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hDREF, a MEF2 sensitive regulator of DNA synthesis

by
Andrea Leah Margaret Rowan

A thesis submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
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Abstract

The Myocyte Enhancing Factor 2 (MEF2) family of proteins have been implicated in a wide variety of cellular mechanisms including: muscle and neuronal differentiation, inhibition of apoptosis, upregulation of c-jun expression, and embryonic and post-natal cardiac development. In the course of my research I have identified a novel MEF2-responsive gene referred to as human DNA Replication Related Element Factor (hDREF). Three putative MEF2 consensus-binding sites have been found within the two untranslated regions (UTRs) of the hDREF sequence (which lie 5' and 3' to the open reading frame). Using CHromatin ImmunoPrecipitation (CHIP) assays I have shown that MEF2 proteins associate with the MEF2 binding site found within the 5'UTR of hDREF under both growth and differentiation conditions. Furthermore, mutation of the MEF2 binding sites results in a failure to repress expression, which indicated that MEF2 acts to negatively regulate hDREF expression during differentiation. Endogenous expression studies indicate that hDREF is highly expressed during growth and downregulated during differentiation. Functional studies suggest that hDREF is directly involved in regulating DNA synthesis, and that overexpression of hDREF leads to aberrant DNA replication in post-mitotic cells leading to polyploidy. Based on my research I propose that hDREF represents a novel MEF2 regulated gene, and that the primary function of hDREF is to ensure DNA synthesis with the cell cycle.

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List of Abbreviations

aa	Amino acid
Ac	Activator -dissociator
ANF	atrial natriuretic factor
BMK1	Big MAP Kinase
BLASTp	Basic Local Alignment Search Tool-protein
BLASTn	Basic Local Alignment Search Tool-nucleotide
BrdU	5-Bromo-2'-deoxyuridine
CHAMP	Cardiac Helicase Activated by
cdk	Cyclin dependent kinase
CA	cardiac actin
CaMKI	Calcium/Calmodulin dependent
CaMKIV	protein kinase I and IV
CBP	CREB binding protein
cdk	Cyclin Dependent Kinase
CHIP	Chromatin Immunoprecipitation
CtBP	carboxyl-terminal binding protein)
DAPI	4,6 Diamidino- 2-phenylindole
DAB	3,3'-Diaminobenzidine
ERK	Extracellular Regulated Kinase
CMV	cytomegalovirus
DR	Direct Repeat
DN	Dominant-negative
DNA	Deoxyribonucleic acid
DREF	DNA Replication Related Element Factor
hDREF	human DNA Replication Related Element Factor
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G418	neomycin
IGF	Insulin like Growth Factor
HDAC	Histone deacetylase
HAT	Histone Acetyltransferase
JNK	c-jun N-terminal kinase

MITR	MEF2 Interacting Transcription Repressor
LINEs	Long Interspersed Elements
MADS	Mini-chromosome maintenance1, Agamous
	Deficiens Serum response factor
MEK5	MAP Extracellular Kinase
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MLC	Myosin light chain
MRF(s)	Muscle regulatory factor(s)
NFAT	Nuclear Factor Activated by T-cells
PI	Propidium iodide
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT	Reverse Transcriptase
PCR	Polymerase Chain Reaction
PCNA	Proliferating Cell Nuclear Antigen
SINES	Short Interspersed Elements
SRF	Serum response factor
TBP	TATA Binding Protein
TCR	T-Cell Receptor
TIR	Terminal Inverted Repeat
TRAMP	Tyrosine Rich Acidic Matrix Protein
UTRs	Untranslated Regions

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Chapter 1

1.0 Introduction

1.1 MEF2 and MRFs, regulators of muscle differentiation.

In order to understand the complex series of events that occurs routinely within the cell, molecular biologists have resorted to constructing complex protein interaction networks that attempt to integrate multiple factors into predictive models. In this thesis, I have chosen to focus my attention on the function and regulation of a novel protein hDREF, in the context of cardiac and skeletal muscle differentiation. I have chosen to work primarily with muscle because it has previously been shown to be an ideal milieu for analyzing the complex series of events that regulate cell fate.

The MEF2 proteins belong to the MADS family of transcription factors, so named for the first four proteins in which this domain was identified: minichromosome maintenance 1 (MCM1), Agamous, Deficiens and Serum response factor (Shore and Sharrocks, 1995). The MEF2 family of transcription factors have diverse function within the cell, and have been implicated in a wide variety of cellular mechanisms. In skeletal muscle, MEF2 has been shown to greatly enhance muscle differentiation when co-expressed with the myogenic regulatory factors myogenin or MyoD (Black and Olson, 1998; Molkentin et al., 1995; Wang et al., 2001). In neurons, MEF2C is active in postmitotic neuronal differentiation, particularly in the cortical layers of the brain (Mao et al., 1999; Mao and Wiedmann, 1999). Expression of MEF2C has also been shown to rescue neuronal cells from apoptosis during neuronal differentiation (Okamoto et al., 2000). In NIH 3T3 and COS cells the MEK5/BMK1 MAP kinase signaling pathway regulates the serum response factor c-jun through a MEF2C mediated signal cascade (Kato, 1997). Likewise

MEF2D has also been shown to regulate the expression of c-jun in HeLa cells (Han and Prywes, 1995). Conversely, in cerebellar granule neurons phosphorylation of MEF2A and MEF2D mediates caspase-regulated apoptosis (Li et al., 2001). In mammals MEF2C is expressed at embryonic day 7.5 (Morin et al., 2000). Embryos that are homozygous null for MEF2C are embryonic lethal at E 9.5 due to severe cardiac abnormalities (Lin et al., 1998), while MEF2C dominant negative animals die prematurely and display attenuated postnatal growth of the myocardium (Kolodziejczyk et al., 1999), indicating that MEF2 expression is required for both prenatal and postnatal cardiac development. Thus the MEF2 family of transcription factors have been implicated in many key cellular functions including growth, differentiation and cell death.

Vertebrates have four MEF2 proteins (MEF2 A-D) which homo- and heterodimerize with each other through the requisite MADS domain (McKinsey et al., 2002; Ornatsky and McDermott, 1996). The MADS domain also mediates heterodimer formation between MEF2 and other proteins (McKinsey et al., 2002). MEF2 DNA binding requires both the MADS and MEF2 domains found at the amino terminus of the MEF2 proteins (McKinsey et al., 2002). The MEF2 binding consensus sequence (CTA (A/T)₄TAG/A) is found in the promoters and regulatory regions of a broad collection of genes (Wang et al., 2001). In cardiac muscle, MEF2 is required for the normal expression of ventricle myosin light chain (MLC2V), cardiac troponin T, cardiac troponin I, α -myosin heavy chain and desmin. (Di Lisi et al., 1998; Kuisk et al., 1996; Molkentin and Markham, 1993; Yu et al., 1992; Zhu et al., 1991). In skeletal muscle, MEF2 is required for

differentiation and forms a regulatory association with the Myogenic Regulatory Factor (MRF) family of proteins. In vertebrates, the MRF family consists of MyoD, Myf5, myogenin and MRF4, and are required for normal muscle differentiation (Olson et al., 1995). The MRFs use the basic helix loop helix domain to heterodimerize with ubiquitous E-proteins and bind to a target sequence (the E-box CANNTG) found in the promoter region of many muscle specific genes including myosin heavy chain IIb, skeletal alpha actin, troponin I and myosin light chain (Lu et al., 2000b). Successful withdrawal from the cell cycle and co-ordination of muscle differentiation requires the expression of the MRFs in combination with the MEF2 family of proteins. Numerous studies have shown that co-expression of MRFs with MEF2 dramatically increases the extent of myogenic conversion, over that observed with either factor alone (Kaushal, 1994; Molkenstein et al., 1995; Naidu et al., 1995; Novitch et al., 1999).

1.2 MEF2 activity is enhanced by association with multiple co-activators.

The association of MEF2 with multiple co-activators may explain the diverse role that this transcription factor plays in regulating cell growth, cell differentiation and cell death. Indirect and direct phosphorylation of MEF2 is one of the multiple ways in which MEF2 activity is regulated. Activated calcium/ calmodulin-dependent protein kinases I and IV (CaMKI and CaMKIV) have been shown to induce a hypertrophic response in cardiomyocytes (Passier et al., 2000). More specifically, CaMKIV has been shown to induce MEF2 mediated cardiac hypertrophy and increase MEF2 activity *in vivo* (Lu et al., 2000a; Passier et al., 2000; Wu et al., 2000). CaMK mediates MEF2 activity through

phosphorylation of class II histone deacetylase (HDACs) proteins, thereby liberating MEF2 from their repressive effects (McKinsey et al., 2002). Once free from their association with HDACs, MEF2 physically associates with the histone acetylases (HATS) p300 and CBP which act to re-acetylate the histone tail, weakening the electrostatic charge between histones and DNA, thereby making the chromatin transcriptionally accessible (Lu et al., 2000a; McKinsey et al., 2000).

The mitogen activated protein kinases (MAPKs) are calcium responsive kinases, which have also been shown to stimulate MEF2 activity. MAPK cascades can be divided into 3 subgroups based on the terminal effector kinase; 1) extracellular signal regulated protein kinase (ERK), 2) c-jun N-terminal kinases (JNK) and 3) p38 kinase (McKinsey et al., 2002). There are four isoforms of p38 kinase (α , β , γ and δ) each of which has been shown to phosphorylate the transactivation domain of MEF2, which results in increased MEF2 transcriptional activity (Han et al., 1997; Han and Molkentin, 2000; McKinsey et al., 2002). Activation of MEF2 by p38 has been linked to activation of the hypertrophic program in cardiomyocytes (Kolodziejczyk et al., 1999), differentiation in muscle (Lu et al., 2000b) and protection of neurons from apoptosis (Mao et al., 1999; Mao and Wiedmann, 1999; Okamoto et al., 2000). The MAPK, ERK5 has also been shown to be a potent regulator of MEF2 activity (Yang et al., 1998). ERK5 regulates MEF2 activity by two separate mechanisms: 1) by binding to the MADs domain of MEF2 and acting as co-activator of transcription and 2) by phosphorylating the transactivation domains of MEF2A, -C, and D (Kato et al., 2000; Kato, 1997; Yang et al., 1998). Phosphorylation of

the MEF2 transactivation domain by ERK5 has been implicated in the signal cascade that activates c-jun expression and cell growth (Kato et al., 2000; Kato, 1997).

In skeletal muscle, the myogenic regulatory factor MyoD forms an auto-regulatory loop with MEF2C leading to complimentary enhancement of expression during muscle differentiation (Lu et al., 2000b; McKinsey et al., 2002; Wang et al., 2001). In addition, both myogenin and MyoD when co-expressed with MEF2C, act synergistically to activate transcription of muscle specific genes (Molkentin et al., 1995). GATA4, a cardiac-specific transcription factor, interacts with MEF2 through a physical association between the MEF2 DNA binding domain and the carboxy zinc finger of GATA 4 (Morin et al., 2000). Recruitment of MEF2 by GATA-4 to target genes has been shown to synergistically activate Atrial Natriuretic Factor, alpha Myosin Heavy Chain and alpha Cardiac Actin (Morin et al., 2000). Yet another example of MEF2 acting as a co-activator of gene expression is in the synergistic activity that results from Nuclear Factor Activated by T-cells (NFAT) and MEF2 protein interactions. Slow myofiber expression, and muscle differentiation have both been shown to be activated by the synergistic activity of NFAT with MEF2 (Wu et al., 2000). In addition pRb has also been shown to up-regulate MEF2 activity through MyoD (Novitch et al., 1999). In this cascade pRb acts not only to suppress the cell cycle by binding E2F and limiting S phase progression, but also promotes cell differentiation through association with the myogenic factors MEF2 and MyoD (Novitch et al., 1999).

