding sequence across different strains of a bacteria species are not yet readily available. Instead, we assume that the different binding sequences (of the same transcription factor) located in different regulatory regions across the genome may be viewed as a sample of the stationary binding sequence distribution. This is clearly not the case if the selection pressure is low, since close to the selection threshold,

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Footnote 9: This modification of the fitness function should in principle also be applied to the case of RNA virus evolution in the vicinity of the phase transition. However, it would not make much of a difference there because the distribution would already be peaked away from small \( r \).
even small differences in selection pressure experienced by the different binding sequences will produce different binding sequence distributions; see Fig. 2. But this should not be a concern for bacteria since \( \bar{x} \gg \bar{z} \) there. An obvious candidate is the binding sequences for the the well-known *E. coli* global regulatory CRP (also known as the catabolite activator protein; CAP), which is activated under a low cellular glucose level (Saier et al. 1996). There are over 100 CRP sites in the *E. coli* genome. We take from the RegulonDB database (Salgado et al. 2000) a list of 28 sites which are biologically confirmed binding sites and appear only once in a given regulatory region. (The case of multiple binding sites is discussed in the following section.) The drawback of using CRP sites is that CRP is hardly ever the only regulator in a target regulatory region, and interaction with other transcription factors can complicate the situation.

Figure 6 shows the histogram of the number of mismatches of these CRP sequences from the consensus sequence TGTGA...TCACA. While it peaks at \( r^* = 2\text{–}3 \), similarly to the corresponding distribution of the infinite-mesa model in Fig. 5, it is clear that the distribution of the CRP sites is broader. The few outliers at \( r = 0, 1, 4, 5 \) may well be due to direct or subtle interaction with other factors which we have not considered in this simple model. The existence of nearly equal peaks at \( r = 2 \) and \( r = 3 \) is more perplexing: According to our model, the distribution should be peaked at the largest possible \( r \). [For the \( L = 10 \) sequence, entropy favors \( r = 3 \) over \( r = 2 \) by a factor of eight.] One possible cause of the discrepancy may be the deviation of the real binding energy matrix \( e_i(S) \) from the two-state model. For instance, suppose that the chemical potential \( \mu \) in the ON state is such that \( r_0 = \mu/\kappa < \kappa \). Then according to the pure two-state model, the maximum number of allowed mismatches is two. However, small deviations in the binding energies from \( \kappa \) will allow a maximum of three mismatches in a subset of the \( L \) positions, thereby producing a distribution peaked at both \( r = 2 \) and \( r = 3 \) as shown in Fig. 6. The actual stationary distribution of \( r \) can be easily computed numerically if the energy matrix is known. However, at present, the authors know of no example of a transcription factor whose binding energy matrix is measured and a large number of binding sequences are available.

A very different explanation of the data in Fig. 6 is the differential selection of each of the CRP motifs as alluded in the Introduction. Specifically, one can envision a situation where the single “ON state” assumption we adopted is not valid, and instead the cell coordinates a graded response to cellular glucose shortage, requiring different operons to turn ON at different (activated) CRP concentrations. [In this case, our assumption that the fitness function \( (1) \) has a simple linear dependence on the binding probability obviously breaks down.] Within this scenario, the distribution in Fig. 6 is solely a result of the functional need of the cell, and its resemblance to the statistical distribution in Fig. 5 would be fortuitous. Distinguishing between the plausibilities is important and can be done by either sequencing the CRP binding sites in a variety of related strains to accumulate statistics on polymorphism for each site or performing site-directed mutagenesis to specific binding motifs and directly measuring the fitness function. In general, one may expect to find that the form of the fitness function depends on the biological function of the binding site. In particular, form \( (1) \) seems more likely to be appropriate for the case of transcriptional repressors than for activators, since repressors need to have a binding probability close to one, to suppress efficiently transcription from the promoter, which is active in the absence of the repressor. In the case of activators, the promoter has a very low basal level of transcription and even an activator with a relatively low binding probability can lead to a large effect on the transcription level.

**Multiple Binding Sites**

It is well known that regulatory binding motifs often occur in doublets or even higher multiplets. For instance, the regulatory regions of the *E. coli* genes *crp, dadA, dxdA, fixA, glpFK, glpTQ, lac, manX, nagE*, and *tss* are some of the many regions that contain two CRP binding sequences. Here, we extend our model to account for the possibility of multiple sites that bind the same protein and regulate the same promoter. We pursue the question of whether we can interpret regulatory regions with multiplets as being under higher selective pressure for factor binding than regulatory regions with single binding sites.

