REGULATION OF THE C-JUN ENHANCER/PROMOTER BY MEF2 PROTEINS DURING MYOGENESIS

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Abstract

Expression of the c-jun immediate early gene is regulated by a variety of physiological and developmental processes. Analysis of the c-jun gene has identified the presence of a Myocyte Enhancer Factor 2 (MEF2) binding site in its regulatory region. The MEF2 site is bound by transcription factors encoded by four genes (MEF2A-D). In this study we assessed the role of the MEF2 site in the c-jun enhancer in myogenic cells. C2C12 myoblasts were transfected with either a reporter gene containing the full length c-jun enhancer (-225 to +150, pJLuc) or the same reporter with the MEF2 site mutated (pJSXLuc), and then allowed to differentiate. Mutation of the single MEF2 site resulted in a 40% decrease in transcriptional activation of the c-jun enhancer in myogenic cells. DNA binding assays demonstrated that MEF2 binding to the c-jun MEF2 site is induced during the transition from myoblasts to myotubes. Occupancy of the c-jun MEF2 site in myogenic cells is predominantly due to MEF2A homodimers, although some MEF2A:MEF2D heterodimers are present. Overexpression of MEF2A and a truncated form of the c-jun enhancer (-80 to +150, pJC9 0FLuc) led to a seven fold increase in reporter activity. Co-expression of MEF2A and the basic helix-loop-helix protein (bHLH) MyoD resulted in a synergistic 90 fold increase. Co-immunoprecipitation studies demonstrated that, *in vivo*, the bHLH protein myogenin can interact with MEF2A, as well as MEF2D. Therefore MEF2 is important for *trans*activation of the c-jun enhancer in

muscle cells, and may interact with other bHLH myogenic factors in order to accomplish this.

Acknowledgements

When it's all done, you begin to realise how many people have helped you along the way. I would like to thank my supervisor Dr. John McDermott, whose knowledge, guidance, and understanding, has been invaluable over the past two years. Thanks to Dr. Olga Ornatskaia for your endless help, and sharing your knowledge with me. My best friend Angie has always been there for me and supported me all the way - thanks bud. I would like to thank all the members of our lab that I have worked with, those who have finished, and the present rookies, Sonya and Padma, and the seasoned veterans Dave, Zoë, and Sandra. I would like to thank my parents and family, Angie's family, my friends, and especially Laila for all their help. Thanks to Dr. Bédard, Natalie, and the rest of the Bédard lab for their help throughout these two years. I would also like to thank Dr. Ron Prywes for providing many of the reporter constructs necessary for this study. I would also like to thank the members of the Kinesiology programme, especially Dr. Cafarelli, Dr. Gledhill, Dr. Hood, Dr. Kelton, and Dr. Malszecki for their support. Finally, I would like to thank God for providing me the opportunity to accomplish my goals.

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Introduction

The differentiated muscle cell is the result of a variety of cellular events, including cell cycle withdrawal, fusion of myoblasts, and the expression of muscle specific genes. The expression of many of these genes is regulated at the transcriptional level and is dependent on transcription factors binding to DNA and altering the rate of transcription. Two families of DNA binding proteins critical for the myogenic process are the Myocyte Enhancer Factor2 (MEF2) and myogenic basic helix loop helix (MyoD) families. Their expression is increased during differentiation and they bind to DNA in a sequence specific manner. The available evidence indicates a molecular network in the control of myogenesis, in which the MEF2 and bHLH genes are central components.

MEF2 proteins are also present in non-muscle cells, and MEF2 cis elements are present in the enhancers of non-muscle specific genes. Their role in this context is only just beginning to be defined. For example, the MEF2 cis element is present in the enhancer of the relatively ubiquitously expressed c-jun gene. c-Jun forms part of the complex of the Activator Protein-1 (AP-1) family of transcription factors. Along with MEF2 there are several other binding sites for transcription factors in the c-jun enhancer which have the ability to influence its transcription (SP1, CTF, NF-Jun, and two AP-1 like binding sites). Although there is a MEF2 binding site in the c-jun enhancer, the role of c-Jun during myogenesis has yet to be determined. The c-Jun protein can form heterodimers with members of the fos (c-Fos, Fra-1, Fra-2) or ATF/CREB family, or dimerize with other members of the Jun family (JunB, JunD). It is believed that the AP-1 complex may control the expression of genes involved in cellular proliferation, however,

in certain cell lines, it is also believed to be important for differentiation. Studies so far have indicated that c-Jun overexpression inhibits differentiation of myoblasts into myotubes . This inhibition is likely due to a direct protein:protein interaction between MyoD and c-Jun. However, these conclusions were based on overexpression studies, which may not necessarily reflect the true 'physiological' role of c-Jun during myogenesis. Although Jun/AP-1 and MyoD properties oppose each other when either is overexpressed, it is possible that their physiological levels during myogenesis are exquisitely counter balanced and that the precise maintenance of their concentrations is an important determinant of the differentiation process. An effective way in which this is accomplished is through the assembly of various transcription factor complexes in the enhancer region of the gene.

Purpose

Since the MEF2 factors are induced during myogenesis, and the regulation of c-jun transcription could be potentially important during myogenesis, we undertook this study in order to assess the role of the MEF2 site, and the proteins that bind to it, in regulating the c-jun enhancer during myogenesis.

Overview of Experimental Strategy

In order to determine if MEF2 can modulate the transcriptional activity at the c-jun enhancer/promoter region, transient transfection assays will be used. Specifically, Hela cells will be transfected via the calcium phosphate co-precipitation technique. The constructs that will be transfected are pMT2MEF2A, pMT2MEF2C, pMT2MEF2D, pJLuc, and p0FLuc. pJLuc is a reporter construct which contains -225 to +150 of the c-jun enhancer/promoter upstream of the firefly luciferase gene (see fig.1).

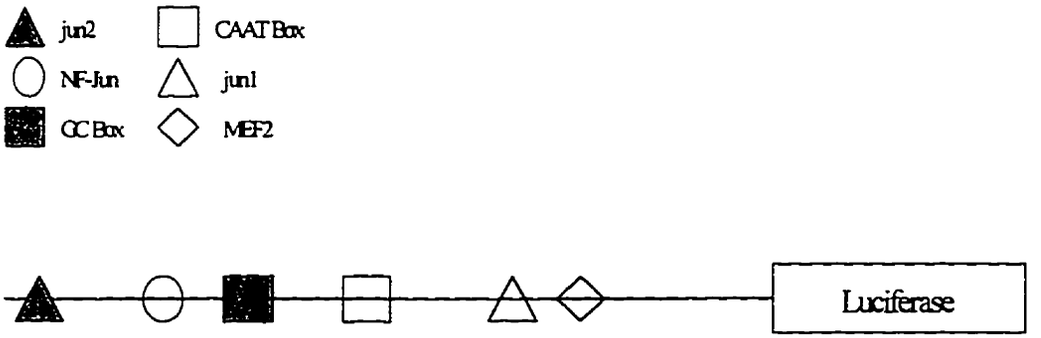

Figure 1. Schematic drawing pJLuc.
pJLuc contains -225 to +150 of the c-jun enhancer/promoter, upstream of the firefly luciferase gene. The known transcription factor binding sites are indicated.

As a control, the reporter construct p0FLuc will be used (see fig.2). This construct contains -53 to +42 of the c-fos promoter.

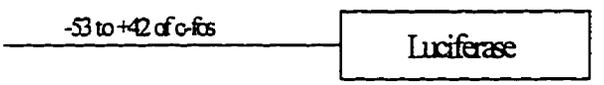

Figure 2. Schematic drawing p0FLuc.
p0FLuc contains -53 to +42 of the c-fos promoter, upstream of the firefly luciferase gene.

pMT2- MEF2A, MEF2C, and MEF2D are expression vectors for MEF2A, MEF2C, and MEF2D respectively. This experiment will allow us to determine if members of the MEF2 family are binding to the MEF2 site in the pJLuc construct and if it they are able to activate transcription of the luciferase gene. Since p0Fluc has no MEF2 site, this will act as a control to determine that MEF2 on its own is not activating the luciferase gene, independent of the presence of a MEF2 site. Once it has been established that MEF2A is binding and activating its site in the enhancer, the pJLuc and p0Fluc reporter plasmids, along with the pJSXLuc reporter will be transfected into growing myoblasts, which will then be induced to differentiate. pJSXLuc (see fig.3) contains -225 to +150 of the c-jun enhancer/promoter, however, there are point mutations on the MEF2 site. The MEF2 site in the enhancer is CTATTTTTAG, which fits the consensus binding site CTA(A/T)$_4$TAG. Instead, pJSXLuc contains the MEF2 site CGATTTTTCG (the underlined bases changed). These mutations have been shown to abolish MEF2 binding to this site (14). The results for this transfection will tell us if MEF2 is affecting the transcription of c-jun during myogenesis.

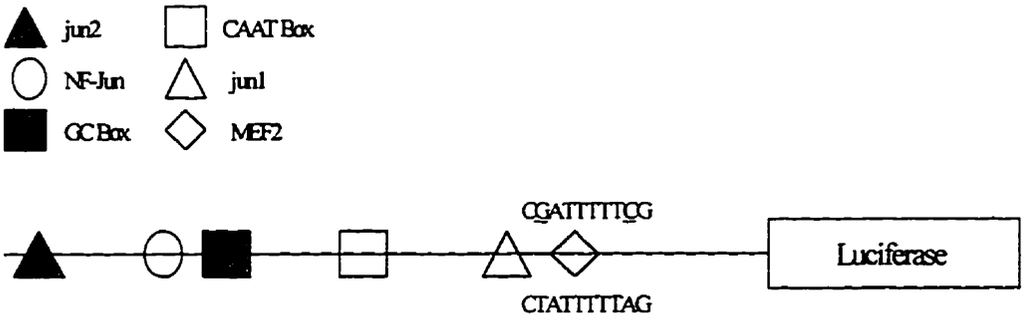

Figure 3. Schematic drawing of pJSXLuc.
pJSXLuc consists of -225 to +150 of the c-jun enhancer/promoter, upstream of the firefly luciferase gene. This reporter is identical to pJLuc, except in this reporter, there are two mutations in the MEF2 site, which are underlined here.

It has been shown that MEF2 proteins and the myogenic bHLH proteins can interact to synergistically increase transcription. In order to determine if this could occur at the MEF2 site on the c-jun enhancer, the reporter construct pJC90FLuc (see fig. 4) will be used. Hela cells will be transfected with pJC90FLuc alone, or along with either pMT2MEF2A, or pMT2MyoD (a MyoD expression vector).

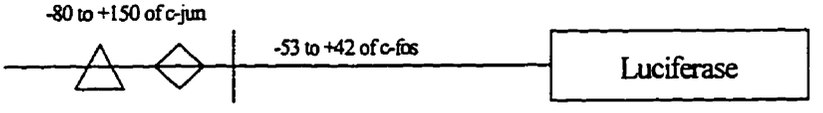

Figure 4. Schematic drawing pJC90FLuc.
pJC90FLuc contains -80 to +150 of the c-jun enhancer/promoter, upstream of p0Fluc. This reporter only contains the binding sites for the upstream AP-1 site, and MEF2.

In order to determine if MEF2 and the myogenic bHLH proteins can interact *in vivo*, immunoprecipitations will be performed. Myogenin proteins will be immunoprecipitated from C2C12 myotubes using a monoclonal α-myogenin antibody. Like MyoD, myogenin is a member of the myogenic bHLH proteins. The

immunoprecipitated myogenin will then be analysed using western immunoblotting to detect the presence of MEF2 proteins complexed with myogenin.

Review of the Literature

The decision of muscle cells to proliferate or differentiate is dictated by a variety of cellular signals. Although the complex network of myogenic signals *in vivo* are presently incompletely characterised, the differentiation of myoblasts can be mimicked in tissue culture (60). A great deal is known about myogenesis *in vitro*, which has led to considerable insight into *in vivo* muscle differentiation. When myoblasts in culture are provided with serum and certain growth factors, the differentiation process is inhibited and the cells are able to proliferate (60). The withdrawal of growth factors and serum below certain thresholds initiates the differentiation program. This process consists of fusion of the myoblasts to form myotubes, the maintenance of a post-mitotic state, and the activation of muscle specific genes (60). The transcriptional activation of genes is a critical process in the myogenic pathway.

Transcription

The flow of genetic information within a cell generally proceeds from DNA to RNA, in a process called transcription, and then from RNA to protein in a process called translation. They are many steps along this path which have the potential to be regulated. It is at the level of transcription where gene expression is frequently controlled. This control is mediated by intra- and extracellular signals which in effect, can regulate transcription (53). The process by which this occurs is through the modulation of the activity of certain DNA binding proteins, called transcription factors (53, 72).

Upstream to a gene's transcription initiation start site is the region where transcriptional regulation occurs (58). This region can be divided into two parts: a promoter, and an enhancer region (See fig. 1), however, there can also be enhancer elements downstream of the transcriptional start site (53). The binding of transcription factors to these regions is necessary for transcription to occur.

Figure 1. Schematic diagram of gene regulation.
The transcriptional start site is indicated by the bent arrow. Although not shown, enhancers can also bind downstream of the transcriptional start site.

Binding to the promoter region are the general transcription factors, which comprise the RNA polymerase II holoenzyme (72, 12). The promoter region often contains the nucleotide sequence TATA, referred to as the TATA box (53). The general transcription factors are thought to assemble at the promoter in a stepwise manner. The first to bind there is Transcription Factor IID (TFIID) (72). TFIID is a complex which consists of the TATA binding protein (TBP), which recognises and binds to the TATA sequence, as well as approximately 10 TBP- associated factors (TAFs) (72). The polymerase II holoenzyme, which contains many of the important transcription factors, such as the Srb proteins, comes as a preassembled complex to the DNA (72). The Srb proteins can phosphorylate the C-terminal domain of RNA polymerase II, which leads to the initiation of transcription by RNA polymerase II (72).

