mef2 activity levels differentially affect gene expression during *Drosophila* muscle development

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Cell differentiation is controlled by key transcription factors, and a major question is how they orchestrate cell-type-specific genetic programs. Muscle differentiation is a well studied paradigm in which the conserved Mef2 transcription factor plays a pivotal role. Recent genomic studies have identified a large number of *mef2*-regulated target genes with distinct temporal expression profiles during *Drosophila* myogenesis. However, the question remains as to how a single transcription factor can control such diverse patterns of gene expression. In this study we used a strategy combining genomics and developmental genetics to address this issue *in vivo* during *Drosophila* muscle development. We found that groups of *mef2*-regulated genes respond differently to changes in *mef2* activity levels: some require higher levels for their expression than others. Furthermore, this differential requirement correlates with when the gene is first expressed during the muscle differentiation program. Genes that require higher levels are activated later. These results implicate *mef2* in the temporal regulation of muscle gene expression, and, consistent with this, we show that changes in *mef2* activity levels can alter the start of gene expression in a predictable manner. Together these results indicate that Mef2 is not an all-or-none regulator; rather, its action is more subtle, and levels of its activity are important in the differential expression of muscle genes. This suggests a route by which *mef2* can orchestrate the muscle differentiation program and contribute to the stringent regulation of gene expression during myogenesis.

muscle differentiation program | transcription factor levels

For several decades it has been appreciated that the controlled regulation of gene expression, including the coordinated activation of batteries of genes, lies behind cell differentiation programs (1–3). It is now clear that a principle tier of control of cell differentiation is through key transcription factors, and an important general question is how these factors coordinate the genetic program of such complex processes. A classic paradigm is muscle, in which the conserved Mef2 transcription factor is a major regulator of gene expression and differentiation (4). Mef2 was first identified in mammalian cell culture (5–7), but because mammals possess four closely related *mef2* genes functional analyses during development are complicated. In contrast, *Drosophila* has a single *mef2* gene and was the first organism to be used to show that *mef2* is required for muscle development *in vivo* (8–10). This highlighted that the analysis of how *mef2* functions is central to understanding how muscle is made. Important characteristics of the underlying genetic program include the temporal coordination of muscle gene expression (11–13) and the regulation of levels and relative stoichiometries of gene expression during myogenesis (14–19). Although these basic features have been known for many years, much remains to be understood about them. However, the identification of Mef2 as a key regulator of muscle gene expression and the more recent development of genomic methodologies provide the opportunity to dissect the mechanisms that underlie these phenomena.

Ideas for how a transcription factor might control programs of cell differentiation have been developed, for example, in the analysis of *Hox* gene function (20–23). It could directly activate many target genes required for the differentiation program, or it could function in a hierarchical system in which it directly regulates only a small number of genes, which then regulate the bulk of the required genes. In the case of Mef2, the former arrangement was suggested by the occurrence of the consensus DNA-binding site for Mef2 in the control regions of many muscle genes (4), together with the observation that ectopic expression of *mef2* is capable of activating ectopic expression of a range of target genes (24–26). This mode of action has recently been strongly supported, and developed further, by two genomic ChIP studies that identified hundreds of *mef2*-regulated genes whose control elements bind Mef2 *in vivo* during *Drosophila* development (27, 28). Consistent with the expression of Mef2 from gastrulation to the end of embryogenesis (9), these genes display a range of expression profiles during muscle development. Among them, there are also groups of genes that are expressed together; for example, there is a burst of gene expression during the early phase of muscle differentiation. It is therefore apparent that to understand the genetic program of myogenesis it is necessary to understand how Mef2 can regulate an extensive array of target genes with diverse temporal expression patterns and, within this, how subsets of genes can be expressed together.