Some factors (e.g., \( \sigma \)-repressor) bind *cooperatively* to binding sites, thereby effectively enhancing their
DNA binding specificity. Cooperative factor binding can play an important and interesting role in transcription regulation (see, e.g., Ptashne 1992), however, it does so only for a fraction of the known multiplets, since many factors (such as CRP) have no binding domain for an attractive interaction between themselves. In the present study we exclude factor-factor interactions and explore possible selective advantages of multiple independent binding sites. This approach is similar in spirit to studies of gene duplication, which consider the evolution of multiple copies of the same gene (see, e.g., Wagner 2000). One scenario could be that several bound transcription factors can simultaneously interact with polymerase to recruit (or repel) it synergistically more efficiently than a single factor would. For the case of CRP, this effect has been observed, and studied in detail, experimentally (Busby and Ebright 1999; Langdon and Hochschild 1999). An individual organism with a multiplet of binding sites for a factor then has a fitness advantage over one with a single binding site, if a strong activation (or repression) is beneficial for the biological function. Consequently, selection would favor multiplets over singlets. On the other hand, random mutations tend to destroy the binding motifs, so that an additional motif is maintained only when its contribution to the fitness is sufficiently high. In the following, we explore this scenario within our model.

Let us assume that there are two binding sites in a certain regulatory region and ask whether they will be maintained by evolution. We begin by constructing a “two-site fitness function” that makes the selection mechanism outlined above explicit. As in the previous sections on the single-site problem, we assume that the state of the bacterium/virus alternates between an ON state, where factor binding leads to a fitness gain, and an OFF state, where factor binding has a negative effect. In the ON state, let the fitness gain due to factor binding be $\delta\phi_{A1}$, $\delta\phi_{A2}$, or $\delta\phi_{A12}$, if a factor is bound to site 1 only, site 2 only, or both sites, respectively. Using the same arguments as for the single site case, the time-averaged fitness becomes

$$
\bar{\Phi} = 1 + \alpha \cdot [P_1(1 - P_2) + \sigma P_2(1 - P_1)] + \omega P_1 P_2
$$

where $P_1$, $P_2$ denote the probabilities that a factor is bound to site 1, 2, which depend on the respective sequences (we neglect the possibility of cooperative binding at this point). The selection pressure, $\alpha$, again has the form (2), with $\alpha = f\delta\phi_{A1}$, while the “synergism factor” $\omega$ describes the fractional fitness advantage of two bound factors over just one, i.e., $\omega = \delta\phi_{A12}/\delta\phi_{A1}$. In the remaining term, the dimensionless coefficient $\sigma$ constitutes an “asymmetry factor equal” to the relative fitness gain $\delta\phi_{A2}/\delta\phi_{A1}$ (i.e., when $\sigma \neq 1$ one may distinguish between a more “important” and a less important site). Note that not only the selection pressure, but also $\omega$ and $\sigma$ may vary between regulatory regions, even when they are controlled by the same factor, since both depend on the location of the binding sites with respect to the promoter and on the sequence of the promoter itself (see the Discussion at the end of this section).

As in the single-site problem, we work in the two-state model approximation (see the section on Binding Probability), so that the binding probabilities $P_1$ and $P_2$ depend only on the number of mismatches $r_1$ and $r_2$ in the respective site and take the form (4). When the selection pressure $\alpha$ is much higher than the mutation rate $v$ (as we typically expect in the case of bacterial evolution), we again invoke the argument that very small differences in the fitness function are hardly resolvable by finite populations, and therefore the fitness function should become neutral, i.e., $r$ independent, at small $r_1$ and $r_2$. This effectively amounts to using step functions for the binding probabilities, i.e., $P(r_{1,2}) = 1$ for $r_{1,2} \leq r_0$ and $P(r_{1,2}) = 0$ for $r_{1,2} > r_0$. The two-site fitness function in $(r_1, r_2)$-space is then

$$
\bar{\Phi}(r_1, r_2) = \begin{cases} 
1 + \alpha & \text{if } r_1 \leq r_0 < r_2 \\
1 + \alpha \sigma & \text{if } r_2 \leq r_0 < r_1 \\
1 + \alpha \omega & \text{if } r_1, r_2 \leq r_0 \\
1 & \text{if } r_1, r_2 > r_0 
\end{cases}
$$

To simplify the discussion in the following, we use the fitness function (20) over the whole range of $\bar{\alpha}$, since it yields a correct description at large $\bar{\alpha}$, and at small $\alpha$, it produces no qualitative changes in the behavior of the stationary distribution compared to the smooth fitness function with $P(r)$ of the form (4).

