Computational Biology: Towards Deciphering Gene Regulatory Information in Mammalian Genomes

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SUMMARY

Computational biology is a rapidly evolving area where methodologies from computer science, mathematics and statistics are applied to address fundamental problems in biology. The study of gene regulatory information is a central problem in current computational biology. This paper reviews recent development of statistical methods related to this field. Starting from microarray gene selection, we examine methods for finding transcription factor binding motifs and *cis*-regulatory modules in co-regulated genes, and methods for utilizing information from cross-species comparisons and ChIP-chip experiments. The ultimate understanding of *cis*-regulatory logic in mammalian genomes may require the integration of information collected from all these steps.

KEY WORDS: *Cis*-regulatory module; ChIP-chip; Comparative genomics; Gene expression; Microarray; Motif discovery; Transcription factor.

1. Introduction

The human genome is composed of about 3 billion letters (A, C, G, or T) arranged in linear molecules called DNA. Distributed within the DNA are about 30,000 coding regions (genes) that encode proteins (International Human Genome Sequencing Consortium, 2001, 2004). These genes serve as blueprints for the synthesis of mRNAs (i.e., transcription of the gene) that in turn are used as templates for the production of the proteins (i.e., translation the mRNA). A more detailed depiction of these processes is presented in Figure 1, and a list of common terminology is given in Table 1.

[Figure 1 about here]

[Table 1 about here]

Both transcription and translation are tightly regulated temporally and spatially. For example, a gene may be transcribed only during one or more specific stages of embryonic development and only in specific cell lineages during those stages. The precise control of when and where to transcribe a gene depends on the interaction among *trans*-acting protein factors and *cis*-acting sequence elements. *Trans*-acting proteins, often transcription factors, are protein products of certain genes that serve as regulators of the expression of other genes. These proteins diffuse in the cell, recognize and bind to certain sequence segments in DNA. Upon binding, they can induce changes of chromatin structure or interact with basal transcriptional machinery, and thereby initiate, repress or modulate transcription of genes close to the binding site. The loci on the DNA where *trans*-acting proteins bind to are called *cis*-acting elements, also referred to as *cis*-regulatory elements or *cis*-elements. A given transcription factor typically recognizes a specific though not totally conserved sequence pattern called a binding motif. A binding motif is usually 6~30 base pairs in length. In the motif, some nucleotides (e.g., A, C, G or T) tend to occur more often than the others in specific positions (Figure 2). Different transcription factors may have different binding motifs,

and multiple transcription factors can bind cooperatively to a *cis*-element that contains several different binding motifs that are closely clustered together. At any time, the particular composition of transcription factors active in the nucleus of a cell determines which subset of *cis*-elements is bound and activated in this cell. This combinatorial binding allows a few hundred transcription factors to control the spatial and temporal expression patterns of tens of thousands of genes. In a very real sense, *cis*-regulatory sequences are the hardwired "control logic" in the genome. The development of a fertilized egg to an advanced embryo with complex body plans and organs may be, to a first approximation, regarded as the successful implementation of the transcription programs encoded in these *cis*-elements by successive lineages of cells during development (Davidson, 2001).

[Figure 2 about here]

Although most genes (coding-regions) in the human genome have been identified and annotated, the *cis*-elements that control their expression are largely unknown. These elements can be very far away from the coding regions, e.g., 10,000 bps away from the transcription start site. The identification of such elements in the non-coding regions, which account for more than 95% of the genome, is thus a challenging problem whose solution requires not only new experimental data but also new statistical and computational methods.

2. Sources of Information and Outline of Review

In this section we introduce the sources of information that could be used for the prediction of *cis*-regulatory elements, as a preparation for the review in subsequent sections of statistical methods for such predictions.

The first type of information is based on sets of co-regulated genes. Such gene sets may be compiled based on existing biological knowledge. Alternatively, they may be obtained through analysis of data from global gene expression profiling experiments. In section 3 we review statistical methods for the selection of co-regulated genes. Such co-

regulated gene sets are important for *cis*-regulatory analysis because, compared to random sequences, the sites bound by a transcription factor are enriched in the set of genes that are co-regulated by this factor. Thus one way to identify the motif is to look for over-represented sequence patterns in the genomic regions near these genes. Section 4 reviews statistical models and algorithms for motif discovery in such genomic sequences.

The second type of information arises from the observation that transcription factor binding sites tend to be clustered together. This clustering facilitates the synergistic interactions of the transcription factors to elevate or decrease the level of transcription. Therefore, if a predicted site is found to be in the vicinity of other binding sites, it is more likely to be real. Thus, the power for *cis*-element prediction can be enhanced by incorporating the combinatorial patterns of DNA motifs (i.e., *cis*-regulatory modules, CRM) into the statistical methods. Methods for CRM discovery are treated in section 5.

A third source of information is provided by evolution. *Cis*-regulatory elements with essential gene regulatory roles are under selection pressure and are likely to be conserved across related species. Hence, even if our primary interest is in human (or mouse), the use of sequence information from multiple mammalian or vertebrate species will enhance the reliability of our predictions. The use of multiple genomes in *cis*-regulatory analyses is reviewed in section 6.

The recent development of the technology of chromatin immunoprecipitation on microrarray (ChIP-chip) provides the fourth source of information for *cis*-regulatory analysis. This technology enables large-scale screening for the binding regions of specific transcription factors. At a resolution of ~1-2 kb, such experiments can identify regions in the genome where a given transcription factor may bind. Thus ChIP-chip experiments have the potential to greatly reduce the search space for motif discovery. Section 7 examines methods for the analysis of this new type of data.

The availability of these different sources of information poses the statistical challenge of how to simultaneously employ them to make predictions of mammalian *cis*-elements a reality. It is our hope that this review will stimulate interest in the statistics community on this central problem in computational biology.

3. Gene Selection from Microarray Experiments

3.1 *Gene Selection by Cluster Analysis*

After a decade of development, microarray technology (Schena et al., 1995; Lockhart et al., 1996) has reached a very high degree of throughput and capacity. Each single microrarray profile can provide measurements on the expression level of almost all known human genes (say G=35,000 genes) for a particular sample. Low-level analyses including expression index computation and array normalization are important for conducting a solid downstream study. These issues were described in Li and Wong (2001), Yang et al. (2002), Bolstad et al. (2003), Irizarry et al. (2003) and Speed (2003) and are not discussed here. Suppose we have N samples collected under N biological conditions (some of them could be biological replicates), the resulting data is represented as a G by N data matrix (Figure 3a). How could one select sets of co-regulated genes based on this data? If the collection of samples (i.e., conditions) is large and diverse, then conceptually the simplest approach is to apply cluster analysis to group genes into clusters (Figure 3b). Each gene is represented by a vector in N-dimensional space and a similarity metric is defined. The clusters are constructed so that a gene vector is more similar to those within its cluster than those outside. Visual inspection of the clustering results by the heat map (Eisen et al., 1998) often reveals further information about the clusters. The most commonly used clustering algorithms are hierarchical clustering and k-means clustering. We will not review these classic methods further, as excellent coverage of them can be found in many books (Hastie, Tibshirani, and Friedman, 2001, pp 453-484; Speed, 2003, pp 159-199).

[Figure 3 about here]

An issue of special importance for *cis*-regulatory analysis is the handling of "scattered genes". In most biological experiments, a gene not relevant to the biological processes under study may nonetheless show substantial variation. By chance, the expression pattern of such a scattered gene may be loosely similar to the pattern shared by a group of tightly co-regulated genes. As a result, the scattered gene may be incorrectly clustered with this group of co-regulated genes and reduce the signal to noise ratio in subsequent *cis*-regulatory analysis. To handle scattered genes, Tseng and Wong (2005) considered the probability for two genes to be co-clustered by k-means on a random subsample of the genes. A group of genes is said to form a "tight cluster" if all the pair-wise, within-group co-clustering probabilities are sufficiently high. The tight clusters are sequentially extracted until no further subset of genes satisfies the tightness criterion. The resulting tight clusters are good starting points for *cis*-regulatory analysis. We note that random subsampling was used earlier by Tibshirani et al. (2001) and Dudoit and Fridlyand (2002) to determine the optimal number of clusters in cluster analysis.

In analyzing microarrays collected from different series of experiments with possibly different array types, it is sometimes desirable to summarize the similarity of gene expression within each series by a similarity index and then analyze the similarity indexes across the different series of experiments. For example, if there are 20 different series of experiments then for each pair of genes we will have a 20-dimensional vector for the 20 within-series correlations (called first-order correlation indexes). One may perform cluster analysis on these first-order indexes to identify clusters of gene pairs with high "second-order" correlations. A group of genes linked through this analysis may be involved in a process that is activated in some but not all of the series of experiments. Zhou et al. (2005) developed this

approach for the analysis of transcriptional programs in yeast based on 618 yeast arrays from 39 different series of experiments.

Finally, we note that a recent and promising development of clustering algorithms is to formulate a parametric model and to perform fully Bayesian analysis (Fraley and Raftery, 1998). When the model is a mixture of Gaussian distributions, the resulting algorithm is closely related to the k-means algorithm. The advantage of modeling is that one can modify the model to handle complications such as different correlation structures in the different clusters. For example, scattered genes can be handled by specifying a very large scaling parameter for one of the components in the mixture. The number of clusters can also be inferred by considering the Bayes factors. Perhaps due to its computational complexity, this promising approach has not yet been used widely in large scale gene expression studies.

3.2 *Gene Selection by Comparative Analysis*

3.2.1 *Introduction, Multiple Testing, and False Discovery Rate*

Comparative analysis is another widely used method to select genes. This analysis compares gene expression levels between different experimental conditions and aims to find genes that show desired variation in expression. Often, the analysis is driven by hypothesis testing. A simple example is to find genes that are differentially expressed between wild type mice and mutant mice where a transcription factor is knocked out. Thus, for each gene, one wishes to test the null hypothesis H_0: "the gene's mean expression level does not depend on genetic background" against the alternative H_1: "the gene's mean expression levels are different between wild type and mutant mice". Genes for which H_0 is rejected are the potential targets of the transcription factor.

The detection of differentially expressed genes is an extensively studied topic (e.g., Kerr, Martin, and Churchill, 2000; Baldi and Long, 2001; Efron et al., 2001; Newton et al., 2001; Tseng et al., 2001; Tusher, Tibshirani, and Chu, 2001; Dudoit et al., 2002; Lönnstedt

and Speed, 2002; Pan, Lin, and Le, 2003). Readers are referred to Cui and Churchill (2003) for a review of early efforts. A special issue here is the adjustment for multiple hypothesis testing (Dudoit et al., 2002). If for each of 1000 genes, we conduct a canonical *t*-test at level 0.05, on average 50 false rejections will be made. Thus, even if none of the alternative hypotheses is true, we may get some "significant" results, all of which are false positives. To prevent these misleading results, Benjamini and Hochberg (1995) proposed to control the false discovery rate (FDR) in the context of multiple testing. The FDR error measure is defined to be the expected fraction of rejections that are false positives. Intuitively, controlling FDR at level 0.05 means that among all rejections, the percentage of false positives is 5% or less on average. Since controlling FDR may allow a few false positives in the rejections, it is less conservative than controlling the family-wise error rate (FWER), which controls the probability of at least one false positive. The FDR concept has been adopted and further developed for applications in microarray analysis (e.g., Efron et al., 2001; Tusher et al., 2001; Reiner, Yekutieli, and Benjamini, 2003; Storey and Tibshirani, 2003). This development has impacted the routine analysis by biologists and has been incorporated into many software packages (e.g., SAM: Tusher et al., 2001). Readers are referred to Benjamini and Hochberg (1995), Benjamini and Yekutieli (2001), Storey (2002, 2003), Storey, Taylor, and Siegmund (2004) for statistical procedures that control or estimate FDR, and to Dudoit, Shaffer, and Boldrick (2003) for a comprehensive review of multiple testing in microarray experiments.

3.2.2 *Increasing Power of Multiple Testing by Pooling Information*

The introduction of FDR to microarray analysis does not completely address the issue of power, i.e., the probability of selecting truly differentially expressed genes. Although at the same nominal error rate level, say 0.05, a procedure that controls FDR tends to have higher power than a procedure that controls FWER, this is only because the FDR procedure has

adopted a different error rate measure and a relaxed rejection cutoff. To make this clear, one can compare the Bonferroni adjustment that controls FWER and the BH procedure (Benjamini and Hochberg, 1995) that controls FDR. In both cases, a raw *p*-value is computed for each individual test, and a null hypothesis is rejected if its *p*-value is less than *c*. If both methods compute the raw *p*-values in the same way, the only difference between the two is the way the cutoff *c* is chosen. BH usually chooses a larger cutoff and therefore rejects more often than Bonferroni. However, if one is only interested in top 100 genes with the smallest *p*-values, the two methods will provide exactly the same list of genes since they are all based on the same set of test statistics. In this sense, BH does not represent a gain in power. To increase the real power, one needs to change the order of test statistics so that among, say, the top 100 genes, the number of truly interesting genes will be increased. This can only be achieved through constructing a set of test statistics with higher discriminating ability.

In microarray experiments, the concern about power mainly arises from the fact that due to cost constraint, the number of replicates is typically small. Given the large number of genes involved and the small number of replicates available, it is not unusual to find genes with very small within-group sample variance just by chance. Much of the noise in gene selection stems from this small variance problem which, for example, can result in extremely large *t*-statistics in two sample comparisons. Borrowing information from multiple genes to stabilize the variance estimates for individual genes provides a good solution to this problem (Figure 3c).