The importance of different levels of regulator proteins in patterning during early animal development is well documented (29, 30). However, much less is known about the possible quantitative requirements for transcription factors later in development when differentiation programs are operating. In muscle, two findings indicate that levels of key transcription factors contribute to the regulation of muscle development. First, the use of allelic combinations showed that skeletal muscle development in mice is sensitive to the levels of myogenin (31). Second, expression of different levels of a *mef2* construct in a *mef2*-null background showed that distinct levels of Mef2 are required in *Drosophila* for different properties of a muscle (32). One explanation for these two findings is that different genes require different levels of the transcription factor for their expression. Here we have addressed this for *Drosophila* Mef2 by combining microarrays with *Drosophila* genetics to determine whether there are genes that respond differently to particular levels of *mef2* activity *in vivo*. We found that some genes do indeed require higher levels for normal expression and that others only need lower levels. Moreover, this differential requirement for *mef2* correlates with when the gene is first expressed during muscle development. These findings suggest one mechanism by which Mef2 can coordinate the expression of its many target genes during the muscle differentiation program.

Results

To investigate whether there are genes with different responses to particular levels of *mef2* activity during muscle differentiation *in*

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE9889).

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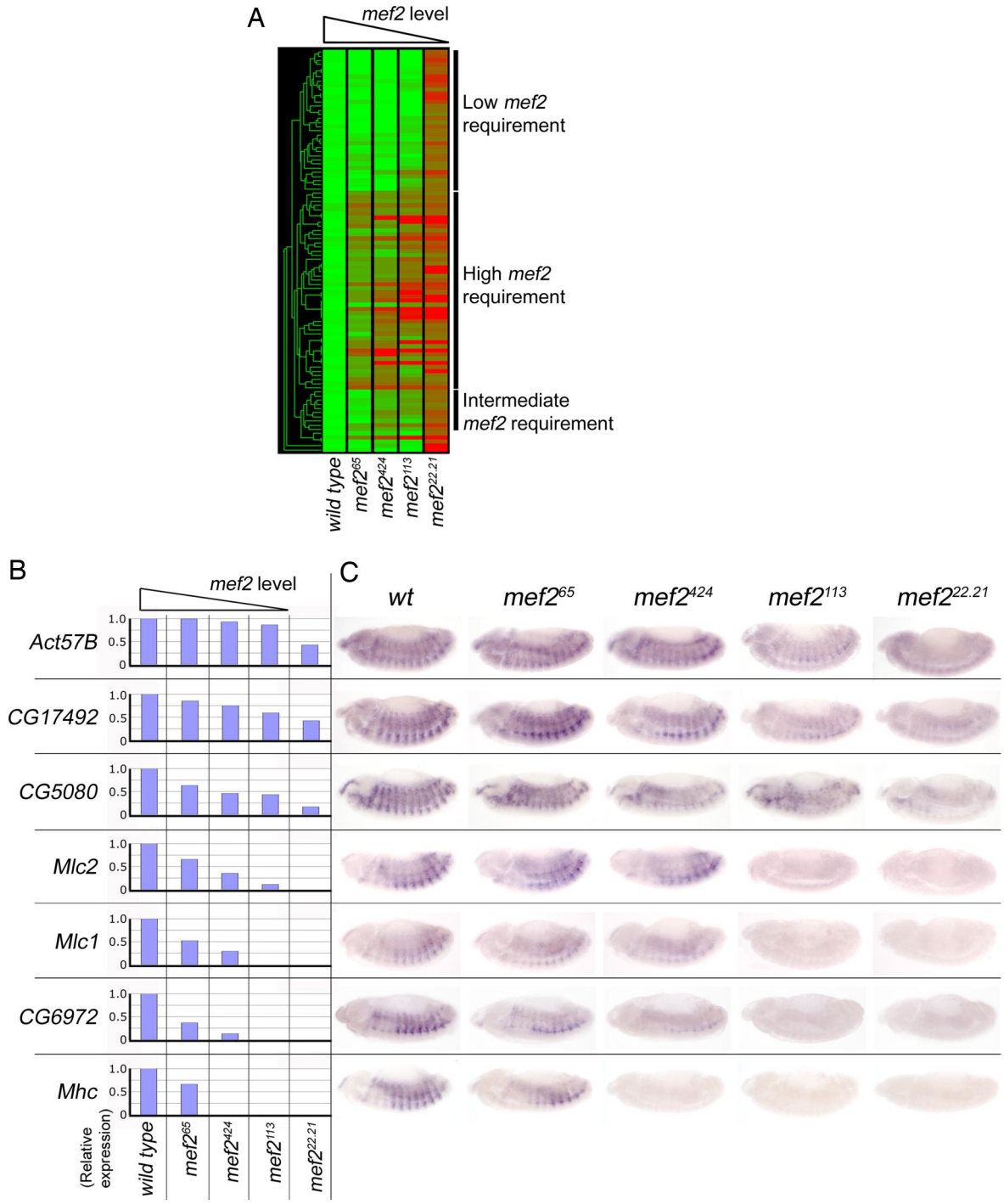