It is straightforward to derive a two-site evolution equation analogous to Eq. (7), which describes the approach of the average distribution of mismatches $N(r_1, r_2, t)$ (neglecting fluctuation effects due to finite population size) to its stationary state $N_0(r_1, r_2)$. One obtains

$$
\frac{\partial}{\partial t} N(r_1, r_2, t) = \bar{\Phi}(r_1, r_2) N(r_1, r_2, t) + v_0 \Delta \left[ (r_1 + 1)N(r_1 + 1, r_2, t) - (r_1)N(r_1, r_2, t) \right] + v_0 \Delta \left[ (r_2 + 1)N(r_1, r_2 + 1, t) - (r_2)N(r_1, r_2, t) \right]
$$

In the continuum limit, Eq. (21) becomes a two-dimensional generalization of the (biased) diffusion equation (9).
Fig. 7. Illustration of the drift-diffusion dynamics for the two-site problem. The arrows indicate the direction and magnitude of the drift velocity $\bar{v} = (v(r_1), v(r_2))$ in the continuum equation (22), while the shading corresponds to the fitness function (dark means high fitness; here we used $\omega = 2$ for the purpose of illustration, and $\sigma = 1$).

\[
\begin{align*}
\frac{\partial}{\partial t} n(r_1, r_2, t) &= \bar{\phi}(r_1, r_2) n(r_1, r_2, t) \\
&+ \frac{\partial}{\partial r_1} \left[ D(r_1) \frac{\partial}{\partial r_1} n(r_1, r_2, t) - v(r_1) n(r_1, r_2, t) \right] \\
&+ \frac{\partial}{\partial r_2} \left[ D(r_2) \frac{\partial}{\partial r_2} n(r_1, r_2, t) - v(r_2) n(r_1, r_2, t) \right]
\end{align*}
\] (22)

where $D(r)$ and $v(r)$ are still given by Eqs. (10) and (11) and $n(r_1, r_2, t)$ and $\bar{\phi}(r_1, r_2)$ are the continuum generalizations of $N(r_1, r_2, t)$ and $\bar{\phi}(r_1, r_2)$, respectively. Figure 7 illustrates the two-dimensional (biased) diffusion dynamics that emerges from Eq. (22). The fitness function has a high plateau or “mesa” at $r_1, r_2 < r_0$ and two strips of lower fitness along the $r_1$ and $r_2$ axis. Hence selection tries to keep $r_1, r_2 < r_0$.

Mutation, on the other hand, drives the distribution toward the average number of mismatches in a random binding sequence, $r_1 = r_2 = (\sigma - 1)L/\sigma$, as indicated by the arrows in Fig. 7. We are interested in the stationary distribution $N_0(r_1, r_2)$ that arises as a balance between selection and mutation. Below we characterize the dependence of $N_0(r_1, r_2)$ on the effective selection pressure $\tilde{\omega} = 2\sigma/v$ and the synergism factor $\omega$ numerically by iterating Eq. (21). However, we first anticipate the qualitative behavior of $N_0(r_1, r_2)$ using the understanding of the single-site problem gained in the last section.

Let us neglect a possible asymmetry between the sites for the moment, i.e., we set $\sigma = 1$. It is clear that if $\tilde{\omega}$ is below a certain threshold value, no motif will be maintained, i.e., the peak of the stationary distribution will be close to $r_1 = r_2 = (\sigma - 1)L/\sigma$. On the other extreme, when $\tilde{\omega}$ is very large, the distribution will certainly be localized on the high fitness mesa, corresponding to two conserved binding motifs. By analogy with the single-site case, we would expect the distribution to be maximally fuzzy in this regime, and hence the peak of the stationary distribution to be close to $r_1 = r_2 = r_0$. What happens when $\tilde{\omega}$ takes on intermediate values? Upon increasing $\tilde{\omega}$, the peak of the stationary distribution may either pass directly from $r_1 = r_2 = (\sigma - 1)L/\sigma$ to $r_1 = r_2 = r_0$ or go through a state with only one conserved motif (see Fig. 8). Intuitively, which of these “pathways” is taken should depend on the value of $\omega$: when $\omega$ is small, the selective advantage of two conserved motifs over one is small and therefore a much higher selection pressure is needed to stabilize two motifs against mutations than just one, i.e., upon increasing the selection pressure the system passes from zero to one to two motifs. Conversely, when $\omega$ is very large, two motifs are actually stabilized at lower selection pressures than a single motif would be, and hence the system passes directly from zero to two motifs. We can estimate the value $\omega_0$ at which the system switches between the two pathways: when $\omega = 1$, the one-motif phase exists in an intermediate range of $\tilde{\omega}$'s, i.e., $\tilde{\omega}_1 < \tilde{\omega} < \tilde{\omega}_2$, where the lower critical value for the transition from zero to one motif is approximately the same as in the single-site problem, i.e., $\tilde{\omega}_1 \sim 1$, and the upper critical value is $\tilde{\omega}_2 \sim (\omega - 1)^{-1}$, since the transition from one to two motifs may be regarded as another single-site problem with $\alpha$ replaced by $(\omega - 1)$.$\tilde{\omega}$. The system switches between the two pathways when $\tilde{\omega}_1 = \tilde{\omega}_2$, and hence $\omega_0 = 2$.