1.3 MEF2 activity is suppressed by association with multiple co-repressors.

The interaction of MEF2 with diverse proteins is also a source for negative regulation. As previously described, the association of MEF2 with the class II family of histone deacetylases (HDACs) results in potent inhibition of MEF2 dependent transcription (Gruffat et al., 2002; Lu et al., 2000a; Lu et al., 2000b; McKinsey et al., 2002; Wu et al., 2000; Zhang et al., 2001b). HDACs 4 and 5 have been shown to physically interact with MEF2 through the MADs domain and inhibit transcription through deacetylation of core histones (Lu et al., 2000b). Overexpression of MyoD can override the MEF2 repression by HDACs, by directly competing with HDACs for their MEF2 binding partners (Lu et al., 2000b). The insulin like growth factor (IGF) can also relieve repression caused by HDACs through activation of calcium signaling and CaMK activity (Lu et al., 2000b).

HDAC7 has also been shown to physically interact with MEF2 proteins and inhibit the activity of MEF2 A, C, and D (Dressel et al., 2001). The MEF2 interaction site found in the N-terminal domain of HDAC7 is necessary to mediate transcriptional repression of MEF2 dependent genes (Dressel et al., 2001). The non-requirement of the C-terminal deacetylase domain of HDAC7 suggests that the N-terminal domain interacts with co-repressors to mediate repression (Dressel et al., 2001). Interestingly HDAC7 has been shown to interact with CtBP, a class I HDAC as well as other HDAC II proteins. This observation suggests recruitment of class I HDACs as co-repressors may also be necessary for transcriptional repression of MEF2 dependent proteins.

The MEF2-Interacting Transcription Repressor protein (MITR), is the predominant cardiac splice variant of HDAC9 (Sparrow et al., 1999; Zhang et al., 2002). Similar to other class II HDACs, MITR / HDAC9 has been shown to repress MEF2 transcriptional activity (Zhang et al., 2002; Zhang et al., 2001b). MITR bears homology with the amino terminal regions of HDACs 4 and 5 but lacks their catalytic domain and is presumed to act in conjunction with co-inhibitors to repress MEF2 dependent transcription (Zhang et al., 2001b). HDAC9 knockout mice are sensitized to hypertrophic stimuli and develop cardiac hypertrophy at an early age, indicating that HDAC9 / MITR expression represses hypertrophic responsive genes and that in its absence MEF2 dependent transcription is unregulated (Zhang et al., 2002).

As previously mentioned, phosphorylation of class II HDACs relieves the repressive effects exerted on MEF2 activity. Phosphorylation of two conserved serines on HDACs 4,5,7 and MITR/ HDAC9 by CaMK1 and IV (calcium/calmodulin dependent kinase) allows for the association of the 14-3-3 protein, which chaperones the HDAC proteins out of the nucleus and relieves the repression on MEF2 activity (Zhang et al., 2001a).

Cabin1 is a calcineurin inhibitor that has also been found to negatively regulate MEF2 activity (Youn et al., 1999). MEF2 is normally sequestered by Cabin1 in an inactive state however, T cell receptor (TCR) signaling leads to an increase in intracellular calcium which results in an increase in calmodulin expression (Youn et al., 1999). Competitive

binding of Cabin1 to MEF2 in addition to calmodulin, allows for liberated MEF2 to activate the steroid receptor Nur77 which mediates thymocyte apoptosis (Youn et al., 1999). MEF2 has also been shown to regulate the cardiac helicase CHAMP (cardiac helicase activated by MEF2 protein). Up-regulation of CHAMP by MEF2C during differentiation, results in the expression of the cyclin dependent protein kinase inhibitor p21^{CIP1}. The p21^{CIP1} kinase inhibitor goes on to prevent cyclin /cyclin dependant kinase interactions which consequentially inhibits cardiomyocyte proliferation (Liu et al., 2001; Liu and Olson, 2002).

Clearly, the co-factor interactions with MEF2 can adversely or positively affect the transcriptional activity of the MEF2 protein complex. MEF2 association with transcription factors such as MRFs, NFAT, and GATA4 results in MEF2 acting as a potent activator, while association with chromatin remodeling proteins such as class II HDACs, and Cabin I results in the silencing of MEF2 transcription. Thus MEF2 can be presented as a global transcription factor capable of regulating multiple interactions with diverse groups of proteins.

1.4 The hDREF sequence contains MEF 2 binding sites.

The MEF2 proteins have thus far been implicated as a key regulators of multiple signaling cascades whereby they act to enhance transcription factors involved in cell cycle, cell differentiation and cell death. Based on these observations I hypothesized that

MEF2 may enhance differentiation by an additional mechanism, i.e. by acting as a transcriptional repressor of mitotic regulatory genes. To test this hypothesis, we initiated a bioinformatics screen of sequence database to identify novel mitotic regulatory genes which contained the MEF2 consensus binding site (CTA[A/T]₄TAG/A]. As expected the search identified multiple promoters and enhancers from known muscle specific genes. However, my search also identified a human gene (hDREF), which met my criteria of a possible mitotic factor. Examination of this gene revealed the presence of 3 putative MEF2 binding sites (nt 761-772, 3657-3668, 4466-4477) and one E-box, the binding site of the Myogenic Regulatory Factor (MRF) family of transcription factors (nt 45-51) (see Figure 1). The discovery of these regulatory binding sites was of interest as the MEF2 and E-box binding sites have been shown to be crucial regulatory elements in the expression of a number of skeletal muscle and cardiac genes (Black and Olson, 1998).

1.5 hDREF nomenclature.

hDREF was originally cloned in 1998 by Nagase et al. in a large scale cloning project of novel human genes. The complete hDREF sequence was later cloned by Esposito et al. (1999) in an effort to identify genes which escaped X inactivation. The hDREF sequence has been mapped to chromosome 2 as well as the X and Y chromosomes, with other partially deleted copies scattered throughout the human genome (Esposito et al., 1999; Nagase et al., 1998). Esposito et al., (1999) originally chose to call their novel gene TRAMP because of its sequence similarity to the Ac family of transposons. However, the name TRAMP had previously been chosen for another completely unrelated protein:

Figure 1: hDREF (tramp) nucleotide sequence with translated amino acid sequence as described in Esposito et. al., 1999. Solid lines indicate the primers used in the RT-PCR assays, and double solid lines indicate the putative promoter sequence (Esposito et al., 1999). Dark and light boxes indicate putative duplicated target sites and terminal inverted repeats respectively. The polyadenylation sequence is indicated in bold nt (5560-5566). Examination of the untranslated regions (which lie three and five prime to the hDREF open reading frame but within the terminal inverted repeats) revealed three putative MEF2 (Myocyte Enhancing Factor 2) binding sites (red lines) and one MRF (Myogenic Regulatory Factor) binding site, the E-box (blue line). The 1.2 Kb 5'UTR contains one of the MEF2 binding sites (nt 762-772) (CTAAAAATAC) as well as the E-box binding sequence (nt 45-51) (CANNTG). The 2.3 Kb 3'UTR contains the other two MEF2 binding sites (nt 3657-3668 & 4466-4477).

Adapted from: Esposito et al. Figure # 3. Hum Mol Genet 1999 Jan;8(1):61-7 A novel pseudoautosomal human gene encodes a putative protein similar to Ac-like transposases.

Tyrosine Rich Acidic Matrix Protein, which made the nomenclature confusing. In order to clarify the situation we have chosen to rename the TRAMP protein as hDREF (human DNA Replication Related Element Factor) after its putative *drosophila* orthologue DREF (DNA Replication Related Element Factor) (see Figure 2).

Expression studies have revealed that hDREF is ubiquitously expressed in all human tissues and is highly expressed in heart, and skeletal muscle (Esposito et al., 1999).

Cloning and sequencing of the hDREF gene revealed a single exon that encodes a 78.2-kDa protein, which is flanked by 5' and 3' by untranslated regions (UTRs) (Esposito et al., 1999). A putative promoter sequence was identified in the 5' UTR using the promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html) and a polyadenylation sequence was found in the 3'UTR (Figure 1) (Esposito et al., 1999).

Further sequence analyses revealed that the 5' and 3' UTRs were flanked by terminal inverted repeats (TIRs) and by direct repeats (DR) suggesting that hDREF represents a human transposable element (Esposito et al., 1999; Pohlman et al., 1984). The amino acid sequence of the hDREF protein as well as the nucleotide sequence of the TIRs and the DRs suggests that hDREF is most similar to the Activator Dissociator (Ac) family of transposable elements (Esposito et al., 1999). The Ac (Activator / Dissociator) family of transposons all have TIRs of 11bp and DRs of 8 bp (Esposito et al., 1999). Gel shift experiments indicated that the mutations found in the TIRs of hDREF have resulted in the inability of the element to bind its own transposase, making it a non-autonomous transposable element (Esposito et al., 1999). hDREF was found to be homologous with

Ac transposase (X05424) of *Zea mays* (corn), the Hobo transposase (M69216) of *Drosophila melanogaster* (fruit fly), the Tam3 transposase (X55078) of *Antirrhinum majus* (snap dragon), and the Tol2 transposase (D84375) of *Oryzias latipes* (fish) (Esposito et al., 1999). Interestingly hDREF also was found to share sequence similarity to a *drosophila* protein named DREF (DNA Replication Related Element Factor) (see Figure 2).

1.6 Transposable elements.

Transposable elements are discrete sequences of DNA that are capable of spontaneously excising themselves out of the genome and reintegrating elsewhere (Campbell, 1999). Transposons were first discovered in the 1930s by Barbara McClintock in *Zea mays* (corn), and have since been found in all organisms studied. There are two main classes of mobile transposable elements, transposons and retrotransposons. Transposons move through a cut and paste mechanism whereas retrotransposons have RNA intermediates and use reverse transcriptase to make a DNA copy of the RNA to insert into a new site. Transposable elements can be further divided into 3 subgroups that consist of LINEs, SINEs and Alu repeats. Long interspersed elements (LINEs) were originally derived from mRNA and constitute approximately 60% of the entire human genome, whereas short interspersed elements (SINEs) were originally derived from tRNA and 5sRNA and make up approximately 11% of the genome (Kazazian and Moran, 1998). Alu repeats consist of a 300 bp sequence that contains the AluI restriction enzyme cut site and are also

Figure 2: BLASTp alignment of hDREF and DREF protein sequences from NCBI May 2002. hDREF and DREF proteins share 30% identical sequence, and 49 % similar sequence over a 81 amino acid stretch in the N-termini of the proteins. Sequence similarity resides entirely within the N-terminal DNA binding and homodimer formation domains of DREF (nt 16-115).

