

Gene expression

Relationship between gene co-expression and sharing of transcription factor binding sites in *Drosophila melanogaster*Antonio Marco^{1,*}, Charlotte Konikoff^{1,2}, Timothy L. Karr^{1,3} and Sudhir Kumar^{1,2}¹Center for Evolutionary Functional Genomics, ²School of Life Sciences and ³Center for Infectious Diseases and Vaccinology, The Biodesign Institute, Arizona State University, Tempe, AZ 85287-5301, USA

Received on March 15, 2009; revised on July 3, 2009; accepted on July 22, 2009

Advance Access publication July 24, 2009

Associate Editor: Joaquin Dopazo

ABSTRACT

Motivation: In functional genomics, it is frequently useful to correlate expression levels of genes to identify transcription factor binding sites (TFBS) via the presence of common sequence motifs. The underlying assumption is that co-expressed genes are more likely to contain shared TFBS and, thus, TFBS can be identified computationally. Indeed, gene pairs with a very high expression correlation show a significant excess of shared binding sites in yeast. We have tested this assumption in a more complex organism, *Drosophila melanogaster*, by using experimentally determined TFBS and microarray expression data. We have also examined the reverse relationship between the expression correlation and the extent of TFBS sharing.

Results: Pairs of genes with shared TFBS show, on average, a higher degree of co-expression than those with no common TFBS in *Drosophila*. However, the reverse does not hold true: gene pairs with high expression correlations do not share significantly larger numbers of TFBS. Exception to this observation exists when comparing expression of genes from the earliest stages of embryonic development. Interestingly, semantic similarity between gene annotations (Biological Process) is much better associated with TFBS sharing, as compared to the expression correlation. We discuss these results in light of reverse engineering approaches to computationally predict regulatory sequences by using comparative genomics.

Contact: amarcoca@asu.edu**1 INTRODUCTION**

Gene expression is typically mediated by transcription factors, which bind to specific DNA sequences (transcription factor binding sites, TFBS). The same transcription factor frequently binds regulatory regions of different genes, inducing their coordinated expression (Davidson, 2001; Latchman, 2005). Therefore, genes containing the same TFBS are often co-expressed. Because the experimental identification of TFBS is difficult, investigators frequently use shared sequence motifs in co-expressed genes to identify putative TFBS (reviews in Chua *et al.*, 2004; Hannehalli, 2008; Wasserman and Sandelin, 2004). This reverse engineering approach uses the co-expression of genes as a tool to predict regulatory sequences, which has been employed in yeasts, plants

and animals (e.g. Cheng and Li, 2008; Dai *et al.*, 2007; De Bleser *et al.*, 2007; Fogel *et al.*, 2004; Perco *et al.*, 2005; Tavazoie *et al.*, 1999).

Are genes with high expression correlation more likely to share TFBS, as compared to those with low expression correlation? This question has been addressed in *Saccharomyces cerevisiae* for which both extensive microarray expression data and experimentally verified TFBS exist (Yu *et al.*, 2003; Allocco *et al.*, 2004; Yeung *et al.*, 2004). In this single-celled organism, only gene pairs with a very high degree of co-expression share significantly larger number of TFBS (Allocco *et al.*, 2004). While these results point to limitations of the use of the reverse engineering approach, they do encourage the use of shared sequence motifs as putative TFBS for some pairs of genes (Allocco *et al.*, 2004).

The regulation of gene expression in animals is far more complex than that in unicellular organisms such as yeast or prokaryotes. At the whole-organism level, the existence of compartmentalization and multiple cell types leads to enhanced complexity in animal regulatory networks. Thus, the experimental characterization of regulatory sequences in multicellular organisms is more difficult, and the use of computational tools to predict putative TFBS more valuable. However, the prediction of regulatory sequences is expected to be generally less successful in animals than in unicellular organisms (see also Tompa *et al.*, 2005; Vavouri and Elgar, 2005). The extent of such limitations in the context of high-throughput microarray expression data remains unknown for animals. Here, we use a compilation of experimentally-verified TFBS and extensive microarray experiment results available for *D.melanogaster* in efforts to (i) quantify the average degree of co-expression for genes containing common TFBS, and (ii) estimate the relative propensity of co-expressed genes to share common TFBS in a multicellular organism.