Binding to the enhancer region is another set of transcription factors which allow for the specificity in the regulation of gene expression by either activating or repressing transcription of that particular gene. There are three important parts to these transcription factors. They are the DNA binding domain, the dimerization domain, and the transcriptional activation domain. (78). An example of one can be seen in the schematic in figure 2. The DNA binding domain recognises specific DNA sequences and makes contact with DNA (78). The dimerization domain allows transcription factors to dimerize, or form partners with other transcription factors. This domain is where the actual physical contact between the partners occurs. The transcriptional activation domain is what allows the transcription factor to alter the transcriptional process. The enhancer region of the gene contains specific nucleotide sequences where these general transcription factors bind to (78). This together, is where the specificity and regulation conferred by these transcription factors lies. Since the DNA binding domain only recognises specific DNA sequences, it is only in those genes containing those specific DNA sequences that that transcription factor will bind to and effect transcription. In other words, different transcription factors will not bind to any enhancer region, but only to those which contain the sequence which it recognises. For example, the transcription factor Myocyte Enhancer Factor 2 (MEF2) binds to the nucleotide sequence $CTA(A/T)_4TAG$ and will bind only to enhancers containing that sequence (60). The dimeric partner can modify the specificity of the transcription factors' effect, as does the transactivation domain, due to its potential to be regulated.

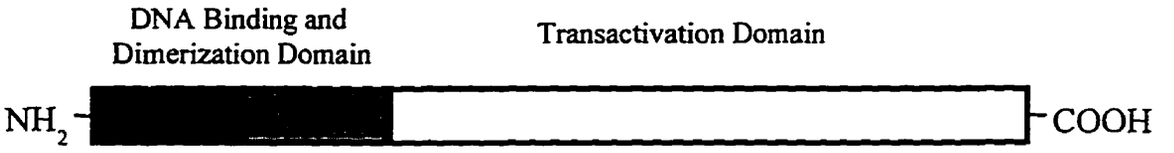

Figure 2. Schematic diagram of MEF2
The above diagram shows the DNA binding, dimerization, and transactivation domain of MEF2. In this case, the DNA binding and dimerization domain are located ion the same region.

Herein exists the association between the transcription factors binding to the enhancers, and those which comprise the basal transcription machinery and bind to the promoter. The activation domains of the transcription factors interact with the basal transcriptional machinery (78). The critical role for transcription factors is in their ability to form protein-protein interactions with the basal transcription machinery (72). They can promote and accelerate the formation of the TFIID complex at the TATA box, and interact with the TAFs and the other proteins associated with the RNA polymerase II holoenzyme (such as Srb), and synergistically activate transcription (72).

Transcriptional Control in Muscle

The importance of the regulation of gene expression by transcription factors is well illustrated during muscle development. The myogenic basic helix-loop-helix family (MyoD family) of transcription factors, which include MyoD, myogenin, MRF4, and Myf5, can activate the muscle differentiation program when expressed in fibroblasts, and an assortment of non-muscle cells (17, 60). This is accomplished by forming heterodimers with ubiquitously expressed E-proteins (60). Once these dimers are formed, this complex can bind to the sequence CANNTG (where N is any nucleotide), referred to as an E-box, which is found in the enhancer of many muscle specific genes (60). Once

bound there, they can activate the transcription of these genes. However, not all muscle genes contain an E-box. Another important myogenic transcription factor family, referred to as the Myocyte Enhancer Factor2 (MEF2) family of transcription factors, can activate the expression of these E-box independent genes (60). The members of this family include MEF2A-D, and they form dimers within the family and bind DNA to activate transcription (60). Enhancers of some muscle specific genes also contain the consensus binding site for both the MyoD family and MEF2 family, leading to activation from both of these sites (see fig. 3). Together, these two gene families are thought to orchestrate the initiation of the myogenic programme.

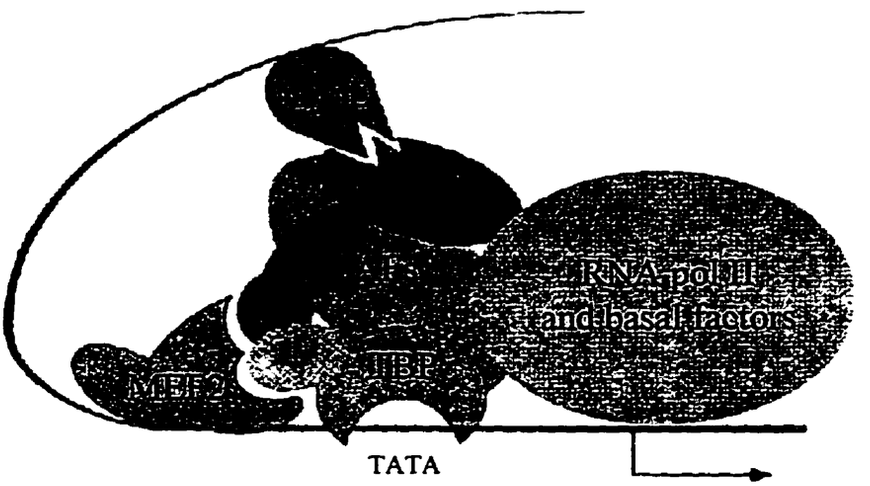

Figure 3. Schematic of the muscle regulatory factors
MyoD and MEF2 are able to interact with the general transcription machinery. As shown, the transcription factors located at a distance from the basal transcription machinery can cause the DNA to bend so that this interaction can occur (adapted from 78).

A more ubiquitous and widely studied transcription factor is c-Jun. As an immediate early gene, c-jun has been implicated in the cellular response to stimuli such as

stress, osmotic shock, and growth factors (38). However, the role of this protein in muscle has not so far been well determined. The c-Jun protein can form heterodimers with members of the fos (c-Fos, Fra-1, Fra-2) or ATF/CREB family, or dimerize with other members of the Jun family (JunB, JunD) (4). The dimerization between these proteins occurs by hydrophobic interactions between their leucine zipper regions. The leucine zipper is an alpha helix in which every seventh amino acid is a leucine (4). The leucine side chains are allocated to one side of the α helix where they form a hydrophobic surface that mediates dimerization (4). Just upstream to this leucine zipper are positively charged residues, known as the basic region, which are responsible for DNA binding. This basic region is highly conserved among the jun and fos proteins, and various CREB and ATF proteins (4). This dimeric complex comprises the transcription factor activity called Activator Protein-1 (AP-1), which can activate or represses the transcription of numerous genes (4). c-Jun has also been shown to be able to interact with CREB binding protein (CBP), thereby increasing the transactivation potential of c-Jun (7,36). It is presently believed that the AP-1 complex may control the expression of genes involved in cellular proliferation. The levels of expression of c-Jun is increased in many cells in response to mitogens, and cells in the exponential phase of growth contain higher levels of c-jun mRNA than serum starved cells (4). Also, micro-injection of anti-fos antibodies, or transfection of c-fos antisense RNA inhibits DNA synthesis or cell proliferation in cultured fibroblasts (4). However, AP-1 may also be involved in differentiation. In PC-12 cells, for example, the expression of c-Jun and c-Fos is increased in response to mitogens, as well as NGF, which leads to neuronal differentiation. The reason for this

difference in function could be due to the cell type, and other factors which may interact with the AP-1 complex (4). The role of c-Jun with regards to its importance in the myogenic process has yet to be determined, however, studies so far have indicated that c-Jun overexpression inhibits differentiation of myoblasts into myotubes (8).

Jun Discovery

The isolation of c-Jun was proceeded by its viral counterpart, v-jun. v-jun was discovered to be a retroviral insert of avian sarcoma virus 17 (ASV 17) (49). ASV 17 was isolated from a spontaneous sarcoma in chickens (14). Computer analysis comparing the amino acid sequence of v-Jun with a data base of amino acid sequences of other proteins found a significant homology between Jun and the yeast transcription factor GCN4 (85). This homology was found to occur between the 66 amino acids in the carboxyl terminal of Jun, and that of GCN4 (85). The 60 carboxyl terminal amino acids of GCN4 is the region responsible for DNA binding (35). GCN4 binds to the sequence ATGA(C/G)TCAT (35). It was therefore then suggested that the Jun protein may also bind to DNA, and possibly to a sequence that is similar to that of GCN4 (85). This was then demonstrated by Struhl, 1988, who demonstrated that Jun could substitute for GCN4 in yeast (73, 84).

The discovery of AP-1, a 47 kd protein (44) which recognised a specific sequence in the human metallothionein gene (42), raised the possibility that a cellular Jun existed, as the consensus sequence recognised by AP-1, TGA C/G TCA (42, 6) is similar to that recognised by GCN4 (9). Experiments which used two different antibodies, the first

against a 17 amino acid sequence in the carboxyl-terminal portion of v-Jun (its DNA binding domain), and a second against 15 amino acid residues in the NH2 domain of v-Jun, were able to recognise purified AP-1 that was subjected to SDS gel electrophoresis and transferred to nitro-cellulose (9). This demonstrated that AP-1 and v-Jun share at least two distinct antigenic determinants (9, 3). This also indicated that the similarity between v-Jun and AP-1 also existed in the amino terminal, which did not exist between v-Jun and GCN4, and also suggested that c-Jun may encode AP-1 (9).

Isolation of the human c-jun proto-oncogene was accomplished by screening a genomic DNA library with a DNA probe consisting of nucleotide 720 to 1601 from v-jun (9). Although the amino terminal amino acid residue of c-Jun had not been identified, and in the first 149 amino acid sequence, c-jun contained a 27 amino acid insertion, and 18 different amino acids when compared to v-jun (9). The least homology was seen in the central 73 amino acids, which had approximately 53 per cent homology with the corresponding 59 amino acids of v- jun (9). The 118 amino acid sequence in the carboxyl terminal of the human c-jun contains only two alterations when compared to v-jun (9). This provided convincing evidence that a cellular homology of v-jun had been identified (9).

In order to identify whether a functional link between c-jun and AP-1 existed, a fusion protein consisting of the carboxyl-terminal (DNA binding) domain of c-jun was constructed. Using deoxyribonuclease I footprint protection experiments, a comparison of the DNA binding specificity of the c-jun fusion protein and AP-1 was made (9, 3). It was determined that AP-1 and the c-jun fusion protein protected the same recognition

sequence within the SV40 enhancer (9), which contains a consensus AP-1 binding site (42). In addition, single base substitutions in the AP-1 site that led to higher affinity binding of AP-1 also led to higher affinity binding of the c-jun fusion protein, and base changes which decreased AP-1 binding also reduced the c-jun fusion protein binding (9). In a separate experiment, peptide fragments were generated by trypsin digestion of purified AP-1 (9). The amino acid fragments of these peptide fragments corresponded to peptides deduced from the nucleotide sequence of c-jun (9). It has also been shown that both c-Jun and v-Jun activate promoters containing AP-1 sites (32). Together these findings suggest that AP-1 is encoded by c-jun (9). It was then shown that Jun can dimerize with Fos to bind to the AP-1 site (68, 6), and activate transcription (15). It has now been shown that c-Jun can homodimerize, as well as heterodimerize with other members of the Jun family, as well as members of the ATF2/CREB family (28).

Like all transcription factors, c-jun regulation can occur through controlling its activity and amount within the cell (38). The activity of c-Jun is manipulated post-translationally through phosphorylation (37). In response to various stressors, eg. U.V., heat, TNF-α, c-Jun is phosphorylated on Ser 63 and, more prominently, Ser 73 in its activation domain by the JNK's (18,34,39). This phosphorylated c-Jun can then interact with co-activators CBP/p300 to increase the transactivation potential of c-Jun (7). Recently, the activation domain of c-Jun has been shown to interact with another protein, JAB1 (Jun-activation domain binding protein 1) (16). JAB1 enhances c-Jun transactivation ability and stabilises its binding to the TRE (16). The abundance of c-Jun is regulated at the transcriptional level, and at the level of protein stability. The half life

of c-Jun is approximately 90 minutes (41), and degradation of c-Jun has been shown to be mediated by the ubiquiton pathway (79). However, phosphorylation of c-Jun by the JNK's decreases c-Jun ubiquitination and increases its stability (57). As the levels of c-Jun rapidly increase in response to various stimuli, it is the transcriptional induction of c-jun which is critical. Understanding how the c-jun regulatory region is controlled will provide clues to the physiological stimuli that regulate the expression of c-jun

Cis-Elements in the c-Jun Enhancer

The c-jun enhancer contains binding sites for many transcription factors (see fig.4). Between base pairs -190 and -183 is the location of the more upstream of two AP-1 binding sites (referred to as jun2) in the c-jun enhancer (71). As shown in figure 1, this site contains the 8 base sequence TTACCTCA, similar to both the classic 7 base pair AP-1 binding site TGA(G/C)TCA, and the 8 base consensus CREB binding site, TGACGTCA (71,82). The AP-1 transcription factor believed to bind here is a heterodimer of c-Jun and ATF-2 (33,82). The downstream AP-1 like site (jun1) is located between positions -71 and -63 of the c-jun enhancer (32). This differs from the classical AP-1 recognition sequence by the presence of an additional A nucleotide TGACATCA (5,32). Two different AP-1 complexes bind to this site. The more abundant is a heterodimer of c-jun and ATF-2, and the other is a heterodimer of c-Jun and Fos (33,82). Both of the AP-1 like binding sites render c-jun inducible to U.V. irradiation and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (20,33,66,71,83). Expression of the adenovirus E1A proteins have been shown to increase the levels of c-jun expression (82).

The jun2 site contributes greatest to E1A induction, however, both AP-1 sites, and the CTF site are necessary for maximal induction (82). MEF2 binds to the cis element CTATTTTTAG at -59 to -51 of the start site in the c-jun enhancer (see fig. 4) (30,32). It is possible that protein-protein interaction occurs between the AP-1 complex binding to the jun1 site in the c-jun enhancer, and the adjacent MEF2 site. DNAase I footprinting of the c-jun enhancer containing point-mutations of the jun1 site, known to abolish AP-1 binding to this site, also decreased protection of the MEF2 site (5). This suggests possible interactions between the AP-1 complex and MEF2 on the c-jun enhancer. The nuclear factor jun (NF-jun) transcriptional enhancer binds to the base pair sequence at positions -139 to -129 of the c-jun enhancer (10). This factor has been shown to bind to its recognition site as a dimer of 55 and 125 kDa (10). Binding to the GC box and the CAAT box are the transcription factors SP1 and CTF, respectively (32).