Fig. 1. Different genes respond differently to changes in *mef2* activity levels. (*A*) Hierarchical clustering of expression data for the 97 *mef2*-regulated genes selected as described. Columns show expression for each gene in wild-type, $mef2^{65}$, $mef2^{424}$, $mef2^{113}$, and $mef2^{22.21}$ embryos as indicated. Green represents genes with no change in expression from wild type, red represents genes with no detectable expression, and intermediate shades represent intermediate values. Labeled bars indicate gene cohorts with high, intermediate, and low requirements for *mef2*. (*B*) Histograms showing the expression array data for seven selected *mef2*-regulated genes (*Act57B*, *CG17492*, *CG5080*, *Mlc2*, *Mlc1*, *CG6972*, and *Mhc*) at stage 13 across the *mef2* allelic series. (*C*) *In situ* hybridizations for the seven genes across the allelic series at stage 13 (anterior is to left and dorsal is to top in these and all other figures).

vivo, we used microarrays in conjunction with a *mef2* allelic series. The allelic series extends from a null mutation, $mef2^{22.21}$, through to the wild type via three hypomorphic alleles, $mef2^{113}$, $mef2^{424}$, and $mef2^{65}$ (10). The phenotype of each hypomorph corresponds to different levels of Mef2, because these phenotypes can be recapitulated by direct manipulation of Mef2 levels (32). $mef2^{113}$ is the strongest of the hypomorph alleles corresponding to relatively low levels, and $mef2^{65}$ is the weakest corresponding to relatively high levels. This experimental design allowed us to determine the gene expression profile in developing embryos at five different levels of *mef2* activity in the following order: wild type → 65 → 424 → 113 → 22.21 (Fig. 1). Throughout this article we use "levels of *mef2* activity" to describe the total activity of the *mef2* gene, which includes the level of expression and the activity of the expressed

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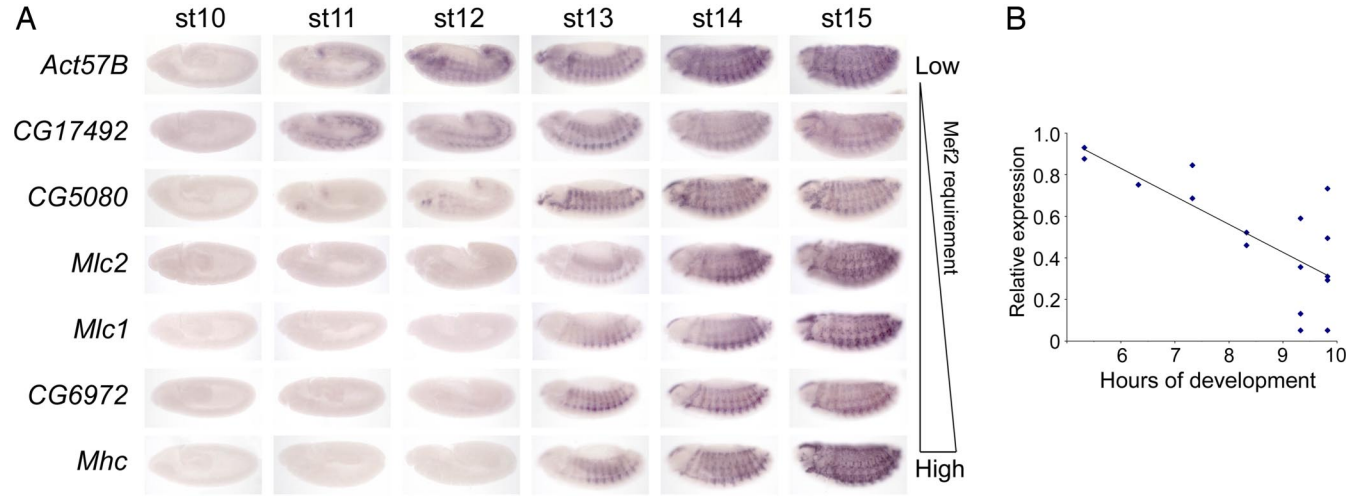