In the one-motif phase, the selection of either motif, at site 1 or site 2, is equiprobable as long as $\sigma = 1$. Correspondingly, $N_0(r_1, r_2)$ has two equal peaks as indicated in Fig. 8. When $\sigma < 1$ the peak associated with site 2 will disappear, but otherwise
the qualitative behavior of the model remains the same. For simplicity, we keep $\sigma = 1$ from here on.

To examine the qualitative picture outlined above and to render it more quantitative, we now characterize the behavior of $N_0(r_1, r_2)$ numerically using the parameters tailored to CRP, i.e., $L = 10$ and $r_0 = 3$. To determine $N_0(r_1, r_2)$, we evolve an arbitrary initial distribution $N(r_1, r_2, t = 0)$ using Eq. (21) until the stationary state is reached. Figure 9 displays three such stationary distributions, one each in the zero-motif, one-motif and two-motif phases (here, we used $\tilde{\omega} = 0.2$, 5, and 50, together with $\omega = 1.1$). Besides justifying the schematic sketch in Fig. 8, it shows that the distributions in both the one- and the two-motif phase are peaked at the “edge” $r_0 = 3$ and are therefore maximally fuzzy as in the single-site problem.

Next we focus on the transitions among the three phases. In Fig. 10, the average total number of matches, i.e., $2L - \langle r_1 \rangle - \langle r_2 \rangle$ (here $\langle \ldots \rangle$ denotes an average over the stationary distribution), is plotted against $\tilde{\omega}$, again with $\omega = 1.1$ (solid line). [Note that in Fig. 10 the y axis is reversed compared to that in Fig. 4.] We observe that the total number of matches rises quite sharply around $\tilde{\omega} = 1$ and $\tilde{\omega} = 10$. These positions agree with our estimates $\tilde{\omega}_c \sim 1$ and $\tilde{\omega}_c \sim (\omega - 1)^{-1}$ based on the qualitative discussion above. (Note that since we work with a small “system size” of $L = 10$, the transitions, which are sharp in the limit $L \to \infty$, appear only as smooth crossovers.) To show that the rises are indeed caused by the transitions from the zero-motif to the one-motif phase and from the one-motif to the two-motif phase, respectively, we also plotted the number of matches in each site at the peak of the stationary distribution (first motif; dots; second motif; diamonds). Note that in this figure the y axis is reversed compared to that in Fig. 4.

In evolution experiments with RNA viruses, this twofold transition should be directly observable [if a suitable operon can be identified where the fitness function (19) is applicable], since according to our estimates above, $\tilde{\omega}$ for these systems can be tuned over a range of 1–100 by varying the fractional exposure to different environmental conditions. On the basis of our model, one would expect, for instance, that one of the sites in a doublet disappears in the course of an evolution experiment, if the selection pressure is sufficiently lowered by reducing the exposure to the environment where binding is beneficial. When the exposure is reduced to zero, both regulatory sites and the gene coding for the transcription factor (if not required for other mechanisms) will be lost.

To complete our characterization of the model behavior, we map out the entire phase diagram in the ($\tilde{\omega}, \omega$) parameter-space. The result is shown in Fig. 11, where $\tilde{\omega}_c$ and $\tilde{\omega}_e$ are plotted as a function of $\omega$. Since $L = 10$ in the present case and the phase boundary is well defined only in the limit $L \to \infty$, the curves $\tilde{\omega}_c(\omega)$ and $\tilde{\omega}_e(\omega)$ are really only crossover lines whose precise location is slightly dependent on their definition (in Fig. 11 they are represented by dashed
would always predict multiple binding sites for bacterial transcription factors. Therefore, within our model, whether one or two motifs are maintained depends almost exclusively on the value of \( \omega \), i.e., \( \omega \leq 1 \) leads to one motif and \( \omega > 1 \) to two motifs. However, there may be cases where maintaining subtle differences in the temporal ordering of turning on/off different operons would give rise to a very small fitness advantage, e.g., flagella assembly and SOS response in *E. coli* (see recent results by U. Alon, submitted for publication). In such cases, the system may respond by keeping one or two motifs according to the theory we presented. And of course there is also the situation of RNA viruses described above where the twofold transition depicted in Fig. 10 could in principle be directly observed.