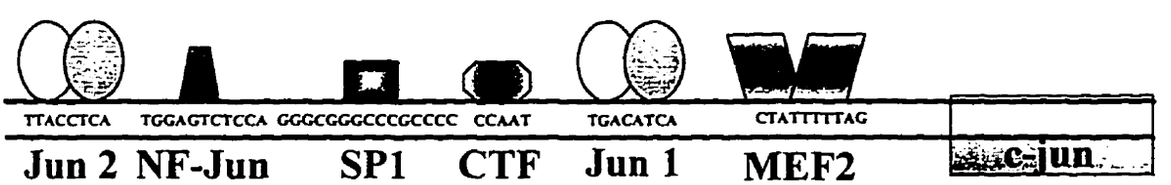

Figure 4. Cis-elements of the c-jun enhancer
Schematic diagram of the known cis-elements from -225 to +150 of the c-jun enhancer/promoter.

SP1 And CTF Sites

With respect to the c-jun enhancer, relatively little is known about GC box, and the CAAT box, where the transcription factors SP1 and CTF bind, respectively. Along with the other jun sites in the c-jun enhancer, CTF is required for full activation of the c-jun enhancer in response to E1A (82). However, without the jun sites, CTF cannot activate transcription (82). This raises the possibility of an interaction between these

bound factors in their transactivation function (82). Both SP1 and CTF may be important for low level basal activity of the c-jun promoter in non-stimulated cells, however, deletion of these sites leads to a greater transcriptional activity in TPA treated HeLa cells (5). Similarly, mutations in the SP1 binding site leads to a greater response in leukemic cells treated with TPA (81). Therefore, it is possible that these two sites function in repressing c-jun transcription (5, 81).

AP-1 Sites

The c-jun enhancer contains two separate cis-elements which AP-1 complexes recognise and bind to. In order to determine the factors which bind to these 2 sites, experiments have been performed on cells which have been exposed to agents such as U.V., TPA, and adenovirus E1A, which lead to increases in c-jun transcription (71,27,66,19,33,82,83,5). c-Jun had been shown to be able to dimerize with ATF-2 resulting in a greater affinity for the 8 base pair CRE sequence than the 7 base pair AP-1 sequence (28), therefore raising the possibility that this heterodimer binds to the jun sites in the c-jun enhancer. Binding assays have been performed using probes of the jun1, jun2, and the 7 base pair AP-1 sequence which is found in the collagenase gene, and is known to bind heterodimers of Fos and Jun (71,33,82,83,27). Two complexes with different mobility's were shown to bind to the jun1 site, while single complexes are present in the jun2 and the collagenase AP-1 site (33). The slower migrating complex of the jun1 probe had the same mobility as the jun2 complex, and the faster migrating complex of the jun1 probe had the same mobility as the collagenase AP-1 probe (33). Cross-competition experiments demonstrated that excess non-labelled jun2 probe

abolishes the slower migrating complex in the jun1 site, and vice versa, excess non-labelled jun 1 probe abolishes the complex formation on jun2 (33). Excess non-labelled collagenase AP-1 probe did not abolish the slower migrating complex complex of jun1, nor the jun2 complex, but did for the faster migrating complex (33). These cross competition analysis demonstrated that the jun1 and jun2 sites bind similar factors, with part of the factors binding to jun1 similar to that of the collagenase AP-1 site (c-Jun/c-Fos) (33,71). Antibodies to c-Jun and ATF-2 were used in an attempt to supershift the complexes (33,82). These experiments demonstrated that the faster migrating complex on jun1 contains a heterodimer of c-Jun/c-Fos (33), whereas at least part of the factors forming the slower migrating complex of jun1 and the complex on jun 2 are heterodimers of c-Jun and ATF-2 (33,82). It was also demonstrated that the factors forming the fast complex (c-Jun/c-Fos) on the jun1 site rapidly dissociate from the DNA, whereas factors forming the slow complex dissociate a great deal slower, due to a higher affinity, and are therefore likely the major factor binding to this site in vivo (33).

Another line of evidence for the regulation of the jun sites by c-Jun/ATF-2 comes from studies with the adenovirus E1A protein (27,82). E1A has been shown to repress the expression of many genes, including the collagenase gene (59). As previously mentioned, the collagenase gene contains an AP-1 site which binds c-Jun/c-Fos heterodimers, as well as c-Jun homodimers, and the repression by E1A is mediated by inhibiting the transactivation by these dimers (27,82). Conversely, the two jun sites in the c-jun enhancer are critical for the observed induction of c-jun by E1A (82). If a c-Jun/c-Fos heterodimer, or a c-Jun homodimer bound to this site, then the increase in c-jun

expression would not occur since E1A has been shown to inhibit the transactivation of these dimers. Taken together with the binding assay experiments it seems that a heterodimer of c-Jun/ATF-2 binds to the jun sites in the c-jun enhancer, and that E1A can distinguish between different AP-1 complexes (27,82).

It has been demonstrated that prior to, and following the exposure of cells to agents known to lead to the induction of c-jun transcription, the c-jun enhancer is fully occupied (33,66,83). This means that induction of transcription is mediated by the post-translational modification of pre-bound factors. A great deal of work has been done on the post-translational control of AP-1, and c-jun in particular.

c-Jun has been shown to be phosphorylated on 5 different sites. There are two in the amino terminal transactivation domain (Serine 63 and Serine 73), and three in the carboxy terminal DNA binding domain (Threonine 231, Serine 243, and Serine 249). Hyperphosphorylation in the carboxy-terminal inhibits DNA binding of c-Jun (37), however this does not seem to be of importance in the regulation of c-Jun by AP-1, since this complex is constituitively bound to the DNA. It is through phosphorylation of the amino-terminal transactivation domain that leads to c-jun expression. Recently, separate efforts have uncovered 2 proteins which preferentially phosphorylate c-Jun at its amino-terminus (34,40). These proteins are referred to as either c-Jun Amino Terminal Protein Kinase (JNK) (34), or Stress Activated Protein Kinases (SAPK) due to their activation in response to intra- and extra-cellular stress (i.e. heat shock, cyclohexamide, and TNF-α) (34). JNK1 and JNK2 are 46 and 55 kD respectively, with JNK1 being the major form (34,18). JNK1 phosphorylates c-jun on Ser 63 and 73 in response to cellular stress, and

increases its transactivation potential (34,18,40). The JNKs have no known effect on the carboxy terminal phosphorylation states (18). In order for JNK to phosphorylate c-Jun, it must bind to c-Jun (34). JNK binds to c-Jun between amino acids 30 to 60 (37,34). Deletions that abolish JNK binding to this site also decrease phosphorylation at Ser 63 and 73 (37,34).

Post-translational modification has also been shown to occur on the amino terminal activation domain of ATF-2 (26,48). Similar to c-Jun, ATF-2 is phosphorylated by the JNKs in response to UV and inflammatory cytokines (26,48). This phosphorylation occurs on Threonine 69 and Threonine 71 in the amino terminal activation domain. (26,48). Like c-jun, there is also a binding site for JNK on ATF-2, between amino acids 47 and 66 (48). There are, however, no similarities in this site between c-Jun and ATF-2 (48). Transcriptional activation of c-jun in response to cellular stress has been shown to be mediated by hyperphosphorylation of c-jun and ATF-2 heterodimers at the jun sites in the c-jun enhancer, and this phosphorylation was due to JNK activity (83).

The signalling pathway leading to JNK activation is now becoming more clear. The murine SEK1, and its' human homologue MKK4 have been found to be strongly activated by stress and inflammatory cytokines (39). SEK1 and MKK4 have been shown to be potent activators of the JNKs, and kinase negative forms of SEK1 and MKK4 inhibits the activation of the JNKs (39). Upstream of SEK1 in the pathway is MEKK1 (39). MEKK1 has been shown to phosphorylate and activate SEK1 in vitro and in vivo (39).

NF-Jun Site

The majority of the present information on the NF-jun transcription factor, and the NF-jun site in the c-jun enhancer has come from studies involving the growth factor tumour necrosis factor-α (TNF-α), and hematopoietic cells (70). TNF-α is important for optimal proliferation of early hematopoietic progenitor cells (70). Experiments have demonstrated that in the presence of an antisence c-jun oligomer, TNF-α had no effect on the proliferation of myeloid leukaemia cells (70). This suggested that TNF-α may influence the transcription of c-jun. TNF-α has been shown to enhance the transcriptional activation of c-jun 3 to 4 fold in myeloid leukaemia cells, which serve as a model cell type for early hematopoietic cells (10,70).

NF-jun is a transcription factor which binds to the 11 base pair sequence TGGAGTCTCCA, found at position -139 to -129 in the c-jun enhancer (10). NF-jun binding can be activated by TNF-α and TPA, both of which activate protein kinase C, and can also be activated by cyclohexamide (10). The NF-jun protein has been shown to bind to its recognition site as a dimer of 55 and 125 kDa (10) (see figure 5). Presently, it is not known whether these proteins can also bind as homodimers as well as heterodimers. The binding of NF-jun to this site seems to be specific, as mutations at base pairs 2 and 3, and, 9 and 10, completely abolish binding (10). However, single base pair mutations on either side of the sequence still result in some binding activity (10).

Reporter assays with the c-jun wild type enhancer/promoter upstream of the human growth hormone (hGH) reporter, transfected into KG-1 cells, resulted in a 5 fold increase in reporter activity in cells treated with TNF-α (10). In a reporter construct with

the c-jun enhancer/promoter with a deleted NF-jun sites, there was no significant increase in reporter activity when treated with TNF-α (10).

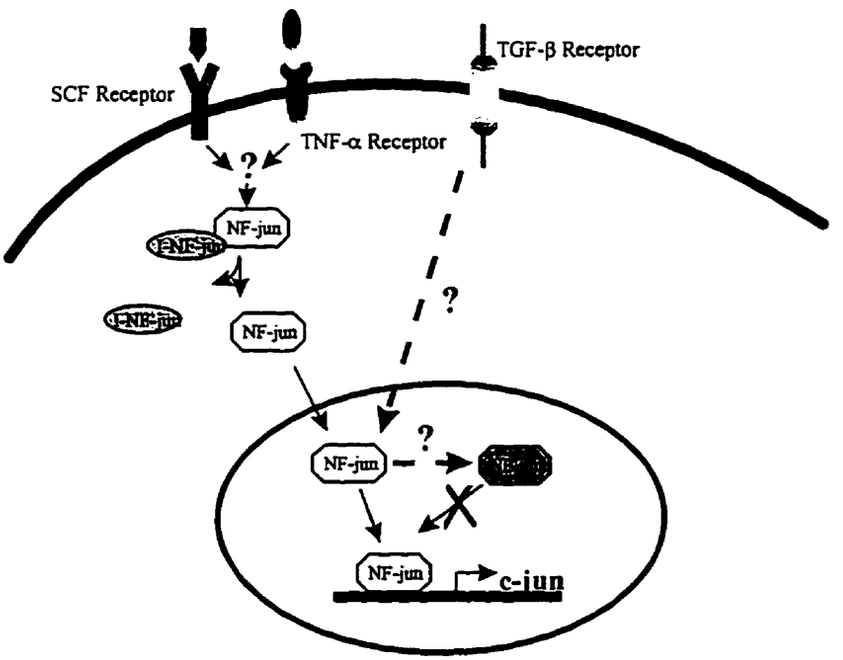

Figure 5. NF-jun regulation
Schematic diagram of the putative mechanism of NF-jun regulation in the regulation of c-jun transcription (70).

There are many similarities between the NF-jun and NF-κB transcription factors. NF-κB is also induced by TPA, TNF-α, and cyclohexamide (10). Similar to NF-κB, the activation of NF-jun includes its translocation from the cytoplasm to the nucleus. The binding site for NF-κB, GGGGGATTTCC, is also similar to that of NF-jun, however, electrophoretic mobility shift assays (EMSA) have shown that the binding sites for both of these proteins are not interchangeable (10). As with NF-κB, the dissociating agents

sodium deoxycholic acid (DOC) or formamide lead to the binding of the NF-jun in untreated KG-1 cells. In the cytoplasm, NF-κB is bound to an inhibitor, I-κB, and it is possible that an interaction exists in the cytoplasm between NF-jun, and an inhibitory protein to regulate NF-jun binding and activity (10).

It is interesting to note that NF-jun expression seems to be restricted to proliferating cells such as myeloid leukaemia cells, and is not detectable in non-proliferating diploid lung fibroblasts, blood monocytes, granulocytes, or resting t-cells (10,66). Since the activation of NF-jun seemed to be restricted to proliferating cells, experiments have been performed to determine the role of NF-jun in response to signals which negatively regulate hematopoietic cell proliferation (70). Transforming growth factor-β (TGF-β), has been shown to be a strong inhibitor of growth factor-stimulated hematopoiesis in normal and leukaemic cells (69). Experiments have demonstrated that in myeloid leukaemia cells, TGF-β interferes with stem cell factor (SCF)-induced proliferation (69). In order for SCF to have an effect on proliferation, c-jun expression is required, as illustrated in antisence experiments (69). SCF enhances the transcriptional activity of c-jun, and this increase is mediated through the NF-jun site (69). EMSA have also shown that SCF enhances NF-jun binding activity (69). In the presence of TGF-β, although, c-jun expression is suppressed, as is NF-jun binding activity (69). However, in the presence of TGF-β, SCF still promotes the translocation of NF-jun to the nucleus, suggesting the TGF-β affects the ability of NF-jun to bind to DNA, but does not interfere with signals leading to nuclear translocation (69). This inhibition in binding caused by

TGF-β may be due to post-translational modifications on NF-jun, or it is possible that NF-jun may form complexes with other nuclear proteins, thereby inhibiting it from binding (70).

MEF2 Site

MEF2 binds to the cis element CTATTTTTAG at -59 to -51 of the start site in the c-jun enhancer (see fig. 1) (30,32). The MEF2 factors have a DNA binding domain which is similar to the DNA binding domain of the serum response factor (SRF) (64). SRF recognises its consensus site ($CC(A/T)_6CG$) via a 90 amino acid binding domain (64). The domain consists of an amino-terminal basic region required for high affinity binding and 50 amino acids in the carboxy-terminal region which is responsible for dimerization (64). The DNA binding domain of SRF has also been shown to be highly homologous with that of the yeast regulator protein MCM1, and the plant homeotic factors, Agamous and Deficiens (61,64,86). This domain of homology between all of these factors has been termed the MADS (for MCM1, Agamous, Deficiens, and SRF) box, and the proteins as a group, the MADS box transcription factors (61,64,86). When originally cloned, the 56 amino terminal residues of MEF2 were noted to be homologous to the MADS box, and this sequence is also necessary (although not sufficient) for DNA binding (64). Hence, MEF2 is also a member of the MADS box superfamily of transcription factors.

