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A Discriminative Model for Identifying Spatial cis-Regulatory Modules[1]

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ABSTRACT

Transcriptional regulation is mediated by the coordinated binding of transcription factors to the upstream regions of genes. In higher eukaryotes, the binding sites of cooperating transcription factors are organized into short sequence units, called cis-regulatory modules. In this paper, we propose a method for identifying modules of transcription factor binding sites in a set of co-regulated genes, using only the raw sequence data as input. Our method is based on a novel probabilistic model that describes the mechanism of cis-regulation, including the binding sites of cooperating transcription factors, the organization of these binding sites into short sequence modules, and the regulation of a gene by its modules. We show that our method is successful in discovering planted modules in simulated data and known modules in yeast. More importantly, we applied our method to a large collection of human gene sets and found 83 significant cis-regulatory modules, which included 36 known motifs and many novel ones. Thus, our results provide one of the first comprehensive compendiums of putative cis-regulatory modules in human.

Key words: cis-regulatory module, probabilistic model, transcriptional regulation.

1. INTRODUCTION

MANY OF THE FUNCTIONS CARRIED OUT BY A LIVING CELL require the coordination of gene expression, to ensure that genes are expressed when they are needed. To understand biological processes, it is thus necessary to understand this transcriptional network. Much of the information that determines when and where genes are expressed is encoded in an organism's genome sequence. Although we now have sequences for many organisms, our understanding of how this cis-regulatory information is encoded is very limited.

In higher eukaryotes, cis-regulatory information is organized into modular units, called *cis-regulatory modules (CRMs)*, where each CRM consists of a few hundred base pairs, and contains multiple binding sites for multiple transcription factors (TFs) (Yuh *et al.*, 1998; Ludwig *et al.*, 1998; Krivan and Wasserman, 2001). Methods for identifying CRMs and their component TFs can thus reveal the organization of the transcriptional network in the cell.

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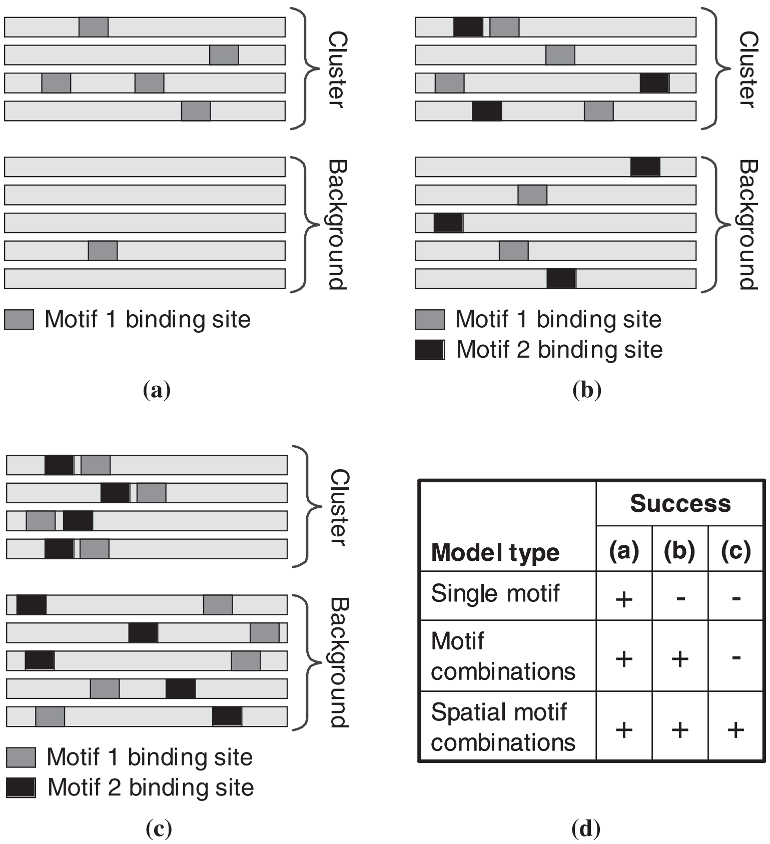
In principle, one could use a two-phase approach for identifying CRMs in a set of upstream regions of co-regulated genes. The first phase would scan for single motifs that are enriched in the upstream regions (see, e.g., Bailey and Elkan [1994], Roth *et al.* [1998]). The second phase would then try to find correlations between these enriched motifs. Such an approach is suitable for discovering some types of CRMs, like the one depicted in Fig. 1(a). However, since each motif is considered in isolation, this approach will fail to discover more subtle CRMs, in which no single motif is enriched, as exemplified in Fig. 1(b). CRMs of the latter type can be found by approaches that look for combinations of motifs that exhibit functional synergism, or tend to co-occur in sequences of interest (Wasserman and Fickett, 1998; Pilpel *et al.*, 2001; GuhaThakurta and Stormo, 2001; Segal *et al.*, 2003; Thompson *et al.*, 2003). However, since these methods do not constrain the occurrences of motifs in each combination to be close together within the upstream region, they will fail to discover CRMs of the type shown in Fig. 1(c). Recently, several methods have been suggested to identify occurrences of known CRMs (Berman *et al.*, 2002; Frith *et al.*, 2001) and to find novel CRMs given a database of known motifs (Sharan *et al.*, 2003; Kel-Margoulis *et al.*, 2002; Aerts *et al.*, 2003), but these methods are restricted to TFs whose binding sites have been previously characterized. To date, we are aware of only one approach that tries to identify novel CRMs and at the same time learn their component motifs de novo (Marsan and Sagot, 2000). A shortcoming of the latter approach is that it is based on a consensus sequence representation of a motif, which has less expressive power compared to the more widely used position weight matrix model.

In this paper, we propose a novel model for transcriptional regulation, based on probabilistic graphical models (Pearl, 1988), and an algorithm to learn this model automatically from data. Our input consists



	Success		
Model type	(a)	(b)	(c)
Single motif	+	-	-
Motif combinations	+	+	-
Spatial motif combinations	+	+	+

FIG. 1. Comparison of the ability of different methods to detect different types of CRMs. Shown for all cases are the gene upstream regions and the locations of binding sites within them, where genes in the "Cluster" contain the CRM, and genes in the background do not. (**a**) CRM consisting of a single motif. (**b**) CRM consisting of a combination of two motifs. (**c**) CRM consisting of a combination of two motifs that are spatially close to each other. (**d**) Methods that search for a single motif can find only CRMs of type (a). Methods that search for motif combinations but disregard their spatial relationships cannot find CRMs of type (c). Our proposed method can find CRMs of all types shown.

of a set of putatively co-regulated genes and their raw sequence data. The model has three components. The first is a motif model that describes the probability that a gene contains a binding site for some motif given the upstream region sequence of the gene. In the second component, we consider sequence windows of a prescribed length along the gene's upstream region. For each window, we model the probability that it contains a CRM that involves k specific motifs, given the binding site occurrences of these motifs. The third component models the probability that a gene contains a CRM given the CRM occurrences in each of the considered windows. We propose an iterative algorithm, based on the expectation maximization (EM) algorithm, for learning the model parameters, and a cross-validation procedure to test the significance of the learned CRMs. Our unified framework generalizes existing approaches for finding CRMs, by integrating both a model for TF binding sites and a model for their organization into modular units. In particular, our method learns motifs de novo and is suitable for identifying all types of CRMs depicted in Fig. 1.