Fig. 2. The *mef2* requirement of target genes correlates with the start of their expression. (*A*) *In situ* hybridizations of wild-type embryos for *Act57B*, *CG17492*, *CG5080*, *Mlc2*, *Mlc1*, *CG6972*, and *Mhc* at different stages of development as indicated. Genes that only need low levels of *mef2* activity (*Act57B* and *CG17492*) are at the top, and those that require the highest levels (*CG6972* and *Mhc*) are at the bottom. (*B*) Graph, with fitted line from linear regression, showing the correlation between the requirement for *mef2* and when the gene is first activated for 16 genes known to be expressed specifically in muscle at stage 13. The requirement for *mef2* is taken as the expression of each gene at stage 13 in $mef2^{424}$ embryos relative to wild type. The start of expression was compiled from *in situ* hybridizations (*A*) (refs. 33 and 34 and data not shown).

protein. For the microarrays, 30-min collections of wild-type and mutant embryos for each *mef2* allele were individually staged, and pools of 150 embryos were then processed at mid stage 13. This corresponds to the early differentiation phase of muscle development and the expression of multiple muscle sarcomeric protein genes. Quadruplicate samples were assayed by using Affymetrix Genechips, and the results were analyzed as described in *Materials and Methods*.

Different Muscle Genes Require Different Levels of *mef2* Activity. We assembled a list of 97 *mef2*-regulated genes. The availability of expression data across the *mef2* allelic series enabled us to assign them with confidence. We included genes that were down-regulated by at least 2-fold in the *mef2*-null allele relative to wild type and whose expression shows a decreasing trend across the series (see *Materials and Methods*). The list contained many characterized muscle genes, e.g., *Act57B*, *if*, *Mhc*, *Mlc1*, *Mlc2*, *Msp-300*, *up*, *nau*, *TpnC73F*, *TpnC47D*, *Scgα*, and *Tig*. Hierarchical clustering of these 97 genes revealed a range of expression profiles across the *mef2* allelic series, i.e., at different levels of *mef2* activity (Fig. 1*A*). For example, genes at the top of the clustering output are expressed at almost wild-type levels in all of the hypomorph alleles and are significantly down-regulated only in the null mutant. They therefore need only relatively low levels of *mef2* activity for normal expression. Other genes further down the figure display a relatively high requirement. They are significantly down-regulated even in the weakest hypomorph allele, $mef2^{65}$, which has the least reduced level of *mef2* activity. There are also genes that display a variety of intermediate requirements for *mef2*. For example, some are expressed at normal levels in the wild type and $mef2^{65}$ allele but are down-regulated in the stronger hypomorph alleles. This expression profiling demonstrates that muscle genes do indeed respond differently to a given level of *mef2* activity.

We then screened the 97 genes for those that had additional evidence implicating Mef2 in controlling their expression. We drew up a list of 16 genes with *in situ* hybridization results showing muscle-specific expression at the start of muscle differentiation (refs. 33 and 34 and data not shown) and that have one or more conserved Mef2 binding sites [supporting information (SI) Table 1]. From this list we selected a group of seven for detailed analysis that contained examples of high, intermediate, and low *mef2*-requirement genes identified in the hierarchical clustering. Each of these genes is ectopically expressed in response to ectopic expression of *mef2* (SI Fig. 4) and also, for those present on the tiling array in a ChIP analysis, binds Mef2 *in vivo* (28). Taken together, these characteristics indicate that Mef2 is a major factor that determines the expression of this group of genes.