To date, there are four members of the MEF2 family, MEF2A, B, C, and D (11,46,51,52,64,86). Between all of these members there is a high homology between the

first 86 amino acids (61,64). There is greater than 80% amino acid homology within the MADS box (61). The next 30 amino acids are referred to the MEF2 domain, of which there is homology between the MEF2 family members, but not other MADS box proteins (61). Beyond amino acid 86 these proteins are more divergent (86).

The MEF2 proteins bind to the DNA sequence (C/T)TA(A/T)4TA(G/A) (64,2,23), however, it has been shown that the flanking sequences may affect binding affinity (86). Binding of the MEF2 proteins is mediated through its MADS box and the MEF2 domain (64). Sequence specificity for DNA binding has been shown to be mediated through the 28 basic amino acids in the amino terminal end of the MADS box (61,64). In binding to DNA, the MEF2 proteins can form homo- or heterodimers with one another (61). Dimerization is specified by hydrophobic amino acids towards the carboxyl end of the MADS box, and by the MEF2 domain (61). It has been suggested that these regions assist in the orientation of the DNA binding domains of the dimeric partners (61).

The binding activity of MEF2A has been shown to increase in differentiating myoblasts (86). Along with its presence in skeletal muscles, MEF2A binding activity has been demonstrated in smooth muscle cells, and primary cardiocytes, both of which lack the myogenic bHLH proteins (86). Other studies have also shown MEF2A binding to be quite ubiquitous, being present in HeLa, fibroblasts, Cos cells, and brain neurons (30,52,62,64). Transcripts of MEF2A have also been shown to be ubiquitous (64).

Unlike other members of the MEF2 family whose transcripts are ubiquitous, mRNAs from MEF2C are only detected at significant levels in skeletal muscle and brain,

with one isoform of the gene being highly brain specific (46,52). MEF2C binding activity and protein expression has been demonstrated in C2C12 myotubes and also in brain neurons (46,52), preferentially in the cerebral cortex (46). As demonstrated by immunofluoresence and binding assays, MEF2C is not seen in myoblasts, and although it is seen in myotubes, its presence occurs late in the myogenic process (52). Experiments on MEF2C have demonstrated that it is phosphorylated at Ser 59, leading to an increase in its DNA binding activity (56). This site corresponds to a Casein Kinase II (CKII) phosphorylation site, and it has been shown to be phosphorylated by CKII in vitro (56). This site seems to be important in the regulation of MEF2, as it is conserved in all four members of the MEF2 family (56).

Studies performed in order to determine the presence of MEF2D transcripts have shown that these transcripts are widely expressed (11,51). However, different splicing patterns reveal that one splice variant is present ubiquitously in cells, while the other is found largely in skeletal muscle, and is specifically induced during myogenesis (11,51). Immunochemical detection studies of MEF2D have revealed that the MEF2D protein is present in differentiated cardiocytes and skeletal myotubes, and also in undifferentiated skeletal myoblasts, where MEF2A and MEF2C are absent (11). Electrophoretic mobility shift assays have also shown MEF2D to be bound to the MEF2 consensus site in C2C12 myoblasts and myotubes, and in HeLa cells (11,30,62).

Original studies had reported that MEF2B did not bind to the consensus MEF2 site, which suggested that MEF2B has separate functions than the other MEF2 family members (64,1). Recent studies have demonstrated that in vitro-translated MEF2B bound

to the MCK MEF2 site with similar affinities as MEF2C (55). The MEF2B MADS box contains a glutamine at position 14 instead of an aspartic acid like the rest of the MADS box proteins (55). The aspartic acid at position 14 has been demonstrated to be important for efficient binding of MADS box proteins. Mutations of MEF2B replacing the glutamine with aspartic acid resulted in an increase in binding, however, the glutamine does not prevent wild type MEF2B from binding to its consensus site. (55). The transcripts of MEF2B have been found in developing cardiac and skeletal muscle, and neuronal cells (55). The MEF2B mRNAs are expressed at highest levels near the end stage in the development of myotubes (55). MEF2B proteins are detectable at low levels in myoblasts, and are detectable in fibroblasts. MEF2B protein expression is increased in differentiated muscle cells (55). It is interesting to note that the expression of MEF2B in myogenic and neurogenic lineage is very similar to that of MEF2C, which suggests that these two factors may have the same functions, or are capable of compensating for one another (55).

Although MEF2 was originally characterised as a muscle specific transcription factor, a contradiction has become evident as MEF2 binding activity has been shown to appear in non-muscle lineages such as HeLa and fibroblasts (11,30,62,64). This controversy is now starting to become better understood, as Ornatsky & McDermott, 1996, have demonstrated that the presence of MEF2 and its DNA binding activity is not necessarily correlated with transcriptional activity at the MEF2 site (62). MEF2 proteins can bind as homo- or hetero-dimers, and there is great potential for post-translational modifications of MEF2 proteins, therefore, regulating transcriptional activity at this level

(62). A model has been proposed based on studies that compare the binding of MEF2 in HeLa to that of C2C12 cells, where MEF2 transcriptional activity is only present in the C2C12 cells, and not in HeLa (62). In this model, MEF2D containing complexes occupy the MEF2 sites of many cell types. In these MEF2D complexes, the transactivational function is inactivated. In order for this site to be activated, these MEF2D containing complexes are replaced with the MEF2A homodimer, which would contribute to activation through the MEF2 site (62).

MEF2 genes have also been implicated in the regulation of the myogenic bHLH genes (61). There is a MEF2 binding site in the enhancer of the myogenin gene (61). This site is necessary for high levels of transcription in cultured muscle cells (61). However, MEF2 is expressed after myogenin in muscle development, therefore, it has been suggested that rather than initiating myogenin expression, MEF2 amplifies and maintains myogenin gene expression (61). Likewise, MEF2 can also regulate expression of the Xenopus MyoD gene (61). Similar to myogenin, MyoD is also expressed before MEF2 during myogenesis, and MEF2 also probably amplifies and maintains MyoD gene expression (61). It has been recently demonstrated that interactions can occur between the DNA binding domain of MEF2 and myogenic bHLH proteins that can lead to the activation of muscle specific gene expression (61). In enhancers which lack an E-box, but which contain a MEF2 site, the myogenic bHLH proteins can activate these genes by protein-protein interactions, with MEF2 bound to its consensus site (54). Conversely, MEF2 can activate E-box dependent genes that lack a MEF2 site through interactions with the bHLH proteins (54). The myogenic bHLH proteins and MEF2 can in some cases

co-operate in the activation of genes that contain an E-box and MEF2 site (55). CBP and p300 have been shown to be important in the myogenic process through interactions with the MEF2 and MyoD family. This interaction allows them to function as co-activators in transcription during differentiation (67,21).

The MEF2 site in the c-Jun enhancer was originally believed to be the binding site for Transcription Factor IID (32). Although consisting of a different sequence, the Xenopus MyoDa (XMyoDa) gene contains a TATA element embedded in a MEF2 site (45). Binding by either TFIID or MEF2 alone were shown to be able to transactivate the XMyoDa promoter in muscle cells (45). However, mutations in the MEF2 site in the c-jun enhancer does not decrease basal transcription levels, suggesting that TFIID does not bind to this site (31). Experiments using specific antibodies to the different members of the MEF2 family have shown that MEF2D is the major MEF2 family member binding to the c-jun MEF2 site in HeLa cells (30). Han and Prywes, 1995, have also shown a small amount of MEF2A in HeLa cells (30). Since MEF2 proteins can heterodimerize, it has been postulated that MEF2A and MEF2D could potentially form heterodimers that may contribute to the control of c-jun transcription (30). Ornatsky & McDermott, 1996, have demonstrated that the predominant MEF2 dimer at the MEF2 site in the c-jun enhancer in HeLa cells is a MEF2A:MEF2D heterodimer (62).

The c-jun MEF2 site has been implicated as an important factor in EGF induction of c-jun transcription (31). This has been shown in experiments where -225 to +150 of the c-jun enhancer/promoter have been linked to the CAT reporter gene (31). Double point mutations in the MEF2 site, which have been shown to abolish MEF2 binding,

leads to only poor induction of the reporter by EGF (31). Uninduced levels were not affected, which supported the suggestion that this site is not serving as a TATA element (31). The same experiment also showed the MEF2 site to be important for serum inducibility of c-jun (31). It was also shown that the MEF2 site is sufficient for induction by EGF, serum, and TPA when placed on a heterologous promoter (31). In NIH 3T3 cells, which contained low MEF2 binding activity and poor serum induction of a c-jun promoter reporter construct and a reporter gene containing a single MEF2 site, transfection of MEF2 was sufficient to reconstitute serum regulation of both of these reporter genes (30). It is interesting to note that transfection of expression vectors of the other MEF2 family members, i.e. MEF2 A, B, and C, were also able to mediate serum induction of the c-jun MEF2 site (30). All of the MEF2 family members are similar in their DNA binding domains. Since all are able to activate a reporter in response to serum, it is possible that the MEF2 DNA binding domain is key to its role in serum regulation (30). Experiments have demonstrated that it is the DNA binding domain of MEF2D that is subject to regulation (30). However, it is only subject to regulation when it is bound to DNA (30). The results of that same study also showed that MEF2D is the major MEF2 site binding molecule in non-muscle cells. It was concluded that MEF2D is the most important MEF2 family member for the induction of c-jun by growth factors (30).

Recently, the MEF2 site in the c-jun enhancer has been shown to be important for c-jun induction in response to lipopolysaccharide (LPS) (29). This study demonstrated that MEF2C can become phosphorylated in its transactivation domain on Thr 293, Thr

300, and Ser387 by p38 (29). This phosphorylation leads to an increase in the transactivation potential of MEF2C (29).

c-Jun and Myogenesis

The transcriptional and post-translational regulation of c-jun is of vital importance in myogenesis. Experiments have demonstrated that v-Jun infected quail myoblasts are prevented from differentiating (25,74). The myoblasts continue to replicate, they do not fuse, and there is inhibition of muscle specific genes such as myosin heavy chain (25,74). C2 myoblasts which have been infected with a c-jun retrovirus are also incapable of differentiating (8). Other studies have shown that v-Fos, c-Fos, and Jun B can also inhibit myogenesis (46). Jun D expression is not regulated during myogenesis, and its levels do not interfere with muscle differentiation (46). This demonstrates the importance of the control of the jun/AP-1 complex during myogenesis.

During differentiation, the levels of c-Jun do not remain constant. In C2 cells induced to differentiate after serum withdrawal, the level of endogenous c-jun mRNA has been shown to decrease until it is undetectable after six days of serum withdrawal (8). One study has consistently observed that the levels of c-Jun in differentiating L6 and C2C12 cells does not change significantly (76,75,77). The reason for this discrepancy is unclear, due to the fact that in this study the same cells which other labs have shown to have the c-jun levels to decrease were used (76,75,77)

Overexpression of c-Jun in myoblasts has been shown to down-regulate the expression of MyoD, myogenin, and MCK gene transcripts (which contains an E-box in

its enhancer) (8,47). Similarly, transfection experiments have demonstrated that MyoD is also able to repress Jun and Fos transactivation of a reporter containing 5 TRE binding sites (8). This is due to a protein:protein interaction between MyoD and c-Jun (8,47). This interaction occurs between the basic region, helix 1, and helix 2 domain of MyoD and the leucine zipper (8) or amino terminal sequences of c-Jun (47). As a result of this interaction between MyoD and Jun, MyoD cannot interact with E12 or E47, and Jun cannot interact with Fos, thereby not allowing these factors to bind to their consensus sequence in the enhancer of certain genes (8). The JunB sequences responsible for repression have not been determined yet. Many of the studies on the inhibition of myogenesis through c-Jun have involved its overexpression, at abnormally high levels, in the cells. These experiments do not, however, elucidate the role of c-Jun at physiological levels.

It is conceivable that Jun and Fos could lead to the induction of factors that lead to the inhibition of myogenesis or the myogenic factors, but this is unlikely. Expression of c-Jun has been shown not to affect levels if Id, the protein which binds to the members of the MyoD family and inhibits their dimerization with the E-proteins (60). Also, studies which have replaced the DNA binding domain of c-Jun with that of GNF1 (a pituitary transcription factor with a different binding site), does not affect the ability of c-Jun to inhibit myogenin and MyoD (47). It is unlikely that the GNF1 would activate the same set of genes as a jun/AP-1 complex would, therefore suggesting that AP-1 is not activating other genes which could be solely responsible for the inhibition of myogenesis (47).

Jun also seems to regulate MyoD at the transcriptional level. At -336 to -329 of the MyoD enhancer is a CRE like binding sequence (63). Under growth conditions, an AP-1 complex of Fos and Jun bind to this CRE like site (63). Transfection experiments have demonstrated that the binding of this AP-1 complex to this site in the MyoD enhancer leads to a decrease in the rate of transcription (63). During differentiation, the binding to this site is downregulated, which would allow for an increase in transcription of the MyoD gene, consistent with the 3 fold increase in MyoD mRNA prior to cell fusion (8,63). Likewise, an E box is present in the enhancer of the fos gene (80). MyoD can act as a negative regulator for c-fos transcription by blocking serum responsiveness through this binding site (80). This provides further evidence of the importance of regulation of AP-1 levels during myogenesis.

An interesting correlation occurs with myoblasts transfected with SV40 T antigen genes. These myoblasts were inhibited from differentiating, and the expression of MyoD and myogenin was suppressed (22). The levels of c-Jun are concomitantly increased (22). This suggests the possibility that large T may inhibit muscle differentiation by inducing c-jun (22). Additional studies in which myoblasts were treated with TPA resulted in induction of c-jun and c-fos mRNAs, which was followed by a temporary reduction in the transcript levels for the MyoD family (22).

Retinoic acid has the ability to enhance MyoD expression, and myogenesis in poorly mitogenic conditions (1). It is also able to stimulate differentiation in myoblasts in high serum concentrations (1). When bound to their ligand, retinoic acid receptors are able to decrease AP-1 activity, possibly due to protein-protein interactions (1,11). Once

again, this illustrates an interesting correlation between the ability of retinoic acid to decrease AP-1 activity, and the promotion of differentiation (1).