A key property of our model is that it is *discriminative* (Segal *et al.*, 2003; Sinha, 2002): Given a set of upstream regions of co-regulated genes and a background set of upstream regions, the model only attempts to find combinations of motifs that *discriminate* between the two sets. This is in contrast to the common *generative* approaches, which try to build a model of the upstream region sequences and train its parameters such that the model assigns the given sequences a high probability. These approaches can often be confused by repetitive motifs that occur in many upstream regions. These motifs have to be filtered out by using an appropriate background distribution (Tavazoie *et al.*, 1999). As we show, our discriminative model allows us to avoid the problem of learning these background distributions and focus on the classification task at hand.

We evaluated the performance of our method on simulated and real data. On simulated data, our method outperformed extant approaches and recovered planted CRMs with high accuracy. On real yeast data, we identified significant CRMs in 11 out of 25 tested gene sets that are putatively regulated by two cooperating TFs. In the majority of the cases in which the motifs for the corresponding TFs were known (7 out of 11), our method recovered them correctly. Finally, we applied our method to a large collection of human gene sets, derived from the gene ontology (GO) process categorization (Ashburner *et al.*, 2000). Overall, we identified 83 significant CRMs that spanned a diverse set of functional annotations. Many of these CRMs consisted of motifs that matched known motifs in the literature, providing additional support that our learned CRMs indeed correspond to true cis-regulatory signals in human.

The rest of the paper is organized as follows: Section 2 presents our probabilistic model of cis-regulation. Section 3 describes the process of learning the model parameters from data. Results of our algorithm on simulated and real data are presented in Section 4.

2. THE PROBABILISTIC MODEL

In this section, we present our model of cis-regulation. We model a CRM that consists of k distinct binding site motifs for k TFs, in the upstream region sequences of a set of genes $\mathbf{G}$, where each gene is either regulated by the CRM or not. Thus, we associate a binary *Regulation* attribute R and an upstream region sequence attribute S with each gene. Since we expect a CRM to span a relatively short region, we partition the upstream region S into n shorter overlapping sequence *windows*, where each window has length L. The model then considers CRM occurrences only within these windows.

Our model has three components. The first is a *motif model*, which represents the motif binding sites that are bound by each of the k TFs. We use the motif model to define n binary attributes for each TF i, $g.M_{i1} \dots g.M_{in}$, indicating whether each of the n windows contains a binding site for the TF. The second component is a *module model* which represents a CRM as a combination of individual motifs. We use the module model to define n binary attributes, $g.W_1, \dots g.W_n$, corresponding to whether the CRM appears in each of the n sequence windows. The last component is a *regulation model* that models the regulation of a gene, $g.R$, by the CRM, as a function of the CRM occurrences in the n different windows. The full model is shown in Fig. 2. In the following, we describe each of the model components in detail.

2.1. Motif model

The first component in our model is a set of variables that represent the binding site motifs for each of k transcription factors. For each gene g, we have a set of binary-valued *Motif* variables, $\mathbf{M} =$

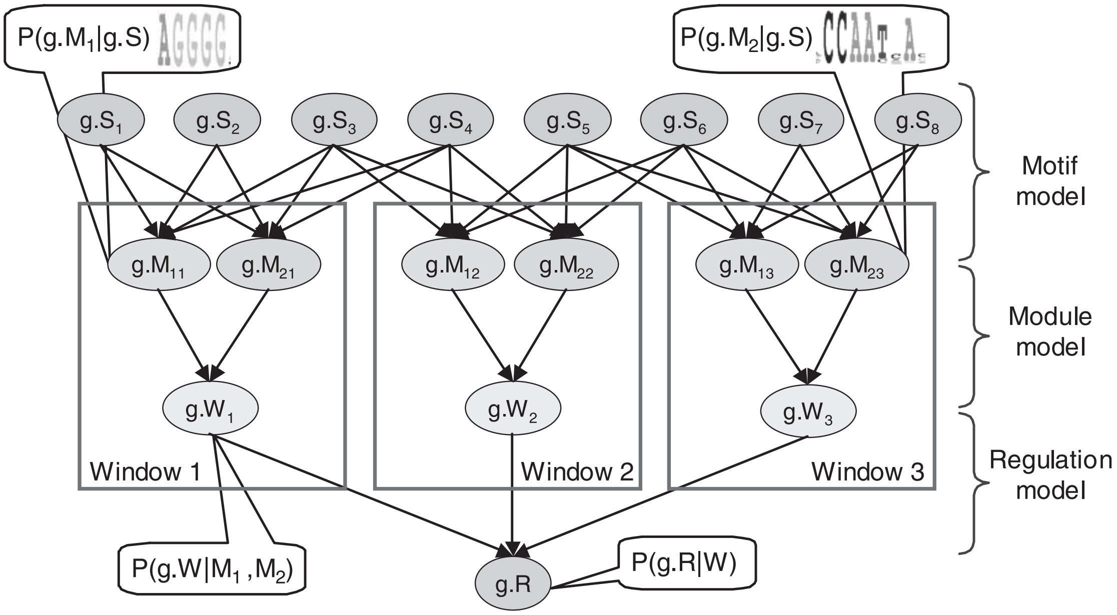


FIG. 2. Illustration of our unified model for a simple example with upstream regions of length eight, windows of length four with two base-pair overlaps, and two motifs. The model contains a total of four distinct conditional probability distributions (CPDs). The CPDs for the first motif are the same, and hence $P(g.M_1 \mid S) = P(g.M_{11} \mid S) = P(g.M_{12} \mid S) = P(g.M_{13} \mid S)$. Similarly, the CPDs for the second motif are the same, and hence $P(g.M_2 \mid S) = P(g.M_{21} \mid S) = P(g.M_{22} \mid S) = P(g.M_{23} \mid S)$. Finally, the same CPD is shared across all windows, and hence $P(g.W \mid g.M_1, g.M_2) = P(g.W_1 \mid g.M_1, g.M_2) = P(g.W_2 \mid g.M_1, g.M_2) = P(g.W_3 \mid g.M_1, g.M_2)$.