2 METHODS

We extracted all experimentally verified TFBS data from the REDfly 2.0 database (Halfon *et al.*, 2008), which contains associations between transcription factors and their target genes from systematic curation of DNase I footprint experiments from multiple developmental stages in *Drosophila* (ranging from antero-posterior to mesoderm and imaginal disk patterning). We retrieved the expression patterns for the target *Drosophila* genes in the dataset from microarrays compiled by Spellman and Rubin (2002). The co-expression level is measured as the Pearson correlation coefficient of their expression values in different stages/experiments.

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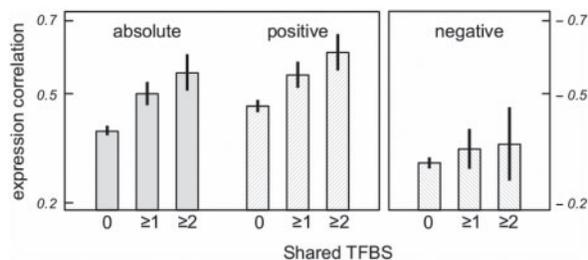


Fig. 1. Number of common TFBS and expression correlation. Height of the boxes represents the average Pearson correlation values for gene pairs sharing different numbers of transcription factor binding sites. The lines over the boxes represent 95% confidence intervals (1.96 times the standard error of the mean). Significance of the differences was assessed by using Mantel tests (Sokal and Rohlf, 1995). Results are shown for absolute, only positive and only negative expression correlation values.

Expression levels in these experiments were measured for the whole organisms.

In addition to REDFLY TFBS, we analyzed data for six transcription factors (*bcd*, *kni*, *Kr*, *hb*, *cad*, *gt*) from Li *et al.* (2008), which enabled us to examine correlations of expression and TFBS sharing in *Drosophila* early development. We used TFBS with a false discovery rate $\leq 1\%$, which were filtered using two additional criteria: the target gene should be expressed during early development; and, if two antibodies were used in the detection, the TFBS should be reported in both experiments. Expression patterns for these TFBS were obtained from Tomancak *et al.* (2002), which captured temporal expression during early development. For these data, we calculated the average expression value for the three experimental replicates in each time window. Alternative analyses using values individually did not change our results.

In further analyses, we also used GO annotation similarity to determine expression correlation instead of microarrays. In order to quantify the GO annotation similarity, we calculated the maximum Resnik semantic similarity between genes (Lord *et al.*, 2003; Resnik, 1999). The calculations were done with the *GOSim* package (Fröhlich *et al.*, 2007) under the R environment (<http://www.r-project.org/>). Analyses using other different measures yielded similar results. In all cases we calculated confidence intervals from 1000 randomly permuted datasets or from standard errors of the mean, as applicable.

3 RESULTS

In an analysis of 2485 gene pairs (71 genes from REDFLY), genes sharing at least one TFBS showed a significantly higher expression correlation than those with no common TFBS (Fig. 1, $P < < 0.01$). The average expression correlation is higher in magnitude for genes sharing at least two TFBS as compared to only one TFBS, although the difference was not statistically significant.

Separate examination of these trends for gene pairs with negative and positive expression correlations showed that genes sharing at least one TFBS had, on average, a 10% more positive expression correlation than those not sharing TFBS (Fig. 1, $P < < 0.01$). We also observed this trend for negative expression correlation, although the differences between categories were much smaller than in the case of positive correlation (Fig. 1).

In these negative correlated gene pairs, the observation that genes sharing TFBS have higher negative co-expression may be taken as an indication of the presence of dual regulators (i.e. transcription factors acting both as activators and repressors). A dual regulator would

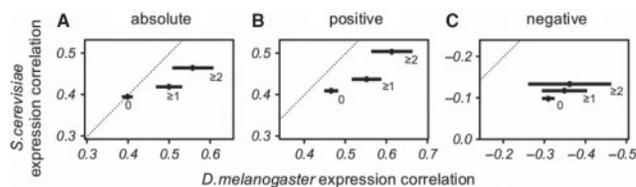


Fig. 2. Number of common TFBS and co-expression correlation comparing *Drosophila* and yeast. The plot compares the average and 95% confidence intervals for yeast and *Drosophila* co-expression correlation values for genes bound by none, more than one or more than two common TFBS. Confidence intervals in yeast are negligible. Dashed line indicates the 1:1 ratio. Different panels show: (A) absolute; (B) only positive and (C) only negative co-expression correlation values.