Conclusion

The importance of regulating gene expression is crucial in developing muscle. This is exemplified by the MEF2 and MyoD families of transcription factors whose role in the transcriptional activation of many genes is critical for myogenesis. The levels of c-Jun, which comprise part of the AP-1 complex, may be important during muscle differentiation, as overexpression inhibits the myogenic process. If the levels of c-Jun do play a role in myogenesis, then the transcriptional regulation of the c-jun enhancer must be tightly regulated. It appears dichotomous that a MEF2 binding site is present in the enhancer since MEF2 levels increase during differentiation, and elevated levels of c-Jun inhibit differentiation. However, one must bear in mind that these studies are overexpression studies and do not reflect the real physiological levels of c-Jun. Therefore, it is plausible that at physiological concentrations c-Jun is actually required during differentiation.

The levels of SP1 decrease during differentiation, and in leukemic cells SP1 binding seems to repress transcription. The levels of NF-Jun in muscle cells has not been determined. Since NF-Jun has so far only been seen in proliferating cells, it would be interesting to compare its levels (if any) in myoblasts and myotubes. It would be intriguing to analyze the two jun sites in the c-jun enhancer to see if there is any change in binding to these sites, or if there are any differences in the factors binding to these sites,

affecting jun's ability to autoregulate itself during the myogenic process. The possibility that other unknown factors binding to the c-jun enhancer during differentiation also exists. There is also the possibility that there is a set of proliferative signals and signals in differentiating cells that are required to induce c-jun. It is possible that MEF2 is responsible for maintiaining the levels of c-jun during differentiation while the other factors binding to the jun enhancer are changing. A combination of DNA footprinting, DNA binding assays, and reporter assays with mutations in the various DNA binding sites would help to answer these possibilities. The question of the importance of c-Jun during myogenesis still remains unanswered. The most logical approach to answering this would be through studies which inhibit the activity of c-Jun during myogenesis *in vitro* and *in vivo*.

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Manuscript

Introduction

The vertebrate MEF2 gene family consists of four members, MEF2A-D (40), and belong to the MADS (MCM1, Agamous, Deficiens, Serum response factor) superfamily of transcriptional regulator proteins (45,59) that now comprises of more than 40 genes from organisms as diverse as yeast and humans (50). The MEF2 activity and function is highly conserved across species from drosophila to humans, consistent with its key role in muscle, and possibly other cell types (40). Between the MADS box of the MEF2 proteins (the first 56 amino acids) there is greater than 80% amino acid identity, and within the next 30 amino acids, referred to as the MEF2 domain, there is high conservation between the MEF2 family members, but not the other MADS box proteins (40). The MEF2 proteins bind to the DNA sequence (C/T)TA(A/T)$_4$TA(G/A) as homo- or hetero-dimers and this dimerization and DNA binding is mediated through the MADS box and MEF2 domain (45,1,15). Although the data regarding MEF2s ability to activate the myogenic program in non-muscle cells is equivocal (37,29), similar to the myogenic bHLH proteins, the MEF2 proteins are critical for muscle formation in drosophila (6), and mamalian cells (35,42). MEF2 proteins bind to and enhance the transcription of many muscle specific genes (reviewed in 40), and they also synergize with the myogenic bHLH proteins to activate transcription (37,29). Therefore, the available evidence indicates a molecular network in the control of myogenesis, in which the bHLH and MEF2 genes are central components.

MEF2 proteins are also present in non-muscle cells (41), and MEF2 cis elements are present in the enhancers of non-muscle specific genes. Their role in this context is only just beginning to be defined (21). For example, the MEF2 cis element is present in the enhancer of the relatively ubiquitously expressed c-jun gene (22). c-Jun forms part of the complex of the Activator Protein-1 (AP-1) family of transcription factors (2). Along with MEF2 there are several other binding sites for transcription factors in the c-jun enhancer which have the ability to influence its transcription (SP1, CTF, NF-Jun, and two AP-1 like binding sites) (23,51). Although there is a MEF2 binding site in the c-jun enhancer, the role of c-Jun during myogenesis has yet to be determined. The c-Jun protein can form heterodimers with members of the fos (c-Fos, Fra-1, Fra-2) or ATF/CREB family, or dimerize with other members of the Jun family (JunB, JunD) (19). It is believed that the AP-1 complex may control the expression of genes involved in cellular proliferation, however, in certain cell lines, it is also believed to be important for differentiation (2). Studies so far have indicated that c-Jun overexpression inhibits differentiation of myoblasts into myotubes (5). This inhibition is likely due to a direct protein:protein interaction between MyoD and c-Jun (5,34). However, these conclusions were based on overexpression studies, which may not necessarily reflect the true 'physiological' role of c-Jun during myogenesis.

Activation of the c-jun enhancer by MEF2 appears dichotomous, since MEF2 protein levels increase during differentiation, but elevated levels of c-Jun may inhibit myogenesis. Therefore, although Jun/AP-1 and MyoD properties oppose each other when either is overexpressed, it is possible that their physiological levels during myogenesis are

exquisitely counter balanced and that the precise maintenance of their concentrations is an important determinant of the differentiation process. One means of precisely regulating transcription factor concentrations in regulatory networks is the existence of cross regulation in which one transcription factor is involved in the control of another's enhancer region. Such a level of regulation has been documented to exist between the MEF2 and bHLH proteins during myogenesis (8,14). We therefore hypothesized that cross-regulatory transcriptional control might be a more common theme that allows the concentration of a number of transcription factors to be finitely co-ordinated, thus enabling a potentially unique network of transcription factors to be established in a particular cell with carefully defined stoichiometries. Since the MEF2 factors are induced during myogenesis, and the regulation of c-jun transcription could be potentially important during myogenesis, we undertook this study in order to assess the role of the MEF2 site, and the proteins that bind to it, in regulating the c-jun enhancer during myogenesis.

Methods

Cell Culture and Antibodies

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) on plastic dishes. C2C12 cells were grown in DMEM + 10% FBS on gelatin coated plastic dishes. In order to induce differentiation of the C2C12 myoblasts, the medium was changed to DMEM + 5% Horse Serum. For immunoblots, the anti-MEF2A and the anti-MEF2D antisera were provided by Ron Prywes (21).

Transfections

For the reporter assays, the appropriate reporter was transfected into C2C12 myoblasts or HeLa cells, which were at 60% confluency, by the calcium phosphate coprecipitation technique. Each plate was transfected with 5μg of the appropriate luciferase reporter construct, and 2 μg of pSV β-gal, which served as an internal control for transfection efficiency. For the overexpression studies, 2.5 μg of pMT2-MEF2A and/or 6 μg of pMT2-MyoD, or the pMT2 vector alone as a control, were transfected into the cell. For the C2C12, the cells were given a 15% glycerol shock 24 hours after transfection and fresh DMEM + 10% FBS. Twenty-four hours later the medium was changed to DMEM + 5%HS. The myotubes were then collected 4 days later. For HeLa, fresh DMEM + 10%FBS was added 48 hours after the calcium phosphate precipitate was added. The cells were then collected 24 hours after the media was changed. The reporter gene constructs used were the following: pJ Luc, which contained -225 to +150 of the c-jun enhancer/promoter upstream of a basal promoter - Luciferase construct; pJSX Luc, containing -225 to +150 of the c-jun enhancer/promoter (the same as pJLuc), except for 2 point mutations in the MEF2 site which inhibit MEF2 binding; pJC90FLuc, which contains -80 to +150 of the c-jun enhancer, containing only the MEF2 and jun1 site, upstream of -53 to +42 of the c-fos promoter; p0FLuc, which contains -53 top +42 of the c-fos promoter; and TATA Luc which contains a TATA box upstream of the luciferase reporter gene. Cell extracts were prepared and luciferase activity was determined as described by the manufacturer (Promega).

Immunoprecipitations and Immunoblotting

Confluent cultures of C2C12 myotubes were grown on 100mm dishes and were washed 3 times with PBS. The cells were freeze thawed once by floating them on liquid nitrogen for 10 seconds, then onto a 37°C water bath for 10 seconds. 300 µl of lysis buffer (50mM tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1mM Sodium Vanadate, 1mM PMSF, 1mM DTT) was added to the dish, and lysed cells were scraped into eppendorph tubes. The lysate was sonicated on ice (2 times, 10 seconds each) and cleared by centrifugation. An additional 500 µl of lysis buffer was added to the eppendorph along with 50 µl of anti-myogenin hybridoma supernatant (F5D) and it was allowed to rock on a nutator for 2 hours at 4°C. This was followed by adding 2µl of Rabbit anti-mouse antibody (Sigma, 2.8 µg/µl) and incubated as above for 2 hours. Protein A-Sepharose (50 µl) was added for overnight incubation. The beads were briefly washed 3 times with lysis buffer and 30 µl per tube of complete 2X Laemmli SDS-sample buffer was used to prepare samples for SDS-PAGE. 10% polyacrylamide mini-gels were loaded with 10 µl of sample per lane, proteins were then transferred to nitro-cellulose and probed with specific antibodies (anti-myogenin and MEF2A or MEF2D).

DNA Binding Assays

The DNA binding assays and extract preparation were carried out as described previously (36). Complementary oligodeoxyribonucleotides were synthesised with an applied Biosystems synthesiser. For the DNA binding assays with various cell extracts, the incubation reaction contained equivalent amounts of protein (based on a Bradford total protein assay), 0.2 ng of probe, 0.45 µg of poly(dI·dC), and 100 ng of single stranded

oligonucleotide in a total volume of 20 µl. The bound fraction was separated from the free probe by electrophoresis on a 4.5% polyacrylamide gel (acrylamide:bis, 29:1) at 4°C. The core nucleotide sequences used in the binding assays were as follows: c-jun MEF2, 5'-tcgagggctatttttagggcc (21); and AP-1 agcttgtgactcattt. Nucleotide in the underlined print conform to the consensus sequence of the MEF2 site, and AP-1 site respectively. For the immuno-gel shift analysis, where appropriate, 1 µl of antiserum or preimmune serum was added to the incubation reaction (in all cases, 0.1 and 1 ul of the antisera was tested to determine that partial supershifts of the complex were not due to limiting amounts of antibody).

Results

Activation of the c-jun enhancer by MEF2A

In order to determine if c-jun transcription could be activated by MEF2A, HeLa cells were transfected with the luciferase reporter pJLuc, which contains -225 to +150 of the c-jun enhancer upstream of the firefly luciferase gene, and with or without a pMT2 MEF2A expression vector. The results indicate that MEF2A overexpression leads to an approximate four fold increase in luciferase activity (see fig.1). As controls, luciferase reporters p0 Fluc, which consists of -35 of the c-fos promoter, as well as TATA-Luc (data not shown), which contains a TATA box upstream of luciferase, were not activated by MEF2A overexpression. This was expected, as there is no MEF2 consensus sequence in either of these constructs.

Activation of the c-jun enhancer by endogenous MEF2 proteins during differentiation

Since the MEF2 family of transcription factors are critical for the myogenic program, we wanted to determine the importance of this MEF2 site in the c-jun enhancer during differentiation. C2C12 myoblasts were transfected with either pJLuc, pJSX Luc, or p0Fluc. Serum was then withdrawn, and the cells were allowed to differentiate. The rationale here is that since pJSX and pJLuc differ only in the MEF2 site, any difference in reporter activity would be due to this site. As can be seen in fig.2, the level of reporter activity with pJSX Luc is only approximately 60% of that of pJLuc. Therefore, MEF2 activity is important for full transcriptional activation of c-jun during differentiation.

Composition of the DNA binding complex at the MEF2 site on the c-jun enhancer in muscle cells

Since both MEF2A and MEF2C, and to a lesser extent, MEF2D can activate the c-jun enhancer, we wanted to find out which MEF2 factors were binding to this site in myogenic cells. In order to accomplish this, extracts from C2C12 myoblasts, and C2C12 myotubes at 2, 4, and 5 days were incubated with a double stranded oligodeoxyribonucleotide comprising the c-jun enhancer MEF2 site. As shown in fig.3A, there was no complex present at the c-Jun MEF2 site in myoblasts. However, as differentiation proceeds, MEF2 binding increases (fig.3A, compare lanes 2 to 4). We then attempted to supershift the complex binding to the MEF2 site in the c-jun enhancer with specific antisera in order to determine its composition. As seen in figure3C, the majority of the complex bound to the c-jun MEF2 site is a MEF2A homodimer, as the MEF2A antibody supershifted the whole complex. Only part of the complex was shifted

by the MEF2D antibody, indicating that MEF2A:MEF2D heterodimers are also present. We also looked at binding to an oligodeoxyribonucleotide consisting of the 7 base pair AP-1 DNA binding sequence. In C2C12 myoblasts there is an AP-1 binding activity present, this increases dramatically after 2 days in differentiation media, then subsequently decreases on day 4 and day 5 when the culture is fully differentiated.

Interaction between MEF2 and MyoD on the c-jun enhancer

It has recently been demonstrated that the MEF2 family and the MyoD family of transcription factors can physically interact to regulate transcription (37). We wanted to determine if this synergistic regulation could occur in the c-jun enhancer. In order to determine this, HeLa cells were transfected with the luciferase reporter, pJC90Fluc, which contains -80 to +150 of the c-jun enhancer. This contains the MEF2 binding site, as well as the jun1 site. There is no E-box, the myogenic basic helix-loop-helix binding site, in this region. PJC90FLuc was used so that the influence from other factors binding to the full length enhancer could be minimised. P0FLuc was used as a control. The reporters were transfected with either MEF2A, or MyoD alone, or with MEF2A and MyoD together. PJC90FLuc along with MEF2A alone led to approximately a 7 fold increase, while MyoD alone led to about a 6 fold increase (see fig.4). Transfection of both MEF2A and MyoD together led to approximately a 90 fold increase over pJC90FLuc alone. This is also 7 fold greater than the additive effect of MEF2A and MyoD transfected alone with pJC90FLuc. These data indicate that MEF2 and MyoD can synergistically increase the level of transcription via the ME2 site in the c-jun enhancer.