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.3

Matrix= blosum 62, **gap open** = 11, **gap extension**=1
x_dropoff = 50, **expect** = 10, **wordsize** = 3 **Filter**

Sequence 1:DNA replication-related element factor [Drosophila melanogaster] Length(1 .. 709)

Sequence 2:hDREF / proposed human homolog of DREF, also referred to as hDREF Length (1 .. 694)

Score = 51.6 bits (122), **Expect** = 4e-05

Identities = 25/81 (30%), **Positives** = 41/81 (49%)

DREF: 14 ELKYEDVSQLNFSKI YSPKM&SVYWRYTGI PSNDNNEVITKQNAVQ IKCHKVI TNHGNII 73
E K + SQ + + P KS WYEGE N + + + C C + GNI

hDREF: 2 LNKSLFSSQTDLKLVAHPRAKSKVWKYEGIDINAEGCH QWKKIYCRICMAQIAYSGNIS 61

DREF: 74 NLRATHQHRHDKDLKELCQEH 94

N L H L H L E E E + +

hDREF: 62 NLSYHHEKNHPIEIEICFVKSN 82

derived from tRNA and 5sRNA (Batzer and Deininger, 2002). To date approximately 1 million Alu repeats have been found in the human genome (Batzer and Deininger, 2002).

Transposable elements act as mutagens through excisions / insertions into introns and exons, and are now becoming recognized as putative causal agents of human disease. Cases of hemophilia, predisposition to colon and breast polyps, Duchenne muscular dystrophy and epilepsy have all been shown to be associated with mutations caused by transposable element insertions (Asch, 1996; Kazazian and Moran, 1998; Miki et al., 1992; Narita et al., 1993). Mobile transposable elements also enhance the probability of unequal cross over due to their repetitive sequences (Olivier et al., 2001). Insertion of transposons into promoters, enhancers and insulators can also disrupt normal gene regulation. Transposable elements may also act to deregulate transcriptional control through insertion of foreign promoters, enhancers, silencers and insulators into regulatory sequences. Conte et al., 2002 recently identified two novel retrotransposons which were capable of directing the expression of the “white gene” in *drosophila*. It was found that the 5’UTR of the ZAM retrotransposon acts as an enhancer, and that the long terminal inverted repeats of the Idefix retrotransposon act as an insulator (Conte et al., 2002). Transposition of ZAM into the promoter of the white gene, which is responsible for eye color, was found to enhance eye color. Transposition of Idefix, into the promoter of the white gene was found to inactivate the promoter of the white gene, which resulted in a decrease in eye color intensity (Conte et al., 2002). Similarly, Ferrigno et al. (2001) found an intact SINE element which carried an active RNA polymerase II promoter within its TIRs, and was responsible for upregulation of LAM3 mouse gene, which

encodes an isoform of the laminin α 3 chain. Thus insertion of transposable elements into any genomic sequence can result in serious alterations in gene expression.

1.7 Untranslated regions as regulatory elements.

Gene expression is generally regulated at the level of transcription via promoters and enhancers and at the post-transcriptional level via transport regulation, translation and transcript stability (Pesole et al., 2001). The UTRs of a gene are important at both levels of regulation, as they often contain elements which can promote, enhance, inhibit or stabilize transcription and translation (Grzybowska et al., 2001). As previously noted, the hDREF transposon contains both 5' and 3'UTRs which contain a putative promoter sequence, the MEF2 consensus binding sequence, E-Box binding site, and the polyadenylation sequence. The presence of these putative regulatory elements in the UTRs of hDREF suggests that the untranslated regions may be responsible for directing the expression of this gene.

1.8 DREF.

As previously stated hDREF (TRAMP) bears amino acid similarity with the *drosophila* protein DREF (DNA Replication Related Element Factor). The *drosophila* protein DREF is a transcription factor that has been shown to be a key regulator of several *drosophila* genes that are involved in DNA synthesis (Hirose et al., 2001). The 86-kDa protein binds as a homodimer to a specific DNA sequence named DRE (DNA

Replication Related Element) (Hirose et al., 1996). DREs are present in the promoter regions of *drosophila* genes such as the DNA polymerase α subunit, E2F, PCNA, cyclin A, thymidine kinase, and the TATA binding protein and has been shown to upregulate the expression of these factors (Choi et al., 2000; Hirose et al., 1996; Hirose et al., 1999; Ohno et al., 1996). Deletional analysis of the DREF protein indicated that it is the N-terminal region (amino acids 16-115) that is essential for both the binding of DREF to the DRE sequence and for homodimer formation (Hirose et al., 1999). Notably it is in this N-terminal region that hDREF shares sequence homology with DREF.

Hirose et al., (1999) examined DREF expression *in vivo* by establishing transgenic flies expressing the N-terminal fragment of DREF under the control of the heat shock promoter, the salivary gland specific promoter, or the eye imaginal disc specific promoter. Overexpression of the N-terminal region of DREF competed with endogenous DREF for the DRE binding site, thereby acting in a dominant negative manner. Heat shock induction of the N-terminal fragment during embryonic, larval, or pupal stages of development caused greater than 50% lethality. This lethality was overcome by co-expression of the full length DREF. Ectopic expression of the N-terminal fragment in the eye imaginal discs reduced the mRNA content of both the 180 kDa subunit of DNA polymerase α and E2F, reduced the extent of DNA replication and endoreduplication (Hirose et al., 1999). Salivary gland cells expressing the N-terminal portion of DREF were visibly smaller than controls and the extent of endoreduplication was significantly reduced (Hirose et al., 1999). Conversely, overexpression of DREF in the eye imaginal

disc of *drosophila* resulted in an increase in apoptosis in cells destined to differentiate. In addition DREF overexpression inhibited the differentiation of photo-receptor cells and induced DNA synthesis in post mitotic cells leading to the rough eye phenotype (Hirose et al., 2001). These results suggest that the DREF-DRE interaction may regulate a large number of genes, many of which are directly or indirectly involved in DNA replication.

1.9 Endoreduplication and cell cycle.

Endoreduplication is a naturally occurring disruption of the mitotic cycle, whereby cells undergo successive rounds of DNA synthesis in the absence of cell division.

Endoreduplication occurs in a wide variety of embryonic and adult cell types in both animal and plants including cardiomyocytes, hepatocytes, megakaryocytes, and trophoblasts (MacAuley et al., 1998). As previously noted, expression of N-terminal DREF in *drosophila* results in a decrease in DNA synthesis and a decrease in the extent of endoreduplication. These results indicate that the expression of DREF is not only required for normal mitotic cell cycle progression, but is also required for the endocycle.

Limited research has been conducted on the molecular mechanisms that regulate the endocycle in mammalian cells. MacAuley et al., (1998) and Hattori et al., (2000) examined endocycle control in mouse giant trophoblasts. They found that the initiation of S phase during endocycles appeared to involve cycles of cyclin E and A synthesis, and that the termination of S phase was associated with an abrupt loss of both of these

cyclins. The cyclin dependent kinase (cdk2) which associates with cyclin E and A was also found to be absent from G phase cells, suggesting that their degradation may be necessary to allow reinitiating of the endocycle and DNA synthesis (MacAuley et al., 1998). By analogy to the mitotic cell cycle, cyclin degradation is presumably necessary to allow the reactivation of the origins of replication (MacAuley et al., 1998). The arrest of the mitotic cycle at the onset of endoreduplication was also associated with a failure to assemble cyclin B/p34cdk1 complexes during the first endocycle, and in subsequent endocycles cyclin B expression was suppressed (MacAuley et al., 1998). As cyclin B failed to activate Cdk1, mitosis was not initiated (Hattori et al., 2000). It was also found that the p57^{Kip2} inhibitor of cyclin A and E associates with Cdks at the end of S phase and is degraded before re-entry into S phase (Hattori et al., 2000) (Figure 3). As previously noted, the *drosophila* transcription factor DREF is responsible for the expression of cyclin A as well as many other key regulators of the cell cycle. It is through the transcriptional control of these regulators that hDREF maintains its control on both the mitotic and endocycle.

1.10 Experimental Rational.

The novel gene hDREF represents an ancient human transposable element that encodes a protein, and bears sequence similarity to the Ac-family of transposons (Esposito et al., 1999). Moreover hDREF bears amino acid similarity to the *drosophila* transcription factor DREF, which has been shown to regulate both the mitotic cell cycle and endocycle. In addition to this, hDREF contains three MEF2 binding sites and one E-box

binding site within the 3' and 5' UTRs of the hDREF sequence. Based on these findings it was proposed that the MEF2 family of transcription factors negatively regulate hDREF expression, and that the novel human version of the *drosophila* DREF protein acts in a similar manner to its *drosophila* ortholog in regulating DNA synthesis.

Figure 3: **A)** Simplified diagram of cell cycle. During G1, cyclins E and D are active. Transition to S-phase is accompanied by increased cyclin E and A, which are degraded at the end of this phase. The onset of the G2 phase marks the beginnings of the CDK1 / cyclin B complex formation, which is prevented during the initiation of the endocycle. The failure of CDK1 to complex with cyclin B in the first and subsequent endocycles prevents mitosis and results in polyploidy cells.

B) The DRE / DREF interaction regulates the expression of multiple genes involved in DNA synthesis, cell cycle and endocycle progression.

A)