The pattern of expression of each of the seven genes was systematically analyzed by *in situ* hybridization with embryos from across the *mef2* allelic series under carefully controlled conditions (see *Materials and Methods*). This both validated the microarray results and showed that each gene was expressed in the differentiating somatic muscle. The expression patterns (Fig. 1*C*) closely matched the trends of expression across the allelic series from the array data (Fig. 1*B*) and emphasize the different responses displayed by the seven genes and hence the data set as a whole. For example, both *CG6972* and *Act57B* are strongly expressed in the wild type at stage 13. However, whereas the $mef2^{113}$ allele has sufficient *mef2* activity for *Act57B* to be expressed at almost wild-type levels, *CG6972* expression is not detected in the $mef2^{113}$ allele. Our findings suggest that these seven genes can be classified according to their requirement for *mef2*. Genes such as *Act57B* only require low levels of *mef2* activity for normal expression, and genes such as *CG6972* require higher levels. Thus, different Mef2 target genes respond very differently to a given level of *mef2* activity in the same group of cells at the same stage of development. This is a significant step forward in understanding *mef2* function in muscle differentiation.

The *mef2* Requirement of Muscle Genes Correlates with the Start of Their Expression During Development. Having found that different muscle genes have different *mef2* requirements, we then asked whether genes that respond to the level of *mef2* activity in a particular way shared any characteristic. We investigated this by undertaking an *in situ* hybridization analysis of the seven genes analyzed in Fig. 1 to determine their gene expression profile during muscle development (Fig. 2*A*). This shows that the requirement of a gene for a certain *mef2* activity level at stage 13 correlates with the start of its expression during development. Thus, genes such as *Act57B* and *CG17492* that need only lower levels of *mef2* activity, i.e., whose expression is hardly affected in the strong $mef2^{113}$