The MEF2 and MyoD family can interact in vivo

In order to determine if the MEF2 family and MyoD family could interact in vivo, immunoprecipitation experiments were performed. In these experiments, the bHLH protein myogenin was immunoprecipitated from C2C12 myotubes. As seen in fig.5A, the monoclonal myogenin antibody successfully immunoprecipitated myogenin (compare lane 1 and 2). These myogenin immunoprecipitates were then probed with MEF2A and MEF2D. MEF2D is present (fig. 5B), as is MEF2A (fig. 5C). Therefore, these data suggest that in C2C12 myoblasts, the MEF2 and myogenic bHLH family of transcription factors do interact *in vivo.*

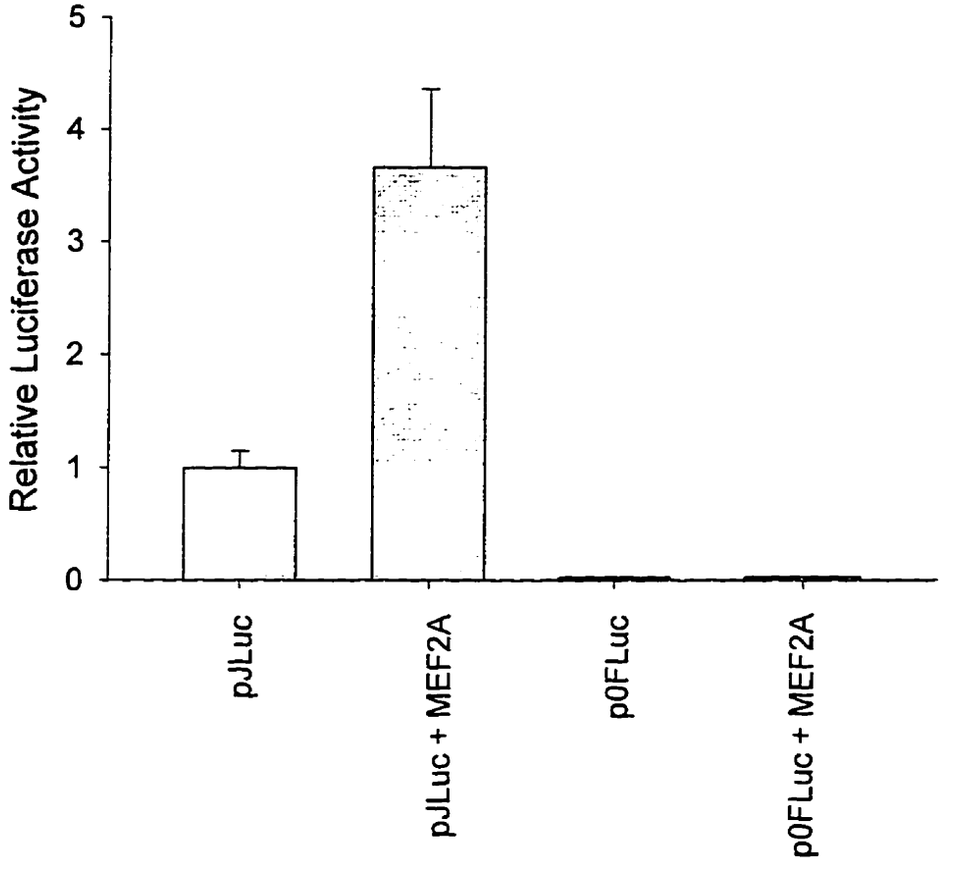

Figure 1. Activation of pJLuc by MEF2A.
HeLa cells were transfected with luciferase reporters, either pJLuc or p0FLuc, alone or with pMT2-MEF2A. Transfection of pJLuc along with MEF2A led to a significant increase in luciferase activity ($p<0.05$).

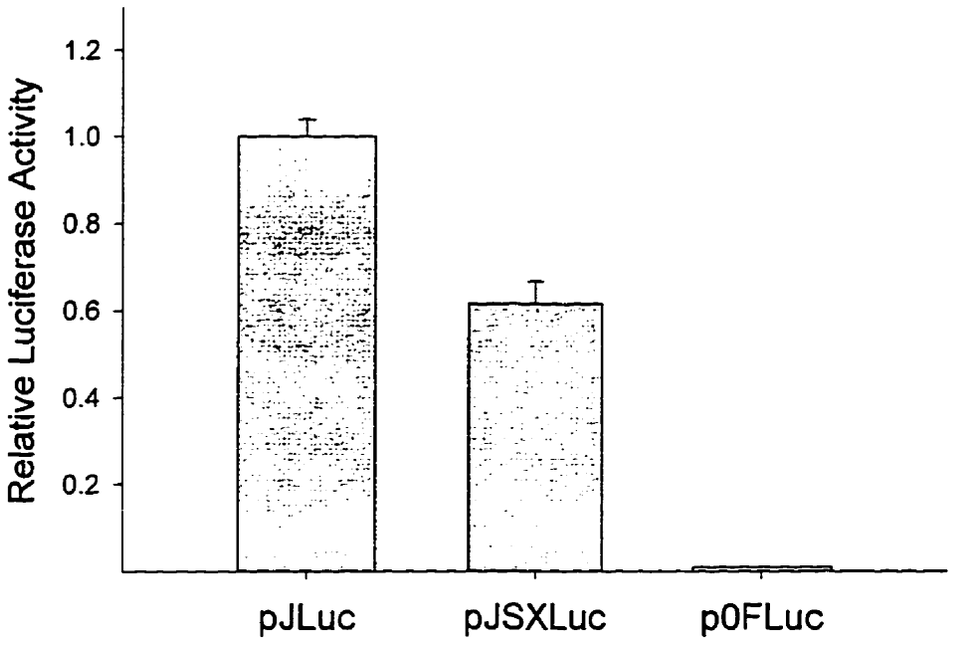

Figure 2. Activation of the c-jun enhancer/promoter during differentiation.

This graph shows the luciferase activity of C2C12 cells which were transfected as myoblasts with pJ Luc, or pJSX Luc, or p0 FLuc, and then allowed to differentiate into myotubes. pJSXLuc luciferase activity is only approximately 60% of that of pJLuc. This difference is due to activation by the MEF2 site in pJLuc ($p<0.05$).

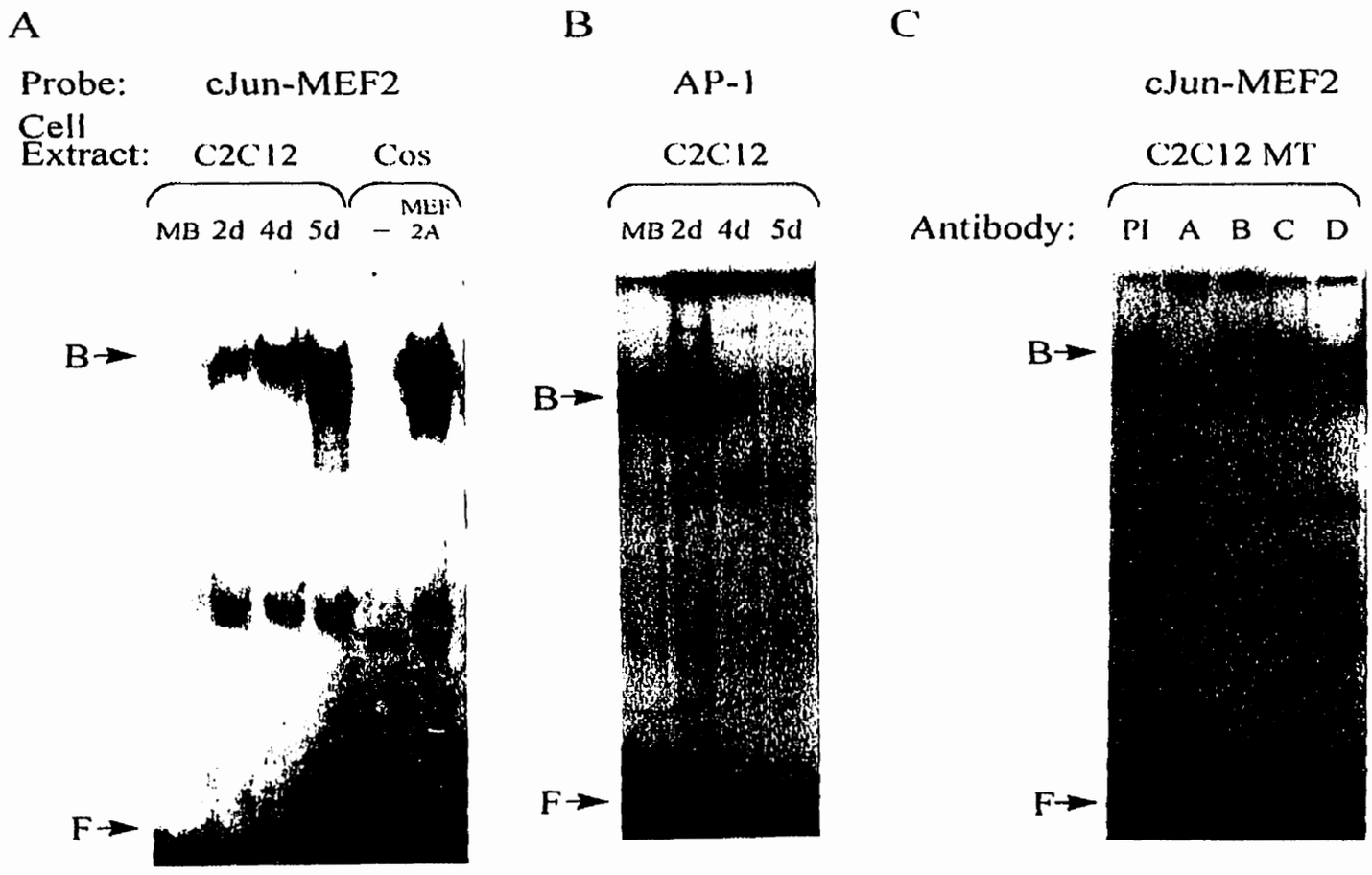

Figure 3. DNA binding and composition of complexes at the c-Jun MEF2 site and DNA binding at the AP-1 site in C2C12 cells.
In the above binding assays (A) C2C12 extracts, either myoblasts, or myotubes at 2, 4, or 5 days were incubated with radiolabelled, double-stranded c-jun MEF2 binding site probe to determine changes in binding during differentiation. (B) C2C12 extracts, either myoblasts, or myotubes at 2, 4, or 5 days were incubated with the radiolabelled, double stranded AP-1 binding site probe to determine changes in binding during differentiation. (C) C2C12 myotube extracts and the c-Jun MEF2 probe were incubated with specific MEF2A-MEF2D immune sera to test whether the endogenous MEF2 was supershifted by the antibodies. B and F refer to bound and free probe, respectively.

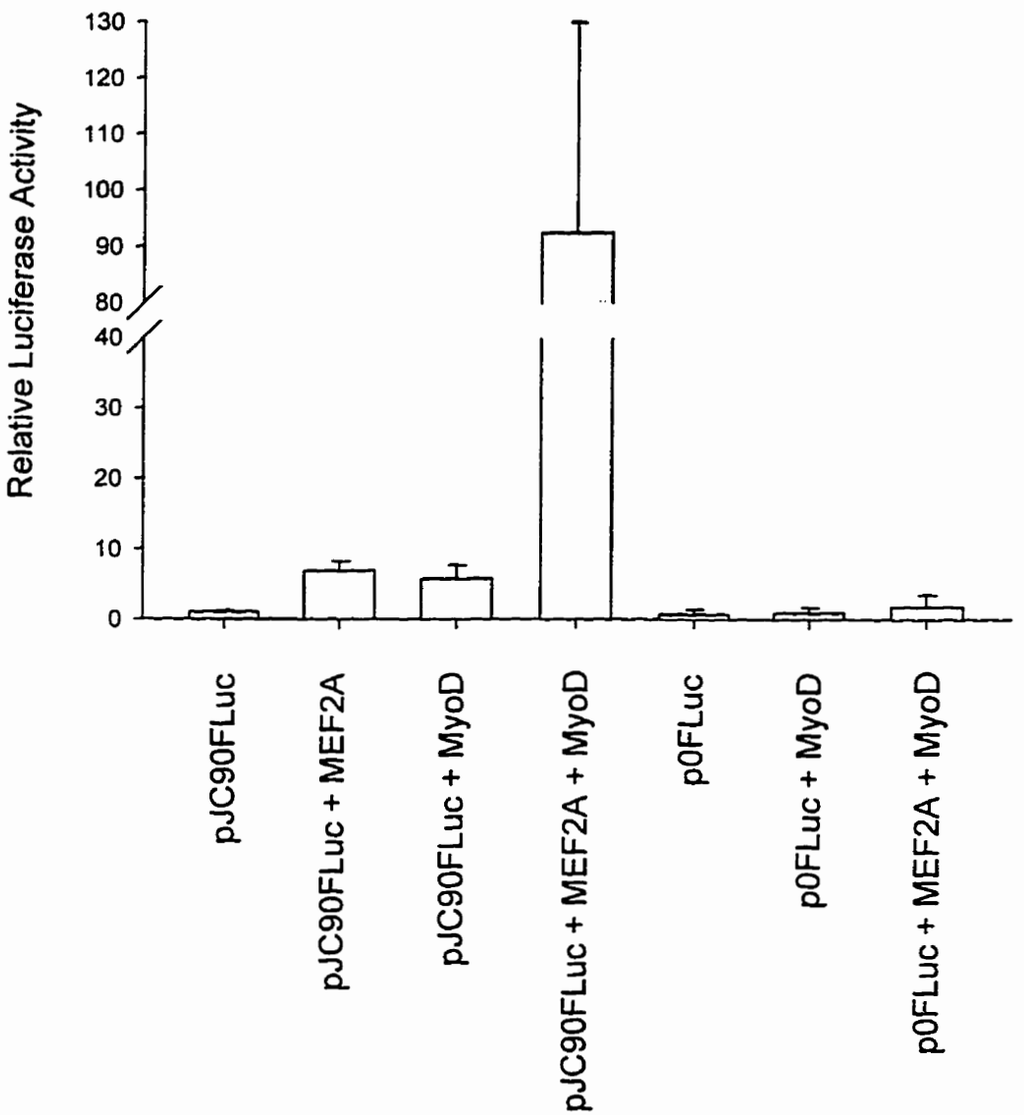

Figure 4. Synergistic transactivation by MEF2A and MyoD.
HeLa cells were transfected with pJC90FLuc reporter along with MEF2A, or MyoD, or MEF2A and MyoD. p0Luc was used as a control. There is a synergism in transactivation when both MEF2A and MyoD are transfected.