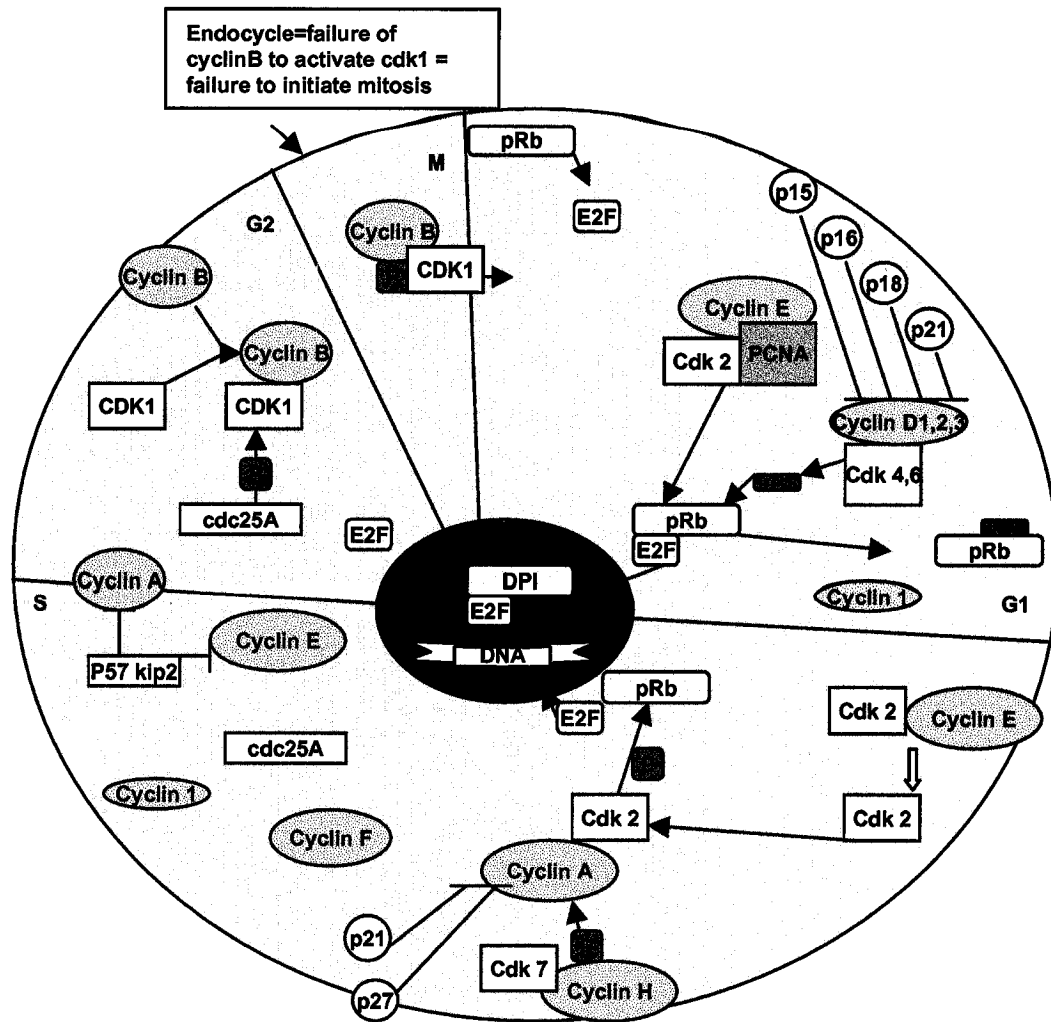


Diagram illustrating the components involved in DNA replication. A central black box represents the DNA replication machinery. Arrows point from this box to several components: **raf**, **Cyclin A**, **PCNA**, **E2F**, **TBP**, and **180 kDa, 73 kDa DNA polymerase α**. A box labeled **Zen** is connected to the central box by a line with a perpendicular bar, indicating inhibition.

1.11 Hypotheses and Objectives.

Based on the experimental results obtained from this body of work my prevailing hypothesis is that:

- 1) The MEF2 family of transcription factors act as negative regulators of hDREF expression.
- 2) The function of the hDREF protein is to ensure DNA synthesis within the mitotic cell cycle and endocycle.

To address the question of hDREF regulation I propose to study A) the expression of hDREF *in vitro* B) the function of the MEF2 binding sites in the UTRs of hDREF using the ChIP (chromatin immunoprecipitation) assay and reporter constructs.

To address the cellular role of hDREF I propose to study; A) the phenomenon of overexpressing hDREF in different cell types; B) growth rates and DNA synthesis of the hDREF expressing cell lines.

Chapter 2

2.0 Materials and Methods

2.1 Preparation of total RNA from cells and tissues.

RNA was collected from C2C12 (mouse myoblasts), H9C2 (rat cardiomyocytes) and human skeletal (SK) muscle cells. Cells were plated at a density of approximately 6.0×10^5 cells per 10cm dish, and cultured in growth media (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Gibco) + 10% Fetal Bovine Serum + 1 % penicillin / streptomycin) for 2 days, or in differentiation media (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 + 2 % Horse Serum + 1 % penicillin / streptomycin) for 5 days. RNA was isolated using RNeasy (Qiagen) isolation kit and treated with DNase I (124.5 U/ul) (Gibco) to eliminate any possible DNA contamination. The samples were then quantified using a spectrophotometer (Beckman) and 260/280 absorbance ratios.

RNA was also collected from the hearts of 3-month old wild type, MEF2C DN and mdx / MyoD (-/-) mice for RT / PCR analysis. In addition RNA from skeletal muscle, brain, liver, and kidney were harvested from the 3-month-old wild type mice. RNA for Northern blot analyses was collected from the hearts of wild type mice aged 0-12 weeks. Harvested tissues were placed in Trizol Reagent (Gibco) on ice, homogenized and RNA extracted according to manufacturer's instructions. RNA was then resuspended in Dep-C water, treated with DNase I (124.5 U/ul) (Gibco) and quantified using the spectrophotometer and 260/280 absorbance ratios.

RNA samples used for Northern blots were resolved on 1 % agarose / 1 x MOPS formaldehyde gel according to standard protocol. Formaldehyde gels were then transferred using the capillary transfer methods to the Hybond-N+ nylon membrane (Amersham Pharmacia Biotech). Membranes were hybridized according to the Hybond-N+ nylon membrane manual.

2.2 Generation of radiolabelled probes used in Northern blot analyses and ChIP assay.

2.2.1 hDREF ORF probe (Northern blots)

The 2.0 Kb ORF of hDREF was cloned into the pcDNA3.1 HIS-myc vector (Invitrogen) (see section 2.6.1). Subsequently, the vector was digested with the EcoRV and Hind III enzymes (Gibco) to liberate the hDREF ORF from the plasmid. The digested probe was electrophoresed on a 0.8% agarose gel and the 2.0 Kb hDREF ORF was excised and gel purified using the Ultra Clean 15 DNA purification kit (MO BIO laboratories Inc.). The probe DNA was then quantified using the spectrophotometer and 260/280 absorbance ratios. The hDREF ORF probe (50 ng) was then radiolabelled with [α - 32 P] dCTP using the Rediprime II random prime labelling system (Amersham Pharmacia Biotech), and the incorporation checked using the Beckman scintillation counter. Membranes were hybridized according to the Hybond-N+ nylon membrane manual and exposed to X-ray film (BioMax film Kodak) for 1-2 weeks.

2.2.2 GAPDH probe (Northern blots).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a catalytic enzyme involved in glycolysis and is constitutively expressed at high levels in almost all tissues and cell types (Ercolani et al., 1988). Due to this ubiquitous expression pattern, GAPDH was used as a loading control in all Northern blots and RT / PCR assays. The GAPDH probe was generated using RT / PCR from total RNA extracted from mouse skeletal muscle. For a complete description of RT / PCR conditions used to generate the GAPDH probe see section 2.3. The 600bp GAPDH PCR product (50 ng) was then radiolabelled with [α -³²P] dCTP using the Rediprime II random prime labeling system (Amersham Pharmacia Biotech), and the incorporation checked using the Beckman scintillation counter (LS6500). Northern blots, which had been previously probed with the hDREF ORF were then reprobed with the GAPDH probe using similar hybridization conditions and exposed to X-ray film (BioMax film Kodak) 1 week.

2.2.3 The hDREF 5' UTR MEF2-specific binding site probe (ChIP assay).

The MEF2 specific binding site found within the 5'UTR of hDREF, along with approximately 100 base pairs of flanking hDREF sequence were amplified by PCR. The primers used to amplify this specific 110 bp region were as follows: *ChIP forward* (5'-3': TTCGAGACCAGCCTGACCAACATGG) and *ChIP reverse* (5'-3':GATCTTC CTGCCTC AGCTTCCTCAG). The 1.2 Kb 5'UTR of hDREF, which had been cloned into pGLOW vector (Invitrogen) (see section 2.6.2 & 2.6.3), was used as the template for generation of the probe. Conditions for amplifications of the MEF2 binding site found in 5'UTR of hDREF were 95°C, 2 min, followed by 25 cycles of 95°C, 1 min, 53°C, 1 min,

72°C, 3 min, and a final elongation of 10 min at 72°C using the (eppendorf Mastercycler personal VWR) PCR machine. The resulting PCR product was then resolved on a 2.0% agarose / 1XTAEgel, purified, quantified and commercially sequenced. The 110bp ChIP assay probe was then radiolabelled with [α - 32 P] dCTP using the Rediprime II random prime labeling system (Amersham Pharmacia Biotech) and the incorporation checked using the Beckman scintillation counter.

2.2.4 Myogenin promoter probe (ChIP assay).

The myogenin promoter (Yee and Rigby, 1993) was used as a positive control in the ChIP assay and was obtained as a gift from Dr. R. Kothary in the pSP72vector. EcoRI and XbaI enzymes were used to liberate the 1.1Kb myogenin promoter, which was resolved on a 1% agarose / 1XTAE gel, gel purified and quantified. The 1.1 Kb myogenin promoter was then radiolabelled with [α - 32 P] dCTP using the Rediprime II random prime labeling system (Amersham Pharmacia Biotech) and the incorporation checked using the Beckman scintillation counter.

2.3 Reverse transcriptase and polymerase chain reaction conditions.

Total RNA was collected from the hearts of 3 month old wild type, MEF2C, and mdx / MyoD (-/-) mice and from the kidneys, livers skeletal muscle and brains of 3-month-old wild type wild type mice. Total RNA (5 μ g) was reverse transcribed into cDNA in buffer containing 1 μ l Oligo (dT)₁₅ (500 μ g / ml, Invitrogen), 1 μ l of 10mM dNTP mix (10 mM

each dATP, dGTP, dCTP and dTTP), 1x First-Strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂), 2 µl DTT (0.1 M, Invitrogen), RNaseOUT Recombinant Ribonuclease Inhibitor (40U/ul, Invitrogen), and SUPERScript II RNase H- Reverse Transcriptase (200U/ul, Invitrogen). The total reaction volume of the reverse transcriptase reaction was 20 µl. A (-) RT reaction was also performed to assess the level of DNA contamination.

Primers were designed to amplify a 200 bp region within the open reading frame of the hDREF sequence. Their sequence is as follows: *hDREF* 5' forward (5'-3':GATATCATGGAGAATAAAAGCCTGGAG) and 5' *hDREF* reverse (5'-3':CTTCCACTGCAGGATGCATCCCTC). PCR amplification was carried out using 10ul of reverse transcribed cDNA. PCR conditions for the amplification of the 200 bp hDREF ORF were as follows: 95°C, 2 min, followed by 25 cycles of 95°C, 1 min, 53°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. A NO RT / PCR reaction was carried out as a control for DNA contamination. A loading control PCR reaction was also carried out using primers specific for GAPDH. Their sequence is as follows: *GAPDH* forward (5'-3':TGACTCCACTCACGCCAAATTCAA) and *GAPDH* reverse (5'-3':TGCCTGCTTCACCACCTTCTTGAT). Using the (ependrof Mastercycler personal) PCR machine, 10ul of reverse transcribed cDNA template was amplified. Conditions for all GAPDH amplification reactions were as follows: 95°C, 1 min, followed by 25 cycles of 95°C, 1 min, 52°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. Samples were analyzed on 1-2 % agarose / 1xTAE gels with ethidium bromide with and visualized under UV light.

2.4 Southern blot analyses.

In order to ensure the specificity of the 5'UTR MEF2 binding site probe, Southern blot analysis was performed using total genomic DNA. Total human genomic DNA was extracted from 50 ml of donated human blood using the DNA Blood Mini Kit (Qiagen). 10 ug of total human genomic DNA was digested with EcoRV enzyme (Gibco) for 3hr at 37 °C. DNA was run on a 1% agarose/1xTAE gel containing ethidium bromide and then processed according to Southern blotting directions found in the instruction for Hybond-N+ nylon membrane. The membrane was then hybridized with the radiolabelled hDREF MEF2 binding site probe according to the Hybond-N+ nylon membrane manual, and exposed to X-ray film (BioMax film Kodak) for 1 week.