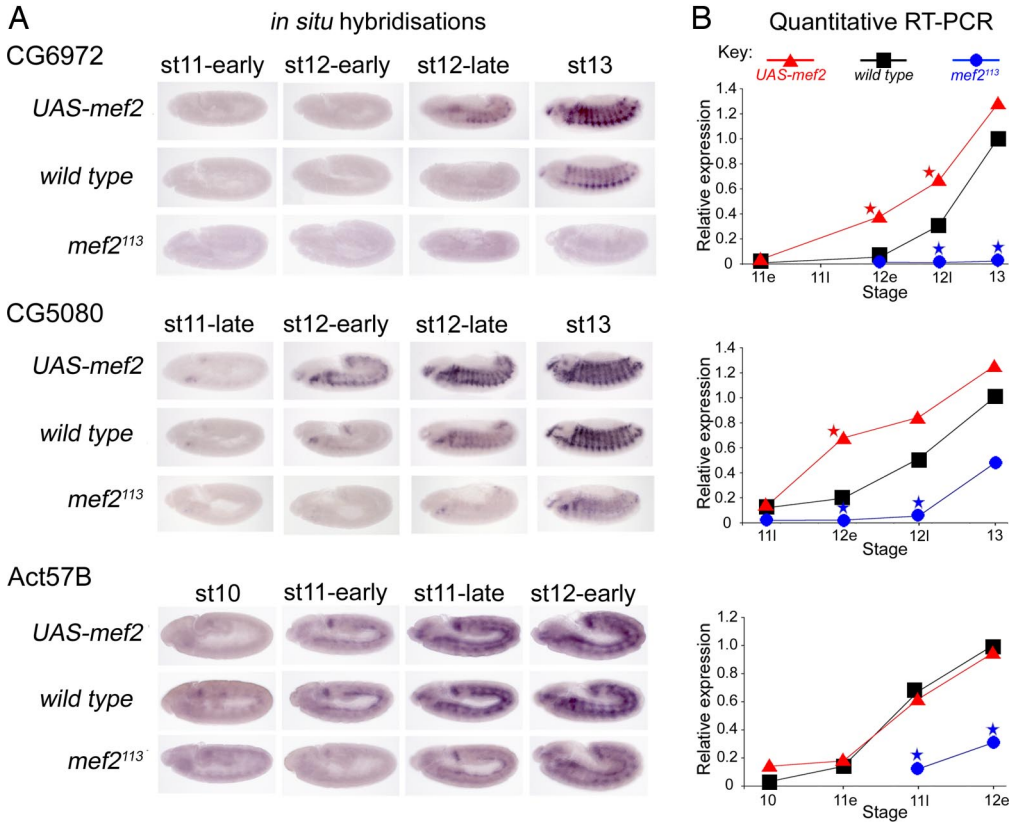

Fig. 3. Increased or decreased levels of *mef2* activity lead to premature or delayed expression of target genes, respectively. (*A*) *In situ* hybridization images of wild-type, *twistGal4;twistGal4* > *UAS-mef2*, and $mef2^{113}$ embryos for *CG6972*, *CG5080*, and *Act57B* at different stages of development as indicated. *CG6972* and *CG5080* expression is premature when *mef2* is overexpressed. *CG6972*, *CG5080*, and *Act57B* expression is delayed when *mef2* activity is reduced. (*B*) Quantitative RT-PCR analysis of *CG6972*, *CG5080*, and *Act57B* transcript levels at the indicated stages. Expression relative to stage 13 levels in the wild type is shown as means from triplicate experiments. Red stars denote values that differ by >1 SD in *UAS-mef2* embryos relative to wild type, and blue stars denote values that differ by >1 SD in $mef2^{113}$ embryos relative to wild type.

hypomorph, are activated early in the muscle differentiation program (stage 11). Genes, such as *CG6972* and *Mhc*, that are activated later in the program (stage 13) are those that require higher levels; i.e., their expression is affected even in the weak $mef2^{65}$ hypomorph. *CG5080*, a gene with an intermediate requirement for *mef2*, is first expressed between these two extremes at stage 12.

To determine whether this correlation is true for other genes, we compared the requirement for *mef2* with the stage of first expression for our list of 16 genes. As a measure of *mef2* requirement we used the expression at stage 13 in the $mef2^{424}$ allele relative to wild type. When this is plotted against the time of onset of expression (Fig. 2*A*, data not shown, and refs. 33 and 34), again there is a clear trend (Fig. 2*B*). Genes that have the higher requirements for *mef2* are activated at later stages. Together these findings suggest that *mef2* influences the temporal expression of these genes and that this may be a widespread characteristic of muscle genes at this stage of development.

Manipulation of *mef2* Activity Levels Can Modulate the Timing of Muscle Gene Expression. If the time of the initiation of Mef2 target gene expression during muscle development depends on its response to a given level of *mef2* activity, then changing the levels should alter the time of gene activation in a predictable manner. To test this we first looked at the effect of decreasing the level by using the $mef2^{113}$ hypomorphic mutant and assessing gene expression relative to wild type in carefully staged embryos. There was a delay in the initiation of gene expression, as shown by *in situ* hybridization for *CG6972*, *CG5080*, and *Act57B* (Fig. 3*A*, compare the bottom two rows of embryos for each gene). These expression profiles were confirmed, and the relative transcript levels in these genetic backgrounds were measured, by using quantitative RT-PCR; this demonstrated a quantitatively significant delay in expression (Fig. 3*A*). Then we increased the level using the Gal4-UAS system to overexpress *mef2* in the developing muscle cells using *twist-Gal4*. This resulted in premature initiation of expression of *CG6972* and *CG5080* (compare the top two rows in Fig. 3*A* for each). However, we did not find this for *Act57B*, and we interpret this as a requirement for another factor(s) in this case. In summary, using genetic manipulations, we could show that *mef2* activity levels directly influence the temporal expression of Mef2 target genes *in vivo* during muscle development. We found that the start of expression of a given Mef2 target gene can be advanced by increases in *mef2* activity levels and delayed by decreases in them. Taken together, these findings suggest a model whereby *mef2* activity levels directly influence the initiation of target gene expression. One simple arrangement that could achieve this is the activation of expression of specific genes at particular thresholds of *mef2* activity.