The most general approach to this problem is based on Bayesian or Empirical Bayes analysis. Let y_{gcr} denote the log-expression value of gene g ($\in \{1, \ldots, G\}$) under condition c($\in \{1,2\}$) and replicate r ($\in \{1, \ldots, R_{gc}\}$), μ_{gc} and $\sigma^2_{gc} \equiv \sigma^2_g$ denote the mean and variance of y_{gcr} for fixed g and c, $\bar{y}_{gc}$ and s^2_{gc} denote the corresponding sample mean and sample

variance. Define $d_g = R_{g1} + R_{g2} - 2$, $v_g = 1/R_{g1} + 1/R_{g2}$, $\beta_g = \mu_{g1} - \mu_{g2}$, $\hat{\beta}_g = \bar{y}_{g1} - \bar{y}_{g2}$, and $s_g^2 = \{(R_{g1} - 1)s_{g1}^2 + (R_{g2} - 1)s_{g2}^2\}/d_g$. Following Lönnstedt and Speed (2002) and Smyth (2004), one may assume:

$$\hat{\beta}_g \mid \beta_g, \sigma_g^2 \sim N(\beta_g, v_g \sigma_g^2) \tag{1}$$

$$s_g^2 \mid \sigma_g^2 \sim \frac{\sigma_g^2}{d_g} \chi^2(d_g) \tag{2}$$

$$\beta_g \mid v_0, \sigma_g^2 \quad \begin{cases} = 0 & \text{with prob. } 1-p \\ \neq 0 & \text{with prob. } p \end{cases} \tag{3}$$

$$\beta_g \mid v_0, \sigma_g^2, \beta_g \neq 0 \sim N(0, v_0 \sigma_g^2) \tag{4}$$

$$\frac{1}{\sigma_g^2} \mid d_0, s_0^2 \sim \frac{1}{d_0 s_0^2} \chi^2(d_0) \tag{5}$$

In this model, the parameters (β_g, σ_g^2) are assumed to be i.i.d. realizations from a prior distribution specified by formula (3)~(5); $\hat{\beta}_g$ and s_g^2 are assumed to be independent given (β_g, σ_g^2). A full Bayesian analysis of the model would introduce a joint prior for hyperparameters v_0, d_0 and s_0^2, and make inference based on the joint posterior of all unknowns. Considerations about computational efficiency usually lead to the use of empirical Bayes approaches, where hyperparameters are replaced by their point estimates obtained from matching observed data to their marginal distributions (e.g., Wright and Simon, 2003; Smyth, 2004). Given hyperparameters, the detection of differentially expressed genes can be based on log posterior odds $B_g = \log\{p(\beta_g \neq 0 \mid \hat{\beta}_g, s_g^2) / p(\beta_g = 0 \mid \hat{\beta}_g, s_g^2)\}$ (Lönnstedt and Speed, 2002). From the fact that $E(1/\sigma_g^2 \mid s_g^2, s_0^2, d_0) = (d_0 + d_g)/(d_0 s_0^2 + d_g s_g^2)$, Smyth (2004) proposed to use another statistic, a moderated *t*-statistic, to rank genes:

$$t_g = \frac{\hat{\beta}_g}{\sqrt{v_g \dfrac{d_0 s_0^2 + d_g s_g^2}{d_0 + d_g}}} \qquad (6)$$

The moderated *t*-statistic can be viewed as a modified version of the canonical *t*-statistic ($\hat{\beta}_g / \sqrt{v_g s_g^2}$) where s_g^2 in the denominator is replaced by a shrinkage estimator $(d_0 s_0^2 + d_g s_g^2)/(d_0 + d_g)$ of the variance σ_g^2. When d_g and v_g do not vary across genes, the log posterior odds B_g is a monotonic increasing function of $|t_g|$. Given d_0 and s_0^2, t_g follows a *t*-distribution with degrees of freedom $d_0 + d_g$ when $\beta_g = 0$ (H_0), and it follows a scaled *t*-distribution $(1 + v_0/v_g)^{1/2} t_{d_0 + d_g}$ when $\beta_g \neq 0$ (H_1). Thus, the information borrowed from other genes allows one to learn what values σ_g^2 usually take. This information introduces additional degrees of freedom to individual tests which can be used to stabilize the variance estimate and to better separate the distributions of the test statistics under H_0 and H_1. This explains why pooling information results in a better ordering of genes (compared to the canonical *t*) and increases power. Notice that in the model above, once t_g is given, changes in v_0 (shrinking the mean component) may change B_g but will not change the relative order of genes as long as d_g and v_g do not vary across genes. Although this property may not hold in other models (e.g., hierarchical models without assuming a conjugate prior), real microarray data analysis suggested that the gain from mean shrinkage is small compared to variance shrinkage (Ji and Wong, 2005a). Since d_0 and s_0^2 are estimated from data in real applications, the null distribution of t_g that is required for FDR control is not readily available. If the model assumptions hold and d_0 and s_0^2 can be estimated accurately, then this distribution can be approximated by $t_{d_0 + d_g}$. When the model assumptions do not hold, one may still use t_g but other techniques (e.g., permutations) may be required to establish the null distribution.

The idea of improving inference by pooling information can be traced back to the James-Stein estimator (James and Stein, 1961). Early applications of this idea to differential gene selection include Baldi and Long (2001), Newton et al. (2001), Tseng et al. (2001), Lönnstedt and Speed (2002), etc. In the context of microarray gene selection, the idea of replacing s_g^2 in canonical *t*-statistics by a variance shrinkage estimator was proposed earlier by Baldi and Long (2001), but their work used an *ad hoc* method to choose hyperparameters. The same idea was also reported in Wright and Simon (2003), Cui et al. (2005) and Ji and Wong (2005a). Compared to Baldi and Long (2001), more principled ways to estimate hyperparameters or construct variance shrinkage estimators were derived in Wright and Simon (2003), Smyth (2004) and Ji and Wong (2005a) using empirical hierarchical Bayes approaches. Cui et al. (2005) proposed a variance shrinkage estimator directly from a James-Stein type estimator. We note that Tusher et al. (2001) and Efron et al. (2001) handled the small replicate problem by adding a constant to the denominator of the canonical *t*-statistics. Their methods are in principle similar to the methods discussed here. The idea of pooling information was generalized to ANOVA with the proposal of modified *F*-statistics with an augmented degree of freedom (Wright and Simon, 2003; Smyth, 2004; Cui et al., 2005). Parallel to the normal hierarchical models, information pooling via Gamma and inverse Gamma models were also developed by Newton et al. (2001) and Newton et al. (2004). Although different methods may differ in terms of the details of how information was pooled, their applications to microarray gene selection all conveyed a consistent message, that is, that the power of multiple testing can be increased by pooling information across individual tests.

3.2.3 *Comparative Analysis with Sophisticated Criterion Matching*

Very often biologists have specific subject matter knowledge that they want to incorporate into the comparative analysis. A study of the sonic hedgehog (*Shh*) signaling pathway in our own group (in collaboration with McMahon lab at Harvard) provides a good

example. In this study, two types of loss-of-function mutant mice – *ptc* and *smo* – were examined. In normal wild type mice, *Smo* (smoothened) is a gene that turns on the transcription factor *Gli*. *Ptc* (patched) is another gene that represses *Smo*. In the presence of the signal molecule *Shh*, *Ptc* is inactivated, *Smo* becomes active and turns on *Gli*. On the other hand, if *Shh* does not exist, *Ptc* represses *Smo*, and *Gli* is turned off. Therefore, when the gene expression profiles were obtained for mutant (*ptc*, *smo*) as well as wild type (*wt*) mice, genes that are activated by *Gli* were expected to show a pattern "*ptc*>*wt*>*smo*", whereas genes that are repressed by *Gli* would show "*ptc*<*wt*<*smo*". Correspondingly, the null hypothesis here is a complex composite null H_0: "Not {*ptc*>*wt*>*smo* or *ptc*<*wt*<*smo*}". Our actual study also involves several different developmental stages and tissue types. Hence criteria such as "*ptc*<*wt*<*smo* and (*limb bud*>*wt* or *head*>*wt*)" are frequently used to screen for genes. Statistical tools that detect general differential expression are not directly applicable. For example, one challenge is controlling FDR for a complex composite null hypothesis. Although permutation tests may be used to obtain the distribution for a null hypothesis of the form "*ptc*=*wt*=*smo*", it is not clear how these tests can be used to obtain the null distribution for H_0 in this case, which contains not only "*ptc*=*wt*=*smo*" but also components such as "*ptc*<*wt*=*smo*", "*ptc*<*wt*>*smo*", etc. As for the construction of test statistics, although ANOVA *F*-tests were proposed to detect differentially expressed genes across multiple conditions, they were mainly used to find genes with any differential expression patterns, and were not used to find a specific pattern as in the present case. If we were to decompose the problem into individual two sample tests (i.e., testing "*ptc*>wt" and "*wt*>*smo*"), we would be faced with the challenge of adjusting for multiple correlated tests resulting from use of the common *wt* data. Thus, comparative analysis with sophisticated criterion matching poses new challenges for computational biologists. A few early efforts towards this direction include Kendziorski et al. (2003) and Ji and Wong (2005a). In Ji and

Wong (2005a), for example, the sonic hedgehog problem was handled using an empirical hierarchical Bayes approach. Log-expressions were described via a normal hierarchical model. Information from all genes was pooled to estimate the model's variance components which were then fixed, and for each gene, the posterior probability that an expression pattern is satisfied was estimated via Monte Carlo. This posterior probability was used as a statistic to order the genes. The use of posterior probabilities provided the flexibility to handle complex patterns in comparative analysis. It was observed in both simulations and real applications that this combined strategy of information pooling and criterion matching significantly increased the power of gene selection.

4. Transcription Factor Binding Site and Motif Discovery

4.1 *Motif Representation*

After a group of co-regulated genes is available, we may wish to look for their common regulatory mechanisms. If co-regulation is induced by the binding of a common set of transcription factors, one would expect that transcription factor binding sites (TFBS) will be enriched in these genes' surrounding DNA sequences. By searching for overrepresented sequence patterns within the group of co-regulated genes (selected as differentially expressed genes, genes clustered according to their transcriptional profiles, etc.), one can infer both the motifs and their positions. This strategy was proven to be successful in studying gene regulation of lower organisms such as *E. coli* (e.g., Stormo and Hartzell, 1989) and yeast (e.g., Roth et al., 1998; Hughes et al., 2000). An appropriate representation of the motif is necessary for any motif or TFBS finding algorithm. A motif is usually described in one of two ways: as a consensus sequence or as a position specific weight matrix (PWM) (Stormo, 2000). The consensus sequence characterizes a motif by the most frequent base at each position. To allow for degeneracy, the characters that are used to describe a motif can be extended from {A, C, G, T} to IUPAC characters (IUPAC, 1986), e.g., "TATRNT" is a

consensus where "R" stands for a purine (A or G) and "N" stands for a base of any type. The PWM characterizes a motif by a matrix, counting the occurrence frequencies of each type of nucleotide at each position (Figure 2). The frequencies in a PWM can be summarized in different ways, either by counting the number of A, C, G, T at each position from a set of known TFBSs (alignment matrix or count matrix), by computing their observed frequencies (frequency matrix), or by taking the logarithm of the likelihood ratio between the observed frequency and the background frequency (weight matrix). Here the background frequency is the frequency of observing a nucleotide in the bulk of non-regulatory genomic sequences. If the positions within a TFBS are assumed to be independent, the alignment matrix can be modeled as a product multinomial, i.e., the observed counts for each position are assumed to follow a separate multinomial distribution $M(\boldsymbol{\theta}_i)$, where $\boldsymbol{\theta}_i = (\theta_{iA}, \theta_{iC}, \theta_{iG}, \theta_{iT})^T$ is the probability that each of the four types of bases occur in the i^{th} position of a TFBS. The weight matrix is only a first order approximation of what is happening in reality. Different positions within a TFBS can be correlated, and examples exist for which incorporating this correlation into the model may increase the performance of downstream analysis (e.g., Zhou and Liu, 2004).

If the binding motif of a transcription factor is known from experiments, we can use it to score sequence patterns and hence to predict TFBSs by scanning genomic DNA sequences (e.g., Quandt et al., 1995; Frith, Hansen, and Weng, 2001). This problem is called *known motif mapping*. Efficient algorithms for computing the exact probability for a sequence pattern to exceed a given score were given in Staden (1989) in the case of independent background sequence models and in Huang et al. (2004) in the case of Markov background models. A more challenging problem is *de novo motif discovery*, i.e., the problem of finding previously unknown motifs and their corresponding TFBSs. This problem is reviewed in the next section.

4.2 *De novo Motif Discovery: Word Enumeration and Weight Matrix Updating*

One approach to *de novo* motif discovery is based on word enumeration. This technique systematically checks all possible words (i.e., sequence patterns like "TATAAT") in co-regulated genes. Word frequencies are evaluated, and overrepresented words whose occurrence is unlikely due to chance are selected as candidate motifs. Methods in this category include van Helden, Andre, and Collado-Vides (1998), Sinha and Tompa (2000, 2002), Hampson, Kibler, and Baldi (2002), etc. For example, in Yeast Motif Finder (YMF) (Sinha and Tompa, 2000, 2002), a third order Markov chain is used to model the random background. The expected number of occurrences of each word and the corresponding variances are computed. Words with high z-scores are then selected and filtered according to additional criteria.

An extension of the word enumeration method is the dictionary model (Bussemaker, Li, and Siggia, 2000), implemented in an algorithm called MobyDick. In this model, frequently used words are compiled into a dictionary, and a sequence is modeled as generated by concatenating words sampled from the dictionary. Each word in the dictionary has a usage probability. The problem of motif finding is transformed into the problems of (i) building the dictionary and (ii) determining the parsing of DNA sequences using the words in the dictionary. To build up the dictionary, MobyDick starts with a set of single-letter words (e.g., A, C, G, T). These words are concatenated to form longer words whose significance is judged by statistical tests based on their predicted frequencies from the current dictionary. Longer words that are significantly overrepresented are added to the dictionary. Given the updated dictionary, sequences are then partitioned probabilistically, and a Newton-Raphson procedure is used to get MLE for word usage frequencies. The procedure is repeated until no new words can be added. Due to the introduction of heuristics to build up the dictionary, MobyDick does

not check all possible words exhaustively, and can be used to parse large scale genome sequences.

In word enumeration methods, since the number of all possible words increases exponentially with word length and the size of the character set used to construct words, brute force enumeration is unrealistic for longer motifs. The MobyDick strategy that concatenates overrepresented words into longer words provides one solution to handle this problem, although there exists the possibility of missing some important motifs. Another possible strategy one can use is to check all words that appear in the sequences rather than all possible words (e.g., Liu, Brutlag, and Liu, 2002). This will change the computational complexity to be linear in sequence length and is more appropriate when total sequence length is much smaller than the size of word space. Like microarray gene selection, word enumeration is a multiple testing problem with special correlation structure (e.g., occurrences of ATTTGCAT and TTTGCATA are not completely independent) for which FDR control is a relevant issue.

Besides word counting, an alternative and perhaps more widely used approach for motif discovery is PWM updating. In this approach, an initial PWM is iteratively refined by alternating steps of TFBS prediction and PWM updating. The logic behind this approach is that, if a motif is enriched in a set of sequences, the alignment of its TFBSs will emerge as a non-randomly conserved pattern as compared to alignments of random segments. Unlike word enumeration methods which mainly report their results as consensus sequences, PWM updating methods report motifs as PWMs which could be easily converted into consensus sequences.

One of the earliest PWM updating methods is based on progressive alignment, exemplified by CONSENSUS (Stormo and Hartzell, 1989; Hertz, Hartzell, and Stormo, 1990; Hertz and Stormo, 1999). To find motifs, CONSENSUS first uses each k-word (word of length k) from the first sequence to construct an alignment matrix. The initial matrices are

used to scan the second sequence and are updated using the best match (or matches) from the scan. Next, updated matrices with lower information content are discarded. The preserved matrices with high information content are the seeds for the next round of scanning and updating which will subsequently be applied to the third sequence. The algorithm continues until all sequences in question are included. As a greedy algorithm, the performance of CONSENSUS will be affected by the order in which sequences are included into the analysis. Multiple runs with different starting points are needed to avoid trapping in local modes.