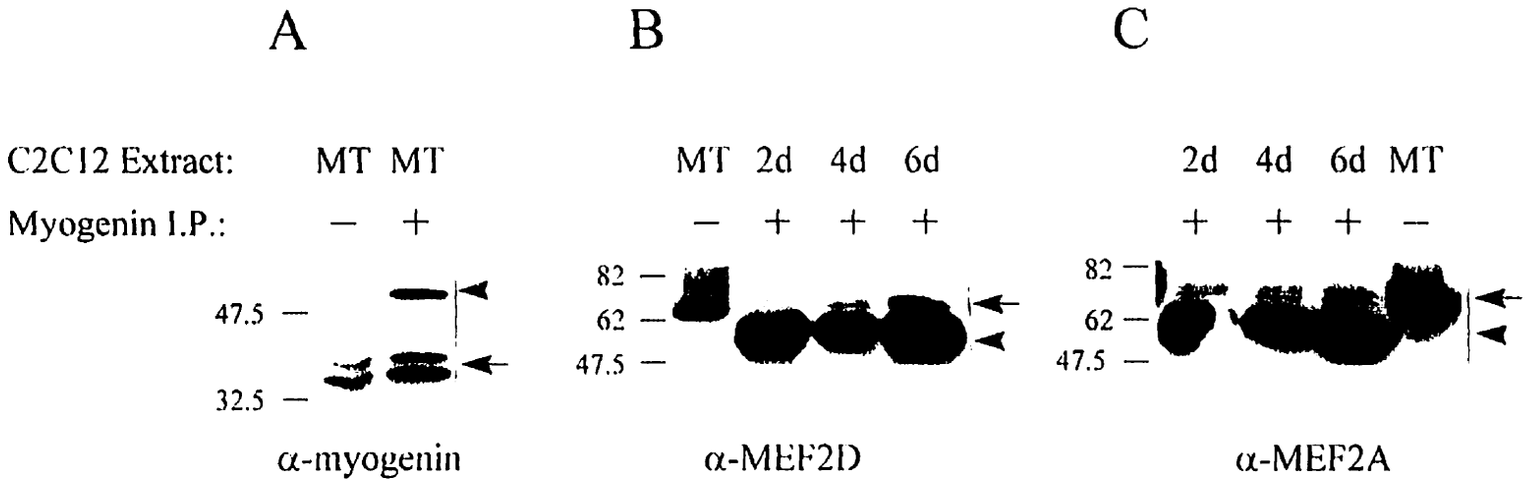

Figure 5. Interaction between MEF2 and myogenin in C2C12 myotubes
Myogenin proteins were immunoprecipitated from C2C12 myotubes at 2, 4, and 6 days after serum withdrawal. The resulting immunoprecipitates were analyzed by immunoblotting with anti-myogenin (A), anti-MEF2D (B), and anti-MEF2A (C). The arrows refer to the specific protein, and the arrowheads indicate the immunoglobulins recognised by the anti-rabbit secondary antibody.

Discussion

Like many transcription factors, c-jun regulation can occur through controlling its activity and amount within the cell (28). The activity of c-Jun is regulated post-translationally through phosphorylation (27). In response to various stressors, eg. U.V., heat, TNF-α, c-Jun is phosphorylated on Ser 63 and, more prominently, Ser 73 in its activation domain by the JNK's (11,25,30). This phosphorylated c-Jun can then interact with co-activators CBP/p300 to increase its transactivation potential (4). Recently, the activation domain of c-Jun has been shown to interact with another protein, JAB1 (Jun-activation domain binding protein 1) (9). JAB1 enhances c-Jun transactivation ability and stabilises its binding to the TRE (9). The abundance of c-Jun is also regulated at the level of protein stability. The half life of c-Jun is approximately 90 minutes (31), and degradation of c-Jun has been shown to be mediated by the ubiquitin pathway (56). However, phosphorylation of c-Jun by the JNK's decreases c-Jun ubiquitination and increases its stability (38). Another prominent feature of c-jun regulation is at the level of transcription. The transcriptional induction of c-jun has previously been demonstrated to increase in response to a variety of stimuli. Stress and DNA damaging agents, such as U.V. irradiation, have been shown to increase the induction of c-jun mRNA (12,58). As well, c-jun induction is increased in response to growth factors and phorbol esters (3,22,21,31). Therefore, as the levels of c-Jun rapidly increase in response to various stimuli, transcriptional induction of the c-jun gene is a critical component of this up-regulation. The cloning and characterisation of the c-jun promoter has allowed studies dissecting the regulatory networks that control its transcription. In this study we provide

evidence that transcription factors induced in terminally differentiating muscle cells potently activate the c-jun enhancer.

In order to analyse the c-jun MEF2 site, HeLa cells were transfected with a c-jun enhancer luciferase reporter gene (-225 to +150 of the c-jun enhancer) along with a MEF2A expression vector. Similar to others (21, 20), we were able to see an increase in MEF2 site dependent reporter gene activity when MEF2 was overexpressed. Therefore, overexpression of MEF2A can transactivate the c-jun enhancer. Han et al. (21) demonstrated this effect for MEF2D, and also implicated MEF2D as important for EGF induction of c-jun. We found MEF2C to be the most potent in activating the c-jun enhancer, followed by MEF2A, and with MEF2D being the least (data not shown). Han et al (1997) have also recently shown that MEF2C does bind to the MEF2 site, as it is important in inducing c-Jun expression in monocytic cells in response to lipopolysaccharide (20).

The activity of the c-jun enhancer is high in growing HeLa cells, and we attribute this to the various sites in the enhancer, i.e. NF-jun, jun1, jun2, SP1, and the CAAT box, which are bound by various transcription factor complexes. However, endogenous MEF2 proteins in HeLa do not contribute to this activation since the c-jun enhancer, which contains a mutated MEF2 binding site, has the same activity as the wild type enhancer when it is transfected. This is in agreement with previous studies (41). It was shown that in HeLa cells, a heterodimer of MEF2A:MEF2D binds to the MEF2 site in the c-jun enhancer and this heterodimer is unable to activate transcription. Therefore in HeLa cells, the basal levels of c-jun transcription do not depend on the MEF2 site. However, in

HeLa and NIH3T3 cells, the MEF2 site is necessary for serum induction of c-jun transcription (22,21). In contrast to the minimal role played in proliferating cells we show that the MEF2 site in the c-jun enhancer is an important regulatory element in myogenic cells, since a mutated MEF2 site in the c-jun enhancer leads to a considerable decrease in reporter activity during differentiation. The residual enhancer activity remaining when the MEF2 site is mutated is due to a contribution from the other transcription factor binding sites, or possibly, as yet, undiscovered cis elements in the c-jun enhancer.

Our binding assays showed that binding to the MEF2 site in the c-jun enhancer increases with the state of differentiation of the cells. We also show that the predominant complex binding to the MEF2 site in the c-jun enhancer in myogenic cells is a MEF2A homodimer. There is also some MEF2A:MEF2D heterodimer binding present. A MEF2A homodimer is also the major complex at the MEF2 site of the MCK enhancer, and it is this homodimer which is responsible for transactivation, as the MEF2A:MEF2D heterodimer is unable to activate transcription (41). Interestingly, the c-jun enhancer has been shown to be fully occupied by transcription factors during un-induced conditions in HeLa, F9, fibroblasts, and human embryonic retinoblasts, and the occupancy does not change in the presence of agents such as UV and TPA which induce c-jun transcription (58,47,24). However, the possibility of a pre-assembled binding complex on the c-jun enhancer has not been tested in muscle cells. Based on our data, one might speculate that in proliferating myoblasts there would be no MEF2 factors bound to the MEF2 site and therefore the existence of a pre-assembled transcription complex containing MEF2 is

unlikely. However, this does raise the possibility that in differentiating cells the formation of a pre-assembled complex containing MEF2, and possibly MyoD, may comprise a unique 'differentiation' specific transcription factor complex capable of activating c-jun transcription. Moreover, recent work has documented that MEF2 factors can physically interact with p38 MAP kinase (20), p300/CBP (4), and the thyroid hormone receptor (32). The recruitment of any, or all of these proteins to the enhancer would add further complexity and specificity to the transcription complex. In addition, the level of SP1, another transcription factor which may repress c-jun transcription (3, 57), decreases in differentiating muscle cells (33). Taken together, this could imply the formation of 'differentiation specific' and 'proliferation specific' transcription factor complexes in the control of the c-jun enhancer.

It has been shown that members of the myogenic bHLH family of transcription factors and the MEF2 family can interact (37). Although the MyoD family of transcription factors can confer the myogenic program to some 'permissive' non-muscle cells (10), the MEF2 proteins alone do not seem to be able to do this, however, there are some conflicting reports on this issue (37 and 29). When the MyoD family members are co-expressed with MEF2, the ability to induce myogenic conversion is reportedly increased (37). In addition, enhancers lacking an E-box, but which contain a MEF2 site, can be activated by MyoD or myogenin, and enhancers lacking a MEF2 site but which contain an E-box can be activated by MEF2 (37). Both sites together on an enhancer can synergistically increase transcription and this seems to be independent of the spacing between the sites (29). Furthermore, MEF2 is able to recover the transactivation ability

of MyoD or myogenin in mutants which have their activation domain deleted, and reciprocally, MyoD and myogenin can do the same with mutated MEF2 (37). However, some caution should be exercised in interpreting these data as most of these interactions have been defined using artificially constructed or multimerized reporter constructs (37). The physical interaction interface between these two families is at the MADS/MEF2 domain of the MEF2s, and the basic region and first helix domain of the MyoD family (37). Also, the amino acids alanine and threonine in the basic region of the myogenic bHLH proteins, which are critical for their myogenic activity, are also important for the physical interaction between MyoD and MEF2 (29).

In the studies reported here we document a synergistic activation of a natural promoter (c-jun) by MEF2 and MyoD despite the absence of an E-box. Since we observed a synergy between the MEF2 family proteins and the MyoD family proteins using reporter assays, we wanted to determine if they physically interact *in vivo*. Most studies to date have relied on *in vitro* and overexpression studies to show this interaction. We assessed this interaction by immunoprecipitating myogenin from C2C12 myotubes and analyzing the immunoprecipitated myogenin for the presence of MEF2A and MEF2D. These experiments demonstrated the presence of MEF2A and MEF2D in the myogenin immunoprecipitate, suggesting a physical interaction between the bHLH and MEF2 proteins within the cell. An interaction between MEF2A and MEF2D was also demonstrated by Kaushal et al. (29). Therefore, the model that we propose for the synergistic activation of the c-jun enhancer is one in which MEF2 binds to its cognate binding site and recruits the activation function of MyoD (see figure 6).

Figure 6. Proposed mechanism for the transcriptional synergism between MEF2 and MyoD on the c-jun enhancer.

Studies concerning the role of c-jun in muscle cells have as yet proven equivocal. The levels of c-Jun have been shown to increase in hypertrophying skeletal and cardiac muscles (43, 49, 16). c-Jun/AP-1 can augment transcription of the skeletal α-actin gene in cardiac hypertrophy by synergizing with serum response factor (SRF) (44). Regenerating rat skeletal muscle also contains elevated levels of c-Jun in satellite cells and newly formed myotubes (26). Conversely, overexpression of c-Jun in myoblasts inhibits their differentiation (5,53,18). However, c-Jun may not necessarily mediate this effect at physiological levels since some studies have shown c-Jun levels to remain relatively constant during muscle differentiation (54,55). Based on the equivocal evidence concerning the role of 'physiological' levels of c-Jun in myogenic cells, and also the potent activation of the c-jun enhancer by myogenic factors reported here, the function of c-Jun in differentiating muscle cells should be re-visited.

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Appendices

Appendix 1 - Methods and Materials

TRANSFECTIONS

For transfections, the DNA calcium phosphate coprecipitation technique was used. The cells were plated 24 hours prior to adding the precipitate. For HeLa, 2 hours prior to adding the precipitate fresh 10% FBS is given to the cells (3 hours for C2C12). The precipitate is then added to the plates.

Plating the Cells

For reporter assays, the HeLa cells were plated at approximately 0.5 $x10^6$ cells per 60 mm dish. The C2C12 myoblasts were plated at 0.3 x 10^6 cells per 60mm dish. The cells were grown in high serum (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum). For the C2C12 myoblasts, 1% gelatin was added to the bottom of the plates in order that the myoblasts attach to the bottom of the plates. The cells were plated 24 hours prior to the transfection.

Preparation of Coprecipitates of Calcium Phosphate and DNA

Solutions Required:

Tris (for 1L of 1M Solution)
Tris Base 121.1g
ddH_2O up to 800ml
cool to room temperature
adjust to desired pH
ddH_2O to 1L

HEBS (for 50ml of 20x)
NaCl 8.18g
Na_2HPO_4 0.1065g
HEPES 5.95g
ddH_2O up to 50ml
pH 7.15

2M $CaCl_2$ (for 20ml)
$CaCl_2$ 4.45g
1M Tris pH 7.5 3.23ml
ddH_2O up to 20ml

In order to make the precipitate, first a solution of the DNA to be transfected, and the $CaCl_2$ is prepared, and then added to the HEBS.

In a 1.5ml microcentrifuge tube, 5 μg of the respective luciferase reporter is added. For the expression vectors, 2.5 μg of pMT2MEF2A was added, and 6 μg of pMT2 MyoD. 2 μg of pSV β-gal was added to control for transfection efficiency. Empty pMT2 vector was used to bring the total DNA to 15 μg. 31.25 μl of 2M $CaCl_2$ was added. Double distilled H_2O was used to bring the total volume up to 250 μl. in order to form the precipitate, this solution was then mixed and added drop-wise to 250 μl of HEBS while gently vortexing. The precipitate solution was then added to its respective plate, and shaken gently to disperse the precipitate evenly.

48 hours after the transfection, fresh 10% FBS was given to the HeLa cells. The cells were then collected 24 hours later.