$\{g.M_{11} \ldots g.M_{kn}\}$, where $g.M_{ij}$ takes the value *true* iff motif i appears in the j-th sequence window of g. Thus, we allow the motif to play a regulatory role in controlling the expression of gene g, by being a part of the CRM in some windows. We model each motif using the standard *position specific scoring matrix* (PSSM) representation (Bailey and Elkan, 1994; Roth *et al.*, 1998), which assumes independence between positions in a binding site. This model assigns a weight to each position in the motif and each nucleotide $\ell \in \{A, C, G, T\}$, representing the extent to which the nucleotide's presence in this position is associated with the motif.

When learning PSSMs, our goal is to estimate the probability that a transcription factor binds a certain gene given its upstream region. Hence, we adapt the discriminative motif model of Segal *et al.* (2002), which is well suited for this purpose. This model is specified using a logistic function with p position-specific weights $w_i[\ell]$, one for each position i and each letter $\ell \in \{A, C, G, T\}$, and a threshold w_0. For a window sequence of length L, we assume that binding occurs once, and with equal probability at each of the $L - p + 1$ possible positions in the sequence. The probability of binding given the sequence is then specified as

$$P(g.M = \mathit{true} \mid g.S_1, \ldots, g.S_L) = \mathit{logit}\left(\log\left(\frac{w_0}{L-p+1}\sum_{j=1}^{L-p+1}\exp\{\sum_{i=1}^{p} w_i[g.S_{i+j-1}]\}\right)\right),$$

where $\mathit{logit}(x) = \frac{1}{1+e^{-x}}$ is the logistic function. We refer the reader to Segal *et al.* (2002) for additional details.

2.2. Module model

The second component in our model describes the composition of a CRM in terms of its component motifs. To capture the notion that some motifs may be more important for a particular CRM than others, we model a CRM as a weighted combination of individual motifs. Specifically, we use the logistic function for representing the probability that a sequence window contains the CRM, given the occurrences of the individual motifs in the sequence

$$P(g.W_j = \mathit{true} \mid g.M_{1j}, \ldots, g.M_{kj}) = \mathit{logit}\left(v_0 + \sum_{i=1}^{k} v_i \cdot g.M_{ij}\right)$$

where $g.W_j$ is a binary variable representing whether the j-th sequence window contains the CRM, $g.M_{ij}$ is a binary variable representing whether the motif bound by transcription factor i is present in the j-th window, and v_i is a weight that specifies the extent to which motif i plays a regulatory role in the CRM. As the probability that a window contains the CRM depends on $\sum_{i=1}^{k} v_i \cdot g.M_{ij}$, the higher v_i the more it contributes to this probability. For interpretability considerations, we restrict the motif weights to be positive (except for v_0). Intuitively, this means that a CRM can depend only on the presence of certain motifs and not on the absence of motifs. We use the CRM model to define n binary window variables for each gene, $g.W_1, \ldots g.W_n$, where the variable for the j-th window, $g.W_j$, depends on the motif occurrences in the j-th window, $g.M_{1j}, \ldots, g.M_{kj}$. Note that the same logistic model is shared across all genes and all windows.

2.3. *Regulation model*

The last component in our model combines the information from each window to specify whether the gene is indeed regulated by the CRM. This model follows our intuition that the probability that a gene is regulated by a CRM increases with the number of windows in its upstream region that contain the CRM. The model describes this regulation probability using a logistic function:

$$P(g.R = \mathit{true} \mid g.W_1, \ldots, g.W_n) = \mathit{logit}\left(p_0 + \sum_{i=1}^{k} p_i \cdot g.M_i\right),$$

where $g.W_i$ indicates whether window i contains the CRM, and p_i specifies the extent to which the presence of the CRM in window i contributes to the overall probability that the gene is regulated. If we expect a priori that certain sequence windows are more likely to contain the CRM than others, then we can assign a higher weight to those windows. For example, when searching for CRMs in human, we might assign a higher weight to those sequence windows that are more conserved between human and mouse. In our setting, we assume that all windows are equally likely to contain the CRM and, thus, use the same weight for all windows. As we show later, this assumption leads to significant computational advantages.

2.4. *Unified model*

We combine the above three components into a unified probabilistic graphical model, shown in Fig. 2. The model defines the following joint distribution:

$$\begin{aligned} &P(g.R, g.\mathbf{W}, g.\mathbf{M} \mid g.S) \\ &\quad = P(g.R \mid g.\mathbf{W}) \prod_{j=1}^{n} \left(P(g.W_j \mid g.M_{1j}, \ldots, g.M_{kj}) \prod_{i=1}^{k} P(g.M_{ij} \mid g.S_j) \right), \end{aligned} \tag{1}$$

where $g.S_j$ is the sequence of window j, and each of the above conditional probability distributions is parameterized as described in the previous sections. Given a model parameterization, we can compute the probability that a gene is regulated by the CRM given the sequence

$$\begin{aligned} P(g.R = true \mid g.S) &= \sum_{\bar{w} \in \mathbf{W}} P(g.R = true \mid g.\mathbf{W} = \bar{w}) \\ &\quad \cdot \sum_{\bar{m} \in \mathbf{M}} P(g.\mathbf{W} = \bar{w} \mid g.\mathbf{M} = \bar{m}) P(g.\mathbf{M} = \bar{m} \mid g.S) \\ &= \sum_{\bar{w} \in \mathbf{W}} P(g.R = true \mid g.\mathbf{W} = \bar{w}) \\ &\quad \cdot \prod_{j=1}^{n} \sum_{\bar{m} \in \mathbf{M}[j]} P(g.W_j = \bar{w}[j] \mid g.\mathbf{M}[j] = \bar{m}) \cdot \prod_{i=1}^{k} P(g.M_{ij} = \bar{m}[i] \mid g.S) \end{aligned}$$

where $\bar{w}$ is a vector that ranges over all possible assignments to each of the n window variables, $\bar{m}$ is a vector that ranges over all possible assignments to each of the $k \cdot n$ motif variables, and $\mathbf{M}[j]$ corresponds to the set of motif variables for window j, $M_{1j}, \ldots, M_{kj}$.