Another class of PWM updating methods is based on explicit sequence modeling. These types of methods include EM based algorithms such as MEME (Bailey and Elkan, 1994, 1995) and Gibbs sampler based algorithms such as Gibbs Motif Sampler (Lawrence et al., 1993; Liu, 1994; Liu, Neuwald, and Lawrence, 1995; Neuwald, Liu, and Lawrence, 1995), AlignACE (Roth et al., 1998) and BioProspector (Liu, Brutlag, and Liu, 2001), etc. In these methods, sequences are viewed as generated from two models – a background model and a motif model. Unknown to us, there is an indicator for each position in the sequence which tells us whether that position is a start of a TFBS or not. Motif discovery is then formulated as a problem of inferring model parameters and the status of unknown motif indicators. By treating the unknown motif indicators as missing data, the problem can be solved in an iterative manner: (i) given the current status of motif indicators, estimate the parameters of the background and motif models; (ii) given current model parameters, re-estimate physical locations of TFBSs and update the status of motif indicators. The next section will provide more details about this approach.

4.3 *De novo Motif Discovery: Explicit Sequence Modeling*

4.3.1 *Model and Likelihood Function*

Using the same notations as Jensen et al. (2004), we let $\mathbf{S} = (S_{ij})$ denote the full set of sequences of total length L, where S_{ij} is the j^{th} nucleotide of sequence i ($i \in \{1, \ldots, I\}$). In the

simplest case, there is only one motif with fixed length W. Each position S_{ij} in the sequence has a prior probability q of initiating a TFBS. If S_{ij} is the start of a TFBS, its associated motif indicator is given by A_{ij}=1, otherwise A_{ij}=0. $\mathbf{A} = (A_{ij})$ is the full set of unknown motif indicators. Assume that different positions in the sequences are independent, and that any base from the background is drawn from a multinomial distribution $M(1;\boldsymbol{\theta}_0)$, and that bases within a TFBS are drawn from a product multinomial $PM(1,\ldots,1;\boldsymbol{\Theta})$. Here, "1" stands for the sample size of the multinomial distribution, $\boldsymbol{\Theta} = (\boldsymbol{\theta}_1, \ldots, \boldsymbol{\theta}_W)$, $\boldsymbol{\theta}_i = (\theta_{iA},\ldots,\theta_{iT})^T$, and θ_{ik} is the probability that base k occurs in the background (when i=0) or at the i^{th} position of a TFBS (when $i \neq 0$). We further let $|\mathbf{A}|$ denote the total number of TFBSs, $\mathbf{S}(\mathbf{A})$ denote the set of bases in all TFBSs, $\mathbf{S}(\mathbf{A}_{(j)})$ denote the set of bases in the j^{th} position of all TFBSs, and $\mathbf{S}(\mathbf{A}^{\mathbf{c}})$ be the set of background bases. Define $\mathbf{N}(.) = (n_A(.), \ldots, n_T(.))^T$ to be a counting function which counts how many times each type of base occurs in its argument, e.g., $\mathbf{N}(\mathbf{S})$ is a vector with four components which counts the occurrence of A, C, G and T in $\mathbf{S}$. For convenience, we use $\mathbf{N}(\mathbf{A}^{\mathbf{c}})$ as a shorthand notation for $\mathbf{N}(\mathbf{S}(\mathbf{A}^{\mathbf{c}}))$ which counts the occurrence of each type of base in the background sequence. Similarly, $\mathbf{N}(\mathbf{A}_{(j)})$ is a vector that counts bases in the j^{th} position of all TFBSs. Given the vectors $\mathbf{v} = (\upsilon_1,\ldots,\upsilon_K)^T$ and $\boldsymbol{\theta} = (\theta_1,\ldots\theta_K)^T$, define $\mathbf{v}+\boldsymbol{\theta} = (\upsilon_1+\theta_1,\ldots,\upsilon_K+\theta_K)^T$, $|\mathbf{v}| = |\upsilon_1| + \ldots + |\upsilon_K|$, $\mathbf{v}/\boldsymbol{\theta} = (\upsilon_1/\theta_1,\ldots,\upsilon_K/\theta_K)^T$, $\boldsymbol{\theta}^{\mathbf{v}} = \theta_1^{\upsilon_1} \times \theta_K^{\upsilon_K}$ and $\Gamma(\mathbf{v}) = \Gamma(\upsilon_1) \times \Gamma(\upsilon_K)$.

Based on the assumptions and notations above, the complete likelihood function of model parameters $\boldsymbol{\theta}_0$, $\boldsymbol{\Theta}$ and q given motif indicators $\mathbf{A}$ and the observed sequence $\mathbf{S}$ is:

$$p(\mathbf{S},\mathbf{A} \mid \boldsymbol{\Theta},\boldsymbol{\theta}_0,q) = p(\mathbf{A} \mid q)\, p(\mathbf{S}(\mathbf{A}^{\mathbf{c}}) \mid \boldsymbol{\theta}_0,\mathbf{A})\, p(\mathbf{S}(\mathbf{A}) \mid \boldsymbol{\Theta},\mathbf{A}) \qquad (7)$$

$$\propto q^{|\mathbf{A}|}(1-q)^{L-|\mathbf{A}|}\boldsymbol{\theta}_0^{\mathbf{N}(\mathbf{A}^{\mathbf{c}})}\prod_{j=1}^{W}\boldsymbol{\theta}_j^{\mathbf{N}(\mathbf{A}_{(j)})}$$

If one further assumes a prior distribution for $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q as $\pi(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, q)$, then the joint posterior distribution of the unknown model parameters and motif indicators can be written down explicitly up to a normalizing constant. Typically, $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q are assumed to be independent a priori, where $\boldsymbol{\Theta}$ follows a Product Dirichlet distribution $PD(\mathbf{B})$ where $\mathbf{B} = (\boldsymbol{\beta}_1, \ldots, \boldsymbol{\beta}_W)^T$, $\boldsymbol{\theta}_0$ follows a Dirichlet distribution $D(\boldsymbol{\beta}_0)$, and q follows Beta(a, b) distribution. Under these assumptions,

$$p(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, q, \mathbf{A} \mid \mathbf{S}) \propto p(\mathbf{A} \mid q)\, p(\mathbf{S}(\mathbf{A}^c) \mid \boldsymbol{\theta}_0, \mathbf{A})\, p(\mathbf{S}(\mathbf{A}) \mid \boldsymbol{\Theta}, \mathbf{A})\, \pi(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, q) \qquad (8)$$

$$\propto q^{|\mathbf{A}|+a-1}(1-q)^{L-|\mathbf{A}|+b-1}\boldsymbol{\theta}_0^{\mathbf{N}(\mathbf{A}^c)+\boldsymbol{\beta}_0-1}\prod_{j=1}^{W}\boldsymbol{\theta}_j^{\mathbf{N}(\mathbf{A}_{(j)})+\boldsymbol{\beta}_j-1}$$

Given the likelihood function or the posterior distribution, the problem of *de novo* motif discovery can then be translated into a problem of finding maximum likelihood estimates or obtaining posterior samples for unknown parameters.

4.3.2 *Search Strategy: EM and Gibbs Sampling*

Both EM and Gibbs sampling based algorithms were proposed to solve the above inference problem. Treating $\mathbf{A}$ as missing data and formulating *de novo* motif discovery as a missing data problem, EM can be used to get MLE estimates of $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q for the likelihood function $p(\mathbf{S} \mid \boldsymbol{\Theta}, \boldsymbol{\theta}_0, q)$. Given the estimates for $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q, one can then infer the status of $\mathbf{A}$ based on its posterior probability. The use of EM in *de novo* motif discovery was introduced by Lawrence and Reilly (1990). Later development includes Cardon and Stormo (1992) who extended the method to allow gaps with variable lengths within a TFBS, and Bailey and Elkan (1994, 1995) who developed the MEME algorithm (Mutiple EM for Motif Elicitation). Multiple motif discovery can be conducted by repeatedly masking motifs found in early iterations in a probabilistic manner. A parallelized version of MEME, ParaMEME, was also proposed (Grundy, Beiley and Elkan, 1996).

Parallel to EM, Gibbs sampling and other Markov Chain Monte Carlo techniques were also proposed to estimate $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$, q and $\mathbf{A}$ (Lawrence et al., 1993; Liu, 1994; Liu et al., 1995; Neuwald et al., 1995). Gibbs sampling iteratively samples from two conditional distributions:

(i) Given $\mathbf{A}$, sample $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q from $p(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, q \mid \mathbf{S}, \mathbf{A})$;

(ii) Given $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q, sample $\mathbf{A}$ from $p(\mathbf{A} \mid \mathbf{S}, \boldsymbol{\Theta}, \boldsymbol{\theta}_0, q)$.

Samples drawn in this way form a Markov chain. Theoretically, after running enough iterations to ensure a sufficient burn-in period, the chain reaches stationarity and the samples drawn at each step will follow the joint posterior distribution given by formula (8) (Geman and Geman, 1984; Tanner and Wong, 1987; Gelfand and Smith, 1990). In reality, however, since the chain can be trapped by local maxima in the posterior distributions, the Gibbs sampler is often used to search for good alignments of TFBSs rather than to obtain posterior samples.

The local maxima problem exists for both the EM algorithm and the Gibbs sampler. To alleviate this problem, these algorithms are typically run from multiple starting points. More advanced sampling methods such as parallel tempering (Geyer, 1991) and equi-energy sampling (Kou, Zhou, and Wong, 2005) have also been used for this problem.

4.3.3 *Examples of Gibbs Sampling*

The first Gibbs sampling algorithm for *de novo* motif discovery is a Gibbs site sampler (Lawrence et al., 1993; Liu, 1994). In the site sampler, each sequence is assumed to have exactly one TFBS, q is irrelevant and formula (8) becomes

$$p(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, \mathbf{A} \mid \mathbf{S}) \propto \boldsymbol{\theta}_0^{\mathbf{N}(\mathbf{A}^c)+\boldsymbol{\beta}_0-1} \prod_{j=1}^{W} \boldsymbol{\theta}_j^{\mathbf{N}(\mathbf{A}_{(j)})+\boldsymbol{\beta}_j-1} \tag{9}$$

Gibbs site sampling first collapses $\boldsymbol{\Theta}$ and $\boldsymbol{\theta}_0$ by integrating them out, resulting in

$$p(\mathbf{A} \mid \mathbf{S}) \propto \frac{\Gamma(\mathbf{N}(\mathbf{A}^{\mathbf{c}}) + \boldsymbol{\beta}_0)}{\Gamma(|\mathbf{N}(\mathbf{A}^{\mathbf{c}})| + |\boldsymbol{\beta}_0|)} \prod_{j=1}^{W} \frac{\Gamma(\mathbf{N}(\mathbf{A}_{(j)}) + \boldsymbol{\beta}_j)}{\Gamma(|\mathbf{N}(\mathbf{A}_{(j)})| + |\boldsymbol{\beta}_j|)} \tag{10}$$

Since there is only one site within each sequence, $\mathbf{A}$ can be reduced to a vector $(\tilde{a}_1,...,\tilde{a}_I)^T$, where $\tilde{a}_i$ is the starting position of the TFBS in sequence *i*. The site sampler then proceeds to update $\tilde{a}_i$ iteratively. Given $\mathbf{A}_{[-i]}$ ($\tilde{a}_1,...,\tilde{a}_I$ except for $\tilde{a}_i$, i.e., the positions of TFBSs in all sequences except for the i^{th} sequence), $\tilde{a}_i$ is drawn iteratively from its conditional distribution $p(\tilde{a}_i \mid \mathbf{S}, \mathbf{A}_{[-i]})$, which can be approximated by

$$p(\tilde{a}_i = x \mid \mathbf{S}, \mathbf{A}_{[-i]}) \propto \prod_{j=1}^{W} \left(\frac{\hat{\boldsymbol{\theta}}_j}{\hat{\boldsymbol{\theta}}_0} \right)^{\mathbf{N}(S_{i,x+j-1})} \tag{11}$$

$\hat{\boldsymbol{\theta}}_j$ is the posterior mean of $\boldsymbol{\theta}_j$ conditional on $\mathbf{S}$ and $\mathbf{A}_{[-i]}$. It can be computed as $\hat{\theta}_{jk} = (n_{jk}(\mathbf{A}_{[-i]}) + \beta_{jk}) / (I - 1 + |\boldsymbol{\beta}_j|)$, where $n_{jk}(\mathbf{A}_{[-i]})$ is the number of bases of type *k* at the j^{th} position of all TFBSs specified by $\mathbf{A}_{[-i]}$. $\hat{\boldsymbol{\theta}}_0$ is the posterior mean of $\boldsymbol{\theta}_0$ conditional on $\mathbf{S}$ and $\mathbf{A}_{[-i]}$. It can be computed in a similar way. Intuitively, given TFBSs in all other sequences, the Gibbs site sampler will infer the motif pattern and use it to update the TFBS in sequence *i*. Such updating will be done for each sequence, and the procedure will be repeated many times until convergence. The reason for collapsing $\boldsymbol{\Theta}$ and $\boldsymbol{\theta}_0$ is that marginalization can reduce autocorrelation between successive samples of MCMC and can speed up the convergence of the sampler (Liu, Wong, and Kong, 1994).

The model used by the Gibbs site sampler does not consider the situation where one sequence has zero or multiple sites. To overcome this drawback, Liu et al. (1995) and Neuwald et al. (1995) proposed to concatenate all sequences into a single long sequence and use formula (8) to formulate the sequence model. Based on the model, Gibbs Motif Sampler (GMS) was developed. GMS is also a collapsed sampler. $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q are integrated out

before **A** is sampled. The sampling of **A** is quite similar to the site sampler, with only a minor modification:

$$\frac{p(a_i = 1 \mid \mathbf{S}, \mathbf{A}_{[-i]})}{p(a_i = 0 \mid \mathbf{S}, \mathbf{A}_{[-i]})} \propto \frac{\hat{q}}{1-\hat{q}} \prod_{j=1}^{W} \left(\frac{\hat{\boldsymbol{\theta}}_j}{\hat{\boldsymbol{\theta}}_0} \right)^{\mathbf{N}(S_{i+j-1})} \tag{12}$$

$\mathbf{A}_{[-i]}$ is now the set of motif indictors for all positions except for the current position *i*, and $\hat{q} = (|\mathbf{A}_{[-i]}| + a)/(L + a + b - 1)$ is the predictive probability that position *i* is the start of a TFBS.