**Discussion and Conclusions**

The fuzziness of regulatory binding motifs is a widely observed phenomenon. The present investigation has shown that the entropic advantage of introducing mismatches from the best binding sequence is sufficient to produce motifs that are maximally fuzzy while still retaining the capability of factor binding. Nevertheless, we cannot exclude that the fuzziness actually bears a selective advantage (in the language of population genetics, this would correspond to a stabilizing selective pressure). The alternative scenario given for the fuzziness of the CRP sites is an explicit example of the latter. It would be very interesting to address this question experimentally by directly measuring the fitness of a bacterium or virus as a function of the sequence of its binding sites: Starting with a wild-type binding site that has several mismatches, what is the effect on the fitness, when the number of mismatches is reduced by site-directed mutagenesis? Does the fitness remain unchanged or is it reduced? Besides answering the question raised above, experiments of that type could also lead to important conclusions on the evolution of genetic regulation.

Another important result of our study is the phase transition associated with the maintenance of motifs. Our general conclusion is that the selection pressure on a single binding motif needs to surpass a threshold value of approximately \( v_0L/2 \) to guarantee maintenance, while the threshold for a second site (for the same factor and in the same regulatory region) is larger by a factor \( (\omega - 1)^{-1} \), where \( \omega \) is given by the ratio of the fitness of the organism with two sites to the fitness of the organism with one site. As pointed out above, this prediction could be tested experimentally by evolving RNA viruses in a fluctuating environment and varying the fractional exposure to the environment where factor binding is beneficial. In this case there would be no need to do site-directed
mutagenesis, since the transition could be observed directly by sequencing.

Our model makes quantitative predictions on the statistics of polymorphisms in binding sites. To test these, it will not suffice to sequence a particular binding site in many isolates from a single, large ($N_{0} \gg 1$) laboratory population, since this population originates from a small, genetically homogeneous population and it takes a time of the order of $v_{c}^{-1}$ to equilibrate the distribution of mismatches in a binding site. Instead, sequencing the same binding sites in several strains should yield the desired data. Besides allowing a comparison to our model, detailed information on polymorphisms in binding sequences would also make it possible to address a number of interesting questions, e.g., How does the selection pressure on binding site sequences compare with the selection pressure on coding sequences? Or can one identify compensating mutations between promoter and binding site sequences? e.g., Could a mutation that weakens the promoter be compensated by a mutation that increases the affinity of an activator to its operator site?

We conclude that the evolution of transcription factor binding sites is a problem that is well suited to establishing a link between the detailed molecular biophysics of a system and its evolutionary dynamics. The theory presented in the present article is meant as a first step, with the hope of stimulating future experiments in this direction. There are many directions for the improvement of the model and the analysis. One important question is the validity of the mean-field analysis described here. Is the finite population size effect important here and how would it change the motif statistics within our model? One can also investigate more elaborate models including, for instance, the effect of a time-dependent environment, the coupled evolution of the polymerase, ad transcription factor and the binding sites, and the interaction among different factors.

Acknowledgments. It is a pleasure to acknowledge useful discussions with Carson Chow, David Moroz, Luca Peliti, and David Thaler. This work is supported in part by the National Science Foundation through Grants DMR-9971450 and DBI-9970199. U.G. is supported in part by a German DAAD fellowship, and T.H. by a Burroughs Wellcome functional genomics award.

Note Added at Proof

Upon completion of the present work, we learned of the work of Sengupta et al. (2002), who also examined the protein–DNA interaction from an evolutionary perspective. While very similar mean-field evolution equations are analyzed in both works, the emphases are quite different, with Sengupta et al. (2002) arguing for a “robustness” criterion based on minimizing the mutational load.

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For example, we would expect that the selection pressure on the coding sequence of the binding region in transcription factors such as csp and lexA, which have many binding sites distributed over the whole genome, is much higher than on individual operator sites, since a change in the sequence of an operator site affects only the regulation of that particular site, while a change in the amino acid sequence of the binding region of the protein affects the regulation of many genes.
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