3. LEARNING THE MODEL

In the previous section, we presented our probabilistic model. We now turn to the task of learning this model from data. Our training dataset D consists of a set of genes $\mathbf{G}$, where for each gene g we are given its upstream region sequence $g.S$ and the value of $g.R$, indicating whether g is regulated by the CRM or not. In learning the models, we need to estimate the model parameters, which include the weights of the k PSSMs, the weights of the logistic distribution $v_0, \ldots, v_k$ for the module model $P(g.W \mid g.\mathbf{M})$, and the weights of the logistic distribution $p_0, \ldots, p_n$ for the regulation model $P(g.R \mid g.\mathbf{W})$.

We follow the standard approach of *maximum likelihood* estimation: Find the parameters $\boldsymbol{\theta}$ that maximize $P(D \mid \boldsymbol{\theta})$. Our learning task is made considerably more difficult by the fact that both the *Motif* variables $g.\mathbf{M}$ and the *Window* variables $g.\mathbf{W}$ are unobserved in the training data. In this case, the likelihood function has multiple local maxima, and no general method exists for finding the global maximum. Thus, we use the *expectation maximization (EM)* algorithm (Dempster *et al.*, 1977), which provides an approach for finding a local maximum of the likelihood function. Starting from an initial guess $\boldsymbol{\theta}^{(0)}$ for the parameters, EM iterates the following two steps. The *E-step* computes the distribution over the unobserved variables, given the observed data and the current estimate of the parameters. The *M-step* then re-estimates the parameters by maximizing the likelihood of the data with respect to the distribution computed in the E-step. This estimation task differs for the different parts of the model.

3.1. E-step: Inferring modules and regulation

Our task in the E-step is to compute the distribution over the unobserved data, which in our setting means computing $P(g.\mathbf{W}, g.\mathbf{M} \mid g.S, g.R)$. As genes are assumed to be independent, this computation can be done separately for each gene, by performing inference in the Bayesian network of Fig. 2. Moreover, since the sequence variables $g.S$ are always observed, the network in which we need to perform inference is effectively a tree. Hence, inference can be performed efficiently using the clique tree algorithm (Pearl, 1988).

In general, the computations carried out by the clique tree algorithm are exponential in the number of parents of each node in the network. In our case, this means that the E-step will be exponential in the number of *Motif* and *Window* variables. As the number of motifs k in a CRM is typically small ($k \leq 5$), our main computational concern is with the number of windows. In a typical setting, we might search for CRMs in upstream regions of length 1,000 bp, using windows of length 200 bp with an overlap of 100 bp between windows. In this case, we have nine windows and the E-step can be computed efficiently.

However, there might be settings in which we wish to search for CRMs in longer upstream regions, or using larger overlaps between windows. In such settings, exact inference becomes infeasible.

When the number of windows is prohibitively large, we propose to use the *hard assignment* version of the EM algorithm. In this version, the E-step computes the most likely assignment to the hidden variables, and the M-step then re-estimates the parameters by maximizing the likelihood with respect to the assignment computed in the E-step. Under the assumption that all sequence windows are equally likely to contain the CRM, it turns out that we can find the most likely assignment to the hidden variables in time that is linear in the number of windows. The algorithm is based on the observation that if the weights p_i of the logistic function $P(g.R \mid g.\mathbf{W})$ are the same for all windows, then the value of the first term in Equation (1) is a function of the number t of window variables whose assignment is *true* and does not depend on which window variables are actually set to *true*. Hence, under the constraint that exactly t of the window variables are assigned to *true*, the problem of finding the most likely assignment can be reduced to finding

$$\begin{aligned}\{\bar{w}, \bar{m}\} &= \text{argmax}_{\bar{w}', \bar{m}'} P(g.\mathbf{W} = \bar{w}' \mid g.\mathbf{M} = \bar{m}') P(g.\mathbf{M} = \bar{m}' \mid g.S)\\ &= \text{argmax}_{\bar{w}', \bar{m}'} \prod_{j=1}^{n} P(g.W_j = \bar{w}'[j] \mid g.\mathbf{M}[j] = \bar{m}'[j]) P(g.\mathbf{M}[j] = \bar{m}'[j] \mid g.S)\end{aligned}$$

where $\bar{w}$ and $\bar{m}$ range over all possible assignments to the *Window* and *Motif* variables, respectively. Thus, the computation decomposes by windows, and the most likely assignment under this constraint is to assign to *true* the t window variables with the highest value of the expression

$$\max_{m'} \frac{P(g.W = \mathit{true} \mid g.\mathbf{M} = m') P(g.\mathbf{M} = m' \mid g.S)}{P(g.W = \mathit{false} \mid g.\mathbf{M} = m') P(g.\mathbf{M} = m' \mid g.S)}.$$

Finally, we choose t as the integer $0 \leq t \leq n$ that yields the assignment with the highest probability.

3.2. M-step: Estimating model parameters

In the M-step, our goal is to estimate the parameters for the distribution of each component of the model so as to maximize the conditional log probability of that component. For the motif model, this means estimating the parameters $P(g.M_i \mid g.S)$ for each motif i of the k motifs that maximize $\sum_{g \in \mathbf{G}} \sum_{j=1}^{n} \sum_{m \in Mij} E[M_{ij} = m] \log P(g.M_{ij} = m \mid g.S)$, where m ranges over the possible assignments to M_{ij}, $\{\mathit{false}, \mathit{true}\}$, and $E[M_{ij} = m]$ is computed in the E-step and is equal to the probability $P(g.M_{ij} = m \mid g.S, g.R)$. Unfortunately, this optimization problem has no closed form solution, and there are many local maxima. Hence, we use a conjugate gradient ascent to find a local optimum in the parameter space.

For the module model, we need to estimate the logistic weights of each motif, $w_0, \ldots, w_k$, in the distribution $P(g.W \mid g.\mathbf{M})$ that maximize $\sum_{g \in \mathbf{G}} \sum_{j=1}^{n} \sum_{m \in \mathbf{M}_j} \sum_{w \in W_j} E[W_j = w, \mathbf{M}_j = m] \log P(g.W_j = w \mid g.\mathbf{M}_j = m)$, where $\mathbf{M}_j = \{M_{1j}, \ldots, M_{kj}\}$, m and w range over the possible assignments to $\mathbf{M}_j$ and W_j, respectively, and $E[W_j = w, \mathbf{M}_j = m]$ is computed in the E-step and is equal to the probability $P(g.W_j = w, g.\mathbf{M}_j = m \mid g.S, g.R)$. Each weight is also constrained to be positive (see Section 2.2). Although there is no closed solution for this constrained optimization problem, the target function is convex, allowing us to find the optimal parameter estimates using gradient ascent on the target function.