GMS was used as a basis to develop other Gibbs sampling based algorithms, examples include AlignACE (Roth et al., 1998) and BioProspector (Liu et al., 2001). AlignACE allows multiple types of motifs to be found. This is done by iteratively masking TFBSs that correspond to motifs already found. BioProspector introduces *ad hoc* heuristics and devises a threshold sampler to help the Gibbs sampler achieve better performance. In the threshold sampler, a position with a score higher than a certain threshold is classified as a motif site with probability one, whereas a position with a score lower than another threshold is not considered as a potential motif site. The two thresholds are adjusted iteratively during the sampling, much like the method of simulated annealing. Other characteristics of BioProspector include: (i) a Markov model which helps to reduce noise from simple repeats, and is used to describe the background sequences; (ii) handling of gapped motifs and motifs with palindromic patterns.

4.3.4 *Various Issues in Gibbs Motif Sampling*

The model in 4.3.1 does not consider gaps within motifs. It is not unusual to observe certain positions within a motif which are not conserved (e.g., a motif with palindromic pattern "CGGNNNNCCG"). To allow gaps in a motif, Liu et al. (1995) and Neuwald et al. (1995) proposed a fragmentation model. A fragmentation indicator $\Delta = (\delta_1, \mathsf{h}, \delta_W)$ is introduced to indicate whether a position should be regarded as part of a motif or as a gap.

The gap is modeled using the background probability $\boldsymbol{\theta}_0$. Δ can be treated as missing data, and its sampling strategies were discussed in detail in Liu et al. (1995).

Another issue that needs to be addressed is how to choose the motif width W. One way to handle this question is to set a large W and use the fragmentation model to choose a subset of the most conserved positions. Another way is to treat W as a random variable, set a prior for it and infer it from the joint posterior distribution of ($\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$, q, W) given the sequences. Examples include Gupta and Liu (2003), and Zhou and Wong (2004). Both put a Poisson prior on W and use a Metropolis move to update it.

It is not unusual for the Gibbs sampler to become trapped in local modes. One such case occurs with phase shift, e.g., the true motif might be "GATCAT", but once the sampler encounters "ATCATA", it may remain in this local mode. To avoid this problem, Liu (1994) proposed a "shift modes" method. This method adds an extra sampling step to the Gibbs sampler, in which "ATCATA" is shifted δ bps to the left or right, and the move is accepted or rejected by the Metropolis rule.

A good background model can increase the sensitivity and specificity of the sampler. A Markov model can partially take into account effects from simple repeats and low complexity sequences (such as "AAAA") which frequently occur in the genome but usually are not real signals. Liu et al. (2001) showed that using a 3rd order Markov model can provide better separation between signal and noise than using a background model that assumes independence between neighboring positions. Although a higher order Markov chain may provide a better background model, the amount of data available puts constraints on the number of parameters one can use. Therefore, one must take into consideration this compromise when choosing the order of the Markov model. Another way to account for simple repeats and low complexity sequences was discussed in Gupta and Liu (2003), where they generalized the dictionary model. Simple repeats and low complexity sequences were

compiled into the dictionary. When calling motifs, these trivial words will not be treated as signals. Since the dictionary does not intend to cover all possible words, the words it compiles can be very long. Therefore, this method can be used to handle higher-order repeats, situations in which the effectiveness of the Markov background model may be limited.

Usually, there is more than one type of motif involved in the co-regulation of genes. Jointly finding those motifs enables one to combine information to improve inference. Multiple types of motifs can be found by iteratively masking TFBSs found previously, such as in AlignACE (Roth et al., 1998). A more principled way is to generalize the model in 4.3.1 directly to multiple motifs and sample motifs simultaneously, such as Liu, Neuwald, and Lawrence (1999) and Thompson, Rouchka, and Lawrence (2003). A related issue is to determine how many motifs exist in the sequences, which is typically handled as a Bayesian model selection problem (Gupta and Liu, 2003; Zhou and Wong, 2004).

In the canonical Gibbs sampler, sampling switches between the two conditional distributions $p(\boldsymbol{\Theta}, \boldsymbol{\theta}_0, q \mid \mathbf{S}, \mathbf{A})$ and $p(\mathbf{A} \mid \mathbf{S}, \boldsymbol{\Theta}, \boldsymbol{\theta}_0, q)$. To speed up the convergence, one can integrate out $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q first and only sample $\mathbf{A}$. This strategy is called "collapsing". Another strategy is "grouping", i.e., sample a group of unknowns jointly (e.g., one can sample an unknown parameter first, then sample the second parameter conditional on the first one, next sample the third parameter conditional on the first two, etc.). Such a strategy was discussed in detail in Liu et al. (1999) in which the block-motif model and HMM was combined to develop a propagation model which adopts a forward summation and backward sampling approach to sample TFBSs. Given TFBSs in other sequences (or given $\boldsymbol{\Theta}$, $\boldsymbol{\theta}_0$ and q), a grouping strategy samples all $\mathbf{A}$ in a single sequence jointly, a procedure closely related to dynamic programming in traditional HMM (Durbin et al., 1998). This method can be easily applied to handle multiple TFBSs of multiple types of motifs in one sequence.

Collapsing and grouping have all been shown to be capable of increasing the efficiency of the Gibbs sampler (Liu et al., 1994).

Inference of Gibbs sampling is based on a large number of posterior draws. These posterior samples need to be summarized in order to be useful for biologists. Reporting a PWM and TFBSs according to, say, the last draw, will result in considerable variation of the results. To provide a better summary, posterior samples need to be averaged. For example, one can infer the most likely motif width and number of motifs first from the posterior draws, and then report a PWM and TFBSs accordingly. The posterior probability that a position falls within a motif site can be used to determine whether the position should be reported as a TFBS. Recently, Jensen et al. (2004) also proposed a Bayesian scoring function approach to improve the results reported by current algorithms (e.g., BioProspector, CONSENSUS). This approach, implemented in BioOptimizer (Jensen and Liu, 2004), triggers a local hill-climbing which moves the result reported by the sampling algorithm towards its neighboring local mode.

4.4 *Combining Word Enumeration and Weight Matrix Updating*

Although word enumeration and PWM updating are discussed as two different strategies, in many cases there is no clear cut difference between these two. For example, if we treat PWMs as stochastic words, then GMS can be thought as some kind of PWM enumeration, which looks for overrepresented PWMs. The only difference is that GMS replaces exhaustive enumeration by probabilistic sampling. Using this idea, Gupta and Liu (2003) generalized Bussemaker's dictionary model to a stochastic dictionary model, and proposed Stochastic Dictionary-based Data Augmentation (SDDA) to build up a stochastic dictionary to infer TFBSs. SDDA adopts a grouping strategy within Gibbs sampling. The main difference between SDDA and the original dictionary model is that (i) PWMs are used as stochastic words; (ii) the new words are not constructed from concatenating existing words

in the dictionary, therefore no assumption is made that a meaningful longer word is always generated from shorter words that are used frequently; (iii) low-complexity words are treated as words in a stochastic dictionary model, although it may have no meaning. By including these trivial words, SDDA discounts repeats from the motif signal. In contrast, over-representation of low complexity words was used to build longer words in Bussermaker's method, and these words were considered as part of the signal.

MDScan (Liu et al., 2002) represents another effort to combine word enumeration with PWM updating. In MDScan, sequences are ranked by using additional information from ChIP-chip experiments (refer to section 7). Sequences with stronger ChIP-chip signals are more likely to contain the desired TFBSs, and these sequences will receive higher priority for downstream processing. MDScan first enumerates all *w*-mers (words of length *w*) in the top *t* sequences and treats them as seeds. For each seed, all *w*-mers in the top *t* sequences which match the seed with at least *m* positions will be collected. These *m*-matches and the seed will be used to construct a PWM. The PWM is then assigned an approximate maximum a posteriori (MAP) score proposed by Liu et al. (1995), and the PWMs with the highest scores are kept as candidate motifs. The candidate motifs are then used to search against all the remaining sequences. Matches which can improve motif scores will be added to the PWMs to enhance those motifs. MDScan refines motifs further by checking *w*-mers already included in a PWM. If excluding a match can increase the motif score, the match will be removed from a PWM. This updating procedure is repeated until convergence. By taking advantage of the external ranking information, MDScan can be more accurate than uninformed *de novo* motif discoveries such as CONSENSUS, BioProspector and AlignACE. Since no sampling is involved, MDScan is usually faster than the Gibbs sampler.

4.5 *Information from Negative Samples*

The algorithms discussed up to now mainly rely on a set of sequences in which TFBSs are enriched. In some situations, such as when ChIP-chip data (section 7) is available, we may have sequences where the motif is expected to be enriched (positive sequences) and sequences where the motif is expected to be depleted (negative sequences). This may help to discriminate between true and false TFBSs. By incorporating information from negative sequences, one can eliminate predicted TFBSs that are enriched in both positive and negative sequences, which are very likely to be false positives. One good example that uses both positive and negative information is REDUCE (Bussermaker, Li, and Siggia, 2001). In REDUCE, upstream occurrences of motifs are assumed to contribute additively to the log fold changes in a gene's expression obtained from a microarray experiment. The algorithm systematically checks all oligomers (up to a specified length) and dimers (two oligomers with a fixed spacing). In each case, a linear regression is fitted to test the association between the occurrences and changes in expression. The one that can most effectively explain the variations in gene expression is then picked up as a potential motif. Additional motifs can be selected in the same way, using residuals from earlier regressions as response variables. Since the regression is fit using all genes in the array, the algorithm uses both positive and negative information from sequences to find motifs. A similar idea was used by Conlon et al. (2003) to develop an algorithm called Motif Regressor. Motif Regressor uses MDScan to generate a candidate list of motifs based on genes with the highest expression fold changes. It then computes a motif-matching score for each gene that takes into account both the number of sites in the sequence and how well each site matches the motif PWM. A regression based on all genes is then fitted to test the association between a gene's upstream sequence motif-matching score and the gene's expression variation measure, and a stepwise variable selection procedure is applied to select candidate motifs. Experimental results showed that the inclusion of low ranking genes (in terms of changes of expression levels) in motif

discovery improves the sensitivity and specificity. For example, when applied to analyze yeast data, Motif Regressor outperformed MDScan, MEME and AlignACE. Although it is not yet clear how the strategy performs in mammalian genomes, the results do suggest that negative information is useful in motif discovery.

Negative sequences can also be used to define the fine structures of motifs. One recent example is DMotifs (Hong et al., 2005). Based on the predictions made by Motif Regressor, DMotifs applies a boosting method to learn multiple motif models by comparing positive and negative sequences. The learning is done in a successive manner. Each new motif model aims to classify samples misclassified by existing models, and the final TFBS prediction is based on the combined decisions of all trained models. In some sense, the PWM method is equivalent to a linear classifier for motif and non-motif sites. By combining multiple different PWMs, DMotifs indeed obtains a non-linear classifier which can capture subtle correlation structures between different positions within a single motif.

5. Module Discovery

The methods in section 4 have proved to be very useful when applied to lower organisms whose genome size is relatively small and whose genome structure is relatively simple (e.g., Roth et al., 1998; Liu et al., 2002; Conlon et al., 2003). However, for mammalian genomes, they are of limited utility. In a mammalian genome with 3 billion base pairs in length, an exact match of a 7-mer, i.e., seven nucleotides in succession, is expected to occur every 16,384 bps, resulting in ~180,000 sites in total. If we allow one mismatch, this number will increase to ~5 million. Degeneracy at multiple positions will further increase the number of matches. Given that human genome has about 20,000-25,000 protein-coding genes (International Human Genome Sequencing Consortium, 2004), we would expect most of the sites to be false positives. For a motif finding algorithm to be practically useful, we need additional ways to reduce false positives.

For most eukaryotic genes, the binding of an individual transcription factor is not sufficient to drive the context-specific transcription. Rather, interaction and cooperation of several transcription factors are needed to affect gene expressions at specific time and locations (Yuh, Bolouri, and Davidson, 1998; Wasserman and Fickett, 1998; Loots et al., 2000; Berman et al., 2002; Banerjee and Zhang, 2003). A *cis*-regulatory module (CRM) is a DNA segment, typically a few hundred base pairs in length containing multiple binding sites, that recruits several cooperating factors to a particular genomic location (Figure 4a). The incorporation of the CRM structure into the search strategy can increase the sensitivity and specificity for *cis*-element discovery. This was first demonstrated by Wasserman and Fickett (1998) in their study of a group of muscle-specific genes. They proposed a Logistic Regression Analysis (LRA) method for classifying regions of fixed length into regulatory modules and non-regulatory backgrounds. In LRA, a set of given PWMs is matched to the sequences in question. The two best hits of every PWM are recorded for each sequence. The scores of all such hits for a sequence are then used as predictors in a logistic regression to predict the regulatory status of the sequence. The model can be fitted using some training data, and then applied to predict the status of unclassified sequences. LRA was applied to analyze a set of muscle-specific genes, and it showed improvement both in sensitivity and in specificity over the simple method where the PWMs were matched to the sequence.

[Figure 4 about here]

The work by Wasserman and Fickett has stimulated several subsequent efforts on CRM discovery (e.g., Krivan and Wasserman, 2001; Frith et al., 2001; Sinha, van Nimwegen, and Siggia, 2003). As an example, the Cister algorithm (Frith et al., 2001) uses a HMM model that treats a sequence as generated from a mixture model. Each nucleotide in the sequence is either drawn from an inter-cluster background or from a CRM. A CRM may contain one or more TFBSs. A nucleotide in a CRM is generated either from a TFBS or from

the intra-cluster background. Inter- and intra-background and the TFBSs evolve as a Markov chain according to some transition probability, and each hidden state has its own emission probabilities to generate nucleotides. The inference of whether a site belongs to a CRM is then based on the posterior probability given the observed sequence, which can be obtained through decoding the HMM.

Both logistic regression and HMM based methods require prior knowledge of the binding motifs (i.e., the PWMs). However, this knowledge is often unavailable. In the latter scenario, the search for a CRM has to rely on *de novo* motif discovery, and the two tasks are tightly coupled – on one hand determination of TFBSs and motifs are essential for CRM detection, while on the other hand knowledge of the CRM will improve the specificity of the TFBS and motif finding. Early attempts to combine *de novo* motif discovery with CRM prediction include Zhou and Wong (2004), Thompson et al. (2004), and Gupta and Liu (2005). In CisModule, developed by Zhou and Wong (2004), a two-level hierarchical mixture (HMx) model is used to model sequences from a group of co-regulated genes. At the first level, the sequences are viewed as a mixture of CRMs, each of length *l*, and pure background sequences outside the modules; at the second level, modules are modeled as a mixture of motifs and within-module background. Each motif is represented as a PWM. The background sequences – both the regions outside modules and the non-site segments within modules – are modeled by a first order Markov chain. A Gibbs sampler algorithm is used to perform Bayesian inference based on the HMx model (Figure 4b). With random initiation, CisModule iteratively cycles through the steps of parameter updating and module-motif detection: (1) Given current modules and motif sites, it updates all the model parameters including PWMs by sampling from their conditional posterior distributions; (2) Given current values of the parameters, it samples modules and motif sites from the conditional distribution. When tested on the 29 skeleton-muscle-specific genes (Wasserman and Fickett, 1998), CisModule found

significantly more experimentally reported sites and fewer false positive sites compared with non-module based motif discovery methods.