Glycerol Shock

Solutions Required:
15% Glycerol
Serum Free Wash Media
10%FBS

24 hours after the transfection of the C2C12, the glycerol shock was performed. The cells were washed in serum free wash media, and then given 15% glycerol for 2 minutes. After the 2 minutes, the cells were washed again with the serum free wash media, and were then given fresh 10%FBS.

Inducing Differentiation of the C2C12 Myoblasts

Solutions Required:
Serum Free Wash Media
5% Horse Serum

24 hours after the glycerol shock, the C2C12 were washed with serum free wash media, and then given 5% horse serum in order to induce differentiation.

Collecting the Cells

Solutions Required:
Collection Buffer (for 100 ml)
1.4M NaCl 10ml
1M Tris 4ml
0.1M EDTA 1.2ml
ddH_2O up to 100ml
PBS (for 1L of 10x)
NaCl 80g

KCl 2g
$Na_2HPO_4 7H_2O$ 14.4g
KH_2PO_4 2.4g
H_2O to 800ml
HCl to pH 7.4
H_20 to 1L

Plates were washed 3 times with 1xPBS. 1ml of the collection buffer was then added to each plate, and the plate put back into the 37°C incubator for 5 minutes. The cells were then scraped off of the plated and into a microcentrifuge tube. The tubes were then spun in the microcentrifuge for 1 minute. The supernatant was then aspirated, and the cells were re-suspended in 80µl of 0.25M Tris pH 7.8, and then frozen for the β-galactosidase assay.

β-galactosidase Assay

Solutions Required:
0.1M NaH_2PO_4/Na_2HPO_4
100x Mg Buffer (for 500 µl)
100 µl 500 mM $MgSO_4$
225 µl ddH_2O
175 µl β-mercaptoethanol
o-Nitrophenyl-β-D-Galactopyranoside (4 g/L)
1M Na_2CO_3

The cells were lysed by freeze thawing the cells 4 times in lysis buffer (0.25M Tris, pH 7.8). The cell extracts were then spun in the microcentrifuge at 4°C for 10 minutes. For each sample to be assayed was added 3 µl of 100x Mg Buffer, 201 µl of 0.1M NaH_2PO_4/Na_2HPO_4, 66 µl of ONPG, and 30 µl of straight cell extract. The samples were then incubated at 37°C until a colour change was detectable. 500 µl of 1M Na_2CO_3 was then added to each tube to stop the reaction. The O.D. of each sample was then taken at 420 nm visible light. A standard number of β-galactosidase units was used for subsequent luciferase determination.

Luciferase Assay

The sample with the lowest β-galactosidase value would have 30 µl of cell extract used in the luciferase assay. The volumes for the rest of the samples were normalised to the B-galactosidase values. In order to make up the volume to 30 µl, 0.25 M Tris, pH 7.8 was used.

The luciferase assay will be carried out using the 9501 Berthold Luminometer. This instrument will inject 100 µl of luciferase assay substrate into the 30 µl of cell

extract/Tris solution, and it will measure the light intensity from this reaction, thereby indicating the luciferase activity in the extract.

For the C2C12 myoblasts, the day after the glycerol shock, the DMEM with 10% FBS will be changed to DMEM plus 5% horse serum. This low mitogen serum will allow cell differentiation to occur. After the cells have differentiated, as indicated by the presence of multinucleated myotubes, the cells will be harvested, and the cell lysis, β-galactosidase assay, and luciferase assay will be carried out the same as for the HeLa cells.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN TRANSFER

Preparation of Cell extracts

Solutions Required:
SDS Sample Buffer (for 500 µl)
- 470µl 2x Laemmli
- 25µl β-Mercaptoethanol
- 5µl PMSF (1M)
- 0.5µl DTT (1M)
- 0.5µl Leupeptin
- 0.5µl Pepstatin

PBS (as above)

Cell extracts for Western were prepared by washing the cells on their dish 3 times with 1x PBS, pH7.5. To each plate, 200 µl SDS Sample buffer was added, and the cells scraped off and put into an eppendorph microtube on ice. Cells were sonicated on ice for 15 seconds on ice, and then boiled at 100^0C for 5 minutes. Total protein concentration was determined by a Bradford assay.

IMMUNOPRECIPITATION

Solutions Required:
1x Lysis Buffer (for 20ml)
- 1M Tris pH 7.4 1ml
- 1.4M NaCl 2ml
- 1% Nonidet P-40 200µl
- 1mM Sodium Vanadate 20µl
- 1mM PMSF 20µl
- 1mM DTT 20µl
- leupeptin 5µl
- aprotenin 5µl

SDS Sample Buffer (see above)

C2C12 myoblasts and myotubes were grown on 100mm dishes. Cells were washed 3x with 1x PBS. The plates were floated on liquid nitrogen for 10 seconds, then in a 37°C water bath for 10 seconds. 300µl of the 1x lysis buffer was added to each dish, and the cells were scraped off and put into a microcentrifuge tube. The cells were then sonicated for 10 seconds on ice, centrifuged, and the supernatant collected into a new tube. To this, 50µl of the anti-myogenin (monoclonal) supernatant was added, and the tube was allowed to rock at 4°C for 1.5 hours. 50µl of 10%PAS was then added, and the tube was left to rock overnight at 4°C.

The sample was then spun (10, 000 x*g*) in the microcentrifuge for 1 minute, and the supernatant was aspirated. 1ml of the lysis buffer was forcefully added in order to wash the beads. The tube was spun for 10 seconds, the supernatant aspirated, and washed with 1 ml of the lysis buffer once again. This was repeated 3 times. Then 30µl of SDS sample buffer was added to the sample. The tubes were then boiled for 5 minutes at 100°C.

15µl of supernatant was then loaded on a 10% SDS PAGE minigel and electrophoresed. The transfer and immunoblotting were performed as above.

Electrophoresis, Western Transfer, and Immunoblot

Solution Required:

Laemnli (for 1L of 10x)
- ddH_2O 800ml
- Tris 30.3g
- Glycine 144.2g
- SDS 10g
- pH to 8.3
- ddH_2O to 1L

10% Running Gel (for 20ml)
- ddH_2O 7.9ml
- 1.5M Tris (pH 8.8) 5.0ml
- 30% acrylamide 6.7ml
- 10% SDS 200µl
- 10% APS 200µl
- TEMED 8µl

Stacking Gel (for 4ml)
- ddH_2O 2.7ml
- 1.0M Tris (pH 6.8) 500µl
- 30% acrylamide 670µl
- 10%SDS 40µl
- 10%APS 40µl

TEMED 4µl

50 µg of total cell protein was electrophoretically resolved on a 10% SDS gel, using 1x Laemmli as the running buffer. The gel was run at 25 mamps. After electrophoresis, the proteins were semi-dry electrophoretically transferred to NitroPlus nitro-cellulose transfer membrane for immunological detection. The filter was blocked for 1 hour in 5% milk. MEF2A and MEF2D are polyclonal antibodies and used at a dilution of 1:1000 in 5% milk. The α-myogenin antibodies used are monoclonal. The filter was then incubated with the antibody overnight at 4^0C. The next day the nitro-cellulose was washed 3 times for 15 minutes in 5% milk. The secondary antibody, goat anti-rabbit IgG HRP conjugated, was applied at a dilution of 1:1000 in 5% milk for the MEF2A and MEF2d probed blots. For the myogenin probed blots, anti-mouse IgG HRP conjugated were used. The nitro-cellulose was incubated at room temperature with the secondary antibody for 2 hours. The nitro-cellulose was then washed 2 times for 15 minutes each with 0.2% NP40 in 5% milk. The nitro-cellulose was then washed 3 times for 30 minutes in 0.2% NP40 in 1x PBS, pH 7.5. The nitro-cellulose was mixed with equal volumes of an enhanced luminol reagent, and an oxidising reagent (peroxide). The HRP catalyses the formation of atomic oxygen from peroxide. This oxygen catalyses the oxidation of luminol, resulting in the emission of light. This light emission is captured on auto-radiography film by the presence of a dark band, which reflects the position of the proteins.

Appendix 2 - Statistics and Raw Data

Raw data for figure 1

	pJLuc	pJLuc + MEF2A	p0FLuc	p0FLuc + MEF2A
Experiment #1				
mean of pJLuc	272397	1166307	6041	5574
261114.5	249832	1211445	6489	6015
Experiment #2				
mean of pJLuc	21166	61333	421	521
15621	10076	25256	400	–
normalised to mean				
Experiment #1				
	1.0432	4.4666	0.0231	0.0213
	0.9568	4.6395	0.0249	0.0230
Experiment #2				
	1.3550	3.9263	0.0270	0.0334
	0.6450	1.6168	0.0256	
mean	1.0000	3.6623	0.0251	0.0259
SE	0.1460	0.6986	0.0008	0.0038

Normalised values were log transformed and analysed by a 1 way Anova

Summary of all Effects; design:
1-Reporter

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	3*	4.482565*	11*	.019828*	226.0714*	.000000*

Tukey HSD test; variable Normalized Luciferase Value
Probabilities for Post Hoc Tests
MAIN EFFECT: Reporter and Overexpression

	pJLuc -0.014828	pJLuc + MEF2A 0.529772	p0FLuc -1.60015	p0FLuc + MEF2A -1.59538
pJLuc		.001105*	.000195*	.000195*
pJLuc + MEF2A	.001105*		.000195*	.000195*
p0FLuc	.000195*	.000195*		0.999969
p0FLuc + MEF2A	.000195*	.000195*	0.999969	

Raw data for figure 2 in manuscript

	Experiment #1		
	pJ LUC	**pJSX LUC**	**p0FLuc**
	638818	368969	1233
	731413	289658	1253
	684569	448054	771
mean	684933		
	Values Normalized to mean of pJLuc		
	0.9327	0.5387	0.0018
	1.0679	0.4229	0.0018
	0.9995	0.6542	0.0011
	Experiment #2		
	634306	328493	2747
	504417	382973	3497
	502623	426595	3115
mean	547115.3333		
	Values Normalized to mean of pJLuc		
	1.1594	0.6004	0.0050
	0.9220	0.7000	0.0064
	0.9187	0.7797	0.0057
	mean of normalized values		
	1	0.6160	0.00364
	standard deviation of normalised values		
	0.0972	0.1256	0.0023
	standard error of normalised values		
	0.0397	0.0513	0.0009

Summary of all Effects; design:
1-
Reporter

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	1.5161	15.0000	0.0084	180.2436	0.0000

Tukey HSD test; variable Normalized Luciferase Value
Probabilities for Post Hoc Tests
MAIN EFFECT: Reporter

	pJLuc 1.000333	pJSXLuc .6160000	p0FLuc .0036333
pJLuc		0.0002	0.0002
pJSXLuc	0.0002		0.0002
p0FLuc	0.0002	0.0002	

Raw Data for figure 4

	pJC90FLuc	pJC9+ MEF2A	pJC9+ MyoD	pJC9+MEF2A+ MyoD	p0FLuc	p0FLuc + MyoD	p0FLuc+MEF2A+ MyoD
Experiment #1							
	1070	6843	4688	36406	1194	1548	3404
	935	8770	4298	29340	1276	1568	3132
mean	1002.5						
Normalize to mean							
	1.0673	6.8259	4.6763	36.3152	1.1910	1.5441	3.3955
	0.9327	8.7481	4.2873	29.2668	1.2728	1.5641	3.1242
Experiment #2							
	368	2997	2550	62024	89	134	130
	571	2617	4047	80874	91	121	133
mean	469.5000						
Normalize to mean							
	0.7838	6.3834	5.4313	132.1065	0.1896	0.2854	0.2769
	1.2162	5.5740	8.6198	172.2556	0.1938	0.2577	0.2833
	mean						
	1	6.8829	5.7537	92.4860	0.7118	0.9128	1.7700
	standard deviation						
	0.1849	1.3472	1.9689	70.9103	0.6015	0.7406	1.7239
	standard error						
	0.0924	0.6736	0.9844	35.4551	0.3008	0.3703	0.8620

Summary of all Effects; design:
1-Reporter

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	6	2.3925	21	0.138789445	17.238	4E-07

Tukey HSD test; variable Normalized Luciferase Value
Probabilities for Post Hoc Tests
MAIN EFFECT: Reporter

	pJC90FLuc	pJC9+ MEF2A	pJC9+ MyoD	pJC9+MEF2A+ MyoD	p0FLuc	p0FLuc+ MyoD	p0FLuc+MEF2A+ MyoD
	-0.006	0.832	0.743	1.846	-0.314	-0.188	-0.020
pJC90FLuc		0.058	0.113	0.000	0.898	0.992	1.000
pJC9+MEF2A	0.058		1.000	0.014	0.005	0.013	0.052
pJC9+MyoD	0.113	1.000		0.007	0.010	0.028	0.102
pJC9+MEF2A+MyoD	0.000	0.014	0.007		0.000	0.000	0.000
p0FLuc	0.898	0.005	0.010	0.000		0.999	0.917
p0FLuc+MyoD	0.992	0.013	0.028	0.000	0.999		0.995
p0FLuc+2A+MyoD	1.000	0.052	0.102	0.000	0.917	0.995	

Appendix 3 - Abbreviations

ABBREVIATION	FULL TERM
AP-1	Activator Protein-1
ASV	Avian Sarcoma Virus
ATF	Activating Transcription Factor
bHLH	Basic Helix Loop Helix
CBP	CREB Binding Protein
CRE	cAMP Response Element
CREB	CRE binding protein
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxy-ribonucleic acid
EGF	Epidermal growth factor
FBS	Fetal Bovine Serum
HS	Horse Serum
JAB1	Jun-activation domain binding protein 1
JNK	c-Jun Amino Terminal Protein Kinases
jun1	Proximal AP-1 site in the c-jun enhancer
jun2	Distal AP-1 site in the c-jun enhancer
MADS	MCM1, Agamous, Deficiens, and SRF
MCK	Muscle Creatine Kinase
MEF2A-D	Myocyte Enhancer Factor2A-D
MKK	MAP Kinase Kinase
mRNA	messenger RNA
NF-jun	Nuclear Factor jun
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
RNA	ribonucleic acid
SAPK	Stress Activated Protein Kinases
SDS	Sodium dodecyl sulfate
TAFs	TBP Associated Factors
TBP	TATA Binding Protein
TFIID	Transcription Factor IID
TGF-β	Transforming Growth Factor Beta
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA Response Element
U.V.	Ultra-Violet Radiation