Finally, we need to estimate the window weight parameters of the distribution $P(g.R \mid g.\mathbf{W})$ that maximize $\sum_{g \in \mathbf{G}} \sum_{w \in \mathbf{W}} E[R = r, \mathbf{W} = w] \log P(g.R = r \mid g.\mathbf{W} = w)$, where w ranges over the possible assignments to $\mathbf{W}$, r indicates whether g is regulated, and $E[R = r, \mathbf{W} = w]$ is computed in the E-step and is equal to the probability $P(g.R = r, g.\mathbf{W} = w \mid g.S)$. This optimization problem is similar to the module model case and, thus, we apply gradient ascent to find the optimal parameter setting.

3.3. Model initialization

In the previous sections, we showed how to apply the EM algorithm to improve the quality of the model in every iteration and converge to a local maximum of the likelihood function. However, the EM algorithm requires an initial model parameterization, which we need to provide. As for all applications of EM, the quality of the starting point has a large impact on the quality of the local optimum found by the algorithm.

We devised a two-phase scheme for the initialization of the motif parameters. In the first phase, we efficiently generate motif seeds of fixed length (6–8 bp) that are abundant in the upstream regions of the regulated genes. We use the identified seeds to initialize motifs by considering occurrences of these seeds with at most one mismatch. These occurrences allow us to initialize a PSSM for each seed and also to possibly extend it at its ends by positions whose information content exceeds a threshold. In the second phase, we score combinations of up to k motif seeds, using the hypergeometric significance test, allowing us to find motif combinations that *discriminate* between the regulated genes and the nonregulated ones. Thus, even in the initialization step, we search for combinations of motifs rather than individual motifs, as this initialization is more suited for the types of CRMs we wish to find.

4. EXPERIMENTAL RESULTS

We applied our module identification method to simulated and real data. The goal of the simulations was to test the ability of the algorithm to recover planted CRMs. In real data, we wished to evaluate the performance of the algorithm in recovering known modules in yeast and to apply it to discover novel modules in human. In all cases, the only input to our program was a set of upstream regions, the window length L, and the list of regulated genes, whose upstream regions are expected to contain the CRM. We designed all models such that a gene is regulated even if only one of its sequence windows contains the CRM, by fixing the weights, p_i, of all windows in the regulation model of Section 2.3 to 12 and setting $p_0 = -6$. By fixing these weights, the learning algorithm tries to find CRMs that do not occur in windows of background genes and occur at least once in the sequence windows of the regulated genes. While this results in more interpretable models, it brings up a practical consideration, which is that most of the sequence window variables, and consequently most of the motif variables, will be set to *false*, leading to an unbalanced optimization problem when updating the weights of each motif. Thus, in practice, we balance this optimization problem by considering the window with the highest posterior probability for each gene.

4.1. Simulated data

As a basic test of our procedure in a controlled setting, we generated random upstream region sequences of length 400 for 50 regulated and 50 nonregulated genes. We then planted CRMs consisting of two motifs of length 8 in a varying fraction of the regulated genes. This gives a known ground truth to which we can compare the learned models. To make the data realistic, we also planted both motifs in 25 of the nonregulated genes, but unlike the motif occurrences in the regulated genes, which were constrained to appear in proximity within the upstream regions, we randomly distributed the two motifs of these 25 nonregulated genes within the upstream regions. Our setting is thus designed so that algorithms that search for a single motif, or algorithms that search for motif combinations but ignore the spatial locations of motifs, will not succeed. Indeed, our algorithm recovered the planted motifs with high accuracy, whereas the above methods did not, as shown in the comparison of the ROC curves of Fig. 3(a). These curves compare the *false positive rate* to the *true positive rate*, when changing the probability threshold at which the *Regulates* variable, $g.R$, is considered to contain the CRM. As transcription factors vary greatly in their binding specificity, it is important that our method can recover CRMs whose motifs exhibit variation in their actual instances. To test this ability, we generated six different datasets, where in each case we varied the information per bit in the PSSM from which we sampled the planted motifs. The results of applying our method to each of these datasets are shown in Fig. 3(b), indicating that most of the planted motifs are recovered even when there is large variation in their instances. The input to our method includes a set of co-regulated genes that are expected to share a CRM. As this input set may contain errors, it is important that we recover CRMs even when only a fraction of the input regulated genes contain it. To test this, we applied our method to six different datasets that varied in the fraction of regulated genes in which we planted the CRM. Our results, in Fig. 3(c), show very good performance even when the CRM was planted in only 30 of the 50 co-regulated genes, slightly more than the 25 confounding occurrences of the motifs in the nonregulated genes.

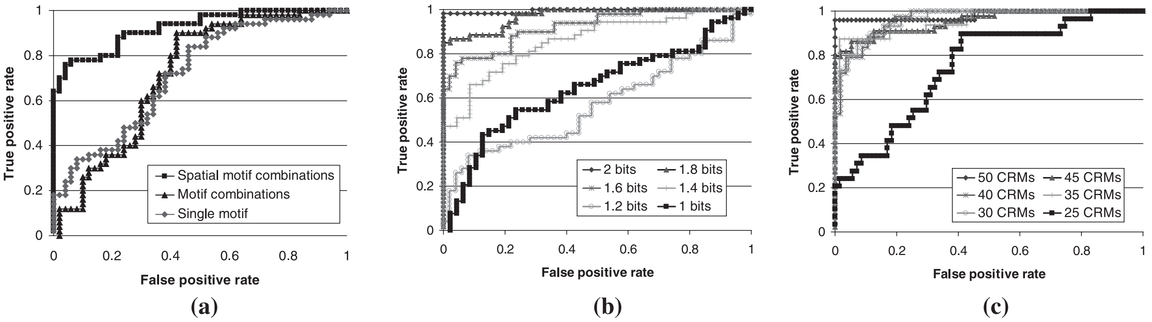


FIG. 3. Performance on simulated data, shown as ROC curves, where the x-axis is the *false positive rate*, $FP/(FP + TN)$, and the y-axis is the *true positive rate*, $TP/(TP + FN)$. In all cases, both motifs were planted in 25 of the nonregulated genes. (**a**) Comparison of different methods when the motifs were planted in all 50 regulated genes and sampled from PSSMs with 1.5 bits of information per each of the eight positions. (**b**) Performance as a function of binding specificity. In each dataset the motifs were planted in all 50 regulated genes, but were sampled from motifs with varying bits of information per position. (**c**) Performance as a function of the fraction of regulated genes in which the motifs were planted, where the planted motifs were sampled from a PSSM with 1.5 bits of information per position.