Another module sampler, Gibbs Module Sampler (Thompson et al., 2004), introduces the module structure into the *de novo* motif sampler by restricting distances between neighboring TFBSs. It models dependencies between neighboring sites using a Markov transition probability matrix. This allows the ordering preferences of TFBSs to be captured which can then be used to reflect the preferences of protein-protein interactions. Based on a model similar to Thompson et al. (2004), Gupta and Liu (2005) proposed a two stage module elicitation algorithm, EMCMODULE. It first uses existing databases or *de novo* motif discovery methods such as BioProspector, MEME or SDDA to generate a list of putative motifs. An evolutionary Monte Carlo (EMC) method is then used to decide which motifs should be included in the CRM, and then updates the corresponding sites and parameters. Like CisModule, both Gibbs Module Sampler and EMCMODULE showed improved performance over the traditional motif sampler in finding clustered TFBSs.

6. Information from Cross-species Comparison

6.1 *Comparative Genomics as a Tool for Identifying Regulatory Elements*

Even with the availability of co-regulated genes and TFBS co-localization information, current methods are still far from being able to predict CRMs and TFBSs precisely. Usually, *de novo* motif discovery is only capable of locating CRMs and TFBSs in sequences of 1-2 kb in length. The typical size of a mammalian gene, however, ranges from less than 10kb to over 100kb (International Human Genome Sequencing Consortium, 2001). This range includes exons, introns and a gene's proximal promoters. CRMs can be located anywhere in this region except for exons. What is worse, CRMs can also be found in enhancers far away from the gene, and we usually do not have any prior knowledge of the locations of those enhancers. If motif discovery algorithms were applied to all regions that

potentially contain CRMs, most of the time the programs would report false predictions. Extra experimental information is thus needed to narrow down the candidate regions before motif discovery algorithms can be confidently applied.

One such experiment has already been performed by nature during the long course of evolution. Functional genomic sequences are subject to selection. Harmful changes in important coding and regulatory regions are eliminated by negative (purifying) selection, and beneficial modifications are propagated via positive selection. Sequences under negative selection evolve slower than non-functional sequences that are shaped by neutral mutations, whereas sequences under positive selection evolve faster. This leaves footprints in the genomes during their long evolutionary history, and comparing genomes from different species allows us to detect functional regions that have undergone negative or positive selection (Miller et al., 2004).

Important *cis*-regulatory elements that share similar functions among different species are usually under negative selection. Conserved non-coding sequences can serve as a guide to identifying such regulatory elements (Hardison, Oeltjen, and Miller, 1997; Hardison, 2000; Pennacchio and Rubin, 2001) (Figure 5a). Comparisons between human and mouse suggest that about 5% of the mammalian genome is under purifying selection (Mouse Genome Sequencing Consortium, 2002). Among them, ~1.5% is estimated to be protein-coding exons, and ~1% is untranslated regions of genes. This leaves ~2.5% uncharacterized, which are potential candidates for conserved regulatory elements as well as other important functional elements such as microRNAs. For CRM study, these numbers have at least two implications. On one hand, the 2.5% of mammalian genome means that there is still a lot of work to do to characterize the regulation of mammalian genomes; on the other hand, the reduction to 2.5% can help greatly to narrow the search space for CRM discovery. By focusing on conserved non-coding regions, we can study subsets of the genome which are most likely to have

important functions. The relatively high signal-to-noise ratio increases our chance of finding important players in gene regulatory networks. This strategy, today known as phylogenetic footprinting (Tagle et al., 1988; Gumucio et al., 1992), was successfully applied to identify regulatory elements in a number of studies (e.g., Emorine et al., 1983; Gumucio et al., 1993; Aparicio et al., 1995; Gumucio et al., 1996; Loots et al., 2000; Göttgens et al., 2000). Other *cis*-regulatory elements may change rapidly in the course of evolution (Ludwig, Patel, and Kreitman, 1998; Dermitzakis and Clark, 2002). To characterize those elements, comparisons of a set of closely related species, termed phylogenetic shadowing (Boffelli et al., 2003), or comparisons among individuals within a single species, termed population shadowing (Makova et al., 2001), are needed. Phylogenetic shadowing can be used to identify lineage specific elements, while population shadowing is used to elucidate species-specific elements. In what follows, we will mainly focus on phylogenetic footprinting.

The power of comparative genomics will be greatly enhanced by the complete sequencing of multiple mammalian and vertebrate genomes. At the time of this writing, the finished or draft vertebrate genome sequences include human, chimpanzee, mouse, rat, dog, chicken and fish (http://www.ncbi.nlm.nih.gov/Genomes/index.html). Additional species such as rhesus, cow and opossum will become available in the near future. The extensive experimental and computational study of a few selected regions such as those covered in the ENCODE project (Collins et al., 2003) will provide detailed annotations for a small subset of the genome. This will provide training and test data crucial for improving CRM finding algorithms. In order to find mammalian CRMs, different types of cross-species comparisons are needed. Comparisons between human and fish which diverged ~450 million years ago mainly identify coding regions (Aparicio et al., 2002). Comparing human and mouse which are diverged ~75 million years ago reveals conservation of both coding and non-coding regions (Mouse Genome Sequencing Consortium, 2002). Utilizing these comparisons,

putative CRMs can be identified as conserved non-coding elements. Human and chimpanzee diverged 5-7 million years ago (Chimpanzee Genome Sequencing Consortium, 2005). Comparisons between these two species can be used to identify regions under positive selection and lineage specific regulatory elements. A recent study of the human CFTR region compared to orthologous regions from 12 vertebrates (Thomas et al., 2003) showed that including other mammalian and vertebrate genomes will promise to further increase the resolving power.

[Figure 5 about here]

6.2 *Aligning Genomes and Calibrating Conservation*

Aligning sequences from different species is the first step to identify evolutionary footprints. In an alignment, sequences are put into a two-dimensional matrix (Figure 5b). Each row of the matrix corresponds to a sequence (e.g., from different species), and bases in the same column ideally would share the same common ancestor. Conserved regions can be identified as contiguous columns in the alignment where bases in different rows share higher similarity compared to neutral background. After defining a scoring scheme to measure base similarities, an alignment algorithm tries to find the optimal construction of the matrix so that the total score of the alignment is maximized. Traditionally, dynamic programming (DP) was used to solve this problem in both global alignment (Needleman and Wunsch, 1970) and local alignment (Smith and Waterman, 1981). The search space for DP is proportional to the products of the lengths of the sequences and grows exponentially as the number of sequences increases. To facilitate the search within large databases and the alignment of multiple sequences, heuristics can be introduced into the DP algorithms. Examples include the most popular tool BLAST (Altschul et al., 1990; Altschul et al., 1997) for searching databases and CLUSTALW (Thompson, Higgins, and Gibson, 1994; Thompson et al., 1997) for constructing multiple sequence alignment. Comparing large genomic sequences, however,

presents new challenges for alignment. This is not only due to the requirement of efficient processing of millions to billions of base pairs at one time, but also due to the difficulty of unambiguously aligning orthologous sequences in multiple species in the presence of repeats and changes in gene order, gene number and gene orientation that are the result of chromosome rearrangements, duplications, deletions and inversions. Moreover, the alignment of neutrally evolving regions from species with moderate or long phylogenetic distance requires algorithms to have high sensitivity in order to put orthologs together; for downstream functional element detection, high specificity is also desired to minimize the adverse effects of misalignment. Specialized alignment tools are needed to handle genome alignment. Currently, both local alignment and global alignment tools exist for aligning long genomic sequences. Examples of the former include BLASTZ (Schwartz et al., 2003a) and SSAHA (Ning, Cox, and Mullikin, 2001). Examples of the latter include MUMmer (Delcher et al., 1999; Delcher et al., 2002), GLASS (Batzoglou et al., 2000), WABA (Kent and Zahler, 2000), AVID (Bray, Dubchak, and Pachter, 2003), and LAGAN (Brudno et al., 2003). Another tool, BLAT (Kent, 2002), allows fast mRNA/DNA and cross-species protein alignment, and the BLAT server in the UCSC genome browser (Kent et al., 2002) links alignment results to detailed genomic annotations. Visualization tools such as PipMaker (Schwartz et al., 2000), VISTA (Dubchak et al., 2000; Mayor et al., 2000), and zPicture (Ovcharenko et al., 2004) are also available to summarize and display alignment conservation. For aligning multiple sequences, MultiPipMaker (Schwartz et al., 2003b), MAVID (Bray and Pachter, 2003), and MLAGAN (Brudno et al., 2003) are available. More detailed discussions of the strategies of alignment can be found in Frazer et al. (2003), Couronne et al. (2003) and Miller et al. (2004). Despite the long list of choices, however, the problem of constructing alignments specifically to support CRM discovery is not completely solved. Because of its short length, a binding site may be misaligned without affecting the overall alignment score

significantly. Therefore standard alignment tools are likely to misalign a significant percentage of conserved binding sites. It is likely that approaches that simultaneously perform alignment and motif discovery may provide better performance.

After the alignment is constructed, the degree of cross-species conservation can be evaluated. The simplest way to do this is to calculate percent identities in a moving window (e.g., Schwartz et al., 2000; Liu et al., 2004). This approach, however, does not take into account variations of genomic features. Comparisons of different species suggested that the neutral mutation rate shows significant variations across the genome (Mouse Genome Sequencing Consortium, 2002; Hardison et al., 2003), and such variations are correlated with variations of GC content, recombination rates and other genomic features. This implies that the same similarity level between multiple species may have higher significance in rapidly changing regions and lower significance in slowly changing regions. By carefully modeling this genome-wide variation, one may increase the sensitivity of conservation-based CRM discovery (Kolbe et al., 2004). Therefore, a conservation measure that takes into account regional variation of evolutionary rate is desired. Li and Miller (2003) provide an example of how this can be done. They first use HMMs to model regional variations. Based on the extent to which two genomes can be aligned, genomic regions are classified according to several different conservation levels. The significance of local gap-free alignments is then evaluated in the context of the broad conservation level of surrounding regions. When evaluating conservation across multiple species, one also needs to consider species' evolutionary relationships. 98% sequence identity between human and chimpanzee, for example, is less significant than the same similarity level between human and mouse. To account for this effect, one can further integrate phylogenetic trees into conservation computations. One example is phylogenetic HMM (Siepel and Haussler, 2004) which computes the "phastCons" score available from the UCSC genome browser (Siepel et al., 2005).

General conservation score calculations detect conservation both in coding regions and non-coding regions. The calculations do not intend to directly discriminate regulatory regions from neutral backgrounds. Since coding regions tend to be more conserved than non-coding regulatory elements, and since these two broad classes may have intrinsic differences, conservation calculations that specifically aim to characterize regulatory DNA may provide more power for CRM discovery. In one such effort (Elnitski et al., 2003), a regulatory potential was calculated based on the human-mouse alignment. The columns of the alignment were first converted into a 5-symbol string (match between A and T, match between C and G, transition, transversion and gap). Next, two 5^{th} order Markov models were fit for two training datasets separately. The first training dataset was composed of known regulatory DNA, and the second dataset consisted of neutral DNA (ancestral repeats). Based on the trained model, a log-odds ratio can then be computed for the classification of each new segment. The resulting ratio represents the segment's regulatory potential. When compared with simple percent identity based methods, this specifically trained model performs better in identifying regulatory DNA. Later, the idea was generalized to three-species comparisons (Kolbe et al., 2004), where a systematic method was used to collapse the three-way alignment to a string with a few symbols; this was then followed by training a model similar to Elnitski et al. (2003). A way to incorporate local variations of the evolutionary rate was also devised in Kolbe et al. (2004). This local adjustment was shown to be able to sharpen the regulatory potential signal. These initial results are promising but are still far from optimal. For example, both methods rely on training data, which may be biased to those well-studied elements or may not be always available. The generalization to more species may introduce additional complications. Both methods first collapse columns into a reduced set of symbols. For two species, such a reduction involves only 24 symbols (pairs between A, C, G, T and gap minus the pair of gap-gap); for three species, the number increases to 124. The number increases

exponentially, making the state space reduction more and more difficult when more species are added into the analysis. Both methods rely on the quality of alignment, and it is not clear how their performance will be affected by the choice of alignment strategies. With further exploration, cross-species comparisons have the potential to provide a good start for incorporating multiple species information into CRM discovery.

6.3 *Incorporating Cross-Species Comparison into Cis-regulatory Module Discovery*

Cross-species conservation has been used both for known motif mapping and for *de novo* motif discovery. An example of known motif mapping is rVISTA – Regulatory VISTA (Loots et al., 2002). For finding TFBSs, rVISTA first identifies all matches of a set of given motifs in sequences from several species. Based on AVID (Bray et al., 2003) alignment of these sequences, it then identifies putative TFBSs that are aligned across species. For each aligned site, a conservation score based on neighboring local alignment is computed. The putative sites are then displayed and clustered into groups by user-specified criteria.

Examples of *de novo* motif discovery which incorporate multiple species information include Wasserman et al. (2000), PhyloCon (Wang and Stormo, 2003), EMnEM (Moses, Chiang, and Eisen, 2004), OrthoMEME (Prakash et al., 2004), PhyME (Sinha, Blanchette, and Tompa, 2004), CompareProspector (Liu et al., 2004), PhyloGibbs (Siddharthan, Siggia, and van Nimwegen, 2005), Ortholog sampler (Li and Wong, 2005) and MultiModule (Zhou and Wong, 2005), etc. Many of them are extensions of the *de novo* motif discovery methods discussed in section 4. For example, PhyloCon generalizes CONSENSUS, EMnEM, OrthoMEME and PhyME generalize MEME (EM-based Motif Finding), CompareProspector generalizes BioProspector (Gibbs Motif Sampler), and MultiModule generalizes CisModule (Module Sampler).

To integrate cross-species conservation with *de novo* motif discovery, different strategies have been proposed. In PhyloCon, conserved regions of orthologous sequences are

first aligned into profiles; profiles from non-orthologous sequences are then compared, and common patterns in these profiles are identified as motifs. This method replaces the sequence progressive alignment in CONSENSUS with a profile progressive alignment strategy.

Wasserman et al. (2000) and CompareProspector (Liu et al., 2004) represent another strategy where cross-species information is passed on to motif discovery via conservation scores. In both methods, a cross-species alignment is constructed, a conservation measure is computed from the alignment, and the measure is then used to guide the motif discovery in the reference species. In Wasserman et al. (2000), a threshold method is used. Regions whose conservation level is below a certain threshold are filtered out, and motif finding algorithms are applied to the remaining sequences. CompareProspector, on the other hand, uses a sliding window percent identity measure to modulate the likelihood ratio that distinguishes motifs from background. In this way, the search for motifs is biased towards conserved regions.