2.5 Chromatin Immunoprecipitation (ChIP) assay.

The chromatin immunoprecipitation assay was used to show a physical interaction between the MEF2 proteins and the binding site found in the 5'UTR of hDREF. The human skeletal muscle cell line SK (Clonetics BioWhittaker) was plated on 10cm dishes at a density of 3.0-6.0 X10⁵ cells / plate and cultured in differentiation media (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 + 2 % Horse Serum + 1 % penicillin / streptomycin) or growth media (SK Bullet Media Clonetics BioWhittaker) for 5 days. Proteins were cross-linked to DNA by adding formaldehyde directly to the media, followed by a 10-minute incubation at 37°C. The media was then removed and cells were washed with ice-cold PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1mg/ml aprotinin and 1mg/ml pepstatin A). Cells were scraped off the

plate and pelleted for 4 minutes at 2000 rpm at 4°C. Cells were resuspended in 200 ul SDS Lysis Buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors 1mM PMSF, 1mg/ml aprotinin and 1mg/ml pepstatin A) for 10 min. The DNA samples were then sonicated using the vibra cell sonicator (Sonic and Materials Inc.) on ice for 10 sec 4X at a sonicator setting of 35 % amplitude. The sonicated DNA was then pelleted for 10 minutes at 13,000 rpm at 4°C, and the debris diguarded. The remaining supernatant was diluted 10 fold in ChIP Dilution Buffer (0.01%SDS, 1.1%Triton X, 1.2mM EDTA, 16.7mM Tris, pH 8.1, 167mM NaCl and protease inhibitors). Approximately 200 ul of the DNA solution was removed from the sample and kept at -20 °C to be used as a positive control.

To reduce nonspecific background, the sample was pre-cleared with 80 ul of salmon sperm DNA / protein A slurry (50% protein A sepharose slurry containing 20 ug sonicated salmon sperm DNA and 1 mg/ml BSA in TE) for 30 minutes at 4°C with agitation. Beads were then pelleted and the supernatant fraction removed. The supernatant was then equally divided into separate microfuge tubes. One tube was treated as the NO ANTIBODY control and the other as the experimental sample. 20ul of MEF2 C-21 antibody (Santa Cruz) or MEF2C antibody (Cell Signaling Technology) was then added to the experimental sample, which was incubated overnight at 4°C with rotation. To collect the protein-DNA complexes, 60 ul of a 50% protein A slurry (20 ug of salmon sperm DNA in 1mg/ml BSA in TE) was added to the sample which was then incubated for 1 hr at 4°C with rotation. The beads were then pelleted by gentle

centrifugation (700 to 1000 rpm at 4°C, ~1min) and the supernatant was removed. The pellet, which contained the protein A beads/antibody/MEF2/DNA complex, was then washed for 3-5 minutes on a rotating platform with 1ml of each of the buffers listed in the order as given below:

- a) Low Salt Immune Complex Wash Buffer, one wash (0.1%SDS, 1%Triton X, 2mM EDTA, 2mM Tris-HCl pH 8.1, 150 mM NaCl)
- b) High Salt Immune Complex Wash Buffer, one wash (0.1%SDS, 1%Triton X, 2mM EDTA, 2mM Tris-HCl pH 8.1, 500 mM NaCl)
- c) LiCl Immune Complex Wash Buffer, one wash (0.25M LiCl, 1%NP40, 1%sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1)
- d) 1X TE, two washes

To liberate the cross-linked DNA from bound proteins, the pellet was resuspended in 20 μ l of 5M NaCl and incubated at 65 °C for 4 hr. DNA was recovered by phenol/chloroform extraction and quantified. The recovered DNA was then slot blotted onto GeneScreenTM membrane (NEN Life Science Products) using a slot blot apparatus (BioRad) and processed according to the GeneScreenTM membrane manual. The blots were then hybridized with the radiolabelled hDREF MEF2 binding site probe and exposed to X-ray film (BioMax film Kodak) for 2-5 days.

After exposure to film one of the ChIP slot blots (blot B Figure 10 III) was allowed to decay for 2 month, and was then reprobbed with the radiolabelled myogenin promoter probe (see generation of myogenin promoter probe). The myogenin promoter was used as a positive control in the ChIP assay as it also contains a MEF2 specific binding site that binds MEF2C proteins during differentiation (Ridgeway et al., 2000).

2.6 Generation of hDREF expression vectors.

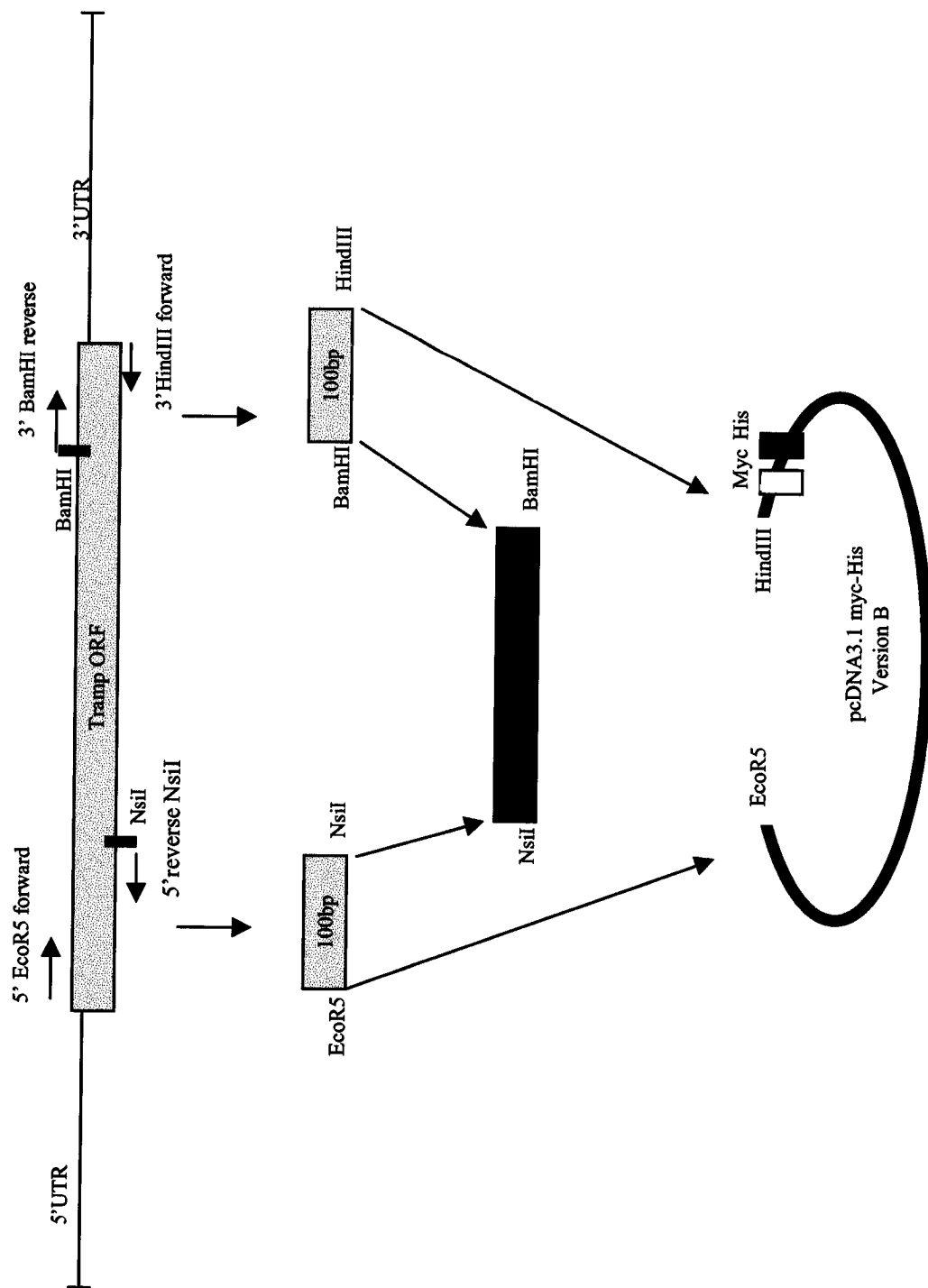
2.6.1 Generation the hDREF ORF /pcDNA3.1 myc-HIS vector.

The hDREF cDNA (clone KIAA0785) was obtained from the Kazuza Institute (Japan) in the pBluescript vector. To generate a construct, which would contain the hDREF open reading frame alone, the following series of cloning reactions were carried out (see Figure 4). The 5' end of the hDREF ORF was amplified using primers which had additional restriction enzyme sites added to their termini. The *forward 5' hDREF ORF* primer had a EcoRV site added for cloning accessibility onto it and read as follows: (5'-3':GATATCATGGAGAATA AAAGCCTGGAG). The *reverse 5' hDREF ORF primer* had a NsiI site added and read as follows: (5'-3':GAGGGATGCATCCTGCAG TGGAAG). The PCR conditions used to amplify the 120 bp 5' fragment of the hDREF ORF are as follows: 95°C, 2 min, followed by 25 cycles of 95°C, 1 min, 53°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. The PCR product was then gel purified and commercially sequenced. Upon confirmation of the correct nucleotide sequence, the 120 bp 5' hDREF ORF fragment was digested with EcoRV and NsiI enzymes (Gibco), and purified.

The 3' end of the hDREF ORF was amplified using primers that had additional restriction enzyme sites added to their termini. The *forward 3' hDREF ORF* primer had a BamHI site added and read as follows: (5'-3':TCTCTTCGGATCCGCCGCCAACGT). The *reverse 3' hDREF ORF* primer had a HindIII site added and read as follows: (5'-3':GCATTAGGACAGCAGCTTCCTGAAGCTTGGG). The PCR conditions used to amplify the 210 bp 3' fragment of the hDREF ORF are as follows: 95°C, 2 min, followed by 25 cycles of 95°C, 1 min, 63°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. The PCR product was then purified and the sequence verified. Upon confirmation of the correct nucleotide sequence, the 210 bp 3' hDREF ORF fragment was digested with BamHI and HindIII enzymes (Gibco), and purified.

Following generation of the 120 bp EcoRV – NsiI fragment and the 210 bp BamHI – Hind III fragment the hDREF / pbluescript construct was digested with NsiI and BamHI enzymes (Gibco), liberating a 1.8 Kb fragment of the hDREF ORF. The 120 bp EcoRV - NsiI fragment and the 1.8 Kb NsiI / BamHI ORF fragment and the 210 bp BamHI - HindIII fragment were ligated together in a directed ligation into the EcoRV - HindIII digested pcDNA3.1 HIS-myc vector (Invitrogen) (Figure 4). Following the generation of the hDREF ORF / pcDNA HIS-myc vector this construct was used for cell transfection experiments.