activate one gene of the pair while repressing the other at the same time. This would produce a negative (complementary) expression correlation between the two genes. Indeed, dual regulators seem to play an important role during *Drosophila* development (Papatsenko and Levine, 2008). In our data, 25% TFBS (22 out of 88) were common to genes with negative expression correlation. The list of potentially dual regulators contained segmentation genes (*bcd*, *kni*, *hb*, *Kr*) and homeotic genes (*abd-A*, *Antp*, *Ubx*), which are known to act simultaneously as repressors and activators during fly development (Lawrence, 1992).

Trends for absolute, positive and negative correlations between *D.melanogaster* and *S.cerevisiae* were similar (Fig. 2), with the exception that all differences were statistically highly significant in yeast analysis presumably due to much larger sample sizes. However, the fraction of negatively co-expressed genes in yeast is much lower than the fraction in *Drosophila* (Fig. 2C), which could suggest a lower frequency of dual regulators in yeast.

Next, we examined the reverse relationship between gene expression correlation and the number of shared TFBS. This is important for evaluating the predictive use of co-expression in detecting TFBS. In particular, we tested whether gene pairs with high (either positive or negative) correlation show an increased probability of sharing TFBS. For positively correlated genes, the number of shared TFBS is 0.20 per pair on average, which is more than two times that observed for negatively correlated genes (0.09). The plot of the proportion of gene pairs sharing TFBS as a function of the expression correlation revealed a mild positive trend (Fig. 3A), with positively correlated genes showing slightly higher propensity of sharing TFBS than the negatively correlated genes. Gene pairs with higher expression correlation shared more TFBS, but the relationship is not statistically significant. Even for genes with expression correlations ≥ 0.75 , only one in five gene pairs shares at least one TFBS (Fig. 3A). Therefore, the use of expression correlation to find genes via comparative genomics of shared motifs may not be very productive.

In contrast to *D.melanogaster*, about 40% of yeast gene pairs with expression correlation above 0.75 shared at least one TFBS and 100% of the genes with expression correlation ≥ 0.90 shared at least one TFBS (Allocco *et al.*, 2004). In *Drosophila*, gene pairs with an expression correlation ≥ 0.90 share a TFBS only 24% of the time. This is a rather large difference, which prompted us to examine whether a small number of TFBS available for *Drosophila* analysis, as compared to yeast (~ 10 times), adversely impacted our investigations. In order to test this possibility, we analyzed