Unlike Wasserman et al. (2000) and CompareProspector, where the summary of cross-species conservation and motif discovery are conducted in two separate steps, EMnEM (Moses et al., 2004), PhyME (Sinha et al., 2004) and PhyloGibbs (Siddharthan et al., 2005) represent a third class of methods where these two steps are combined into an integrated model. In these methods, evolution models are introduced to describe orthologous sequence alignments across species. A group of orthologous sequences are viewed as generated from an ancestral sequence. The ancestral sequence is a mixture of background and motifs, and it evolves into observed sequences under restrictions of phylogenic structure and certain mutation models. Compared to Wasserman et al. (2000) and CompareProspector, the integrated model makes more efficient use of multiple species information, since all sequences from all species are now contributing to the motif discovery. The use of evolution models has the additional advantage of handling species with different lengths of divergence. If all sequences were treated equally in *de novo* motif discovery, the highly similar

orthologous sequences collected from closely related species may have unduly high weight in choosing motifs. By taking into account the structure of phylogenetic trees, evolution models can properly handle this problem. In theory, evolution models can also be incorporated into PhyloCon and CompareProspector, although currently these two methods do not take advantage of this information.

Most methods discussed so far rely on a pre-computed cross-species alignment. Since it is still unclear how well functional sites with only 6-8 bps in length can be aligned by current alignment algorithms, a potential problem one may encounter in these methods is that incorrect alignment may misguide the finding of true TFBSs. To reduce the possible effects of misalignment, one may further integrate the alignment procedure into motif discovery models. One effort in this direction is Zhou and Wong (2005). In their MultiModule algorithm, sequence alignment is modeled through a HMM. Given the alignment, evolution models are then introduced to describe aligned nucleotides from different species. The ancestral sequences are modeled as mixtures of background and CRMs. This method combines alignment procedure, cross-species comparisons and module discovery into a single model. A Gibbs sampler is proposed to infer parameters from the model. The sampler iterates through three steps: (1) given alignments and all other missing data, update motif parameters; (2) given motif parameters, update alignments for each ortholog group; (3) given alignments and motif parameters, update module and motif locations, ancestral sequences and evolutionary bonds. Despite these efforts here, whether or not modeling alignment and *de novo* motif discovery simultaneously is significantly advantageous rather than modeling them separately has not yet been definitely settled. Objective comparisons between these two strategies are of great interest. Currently, arguments exist both in favor and against each of these strategies. For the first strategy, since alignment is constructed during motif discovery, information about putative motifs can be used to guide correct alignments. This may allow

sites that are less conserved to be detected. The disadvantage is that by coupling the alignment with iterative procedures such as EM or the Gibbs Sampler, the computational complexity will be increased. Advantages of doing alignment and *de novo* motif discovery separately include the ease of combining tools developed by different labs (e.g., LAGAN can be easily pipelined with BioProspector), and the lower computational complexity, although it may suffer from the misalignment problems. Finally, when a set of distant species (e.g., species that diverged ~250 million years ago) are used in motif discovery, no method which depends on alignments to pin down locations of TFBSs may be efficient enough due to the rapid change of CRM structures, TFBS duplications, inversions and translocations. Methods that sample motifs directly across the phylogenetic tree could be a more appropriate choice (e.g., Li and Wong, 2005).

7. Information from ChIP-chip Experiments

Although cross-species conservation can help to narrow down the search space, it does not provide direct evidence that a conserved non-coding region is indeed a CRM. Furthermore, some lineage-specific CRMs may be missed by cross-species conservation. Thus, increasingly, researchers are conducting experiments, such as ChIP-chip assays, that provide direct information on the binding locations of specific transcription factors. In a ChIP-chip experiment (Ren et al., 2000; Harbison et al., 2004; Boyer et al., 2005), a transcription factor of interest is cross-linked to DNA (Chromatin). The chromatin is fragmented into small pieces by a sonicator, and the pieces bound by the transcription factor are precipitated using antibodies for the transcription factor. This step is called the Chromatin Immuno-precipitation step (ChIP step). After dissociating the transcription factors from the chromatin fragments and amplifying the DNA by PCR, the ChIP sample is hybridized to a microarray, and a comparison between the ChIP sample and a control sample then allows the identification of candidate genomic regions that may be bound by the transcription factor.

This second step is called the "chip step" as it relies on the use of a microarray that tiles the relevant genomic regions. In a tiling array (Kapranov et al., 2002; Kapranov et al., 2003; Cawley et al., 2004; Kampa et al., 2004), short DNA segments (probes) are selected from target genomic regions at a density of one probe for every 10 to 100 bps. These probes are printed in the array. By hybridizing ChIP/control samples to these probes, one can detect which part of the genome shows enrichment in ChIP samples as compared to control (Figure 6). Such a ChIP-chip experiment can localize the binding location to within 1-2 kb. Therefore, ChIP-chip experiments can provide valuable information for high-throughput computational methods to eventually locate important *cis*-regulatory elements in the genome.

[Figure 6 about here]

ChIP-chip experiments have already been shown in various studies to be useful for locating TFBSs. Examples include MDScan (Liu et al., 2002) and DMotifs (Hong et al., 2005). To incorporate ChIP-chip information, one needs to glean it first from raw hybridization data. This is a problem very similar to the gene selection problem with the exception that an additional spatial correlation structure can be used to improve inference. Since the precipitated DNA fragments bound by transcription factors are usually 1-2kb in length, they will bind to multiple probes that are close to each other in the genome. The signals provided by all those probes can be used collectively to determine if a region is bound by a TF. Current methods to call binding regions include GTRANS (Kampa et al., 2004; http://www.affymetrix.com/support/developer/downloads/TilingArrayTools/index.affx), Keles et al. (2004), Li, Meyer, and Liu (2005), TileMap (Ji and Wong, 2005b), etc. They can be roughly divided into two classes: window-based methods and HMM-based methods. Window-based methods usually set a window around a probe, and then construct test statistics using all probes within the window. Examples include GTRANS where statistics are constructed based on Wilcoxon signed-rank and rank sum tests, and Keles et al. (2004) where

t-statistics are averaged in a sliding window. As in gene selection, the detection of "binding" windows from tiling array data is a large-scale multiple testing problem. Principles discussed in section 3 are also useful here. An example is the improvement of Keles' method by introducing a variance shrinkage estimator to the test statistics (Ji and Wong, 2005b). In HMM-based methods, two distributions are constructed to characterize binding and non-binding probe intensities. The HMM model is then applied to detect the binding region. Examples include Li et al. (2005) and TileMap. All these methods provide a good start for using information from ChIP-chip experiments. However, much remains to be done. Many important problems such as how to combine ChIP-chip experiments with motif/CRM discovery and cross-species conservation, are awaiting careful investigation. More importantly, ChIP-chip experiments provide a potential way for us to really understand the temporal and spatial patterns of DNA binding by transcription factors. Locations and combinatorial patterns of TFBSs will be eventually translated into their functional annotations. There is an urgent need for the development of statistical tools that can address these related issues.

8. Discussion

In the past few years, we have witnessed tremendous progress in the computational analysis of gene regulation. A large effort was made to collect multiple genomes and microarray gene expression data. Much knowledge about transcription factors and their weight matrices has been accumulated. New tools for processing and utilizing different types of data were added to our toolbox. Information from ChIP-chip experiments is becoming available. These results have provided a good infrastructure for the study of complex mammalian gene regulation. For computational biologists, however, the work has just begun.

8.1 *Additional Questions that need to be answered*

The methods we discussed in this paper mainly aim to characterize the genomic locations of *cis*-regulatory elements. They do not directly tell us the functions of these *cis*-elements. A necessary step to delineate functions of a CRM is to learn which transcription factor binds to which site in a CRM. Although in studies such as ChIP-chip experiments the identities of the key transcription factors are known, in many other studies (e.g., many microarray studies where clustering is followed by CRM discovery), it is common that a list of TFBSs is predicted without knowing exactly which transcription factors bind to them. Even in ChIP-chip experiments, collaborating motifs that do not correspond to key transcription factors may be found, and auxiliary factors that bind to these new motifs need to be identified. Computational methods that can help fill this gap warrant further investigation.

To fully characterize functions of CRMs, we have to answer when and where each CRM is activated or inactivated. Development of methods that correlate combinatorial TFBS patterns back to the temporal and spatial patterns of transcription factor binding and gene expression is still in its infant stage.

Finally, it is a common theme that a complex biological process is initiated by a few master transcription factors. These master regulators turn on secondary transcription factors which in turn pass the regulatory commands to downstream genes. Reconstruction of these regulatory cascades based on the CRM information obtained from expression, sequence and ChIP-chip analysis is another important topic for future study.

8.2 *Additional Information that can be incorporated to Transcriptional Regulation Study*

Methods discussed in this paper mainly rely on information collected from DNA sequences and measurements of RNA abundance. Information contained in protein sequences and the three dimensional structure of proteins are seldom used. Potentially, this type of information can be used to answer the question, “What confers the binding specificity of transcription factors?”, which could be especially helpful for linking *cis*-regulatory elements

to their corresponding transcription factors (e.g., Kaplan, Friedman, and Margalit, 2005; Morozov et al., 2005).

The binding of transcription factors to TFBSs is not the only way to modulate gene expression. Transcriptional activities are also regulated by other mechanisms. One example is epigenetic control which involves DNA methylation and histone modifications, etc. Histone acetylations are correlated with active transcription, and DNA methylation helps to establish a silent chromatin state (Jaenish and Bird, 2003; Verdone, Caserta, and Di Mauro, 2005). Knowledge about epigenetic regulation thus can help us to identify CRMs which have the same structure but different activation status. With the application of high-throughput technologies such as ChIP-chip, epigenetic control can now be studied at the genome level (van Steensel, 2005). This offers another layer of information we could use to define the temporal and spatial events of CRM activation.

Just as systematic mutations can be used to dissect biological processes in model organisms such as fruitfly, genetic variations among individuals provide invaluable sources of information to study our own genome. Large scale human variation studies such as HapMap project (The International HapMap Consortium, 2003, 2005), when combined with structural information of CRMs, may also shed light on the gene regulation study that is closely related to human evolution and disease.

8.3 *Towards Validation*

One bottleneck we are now facing is the validation of the results generated by various computational analyses. Many experimental assays (e.g., reporter assay for predicted enhancers) are time-consuming and costly. Therefore, at the current stage, cross-validation using different sources of information is important (e.g., Parmigiani et al., 2004).

Without large scale experimental validations, the comparisons of different methods are extremely difficult. For example, most motif discovery methods judge their performance

based on a few known motifs and TFBSs, and the majority of new predictions are hard to assess. We thus have limited ability to tell which methods are really good in predicting new motifs from mammalian genomes. Systematic comparisons of various methods are of great interest. Ideally, such comparisons should also compare computational efficiencies of different methods which we did not pursue here due to our limited knowledge of their implementation details. Similar validation and comparison issues exist for the problem of microarray gene selection. Some valuable initial efforts towards this direction was made by several groups, e.g., Tompa et al. (2005) compared different *de novo* motif discovery methods (mainly those in section 4), and Irizarry et al. (2005) compared gene selection results from different labs based on the same set of RNA samples (to test platform and lab effects in microarray studies). However, it is fair to say that at present we still lack large-scale, systematic studies that would ground statistical methodologies, suggest how to improve various methods, and identify caveats in each step of data analysis.

8.4 *Toward Integration*

Owing to the complex nature of the problem, the eventual elucidation of the regulation program in mammalian genomes would require us to assemble different pieces of information together to form a global picture. Table 2 summarizes several motif and CRM discovery methods discussed in the paper. It is clear that even for the most recent tools that make the most extensive use of the available data, not all information from microarray transcriptional profiling, clustering of TFBSs, cross-species conservation and ChIP-chip binding is used, let alone information from protein sequences, epigenetic control, etc. CRM discovery methods that integrate all types of information still remain to be developed. Integration does not simply mean the use of all pieces of information; it also means that the information should be used in an efficient way. For example, current CRM discovery methods often rely on clusters of co-regulated genes constructed from transcriptional

profiling. A natural question is whether CRMs can also be used to refine the clusters of co-regulated genes. If so, whether an iterative procedure that switches between clustering and CRM discovery can help to get better predictions of CRMs? Clear answers to these questions are still not available, though research efforts in this direction have been initiated (e.g., Wang et al., 2005).

[Table 2 about here]

Ideally, in the future, different methods will be pipelined (a conceptual framework is shown in Figure 7). Information from DNA sequences, microarrays, cross-species comparisons and ChIP-chip experiments, etc., will be used together to infer locations and combinatorial patterns of CRMs, information on CRMs will then be used to explain observations from microarray experiments, comparative genomics and ChIP-chip data. It is our belief that statistics will continue to play a central role in the computational analyses of gene regulation. We have already seen how statistical tools such as multiple testing, hierarchical modeling, Markov chain Monte Carlo are successfully used in answering different questions at different stages of our study, and how problems in biology promoted the development of statistics (e.g., FDR and power). We are optimistic that the synergic progress made in statistics, mathematics, computer science and biology will eventually allow us to decipher the secrets of the genome.

[Figure 7 about here]

ACKNOWLEDGEMENTS

We thank the associate editor and five anonymous reviewers for their insightful comments. The authors also thank Qing Zhou and Karen Kapur for helpful discussions.

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TABLES AND FIGURES

Table 1. Common biological terminology

Vocabulary	Explanation
Cis-acting element	A regulatory sequence in DNA that can control the expression of a gene on the same chromosome.
Chromatin	A complex of DNA and proteins (histones and nonhistone proteins). When a cell divides, chromatin condenses into visible chromosomes.
Chromosome	The structural unit of genetic material, consisting of DNA and associated proteins. Each cell (except for germ cells) in a particular organism has a fixed number of chromosomes. This number remains the same within an organism but varies from species to species. Humans have 46 chromosomes, including 22 pairs of autosomes and 2 sex chromosomes.
DNA	Deoxyribonucleic acid. The fundamental molecule that stores genetic information. Each DNA molecule consists of two polynucleotide strands that are built from four types of nucleotides – A (adenosine), C (cytidine), G (guanosine), and T (thymidine). Nucleotides within each strand are linked in a linear fashion by covalent bonds. Nucleotides between strands are paired through hydrogen bonds. The pairing is specific: A only pairs with T, C only pairs with G, and vice versa. Owing to this complementarity, the total information carried by DNA can be restored from any single strand.
Exon	DNA segments that are present in mature RNA.
Gene	Segments of DNA that serve as a functional unit by encoding RNAs or proteins. In eukaryotic genomes, most genes are protein coding genes.
Gene expression	The process by which a gene's information is converted into RNA and then (for protein-coding genes) into protein.
Genetic code	The set of rules that specify amino acids in proteins through nucleotide triplets (codons) in mRNAs. Of the 64 possible codons, only 20 different amino acids are specified. Some amino acids are specified by more than one codon, a property called degeneracy.
Genome	The total genetic information carried by a cell or an organism.
Intron	DNA segments that are present in pre-RNA but not in mature RNA.
mRNA	Messenger RNA. A type of RNA that encodes proteins.
Protein	A linear polymer composed of 20 types of amino acids connected by peptide bonds. Proteins are basic building blocks of life and participate in nearly all cellular activities.
RNA	Ribonucleic acid. A linear single-stranded molecule that is synthesized by transcription of DNA. RNA consists of four types of nucleotides A, C, G and U (uracil), with U replacing the role of T in DNA.
Splicing	A process that a primary transcript, or pre-RNA, is converted to mature RNA. Certain segments (introns) are removed from the primary transcript, while the remaining parts (exons) are joined to form the mature RNA.
Trans-acting	Diffusible protein that control genes on the same or different

protein	chromosomes.
Transcription	A process by which a cell copies information from DNA to a complementary RNA.
Transcription factor	A protein required to initiate or regulate transcription in eukaryotic cells.
Translation	A process by which a cell uses mRNA as a templates to synthesize a protein. The protein sequence is determined by mRNA sequences through genetic code.