Figure 4: The cloning of hDREF ORF into pcDNA3.1 vector. The hDREF cDNA (clone KIAA0785) was obtained from the Kazuza Institute in the pBluescript vector. To generate a construct which would contain the hDREF open reading frame alone, the following series of cloning reactions were carried out: The 5' end of the hDREF ORF was amplified using a primer set that had a EcoRV site and a NsiI site added. The 3' end of the hDREF ORF was amplified using a primer set that had a BamHI site and HindIII site added. The PCR products were then purified and the sequencing verified. The 120 bp 5'hDREF ORF fragment was digested with EcoRV and NsiI and the 210 bp 3' hDREF ORF fragment was digested BamHI and HindIII enzymes. Following generation of the 120 bp EcoRV – NsiI fragment and the 210 bp BamHI – Hind III fragment the hDREF / pbluescript construct was digested with NsiI and BamHI enzymes (Gibco), liberating a 1.8 Kb fragment of the hDREF ORF. The 120 bp EcoRV - NsiI fragment and the 1.8 Kb NsiI / BamHI ORF fragment and the 210 bp BamHI - HindIII fragment were directionally ligated to the EcoRV – HindIII digested pcDNA3.1 HIS-myc vector (Invitrogen).



2.6.2 Generation of the 5'UTR/pGLOW vector.

The original cDNA hDREF/ pBluescript clone lacked approximately 1.0 Kb of sequence on the 5' end. This missing region included the direct repeats, the terminal inverted repeats, the E- box binding site and the MEF2 binding site. However, the nucleotide sequence of the missing 5' terminus was available through PUBMED NCBI (Esposito et al., 1999). Using the published sequences specific primers were designed to amplify the entire 5'UTR, including the missing region.

Total Human genomic DNA was extracted from 50 ml of donated human blood using the DNA Blood Mini Kit (Qiagen) and used as the template for the PCR reaction. Specific primers were designed to amplify the entire 1.2 Kb region of the 5'UTR. The *forward 5' UTR* primer had a Nhe I site added and read as follows: (5'-3':CTAGCTAGCCTGTACTGGACACTT. The *reverse 5' UTR* primer had an XhoI site added onto it and read as follows :(5'-3':CCGCTCGAGTGG CTTCTCCACCGGAG CC). The following conditions were used to generate the 1.2 kB 5'UTR fragment: 1 min at 95°C, followed by 25 cycles of 95°C, 1 min, 52°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. PCR products were run out on a 0.7% agarose/1XTAE gel; the gel band was excised and purified, followed by sequencing verification. The newly generated 5'UTR was then cloned into the pGLOW vector (Invitrogen) using T / A ligation according to TOPO pGLOW vector manual (Invitrogen). Clones were then screened and sequenced and one positive clone was chosen to inoculate a large-scale

culture whose plasmid DNA was extracted using the Midi extraction kit (Qiagen).

2.6.3 Generation of the 3'UTR/pGLOW vector.

The 3'UTR of hDREF was generated using the original cDNA hDREF / pBluescript construct obtained from the Kazuza Institute in Japan. Specific primers were designed to amplify the entire 2.3 Kb region of the 3'UTR. The *forward 3' UTR* primer had a BamHI site added and read as follows : (5'-3':CGCGGATCCCAGAGGCGGAACCC). The *reverse 3' UTR* primer had a HindIII site added onto it and read as follows:(5'-3:CCCAAGCTTTTATTGAACAACA). The following conditions were used to generate the 2.3 Kb 3'UTR fragment: 1 min at 95°C, followed by 25 cycles of 95°C, 1 min, 51°C, 1 min, 68°C, 3 min, and a final elongation of 10 min at 68°C. PCR products were purified and the sequence verified. The newly generated 3'UTR was cloned into the pGLOW vector (Invitrogen) using T / A ligation according to the Invitrogen manual. Selected clones were screened and sequenced, and one positive clone was chosen to inoculate a large-scale culture for plasmid DNA extraction (Midi extraction kit Qiagen).

2.6.4 Generation of the CMV/pGLOW vector.

The CMV promoter was PCR amplified using 500ng of the pCDNA3.1 (Invitrogen) vector as a template, and specific primers designed to amplify the entire 654 bp of the CMV promoter. The sequence of those primers are as follows: *CMV forward* (5'-3':CGATGTACGGGCCAGATATACGCGT) and *CMV reverse* (5'-3:TCCCTATA

GTGAGTCGTATTAATTT). The conditions of the PCR are as follows; 1 min at 95°C, followed by 25 cycles of 95°C, 1 min, 63°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. PCR products were purified as above. The newly generated CMV promoter was then sub-cloned into the pGLOW vector (Invitrogen) using T / A ligation according to the Invitrogen manual. Clones were screened and sequenced, and one positive clone was chosen to inoculate a large scale culture for plasmid DNA extraction (Midi extraction kit Qiagen).

2.7 Mutagenesis of GFP reporter constructs.

The MEF2 binding site found in the 5'UTR / pGLOW construct was mutated using the Quick Change Site Directed Mutagenesis kit (Qiagen) to produce a reporter construct which contained the 5'UTR but lacked a functional MEF2 binding site. Similarly, the proximal MEF2 binding site found in the 3'UTR of hDREF, was mutated using the Quick Change Site Directed Mutagenesis kit (Qiagen) to produce a construct which contained the 3'UTR with one mutated and one functional MEF2 binding site. Mutations in the 5'UTR / pGLOW and 3'UTR / pGLOW constructs were confirmed through sequence analysis and the constructs were then transiently transfected into C2C12 cells (see section 2.8 for transfection protocol). The CMV / pGLOW construct was also transiently transfected into C2C12 cells as a positive control. The CMV / pGLOW transfected cells were also cultured under both growth and differentiation conditions. Cells which had been transiently transfected with the pGLOW constructs were cultured in

growth or differentiation media for 2 days, fixed with 4% paraformaldehyde, washed 3X in PBS and visualized using a Zeiss fluorescent microscope on the FITC channel with filters which emitted light at an excitation wavelength of 490λ.

2.8 Transient and stable transfections of hDREF expression vectors into C2C12 and H9C2 cells.

Transfections were carried out using the lipopectamine reagent and protocols (Invitrogen). Stable clones were generated by adding the appropriate antibiotic (neomycin) at a dose of 800 ug/ml of media. Antibiotic selection was carried out for 2 weeks before resistant colonies began to form in the selective media. Individual colonies were picked using an inverted light microscope (Zeiss) that was placed into a sterile flow hood (Class II A/B3 Biological Safety Cabinets). Selected colonies were then cultured, and total protein was collected using PLC gamma buffer: 0.05M HEPES pH 7.5, 0.15 M NaCl, 10 ml of 100% Glycerol, 1 ml of 100% Triton X-100, 0.01 M MgCl₂, 0.02 M NaF, 0.01 M Na Pyrophosphate, dH₂O to 100 ml. Protease inhibitors [0.01M of PMSF, Aprotinin, Pepstatin, Leupeptin NaV] were added prior to use.

2.9 Western blot analysis.

To detect the HIS-myc tagged hDREF fusion protein Western blot analyses were conducted. Total protein was measured using the BSA protein assay (Pierce) and then

run on a 10% acrylamide (Diamed) gel. Proteins were transferred to a nylon membrane (HybondN+ Amersham Pharmacia Biotech) using a semi-dry transfer apparatus (BioRad). Membranes were blocked for 1hr in 0.1x TBST / 5 % skim milk, then incubated for 1hr with the myc primary antibody (Initrogen), which was diluted to a concentration of 1:5000 ratio in 0.1 x TBST / 5% skim milk. Membranes were washed 3 X for 5min in TBST, and then incubated for 1 hr with the secondary antibody (goat anti-mouse conjugated to horse radish peroxidase -BioRad) which was diluted to a concentration of 1:2000 in 0.1 x TBST / 5 % skim milk. After several washes in TBST, membranes were incubated with chemiluminescence reagents (Amersham Pharmacia Biotech) according to manufactures instructions, exposed to Kodak X-ray films (Kodak) and developed.

To ensure equal loading of protein samples, all Western blots were re-probed with the tubulin antibody (Developmental Studies Hybridoma Bank). Membranes were blocked for 1hr in 0.1 x TBST / 5 % skim milk, then incubated for 1hr with the primary antibody, which was diluted to a final concentration of 1:10 ratio in 0.1x TBST / 5% skim milk. Membranes were washed 3 X for 5min in TBST, and then incubated for 1 hr with the secondary antibody (goat anti mouse conjugated to horse radish peroxidase -BioRad) which was diluted to a concentration of 1:2000 in 0.1 x TBST / 5 % skim milk. After several washes in TBST, membranes were incubated with chemiluminescence reagents (Amersham Pharmacia Biotech) according to manufactures instructions, exposed to Kodak X-ray films (Kodak) and developed.

2.10 Immunocytochemistry.

2.10.1 Myc/ DAB and myosin heavy chain staining of cells.

Myc staining was used to determine the percentage of cells in the stable hDREF clones that expressed the hDREF protein. The myosin heavy chain staining was used to determine the degree of cell differentiation. Both of these staining reactions followed the same basic staining protocol. Cells were fixed in 90% methanol for 5 minutes, rinsed three times with cold PBS and blocked for 1hr in 5% skim milk in PBS. The blocking solution was then removed and replaced with the primary antibody (myosin heavy chain- Developmental Studies Hybridoma Bank or myc antibody - Invitrogen) diluted to a concentration of 1:10 in 5% skim milk / PBS. Cells were incubated for 1 hr in primary antibody then washed three times in PBS. Cells were then incubated for 1 hr in the secondary antibody (goat anti mouse antibody conjugated to horseradish peroxidase - BioRad) which was diluted to a concentration of 1:2000 dilution in 5% skim milk / PBS. After removal of the secondary antibody, cells were washed 3 X with PBS then incubated in the 3,3'-Diaminobenzidine / DAB solution, which consists of the following: (0.05 mg/ml DAB powder, 50mM Tris-HCL pH 7.6, 0.03% hydrogen peroxide). The staining was carried out for 20-30 min in low light conditions. Following the visible color development of the DAB, cell nuclei were stained with Harris Modified Hematoxylin (Fischer) by addition of 2ml of hematoxylin concentrate to the cells. Cells were then washed 5 X with PBS and were photographed under a Zeiss inverted light microscope.

2.10.2 Myosin heavy chain, myogenin and MEF2C – FITC staining of cells.

To assess cell differentiation in the hDREF / C2C12 cell line, cells were cultured in differentiation media for 5 days and stained for the presence of myosin heavy chain, myogenin, and MEF2C, all of which are markers of cell differentiation. Cells were fixed in 4% paraformaldehyde (Fisher) in PBS for 10 minutes; wash 3-4 X with PBS, and permeabilized for 5 minutes with 0.3% TritonX (Fisher) in PBS. Cells were then washed 3-4 X with PBS, and blocked with 4% BSA in PBS with 0.3% TritonX for 1hr. The primary antibodies myosin heavy chain (MF20) (Developmental Studies Hybridoma Bank), myogenin (Developmental Studies Hybridoma Bank) or MEF2C (Cell Signaling Technology) were diluted to a concentration of 1:25 in 0.1% BSA in PBS and incubated with the cells at room temperature for 1hr. The cells were then washed 3-4 X times with PBS and incubated in the secondary antibody sheep anti-mouse conjugated to FITC (Stressgen) at a dilution of 1:200 in 0.1% BSA in PBS, at room temperature for 45 min to 1hr. Cells were then washed 3-4 X with PBS and 1ul DAPI (4,6 Diamidino- 2-phenylindole) was added to the cells along with 10 ml of PBS. Cells were then visualized and photographed under a Zeiss fluorescent microscope using the DAPI and FITC filters which emit light at an excitation wavelength of 345 and 490 respectively

2.10.3 DAPI staining.

Cells that had been previously fixed and stained for myosin heavy chain and Harris Modified hematoxylin were also fluorescently stained with 4,6 Diamidino- 2-phenylindole (DAPI) (Sigma) (Figure 22). Approximately 1 ul of DAPI and 5ml of 1 X

PBS was added to each plate of cells. Cells were then visualized and photographed under a Zeiss fluorescent microscope using the DAPI filter which emit light at an excitation wavelength of 345.