Discussion

An important question for understanding Mef2 function, and more generally the orchestration of genetic programs during development, is how hundreds of genes are regulated in a controlled way by a single transcription factor to coordinate the complex process of differentiation. In this study we have uncovered a facet of Mef2 function that offers one explanation for this. We found that Mef2 is not an all-or-none regulator. Rather, its action is more subtle, and levels of its activity differentially affect the expression of muscle genes. By combining *Drosophila* ge-

netics with DNA microarrays, we found that groups of muscle genes have different expression profiles across a *mef2* allelic series. The phenotypes of the *mef2* hypomorphs in this allelic series correspond to different levels of Mef2 protein (32). Moreover, Western and immunohistochemistry analyses show that these alleles actually express different levels of the Mef2 protein, although there may also be additional effects on its activity (10). We have therefore referred to "levels of *mef2* activity" to include both of these aspects. Regardless of how changes in the total levels of *mef2* activity occur, our findings demonstrate that Mef2 can affect different muscle genes independently. Some require higher levels for their expression than others. This is a significant step forward in understanding the role of this transcription factor in the regulation of the genetic program of muscle differentiation. Furthermore, this differential requirement for *mef2* correlates with when the gene is first expressed in this program, suggesting that levels of *mef2* activity are implicated in temporal regulation. In support of this, we found that increased or decreased levels could lead to premature or delayed transcription of target genes, respectively.

A model for muscle development during *Drosophila* embryogenesis is suggested by these findings in which the level of *mef2* activity increases and results in the sequential activation of Mef2 target genes: those that only need low levels would be expressed early in myogenesis, and those that require higher levels would be expressed later. An increase in the total level of *mef2* activity during muscle development could in principle be achieved by two routes, and there is evidence for both. First, it could be more highly expressed and consistent with this, *mef2* transcript levels increase during the early phase of muscle development (34). Second, there could be an increase in the effective activity of the Mef2 protein. One possible route is through the decrease in the expression of the Mef2 inhibitor Him during the period when many of the genes analyzed in this study are first expressed (35). Other regulators may also contribute to changes in Mef2 activity during *Drosophila* development because studies of mammalian Mef2 show that it is subject to an array of regulatory modifications (36–39).

The use of Mef2 in this way to regulate muscle genes during differentiation represents a simple mechanism to coordinate the relative timings and expression of these genes and offers an explanation for a number of features of the muscle differentiation program. For example, the existence of temporal patterns of muscle gene expression is long established (11–13), and our findings indicate how batteries of muscle genes might be expressed together. These would be genes that have a similar requirement for a particular level of *mef2* activity. Our model can also explain the sequential activation of different genes, because those activated at low thresholds of *mef2* activity would be expressed before genes that are activated at higher thresholds. Last, this temporal regulation by Mef2 may also contribute to the absolute level and relative stoichiometry of expression of sarcomeric proteins, which is known to be important in myogenesis (14–18).

Generally in animal development the importance of the level of regulators has been explored in early patterning events. However, an example later in development is the *Caenorhabditis elegans* PHA-4 transcription factor (40). It regulates the expression of a large number of target genes activated at different times throughout pharynx organogenesis, and a key feature in current understanding is an increase in PHA-4 levels that sequentially activates different targets. This parallels our findings with Mef2 in the muscle differentiation program. Nevertheless, other mechanisms may also contribute to temporal programs of muscle gene expression. For example, in a mammalian muscle cell culture model there is a MyoD-activated feed-forward circuit (41). Moreover, even though Pha4 has a dominant role in pharynx development, other factors modulate its action (42), and it is very likely that there are also additional inputs for Mef2 and muscle. Indeed, it has been suggested that the helix–loop–helix transcription factor Twist modulates Mef2 action, albeit on a cohort of genes expressed earlier than those we analyzed (28). Further progress on the orchestration of muscle development by Mef2 will require analysis of the mechanism(s) by which Mef2 differentially activates target gene expression, which in turn will necessitate studies of these other putative factors and the enhancer architecture of muscle genes.

In summary, our findings highlight a previously undescribed aspect to understanding muscle development and suggest one mechanism by which Mef2 can orchestrate multiple events in muscle differentiation. Rather than Mef2 working as a simple on/off switch of muscle differentiation, our work leads to a model in which the level of *mef2* activity increases during muscle differentiation and results in the sequential activation of muscle genes.