4.2. Cis-regulatory modules in yeast

To evaluate the performance of our method in a more realistic setting, we tested its ability to detect putative cis-regulatory modules in yeast. As the collection of CRMs in the literature is limited, we used the genomewide location data of Lee *et al.* (2002) to compile a collection of gene sets for which strong experimental evidence suggests that the genes in each set are regulated by the same two transcription factors. We hypothesized that the genes in each such set should thus contain a CRM consisting of the binding sites for the two TFs. Specifically, the location data contains genomewide chromatin-immunoprecipitation experiments for 106 yeast TFs, where each experiment measured the relative occupancy of the upstream regions of all yeast genes by the TF. We considered measurements with $p < 0.001$ as indicating that the TF binds the upstream region of the corresponding gene. Thus, with each TF, we associated a set of target genes to which the TF binds in vivo. To obtain gene sets that are regulated by two TFs, we computed the intersection of the targets of every pair of TFs and kept only those intersections with at least 25 genes, such that the size of the intersection was greater than would be expected by chance (scored using a hypergeometric distribution). Altogether, we found 25 such gene sets. We hypothesized that each such set contains a CRM corresponding to the two TFs and applied our method to each set using 100 bp windows with 50 bp overlaps, and 500 bp–long upstream region for each gene.[1] In each case, we took the genes in the intersection set to be regulated ($g.R = true$) and selected 100 random genes for which we assumed regulation does not take place ($g.R = false$).

To evaluate the quality of the learned CRMs, we tested whether they captured some characteristics that are specific to the regulated genes. To this end, we performed leave-one-out experiments, where in each experiment we learned a CRM using all the genes except for one, and then used the learned CRM model to compute the probability that the held-out gene is regulated by the CRM. If the CRM is indeed specific to the regulated genes, then regulated genes that are held-out should receive a higher probability for being regulated than the held-out genes that were selected at random. We measured this by computing the *classification margin*: The largest difference between the fraction of held out regulated genes whose regulation probability is above some threshold t and the fraction of held-out nonregulated genes whose regulation probability is above t, for different values of t. To evaluate the significance of the margins we obtained, we compared them to those obtained on 100 datasets, in which random yeast genes were assigned random labels (50 regulated and 50 nonregulated).

We detected significant CRMs in 11 out of our 25 sets ($p < 0.01$). These CRMs are summarized in Table 1. Since each input gene set is the intersection of the targets of two TFs, we expect the CRM to

[1] Similar results were obtained for other settings to the window size and the amount of overlap between windows.

Table 1. Significant CRMs Discovered in Yeast ($p < 0.01$)[a]

TF pair	*Margin*	*Known motifs*	*Predicted motifs*	*% correct*
FHL1, RAP1	0.654	ACACCCATACATTT (RAP1)	AATGTATG, CCATACAT (RAP1)	1/1
FHL1, YAP5	0.637	—	ATGTAAGG, CCGTACAT	—
SWI4, SWI6	0.58	TTTTCGCG (SWI4), ACGCGT (SWI6)	TTTTCCCG (SWI4), AACGCGAA (SWI6)	2/2
MBP1, SWI6	0.535	ACGCGTnA (MBP1), ACGCGT (SWI6)	GACGCGTA (MBP1), CGACGCGA (SWI6)	2/2
ACE2, SWI5	0.505	GCTGGT (ACE2), KCGTGR (SWI5)	ACACACACACA	0/2
FKH2, MCM1	0.48	TTGTTTACST (FKH2) TTWCCCnWWWRGGAAA (MCM1)	CCCTTTTC (MCM1), AGTAAACA	1/2
RAP1, YAP5	0.448	—	ATTTATGG, TCCATCAC	—
NDD1, SWI4	0.447	TTTTCGCG (SWI4)	TGTGCGTG, CACTCACAC	0/1
GAT3, YAP5	0.428	—	CTCAACTA, CTATCTGA	—
NRG1, YAP6	0.414	—	ATACGAAA, GATAGGCA	—
GAT3, PDR1	0.4	CCGCGG (PDR1)	AAGCGGCTGA (PDR1), TCGTTGCTC	1/1

[a] For each module, shown are the two TFs that putatively regulate the genes in each input set, their binding site consensus, the consensus of the learned motifs, and the correspondence between the known motifs and learned ones. We considered two consensus sequences as matching, if one was a subsequence of the other with at most two mismatches.

consist of the binding sites for the corresponding TFs. Thus, we further validated our learned CRMs by comparing the consensus sequence of their motifs to their consensus according to Kellis *et al.* (2003). Our learned motifs matched the known ones very well, recovering 7 out of the 11 known motifs. On four additional sets, our method produced CRMs that were at the 0.05 significance level. For the other 10 sets, we did not discover a significant CRM. This may be explained by the small size of the gene sets (most sizes ranged between 25 and 30) and by the fact that multifactorial regulation does not necessarily involve modular structures. Overall, the results on the location dataset demonstrate the ability of our method to detect true signals in real data.

We also applied the variant of our approach that searches for motif combinations but ignores the spatial information to this data. Our goal was to see whether such an approach can miss true signals also on real data and not only on simulated data. Interestingly, this method managed to capture most of the combinations that were previously discovered, with one important omission: No significant CRM was found on the set of genes that are bound by FKH2 and MCM1, a known true positive (Kumar *et al.*, 2000).

4.3. Cis-regulatory modules in human

Discovering cis-regulatory information in human is hard compared to yeast, as genes are typically regulated by a combination of several TFs and the sequence regions involved in the regulation are often farther from the transcription start site. We tested whether our method, which is designed for discovering these more complex regulatory signatures, can detect true CRMs in human. As the input gene sets, we used sets of genes that are known to be involved in the same process according to the GO database (Ashburner *et al.*, 2000). We hypothesized that such sets are likely to be regulated by several TFs and thus their upstream regions might contain a CRM. Specifically, we extracted all GO annotations with 25–150 genes and applied our method to each of the 381 such annotations, using 200 bp windows with 100 bp overlap between windows, and 1,000 bp upstream region for each gene.[2] For each GO process category, we treated its member genes as regulated by a common CRM ($g.R = \mathit{true}$) and selected 100 random genes to serve as a negative set ($g.R = \mathit{false}$).