2.11 Characterization of myotube fusion and multinucleated cells.

A fusion index was calculated as the number of nuclei in myotubes divided by the number of total nuclei. A myotube was defined as an elongated multinucleated fused cell that expressed myosin heavy chain. To calculate the fusion index, cells were stained with myosin heavy chain (MF20) and hematoxylin (see section 2.10.2) then visualized using a Zeiss inverted microscope. Each calculated fusion index was the average of 3 different cell count experiments. Cells were determined to be abnormal bag like myotubes if they met the following criteria: 1) had multiple nuclei (>2 nuclei) 2) maintained a rounded structure and did not elongate to a standard myotube structure 3) and were positive for myosin heavy chain expression.

2.12 Cell Counts.

To examine the effects of hDREF overexpression on the cell cycle, cell counts were performed. Cells were plated at a density of 2.0×10^5 cells / plate and cultured in growth media for 5 days or were plated at a density of 6.0×10^5 cells / plate and cultured in differentiation media for 5 days. Each day 3 plates from the growth treatment and three plates from the differentiation treatment were collected and counted using a

hemacytometer (Hausser Scientific). Each plate of cells were counted in 4 different fields of the hemacytometer. Cells that were floating in the media were counted and designated as dead cells. Adherent cells were trypsinized, counted and considered as the viable cell population. The numerical results from each day were averaged and the death and growth rates calculated.

2.13 Fluorescent Activated Cell Sorting (FACS) analyses.

FACS analyses was carried out on cells that had been cultured in growth or differentiation media, to ensure that cells that had been counted as living were in fact viable, and cells that had been counted as dead were non viable. After 5 days in growth or differentiation media, adherent cells and cells floating in suspension were collected separately and stained with Annexin V-FITC and propidium iodide (PI) as described in the annexin V-FITC Apoptosis Detection Kit (Pharmingen). All samples were analyzed using the Beckman Coulter-Ultra flow cytometer. The flow cytometer used an argon laser with an excitation wavelength of 488 nm and a FITC and PI detection wavelength of 525 nm and 610 nm respectively. Distributions of viable apoptotic and necrotic cells were graphed and visualized using Expo 32 Cytometer Software, Version 1 (Applied Cytometry Systems). The same cytosettings were maintained throughout the entire experimental time-course.

2.14 Multinucleated single cells.

Cells were plated at a low density (approximately 10 cells / 10 cm dish) or at a density of one cell / well, through serial dilutions. Cells were then visually tracked using the inverted Zeiss light microscope over a 5-day time course while incubated in low serum media. At the end of the 5-day time period the cells were fixed in 90% methanol for 5 minutes and then rinsed three times with cold PBS. Nuclei were stained with Harris Modified Hematoxylin (Fischer) washed several times with PBS and then photographed under a Zeiss inverted light microscopy.

2.15 BrdU (5-Bromo-2'-deoxyuridine) incorporation assays.

In order to determine if the hDREF over-expressing cell lines were capable of continued DNA incorporation when cultured in differentiation media BrdU incorporations assays were carried out. Cells were cultured for 3 days in differentiation media, and on the third day the media was replaced with 10 ml of fresh differentiation media containing 75ug of BrdU. Cells were cultured for an additional 2 days, then fixed in 70% ethanol for 10 minutes and then rinsed three times with cold 1 X PBS. 5ml of 2N HCl was added to the plates, which were then incubated for 20 min at 37 °C. The HCl was removed and 0.1M Tris pH 8.4 was added for 10 min. The cells were then washed with 1X PBS+0.1% TWEEN followed by a 1 hr incubation in 5 % skim milk / PBS and then another 1hr incubation in anti-BrdU (Amersham) at a dilution of 1:200 in 5 % skim / PBS. Cells were washed three times in 1 X PBS and incubated for 1hr in the secondary antibody (sheep anti mouse antibody conjugated to FITC -Stressgen) at a concentration of 1:200 in 5 % skim milk / PBS. Cells were washed 3 X in 1 X PBS and 1ul of DAPI (Sigma) was

added to the cells along with 10 ml of fresh PBS. Cells were then visualized and photographed under a Zeiss fluorescent microscope using the DAPI and FITC filters which emit light at an excitation wavelength of 345 and 490 respectively.

Chapter 3

3.0 Results

3.1 BLAST search for *drosophila* and murine orthologs of hDREF.

The (TRAMP) hDREF protein was discovered in a blast search for human genes that contained the MEF2 consensus-binding sequence. This gene sequence was submitted to the NCBI database by Nagase et al. (1998) and Esposito et al. (1999). Esposito et al. (1999) reported that the novel gene represented an ancient transposable element complete with an active transposase, terminal inverted repeats and direct repeats. Based on the similarities of the nucleotide sequence to the Ac- element (Activator Dissociator elements) family of transposons, this novel gene was named TRAMP. BLASTp (Basic Local Alignment Search Tool protein) (NCBI) searches revealed that the so-called TRAMP protein bears amino acid similarity to a *drosophila* protein called DREF (DNA Replication Related Element Factor). The name “TRAMP” has also been used to describe an unrelated protein (Tyrosine Rich Acid Matrix Protein). Because of the confusing nomenclature, the Ac-like transposable element “TRAMP” will be referred to as human DREF (hDREF).

Using the BLASTp algorithm, the hDREF and DREF protein sequences were aligned (Figure 2). It was found that hDREF and DREF proteins share 30% identical sequence, and 49 % similar sequence within a N-terminal stretch of 81 amino acids. The sequence similarity seen between hDREF and DREF resides entirely within the N-terminal domains of the proteins. Outside of these domains sequence similarity drops off completely. Interestingly, it is the N-terminal domain of *drosophila* DREF that the DNA binding and homodimer formation domains are found (nt 16-115).

BLAST searches using the available mouse genome did not result in any significant matches to hDREF. However using the BLASTn (Basic Local Alignment Search Tool nucleotide) (NCBI) algorithm and the mouse EST database there were over 260 hits against the hDREF (tramp) cDNA sequence, some of which had greater than 80 % identical sequence (Figure 5).

3.2 The untranslated regions of hDREF contain three putative Myocyte Enhancer Factor 2 (MEF2) binding sites and one Myogenic Regulatory Factor (MRF) binding site, the E-box.

Examination of the untranslated regions (which lie three and five prime to the hDREF open reading frame but within the terminal inverted repeats) revealed three putative MEF2 (Myocyte Enhancing Factor 2) binding sites and one MRF (Myogenic Regulatory Factor) binding site, an E-box (Figure 1). The 1.2 Kb 5'UTR contains one MEF2 binding site (nt 762-772) (CTAAAAATAC) as well as an E-box binding sequence (nt 45-51) (CANNTG). The 2.3 Kb 3'UTR contains the other two MEF2 binding sites (nt 3657-3668 & 4466-4477). The discovery of these regulatory binding sites was of interest as the MEF2 and E-box binding sites have been shown to be crucial regulatory elements for the expression of a number of skeletal muscle and cardiac genes (reviewed in Molkentin et al., 1995). The presence of the MEF2 binding sites and an E-box binding site in the putative regulatory regions of hDREF, suggested that hDREF expression might be regulated by muscle transcription factors.

Figure 5: BLASTn alignment of hDREF and the murine EST database (NCBI) June 2002. There are over 260 hits against the hDREF (tramp) (4.48 Kb cDNA) sequence, some of which had over 80% identical sequence. **A)** Soares_NMEBA_branchial_arch Mus musculus (eastern European house mouse) cDNA clone 475 bp shares 86 % identities with hDREF **B)** Soares_NMEBA_branchial_arch Mus musculus (eastern European house mouse) cDNA clone 509 bp shares 85 % identities with hDREF.

Query= (hDREF nucleotide sequence)
Database: GenBank Mouse EST entries

```
>gi|12021477|gb|BF720475.1|BF720475 mab54g07.y1 Soares_NMEBA_branchial_arch
Mus musculus cDNA clone IMAGE:3974220 5'.Length = 475
Score = 202 bits (102), Expect = 5e-49 Identities = 198/228 (86%),
Gaps = 4/228 (1%)Strand = Plus / Minus
```

Query: 2633 cagagaatcgcttgaacctgggaggtggaggttgacagtaagctgagat 2680
 |||||||
 Sbjct: 145 aggagaatcgcttgaaccagggagctggaggttgacagtgaagccgagat 98

Query= (hDREF nucleotide sequence)
Database: GenBank Mouse EST entries

>gi|12021426|gb|BF720424.1|BF720424 mab54b05.y1 **Soares_NMEBA_branchial_arch**
Mus musculus cDNA clone IMAGE:3973976 5'.Length = 509
Score = 190 bits (96), Expect = 2e-45 **Identities = 216/253 (85%),**
Gaps = 10/253 (3%)Strand = Plus / Minus

```
Query: 2705 agagcgagactct 2717
          |||||
Sbjct: 207 agagcgagactct 195
```

3.3 Endogenous hDREF expression.

3.3.1 hDREF expression *in vivo* and *in vitro*.

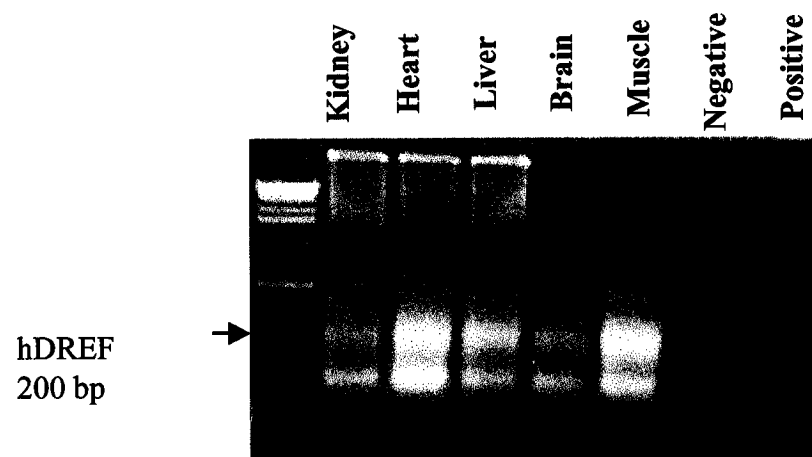
Using RT/PCR analyses it was found that hDREF is expressed in the kidney, heart, liver, brain, and skeletal muscle of wild type mice (Figure 6). hDREF expression was next assessed in the hearts of wild type mice aged 0 to 12 weeks. During early stages of postnatal growth, the heart switches from a hyperplastic growth mechanism to a hypertrophic growth mechanism i.e. cells increase in size but not number (Kolodziejczyk et al., 1999). The time course results indicate that as the heart switches to hypertrophic growth, endogenous hDREF expression decreases (Figure 7 A).