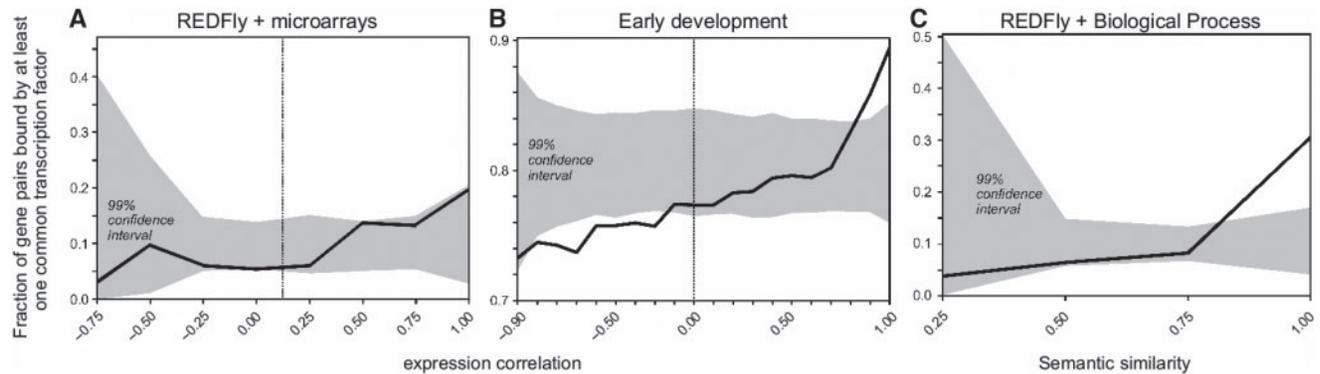


Fig. 3. Relationship between degree of co-expression or semantic similarity, and fraction of common TFBS. The fraction of gene pairs sharing at least one common TFBS (y-axis) is plotted for intervals of co-expression levels in (A) and (B), and semantic similarity in (C) (x-axis). A 99% confidence interval for random expectation is represented in gray. Positive and negative correlation values are separated by a dashed vertical line. (A) Comparison between REDFLY 2.0 reported TFBS and Spellman and Rubin (2002) compilation of microarray expression data. (B) Comparison between early development dataset of TFBS generated by Li *et al.* (2008) and Tomancak *et al.* (2002) microarray dataset. (C) Fraction of gene pairs sharing at least one common TFBS in REDFLY 2.0 for different intervals of semantic similarity.

randomly selected subsets of the yeast TFBS data, such that the random yeast subsets had the same number of transcription factors as those present in our *Drosophila* dataset. The yeast subsets still showed a significant enrichment for highly co-expressed genes in 80% of the analyses, which suggests that the smaller number of TFBS available in *Drosophila* is not expected to obscure the relationship between expression correlation and the number of genes with common TFBS.

Next, we compared the temporal microarray results with those observed in the analysis of adult tissues by using the FlyAtlas data (Chintapalli *et al.*, 2007), which contain genes expressed in 26 tissues. We calculated the proportion of tissues in which genes sharing TFBS were co-expressed and the proportion of co-expressed genes not sharing TFBS. Genes sharing TFBS are co-expressed, on average, in 55% of the analyzed tissues, which was similar to the proportion of co-expressed genes not sharing TFBS (52%). Thus, the expression correlations based on the proportion of tissues in which genes are co-expressed will not be effective as a tool for finding TFBS. On the other hand, genes were enriched for common TFBS in specific tissues. For instance, genes expressed in the brain, share many more common TFBS than expected by chance (2.9 times). For fat body this ratio is 3.1, and for the heart organ as high as 4.7.

The overall lack of relationship between the extent of co-expression and the number of shared TFBS may be related to the greater diversity of tissues and cell types resulting from the complex developmental pathways in higher metazoans. This attribute is less severe in the earliest stages of *Drosophila* embryogenesis. In order to assess the relationships between co-expression and shared TFBS in the early development, we took advantage of a recently published genome-wide experimental detection of TFBS for six transcription factors acting during early development (Li *et al.*, 2008). The expression correlation values were measured for the temporal expression pattern during the first 6 h of development (Tomancak *et al.*, 2002). We examined the fraction of shared TFBS as a function of the degree of co-expression, and found that gene pairs with expression correlations ≥ 0.80 were statistically enriched for common TFBS (Fig. 3B). Genes showing substantial negative

correlation had a smaller fraction of common TFBS. The positive association between co-expression and shared TFBS is likely due to two primary factors. First, there are no differentiated tissues at these early stages. Second, the embryonic chromatin is not organized in heterochromatic structures prior to the third hour of development which, potentially, makes all genomic DNA accessible to the action of transcription factors (Lu *et al.*, 1998; Vlassova *et al.*, 1991). These two factors make the early *Drosophila* embryo to exhibit higher similarities with those observed in yeast. Therefore, expression correlation is expected to be more useful to find TFBS during early development.

Next, we examined whether genes involved in the same biological processes show an overabundance of shared TFBS. Annotations in the Biological Process category in Gene Ontology (GO; Ashburner *et al.*, 2000) reflects our knowledge on tissues and cell types in which genes are found to be co-expressed. This information is more comprehensive (and of different type) than that of selected adult organs in FlyAtlas, and covers embryonic tissues as well. We calculated an index that reflects the degree of similarity in the GO annotation of two gene products (Resnik similarity measure; Lord *et al.*, 2003). This index ranges from 0 to 1 (from completely unrelated annotations to identical annotation for genes). The average level of annotation similarity between gene pairs was higher for genes sharing more TFBS (0.68 versus 0.56). In reverse, gene pairs with very similar GO annotation were more highly enriched in common TFBS (Fig. 3C). The percentage of gene pairs sharing TFBS, when the similarity in the annotation was over 0.75, was $\sim 30\%$ and significantly higher than expected.