As few CRMs are known in human, we evaluated the quality of the CRMs that we learned using the leave-one-out procedure described above. For each CRM, we measured the classification margin of its leave-one-out experiment and compared it to the classification margin obtained on 100 sets of random human genes. Overall, we found 83 significant CRMs, spanning 71 GO categories ($p < 0.01$), where 46 of these CRMs consisted of two motifs, and 37 consisted of three motifs, for a total of 203 motif

[2] We experimented with several parameter settings. The choice of 200 bp windows with 100 bp overlap gave the best results, though other similar settings yielded similar results.

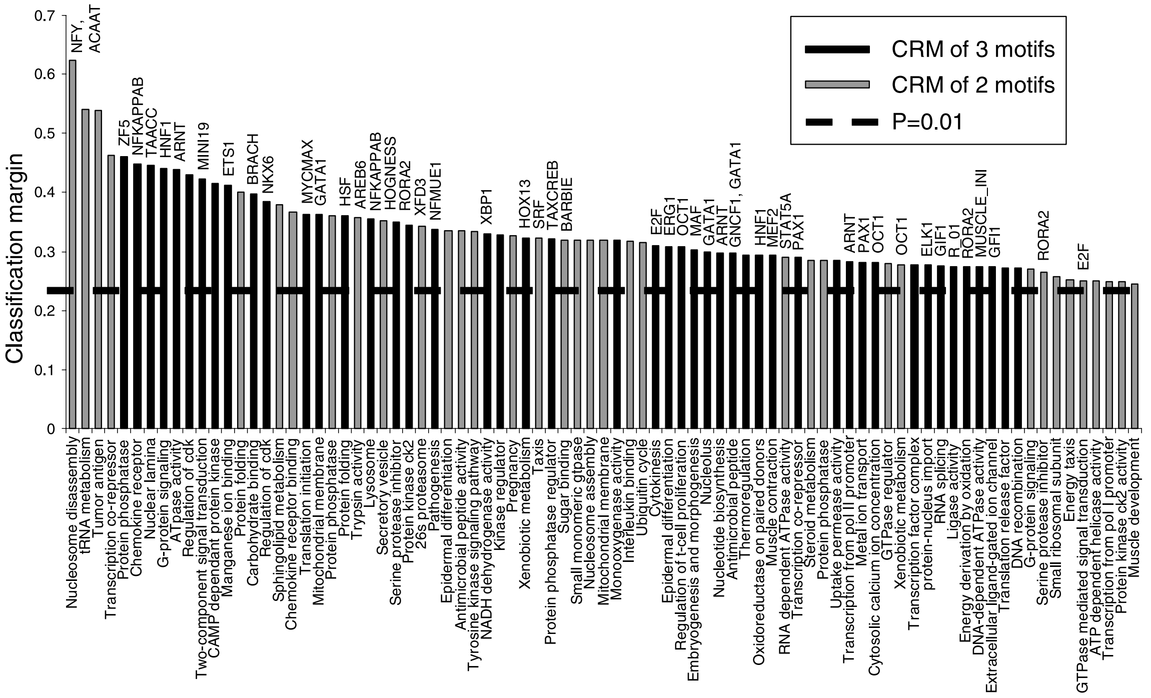


FIG. 4. Summary of the 83 significant CRMs ($p < 0.01$) that we learned on sets of human promoters, sorted by the classification margin (y-axis) obtained for each CRM in leave-one-out experiments. The x-axis indicates the GO category that was used as the input gene set when learning the CRM. In cases where one or more of the motifs that we learned for the CRM matched a known motif, we list the latter above the bar of the corresponding CRM. The dashed black line indicates the best classification margin obtained from applying our method to 100 sets of random human genes and, thus, corresponds to a p-value of 0.01.

instances. We matched this list of motifs against a list of 414 known motifs from Wingender *et al.* (2001), using the comparison method of Pietrokovski (1996). Out of the 203 motif instances that we learned, 54 corresponded to known motifs, spanning 36 distinct motifs. The leave-one-experiments, combined with the recovery of known motifs, provide strong evidence that our method indeed detected a large number of putatively true CRMs in human. A summary of all the significant CRMs that we found, including the GO category that was used as input and the known motifs that were recovered, is shown in Fig. 4.

A more detailed inspection of our results showed many GO classes for which at least one of the motifs that we learned was known in the literature to be bound by a TF that regulates the genes associated with

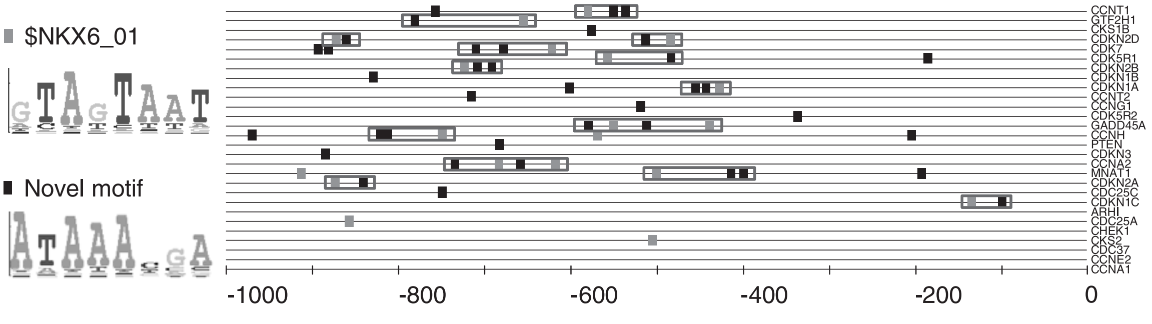


FIG. 5. Visualization of the two-motif CRM that we learned on genes that belong to the "regulation of CDK activity" class. Shown are the 1,000 bp upstream regions of all 28 genes that are assigned to this annotation according to GO. The occurrences of the two motifs in each upstream region are shown as red (NKX6 motif) and black (novel motif) rectangles, where we highlighted (blue rectangle) all 13 genes in which the two motifs occurred within 200 bp of each other in an upstream region.

that class. For example, we learned a significant CRM for protein folding genes, in which one of the motifs matched the binding site model for HSF (heat shock factor), a known activator of protein folding genes under stress and heat shock conditions. As another example, we learned a significant CRM on a set of mitochondrial membrane genes, in which one of the motifs matched the GATA PSSM. Indeed, the GATA TF is known to induce mitochondrial membrane genes. We also inspected the learned CRMs visually and found that they indeed consisted of motifs whose occurrences were close to each other in the upstream regions of the regulated genes, whereas these motifs did not occur very often in the nonregulated genes. An example is shown in Fig. 5 for the CRM learned from the "regulation of CDK activity" class. As can be seen, for this category, 13 of the 28 genes contain the CRM. In contrast, this CRM appears in only 4 of the 100 nonregulated genes (data not shown). As further support for this CRM, one of the motifs composing this CRM matches the binding site model for NKX6, a regulator of insulin biosynthesis, which also has some known role in regulating cyclin dependent kinase (CDK) genes.