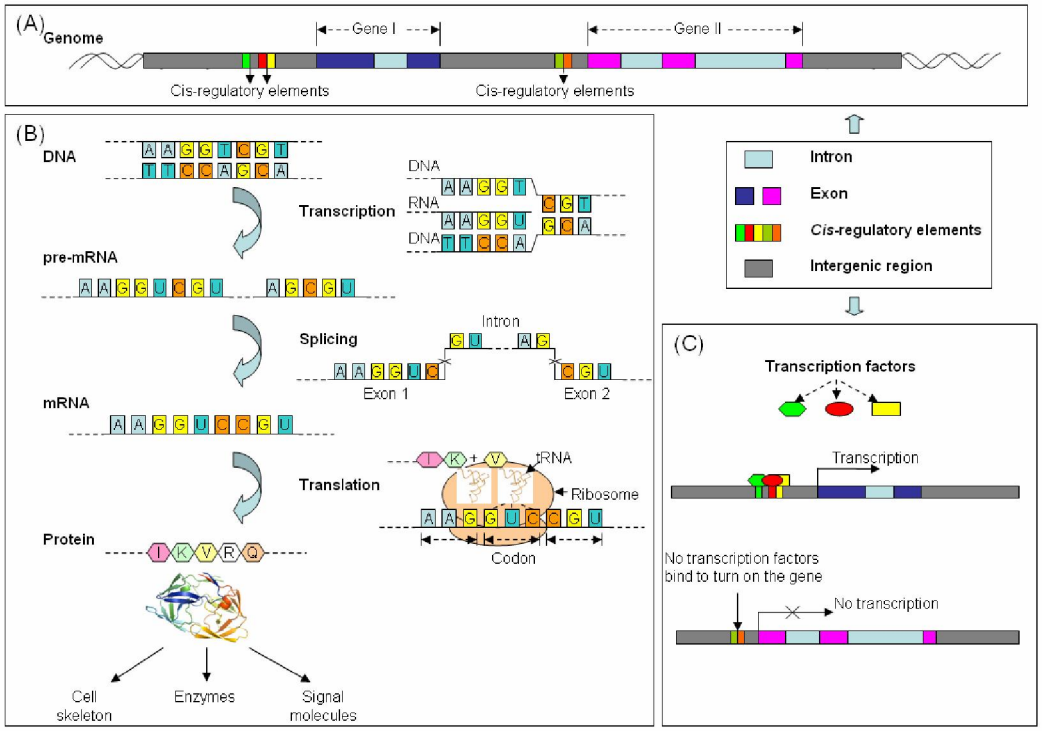
Table 2. Tools for motif/CRM discovery

Tools	De novo	Abun-dance	CRM	Cross-species	ChIP-chip
Cister (Frith et al., 2001)		√	√		
LRA (Wasserman and Fickett, 1998)		√	√		
rVista (Loots et al., 2002)			√	√	
YMF (Sinha and Tompa, 2002)	√	√			
CONSENSUS (Stormo and Hartzell, 1989)	√	√			
MEME (Bailey and Elkan, 1994)	√	√			
GMS (Liu et al., 1995)	√	√			
AlignACE (Roth et al., 1998)	√	√			
BioProspector (Liu et al., 2001)	√	√			
MobyDick (Bussemaker et al., 2000)	√	√			
SDDA (Gupta and Liu, 2003)	√	√			
REDUCE (Bussermaker et al. 2001)*	√	√			√
MDScan (Liu et al., 2002)*	√	√			√
MotifRegressor (Conlon et al., 2003)*	√	√			√
DMotif (Hong et al., 2005)*	√	√			√
CisModule (Zhou & Wong, 2004)	√	√	√		
Gibbs Module Sampler (Thompson et al. 2004)	√	√	√		
EMCModule (Gupta and Liu, 2005)	√	√	√		
PhyloCon (Wang and Stormo, 2003)	√	√		√	
EMnEM (Moses et al., 2004)	√	√		√	
OrthoMEME (Prakash et al., 2004)	√	√		√	
PhyME (Sinha et al., 2004)	√	√		√	
CompareProspector (Liu et al., 2004)	√	√		√	
PhyloGibbs (Siddharthan et al., 2005)	√	√		√	
Ortholog Sampler (Li and Wong, 2005)	√	√		√	
MultiModule (Zhou and Wong, 2005)	√	√	√	√	

* In principle, both microarray expression changes and ChIP-chip binding intensities can be used by these algorithms.

The table lists motif/CRM discovery algorithms discussed in the paper. Methods are classified according to the following criteria: (i) *De novo*, whether the method finds previously unknown motif or uses currently available motif; (ii) Abundance, whether overrepresentation of motifs in co-regulated genes is used; (iii) CRM, whether the information from TFBS clusters is used; (iv) Cross-species, whether cross-species conservation is used; (v) ChIP-chip, whether ChIP-chip binding intensities can be incorporated.

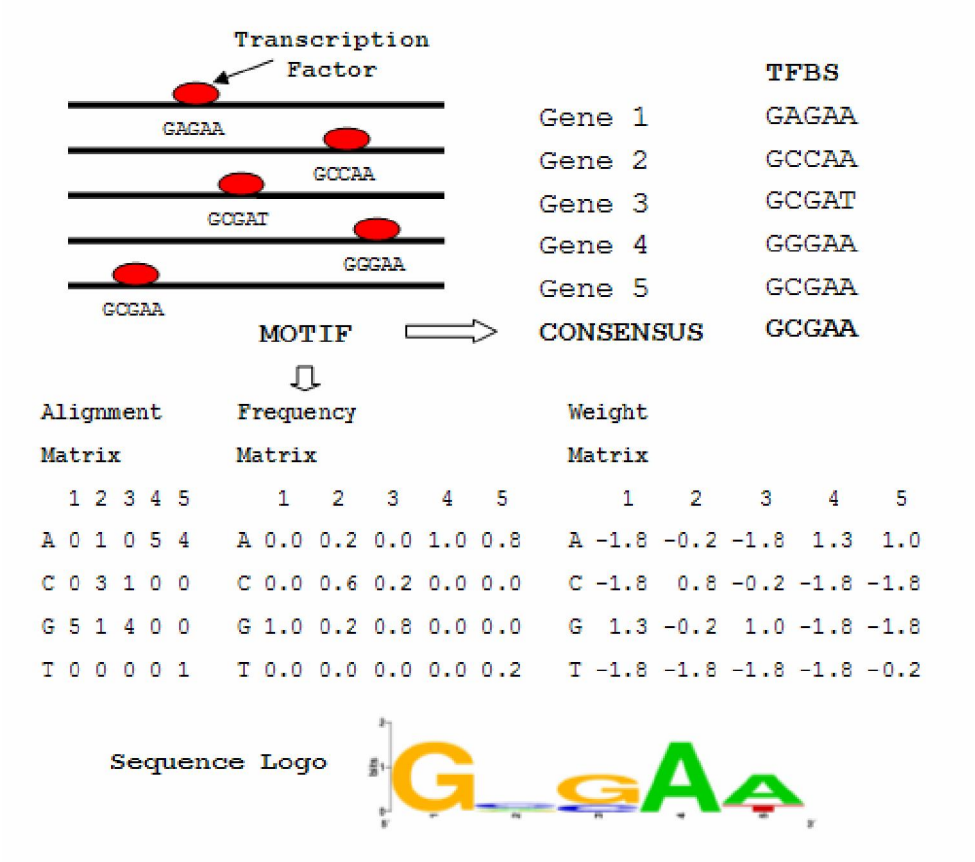
Figure 1. Basic concepts in biology.



(a) The genome is the total genetic information carried by a cell or an organism. The double helix DNA is the main carrier of genetic information. Genes are segments of DNA that encode RNAs or proteins. *Cis*-regulatory elements are DNA segments that control when, where and at what level nearby genes are turned on/off to produce RNAs or proteins.
(b) DNA consists of two complementary strands. Each strand is a linear polymer made up from four types of nucleotides: A, C, G and T. Nucleotides from the two strands form base pairs through hydrogen bonds. The pairing is specific, A only pairs with T, C only pairs with G, and vice versa. The linear DNA sequences in the form of "AAGGTCGT…" provide the blue prints for making living organisms. Genes execute their functions by producing RNAs and proteins. This is achieved through a multiple step procedure. The information carried by double stranded DNA is first copied to single stranded RNA through a procedure called transcription. The RNA produced is premature and needs to be edited through a procedure called splicing. Certain segments (introns) are removed from the premature RNA, and the remaining parts (exons) are linked together to form a mature RNA. One type of mature RNA, messenger RNA (mRNA), is used as a template to synthesize proteins. Proteins are the basic building blocks of life. They have a wide range of functions and can serve as enzymes, signal molecules, components of the cell skeleton, etc. Like DNA and RNA, proteins are also linear molecules, but they are built up from 20 different types of amino acids instead of 4 types of nucleotides. The amino acid sequence of a protein is uniquely determined by its coding mRNA. Three consecutive bases (codon) in an mRNA molecule uniquely determine an amino acid in the product protein sequence. The map between all 64 possible codons and 20 possible amino acids is called the genetic code. The procedure by which a cell synthesizes proteins from their coding mRNAs is called translation. After translation, proteins can be modified further and fold into certain structures in three dimensional space before they execute their function. The whole procedure by which a gene executes its function through transcription and translation is called gene expression.

(c) Complex biological processes often involve coordinated control of the expression of many genes. Certain regulator genes make proteins called transcription factors. Transcription factors can diffuse in the cell and can bind to certain locations in genomic DNA. The DNA sequences where transcription factors bind to are called *cis*-regulatory elements. When bound to DNA, transcription factors may change the chromatin structure or interact with basal transcriptional machinery, therefore modulating the transcription of nearby genes. Expression of a single gene can be modulated by synergic interactions among several types of transcription factors. Through transcriptional control, genes form a regulatory network. The expression of certain genes (transcription factors) turn on/off the expression of certain other genes (primary targets), which in turn may influence the expression of genes further downstream (secondary targets). Although not shown here, gene expression can also be regulated in other ways, e.g. alternative splicing, protein modification, etc.

Figure 2. Transcription factor binding motif

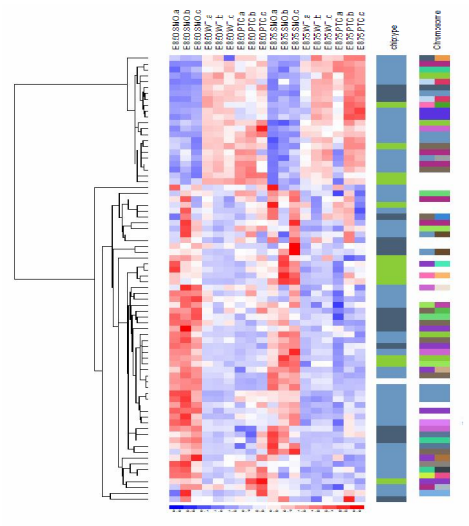


Transcription factors (red) bind to DNA elements to modulate gene expression. For many transcription factors, their binding sites (TFBS) show conserved sequence patterns called motifs. A motif can be represented either by a consensus sequence, an alignment matrix, a frequency matrix or a weight matrix. The consensus sequence gives the most frequent nucleotide in each position. The alignment matrix counts the occurrence of each base type. The frequency matrix represents the frequencies of each base type at each position. The weight matrix computes a log-ratio between observed frequencies in the frequency matrix and base occurrence frequencies in random DNA (background frequency). To avoid taking the logarithm of zero, usually a pseudo-count is added to an alignment matrix before computing the weight matrix. Since an alignment matrix can be described by a Product Multinomial distribution, this operation is equivalent to putting a Product Dirichlet prior on base frequencies. The weight matrix here is obtained by adding 0.25 to each cell of the matrix. Sequence logo (Schneider and Stephens, 1990; Crooks et al., 2004) can be used to visualize the motifs. Each logo consists of stacks of symbols, one stack for each position in the motif. The height of each symbol is proportional to its frequency, and the symbols are sorted so that the most common one is on top. The height of the entire stack is then adjusted to signify the information content of the sequences at that position. Motif discovery aims to find both the motif pattern and the location of TFBSs in DNA sequences.

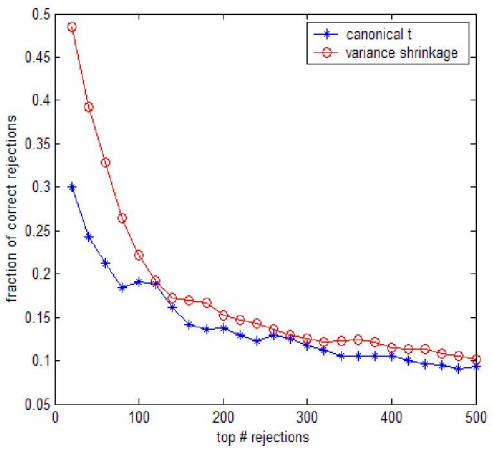
Figure 3. Gene selection from microarray experiments

Condition	Mutant			Wild Type		
Replicate	1	2	3	1	2	3
Gene 1	132.724	112.445	128.478	154.888	122.215	138.303
Gene 2	161.825	163.304	210.121	159.003	172.366	163.199
…						
Gene G	1988.66	2063.48	1899.91	1997.77	2156.19	1977.75

(a)



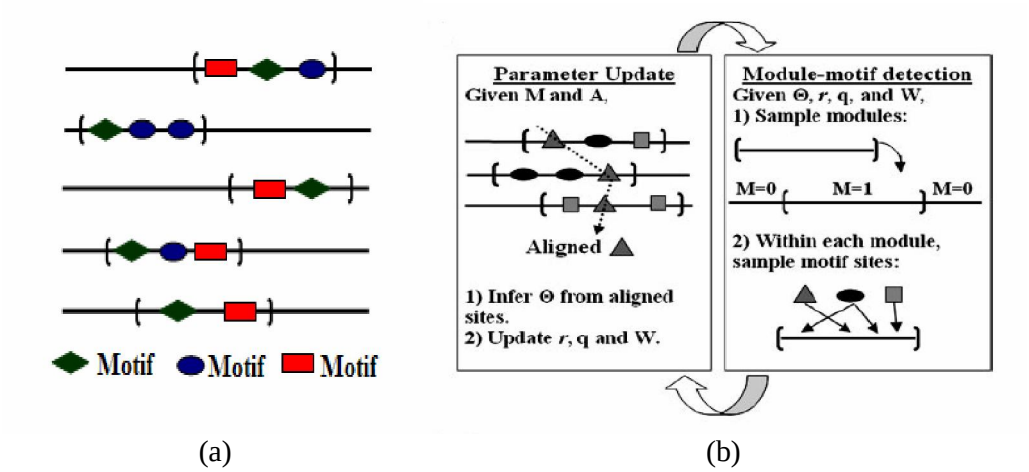
(b)



(c)

A microarray experiment measures expression levels of tens of thousands of genes under different conditions (e.g., mutant vs. wild type). Differentially expressed genes – genes that show different expression levels among different conditions – or clusters of genes that show similar expression patterns are usually selected as targets for further analysis. (a) The typical data structure of a microarray experiment. (b) Clusters of gene expression patterns. (c) By pooling information (shrinking variance) from all genes in the array, one can achieve higher power in gene selection than the canonical *t*-test. The figure shows the fraction of correctly selected genes among the top 20, 40, 60, … rejections.