These results indicate that the spatial patterns of expression captured in gene annotations have a better association with the number of shared TFBS as compared to the expression correlation obtained from *Drosophila* microarray experiments. In contrast to Biological Process, analyses using the other two GO categories (Molecular Function and Cellular Component) yielded no significant association with the number of TFBS shared (data not shown). Therefore, Biological Process semantic similarity could be a useful tool for finding genes with common TFBS.

We also examined the relationships between the microarray-based gene expression and semantic similarity. The overall correlation coefficient was rather small ($R^2 \approx 0.1$), however, gene pairs sharing TFBS with expression correlation >0.75 showed high semantic similarities. On the other hand, pairs of genes sharing TFBS with a semantic similarity >0.75 had a lower average expression correlation. Therefore, many gene pairs with high semantic similarity have low expression correlations. For example, *slbo* and *btl* are co-activated during cell migration processes (Murphy et al., 1995), and have semantic similarity value of 0.77. But, the co-expression correlation is <0.01 in the microarray experiments. The expression of *rho* and *sim* provide another example, they are both expressed in the neuroectoderm (Martín-Bermudo et al., 1995; Nibu et al., 1998) and have a semantic similarity of 0.94. However, the co-expression correlation is only 0.21. Many such gene pairs are significantly enriched for common TFBS.

4 DISCUSSION

Computational genomics studies often predict TFBS assuming that co-expressed genes are more likely to share binding sites than those not co-expressed (Chua et al., 2004; Hannehalli, 2008; Wasserman and Sandelin, 2004). However, one can begin to assess the underlying principles of these studies only by comparing experimentally verified TFBS and expression patterns. Here we addressed this question in one model animal, *D.melanogaster*, and compared the results to those observed in the yeast. We find that genes bound by the same TFBS have expression correlations greater than those not sharing TFBS. Nevertheless, unlike yeast, gene pairs with a high degree of co-expression in DNA microarray experiments are not statistically more likely to share a TFBS (except in the early development). We obtained similar results by using other microarray experiments (Arbeitman et al., 2002; Manak et al., 2006; Tomancak et al., 2002).

Many *Drosophila* microarrays deal with whole organism gene expression level at different time periods (Arbeitman et al., 2002; Manak et al., 2006; Stolt et al., 2004; Tomancak et al., 2002). Given that genes can be transcribed in different tissues simultaneously in multicellular organisms, it is reasonable to expect that the spatial expression patterns of gene products will be more informative than temporal patterns in order to detect TFBS. This may be the reason why there is no statistical enrichment of TFBS in even highly co-expressed genes (Fig. 3A). Of course, other causes might explain the partial uncoupling between microarray (temporal) expression patterns and regulation by common transcription factors: including differential affinity to binding sites (see Discussion in Pankratz and Jackle, 1993); or regulation by miRNA (Sempere et al., 2003; Sokol et al., 2008). The presence of indirect regulatory interactions after early development might also obscure the association between co-expression and TFBS.

Our results suggest that most collections of DNA microarray data in *Drosophila* may not be useful to predict TFBS, unless we study the action of transcription factors during very early development. These findings are consistent with the success in mammals of reverse engineering approaches to infer regulatory sequences when microarrays measure expression levels in different tissues, that is, spatial expression, instead of quantifying global transcript levels in the whole organism (e.g. De Bleser et al., 2007; Perco et al., 2005). The use of cell cultures in mammals has been also useful to detect

TFBS involved in cell-cycle control (e.g. De Bleser et al., 2007; Elkon et al., 2003). Further analyses in *Drosophila* cell lines could be of interest.

We could not establish a quantitative relationship between the number of tissues in which two genes are co-expressed and TFBS sharing. However, exploration of individual tissues reveals that genes co-expressed in the same organ are indeed enriched for shared TFBS. The fact that genes with similar Biological Process annotations are statistically enriched for common TFBS further supports the above conclusion and suggests that the correlations of spatial patterns of gene expression will provide invaluable for finding TFBS via comparative analysis in multicellular eukaryotes. In particular, correlations in spatial patterns of gene expression captured in high-throughput *in situ* RNA hybridization techniques in *Drosophila* may be useful in identifying TFBS using a comparative approach (Tomancak et al., 2002; Lécuyer et al., 2007).

ACKNOWLEDGEMENTS

The authors thank Fabia Battistuzzi and two anonymous reviewers for useful comments on this manuscript.

Funding: National Institutes of Health grant (to S.K.).

Conflict of Interest: none declared.

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