Materials and Methods

***Drosophila* Genetics.** The stocks used were $mef2^{65}$ (10), $mef2^{424}$ (10), $mef2^{113}$ (10), $mef2^{22.21}$ (8), *UAS-mef2* (8), *da-Gal4*, *69B-Gal4*, and *twi-Gal4*; *twi-Gal4* (43). For mutant selection by the absence of GFP in the microarray and quantitative RT-PCR analyses, the *mef2* alleles were balanced over *CyO P(Gal4-twi)P(UAS-2eGFP)* (44). For mutant selection by absence of lacZ for *in situ* hybridization, the *mef2* alleles were balanced over *CyO ftzlacZ*. All Gal4-UAS crosses were at 25°C. The wild-type stock for the microarray analysis and the *in situ* hybridizations in Fig. 1 was *dp cn a px sp* (the stock used for the mutagenesis that produced $mef2^{65}$, $mef2^{424}$, and $mef2^{113}$ and kindly provided by Janis O'Donnell, University of Alabama, Tuscaloosa, AL). The wild type for the *in situ* hybridization analyses in Figs. 2 and 3 and SI Fig. 4 was *Oregon R*.

Microarray Analysis. After two 1-h prelays, 30-min embryo collections were aged at 25°C for 6.5 h before sorting. Mutant embryos were individually selected by the absence of the GFP balancer chromosome. Both mutant and wild-type embryos were accurately staged at mid 12 by using autofluorescence to visualize the germ band to ensure that all were within a 30-min window. Embryos were allowed to develop until mid stage 13, dechorionated, inspected to ensure that normal development had continued, and immediately homogenized in TRIzol before storage at −80°C. RNA was isolated, labeled, hybridized to Affymetrix Genechip 1 arrays, and scanned by the Flychip *Drosophila* microarray resource (www.flychip.org.uk). Data were supplied in MAS5.0 normalized form and analyzed by using Genespring software (Agilent Technologies). A total of 97 *mef2*-regulated genes (available on request) were selected by using the following criteria: (*i*) four of four measurements flagged present in wild-type embryos; (*ii*) >2-fold down-regulation in the $mef2^{22.21}$ allele relative to wild type or flagged absent in the $mef2^{22.21}$ allele; (*iii*) expressed with a decreasing trend across the allelic series (decreases, or increases by <1.5-fold, between each successively stronger allele); (*iv*) mutant alleles show statistically significant differences relative to wild type by one-way ANOVA ($P < 0.05$). Hierarchical clustering used the Gene Tree function of Genespring with the standard correlation similarity measure.

Phylogenetic Footprinting. Precomputed pairwise *Drosophila melanogaster–Drosophila virilis* (SLAGAN) alignments were viewed by using VISTA (45) (http://genome.lbl.gov/vista/index.shtml). Mef2 sites were identified by using the degenerate consensus YTAWWWWTAR (8).

***In Situ* Hybridization.** For RNA probe synthesis, the following cDNA clones were obtained from the *Drosophila* Genomics Resource Center: *CG5080*, clone LD34147; *CG17492*, clone GH28686; *CG6972*, clone RH25557; *Mlc1*, clone RE07220; *Mlc2*, clone RE35841; *Mhc*, clone LD31809; *Act57B*, clone LD04994. *In situ* hybridizations and antibody stainings were undertaken as previously described (26). The responses to *mef2* activity levels were analyzed by comparing embryos processed in parallel using common reagents and the same incubation times. At least 10 embryos for each genetic condition at each stage were analyzed, and representative images are shown.

Quantitative RT-PCR. Embryos from 1-h collections were individually inspected and sorted to ensure that all were the correct stage. For mutant selection they were also sorted for the absence of GFP fluorescence at mid stage 12. Embryos were allowed to develop at 25°C to the appropriate stage and rinsed before snap freezing. Embryos were homogenized, and total RNA was isolated by using an RNeasy protect mini kit (Qiagen). First-strand cDNA was primed with poly d(T) and used SuperScript III (Invitrogen) under the manufacturer's standard conditions. Quantitative PCR used SYBR green (Bio-Rad) in the manufacturer's standard conditions in a Chromo4 instrument (MJ Research).

Cycle conditions were as follows: 95°C for 15 min, 35 × 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 30 s. Measurements in triplicate were made by using *rp49* as the reference, and mean results were plotted as $2^{-\Delta\Delta C(T)}$ relative to the peak level of expression in the wild type (46).

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