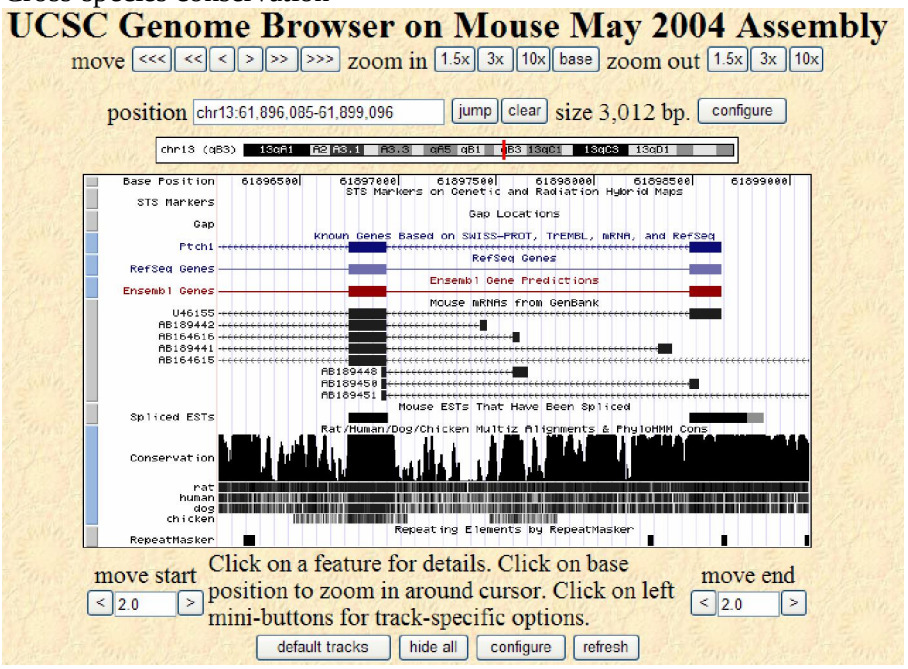
Figure 4. Clustering of transcription factor binding sites



(a) (b)

(a) TFBS tend to be clustered together. (b) The strategy used by CisModule (Zhou and Wong, 2005) to incorporate this information into motif discovery. CisModule iterates between two sampling steps: (i) given the module location indicator **M** and motif location indicator **A**, it infers the parameters **Θ** for characterizing motifs, the module abundance **r**, the motif abundance **q** and the motif widths **W**; (ii) given **Θ**, **r, q** and **W**, it samples module indicator **M** and motif indicator **A**.

Figure 5. Cross-species conservation



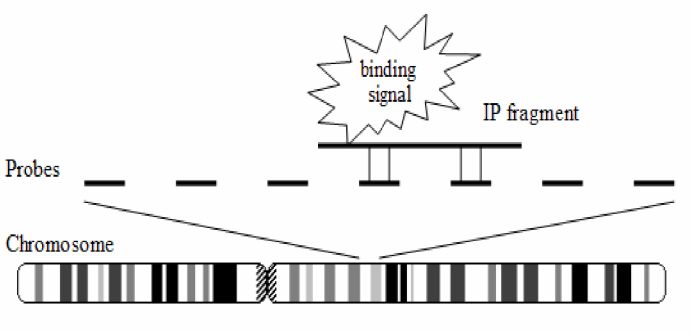
(a)

```
mm5.chr13           -ctgat-cgcttacCTTCCACCCACAGCTCCTCCACGTTGGTCTCGAGATTAGCTGCCTTTAATCCCACA
rn3.chr17           -cgggt-cgcttacCTTCCACCCACAGCTCCTCCACGTTGGTCTCGAGATTAGCTGCCTTTAATCCCACA
hg17.chr9           -cgggcgctcttacCTTCCACCCACAGCTCCTCCACGTTGGTCTCGAGGTTCGCTGCTTTTAATCCCACC
canFam1.chr1        gctggt-ctcttacCTTCCACCCACAGCTCCTCCACGTTGGTCTCGAGATTAGCTGCCTTTAATCCCACA
galGal2.chrZ_random -cg----cactcacCTTCCACCCACAGCTCCTCTACGTTGGTCTCTAGGTTGGCCGCCCGTAGTCCCACC
                     *     * ** ********************** *********** ** ** ** **   ** ******

mm5.chr13           GCGAAGGCCCCAAATATGAGGAGACCCACAACCAAAAACTTGCCGCAGTTCTTTTGAATGTAACAACCCA
rn3.chr17           GCGAAGGCCCCAAATATGAGGAGACCCACAACCAAAAACTTGCCGCAGTTCTTTTGAATGTAACAACCCA
hg17.chr9           GCGAAGGCCCCAAATATGAGGAGGCCCACAACCAAGAACTTGCCGCAGTTTTTTTGAATGTAACAACCCA
canFam1.chr1        GCGAAGGCCCCAAATATGAGGAGGCCCACAACCAAGAACTTGCCGCAGTTTTTTTGAATGTAACAACCCA
galGal2.chrZ_random GCGAACGCGCCGAATATCAGCAGGCCGACCACCAGGAACTTGCCGCAGTTTTTTCTGAATGTAGCAGCCCA
                    ***** ** ** ***** ** ** ** ** ****  ************** ** ******** ** ****

mm5.chr13           GTTTAAATAAGAGTCTCTGAAACTTCGCTCTCAGCCACAGCGGCGCTTTCCGGCCAGTAGCCTTCCCctg
rn3.chr17           GTTTAAATAAGAGTCTTTGAAACTTCGCTCTCAGCCACAGCGGCGCTTTCCGGCCAGTAGCCTTCCCctg
hg17.chr9           GTTTAAATAAGAGTCTCTGAAACTTCGCTCTCAGCCACAGCGGCGCTTTCCGGCCAGTAGCCTTCCCctg
canFam1.chr1        GTTTAAATAAGAGTCTCTGAAACTTCGCTCTCAGCCACAGCGGCGCTTTCCGGCCAGTAGCTTTCCCctg
galGal2.chrZ_random GGTTAAAGAGCAGTCTCTGAAACTTCGCCCGCAGCCACAGCGGCGCTCTCCTCCCGGTAGCTCTCCCctg
                    * ***** *  ***** *********** * ***************** ***  ** *****  *******

mm5.chr13           gggacgaagcagaag-ggaggagtgaacgccggg------gaatcgctgtcccc----------------
rn3.chr17           gggacgaagcagaag-ggaggagtgaacgccggg------gaatcgctgtctcc----------------
hg17.chr9           gggacgaagcagaag-ggaggagtgagcgccggg------gagtcgcggcccgcgcgccca---------
canFam1.chr1        aggacgaagcagaa--ggaggagtgagcgccggg------gagtcgcggcccgcgcgtccacgcccgcct
galGal2.chrZ_random ----caaggcaagcgcggatcagtcagcgatgggccccacgggcagggggcccctaggccg----------
                        * * ***     ***  *** * **  ***      *    *  * *
```

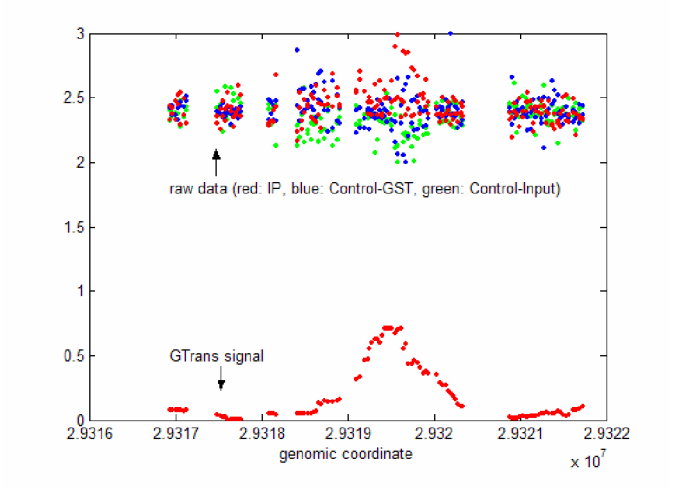
(b)

Functional sequence elements tend to be conserved across multiple species. (a) A region at the beginning of the Ptch1 gene (http://genome.ucsc.edu). The colored thick blocks in panels labeled by “Ptch1” “Refseq Genes” and “Ensembl Genes” are exons, which contain protein-coding sequences. The remaining parts are non-coding regions. The panel labeled by “Conservation” is the conservation score computed from mouse-rat-human-dog-chicken comparisons (Siepel et al., 2005). Exons are functional regions, and their conservation level is high. Some non-coding regions also show high conservation level. These conserved non-coding regions are candidates for functional regulatory elements. (b) Part of the alignment between mouse Ptch1 gene and its homologs in other species. The sequences of exons (blue) are nearly identical in all species compared here.

Figure 6. ChIP-chip experiment



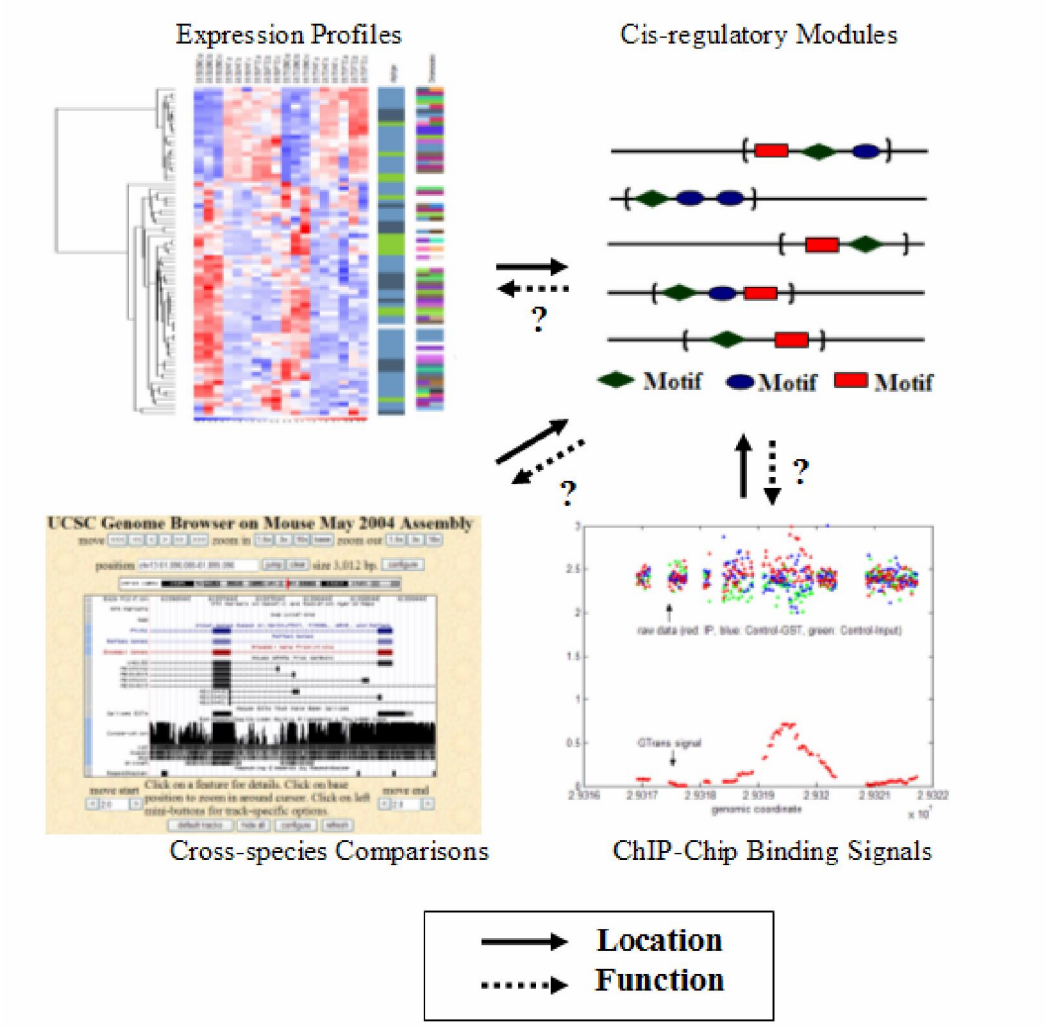
(a)



(b)

(a) The ChIP-chip tiling array experiment tries to locate where transcription factors bind to the genome. Immuno-precipitated (IP) DNA fragments are hybridized to probes evenly spaced across the chromosome. Since IP fragments are amplified from DNA bound by transcription factors, the hybridization signal will tell which part of the genome contains sites for transcription factors to bind. (b) A typical data structure of a ChIP-chip tiling array experiment. Each point represents a signal for a probe. Probes are arranged by their physical locations in the genome. The top panel shows raw hybridization signals for the IP samples (red) and the control samples (blue, green). The bottom panel shows the binding signal computed by GTRANS software.

Figure 7. Integrating various sources of information



For identifying *cis*-regulatory modules in higher organisms, one needs to integrate different types of information. Expression profiles from microarray data can be used to cluster co-regulated genes. Cross-species comparisons and ChIP-chip experiments can help to narrow down the search region. Module discovery algorithms that take clustering information of TFBSs into account can then be applied to search for transcription factor binding sites and motifs. After locating CRMs, the more challenging problem is to relate the locations and combinatorial patterns of TFBSs back to gene functions. This area is largely unexplored.