CONCLUSIONS

In this paper, we have presented a novel probabilistic model for the mechanism of cis-regulation, which captures many aspects of this process, including the presence of multiple binding sites for multiple transcription factors in short DNA sequences. We presented an algorithm to learn this model from data, which allows us to predict cis-regulatory modules and their component motifs using only the raw sequence data as input. Our results demonstrated the ability of our method to find known signals in simulated data and in yeast and showed its utility for detecting cis-regulatory modules in human.

There are several directions for refining and extending our approach. First, our model requires a specification of the sequence windows in which we expect to find the CRM. We are now working on modifications to the model that will treat the entire upstream region as one sequence, but still bias the search toward finding motifs whose occurrences cluster together. Second, we are exploring the use of our approach as part of a richer probabilistic framework that combines gene expression measurements (Segal *et al.*, 2003). Finally, in some cases, we did not detect significant CRMs. While some of these may be due to limitations of our approach, others may be due to different regulation mechanisms that depend on more subtle sequence signals. Understanding the reasons for not detecting modules in such cases may reveal novel characteristics of cis-regulation.

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REFERENCES

Aerts, S., Van-Loo, P., Thijs, G., Moreau, Y., and De-Moor, B. 2003. Computational detection of cis-regulatory modules. *Bioinformatics* 19(Suppl. 2), 5–14.

Ashburner, M., *et al.* 2000. Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nature Genet.* 25, 25–29.

Bailey, T.L., and Elkan, C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 2, 28–36.

Berman, B.P., Nibu, Y., Pfeiffer, B.D., Tomancak, P., Celniker, S.E., Levine, M., Rubin, G., and Eisen, M.B. 2002. Exploiting transcription factor binding site clustering to identify cis-regulatory modules involved in pattern formation in the drosophila genome. *PNAS* 2, 757–762.

Dempster, A.P., Laird, N.M., and Rubin, D.B. 1977. Maximum likelihood from incomplete data via the EM algorithm. *J. R. Statist. Soc. B* 39, 1–39.

Frith, M.C., Hansen, U., and Weng, Z. 2001. Detection of cis-element clusters in higher eukaryotic DNA. *Bioinformatics* 17(10), 878–889.

GuhaThakurta, D., and Stormo, G.D. 2001. Identifying target sites for cooperatively binding factors. *Bioinformatics* 17(7), 608–621.

Kel-Margoulis, O.V., Ivanova, T.G., Wingender, E., and Kel, A.E. 2002. Automatic annotation of genomic regulatory sequences by searching for composite clusters. *Pac. Symp. on Biocomputing*, 187–198.

Kellis, M., *et al.* 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* 423, 241–254.

Krivan, W., and Wasserman, W.W. 2001. A predictive model for regulatory sequences directing liver-specific transcription. *Genome Res.* 11(9), 1559–1566.

Kumar, R., *et al.* 2000. Forkhead transcription factors, Fkh1p and Fkh2p, collaborate with Mcm1p to control transcription required for M-phase. *Curr. Biol.* 10(15), 896–906.

Lee, T.I., Rinaldi, N.J., Robert, F., *et al.* 2002. Transcriptional regulatory networks in *Saccharomyces cerevisiae*. *Science* 298(5594), 799–804.

Ludwig, M.Z., Patel, N.H., and Kreitman, M. 1998. Functional analysis of eve stripe 2 enhancer evolution in drosophila: Rules governing conservation and change. *Development* 125(5), 949–958.

Marsan, L., and Sagot, M.-F. 2000. Extracting structured motifs using a suffix tree—algorithms and application to promoter consensus identification. *Proc. 4th Ann. Int. Conf. on Research in Computational Molecular Biology (RECOMB)*, 210–219.

Pearl, J. 1988. *Probabilistic Reasoning in Intelligent Systems*, Morgan Kaufmann, San Francisco.

Pietrokovski, S. 1996. Searching databases of conserved sequence regions by aligning protein multiple-alignments. *Nucl. Acids Res.* 19, 3836–3845.

Pilpel, Y., Sudarsanam, P., and Church, G.M. 2001. Identifying regulatory networks by combinatorial analysis of promoter elements. *Nature Genet.* 29(2), 153–159.

Roth, F.P., Hughes, P.W., Estep, J.D., and Church, G.M. 1998. Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. *Nature Biotechnol.* 16, 939–945.

Segal, E., Barash, Y., Simon, I., Friedman, N., and Koller, D. 2002. From sequence to expression: A probabilistic framework. *Proc. 6th Ann. Int. Conf. on Research in Computational Molecular Biology (RECOMB)*, 263–272.

Segal, E., and Sharan, R. 2004. A discriminative model for identifying spatial cis-regulatory modules. *Proc. 8th Ann. Int. Conf. on Research in Computational Molecular Biology (RECOMB)*, 141–149.

Segal, E., Yelensky, R., and Koller, D. 2003. Genome-wide discovery of transcriptional modules from DNA sequence and gene expression. *Bioinformatics* 19(Suppl. 1), S273–S282.

Sharan, R., Ovcharenko, I., Ben-Hur, A., and Karp, R. 2003. Creme: A framework for identifying cis-regulatory modules in human-mouse conserved segments. *Bioinformatics* 19(Suppl. 1), S283–S291.

Sinha, S. 2002. Discriminative motifs. *Proc. 6th Ann. Int. Conf. on Research in Computational Molecular Biology (RECOMB)*, 291–298.

Tavazoie, S., Hughes, J.D., Campbell, M.J., Cho, R.J., and Church, G.M. 1999. Systematic determination of genetic network architecture. *Nature Genet.* 22(3), 213–215, 281–285.

Thompson, W., Rouchka, E.C., and Lawrence, C.E. 2003. Gibbs recursive sampler: Finding transcription factor binding sites. *Nucl. Acids Res.* 31(13), 3580–3585.

Wasserman, W.W., and Fickett, J.W. 1998. Identification of regulatory regions which confer muscle-specific gene expression. *J. Mol. Biol.* 278(1), 167–181.

Wingender, E., *et al.* 2001. The TRANSFAC system on gene expression regulation. *Nucl. Acids Res.* 29, 281–283.

Yuh, C.H., Bolouri, H., and Davidson, E.H. 1998. Genomic cis-regulatory logic: Experimental and computational analysis of a sea urchin gene. *Science* 279, 1896–1902.

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