Characterization of endogenous hDREF expression in the rat cardiomyocyte cell line H9C2, the mouse skeletal muscle cell line C2C12 and the human skeletal muscle SK cell line revealed that hDREF expression is highest during growth when the cells are actively dividing, and is substantially decreased during differentiation as cells withdraw from the cell cycle (Figure 7 B). These observations suggest that as cells switch from a state of growth to differentiation there is a substantial decrease in hDREF expression.

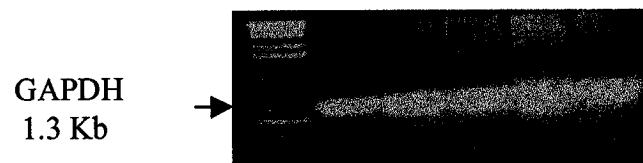
3.3.2 hDREF expression is downregulated in the presence high MEF2 activity.

As suggested by the presence of the MEF2 binding sites in the UTRs of hDREF and the decrease in hDREF expression during periods of high MEF2 activity (i.e. during

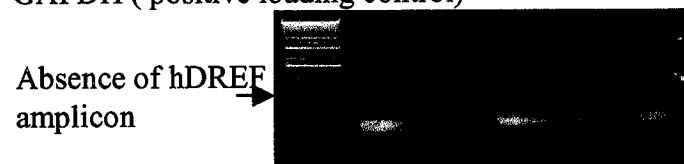
Figure 6: hDREF expression in several tissues from wild type 3-month-old mice. **A)** hDREF is highly expressed in the heart and skeletal muscle. 5 ug of RNA were reverse transcribed into cDNA, and treated with DNase I. Total cDNA was then used in a PCR reaction to identify a 200 bp hDREF fragment. **B)** GAPDH primers were used to amplify a 1.3 Kb fragment. The GAPDH PCR product was used as a loading control to ensure all samples were equally represented. **C)** To control for DNA contamination, a reaction with no reverse transcriptase was carried out.



A) RT/PCR of hDREF. 3 month old mouse

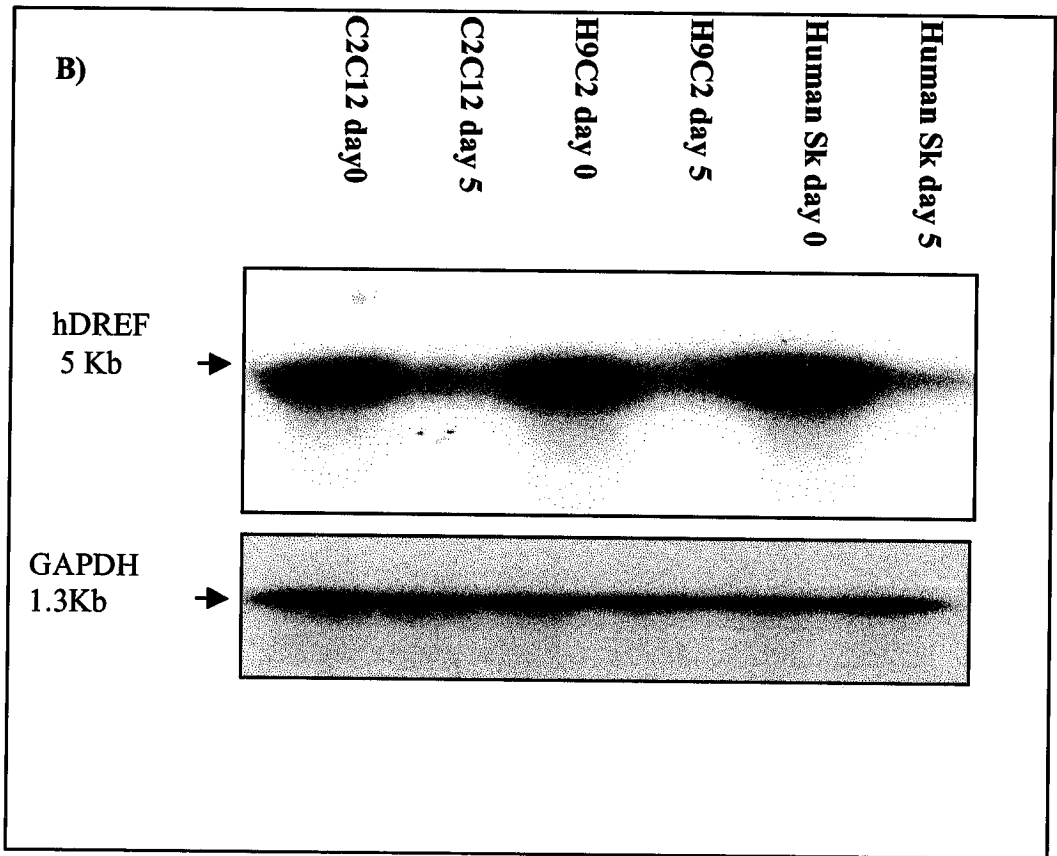
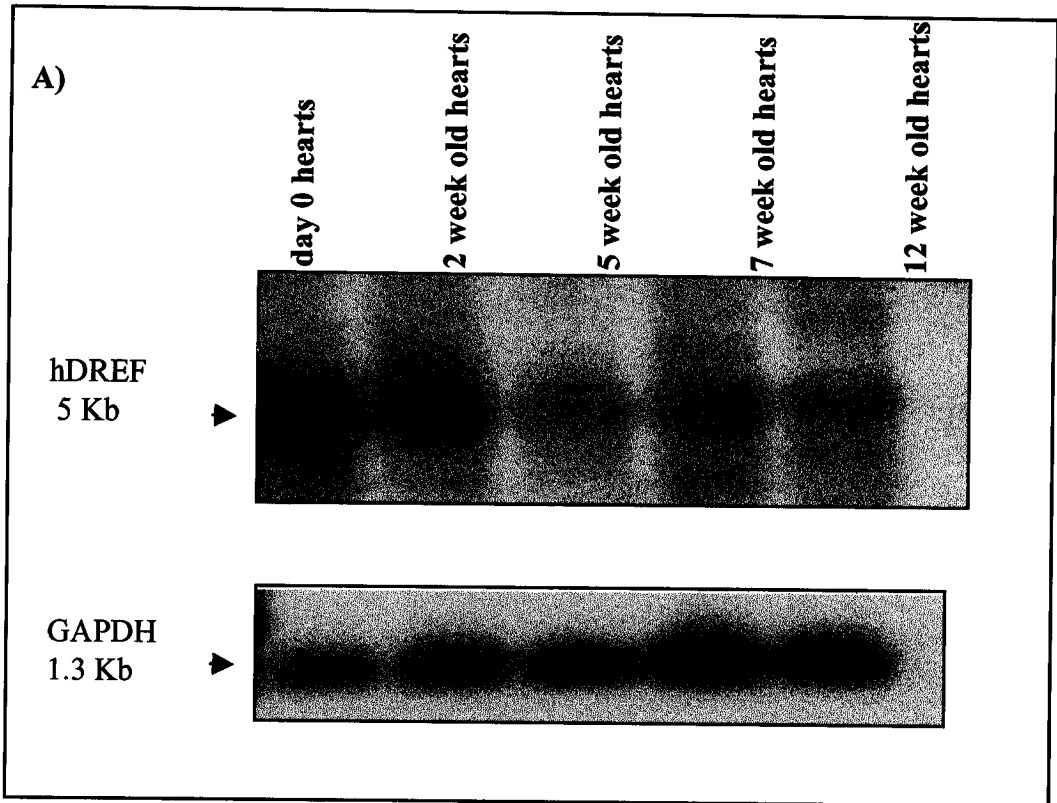


B) GAPDH (positive loading control)



C) no RT (- control)

Figure 7: **A)** Characterization of endogenous hDREF expression. Northern blot of wild type murine hearts from birth to 12 weeks of age. 30 ug of total heart RNA was run out on a formaldehyde gel and transferred to a membrane. Probing the membrane with the radiolabelled hDREF ORF revealed that endogenous hDREF is highly expressed immediately after birth, but decreases as the animal matures. The membrane was reprobed with GAPDH as a loading control. **B)** Northern blot analysis of hDREF expression in the rat cardiomyocyte cell line H9C2, the mouse skeletal muscle cell line C2C12 and the human skeletal muscle SK cell line. 30 ug of total RNA from cells was run out on a formaldehyde gel and transferred to a membrane. Probing the membrane with the radiolabelled hDREF ORF revealed that hDREF expression is highest during cell growth, and decreases during differentiation. The membrane was re-probed with GAPDH as a loading control.



differentiation) it was hypothesized that hDREF expression may be negatively regulated by MEF2. To test this hypothesis, hDREF expression was examined in wild type and MEF2C dominant negative hearts (MEF2C DN). The MEF2C DN model expresses a transgene under the control of a cardiac specific promoter (myosin heavy chain). This transgene contains the MADS and MEF2 DNA binding and homodimer formation domains, but lacks the transactivation domain. The transgene can bind to endogenous MEF2 proteins, and the MEF2 DNA binding site, but lacks the transactivation domain that is necessary for MEF2 activity. As a result MEF2 activity is severely downregulated leading to a reduction in the size of the post-natal myocardium (Kolodziejczyk et al., 1999). Comparison of MEF2 DN animals and wild type animals revealed increased hDREF expression in the hearts of MEF2DN over that of wild type hearts (Figure 8A).

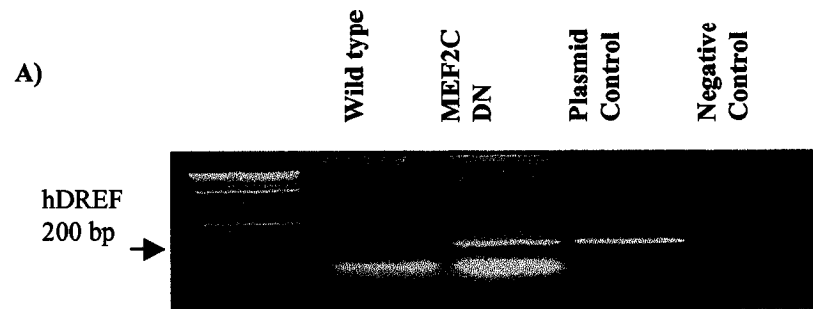
The mdx / MyoD (-/-) mouse contains point mutations in both the dystrophin and MyoD genes, and represents one of the working models for Duchenne Muscular Dystrophy. The mdx / MyoD (-/-) mouse displays increased MEF2 expression / activity, dilated cardiomyopathy, and severe skeletal myopathy leading to dystrophic muscle and premature death around 1 year of age (Megeney et al., 1999; Kolodziejczyk et al., 1999). The severity of the phenotype results from an inability of the muscle to regenerate, due to impaired satellite cell self renewal. Although MyoD is not expressed in the heart its absence has been shown to evoke hypertrophic growth, indicating that skeletal muscle damage contributes to progressive cardiomyopathy (Megeney et al., 1999). Interestingly, endogenous hDREF expression in the mdx / MyoD (-/-) hearts was found to be virtually

non-existent (Figure 8B). The increased MEF2 activity seen in the mdx / MyoD (-/-) mice correlates with a substantial decrease in hDREF expression. The results from the expression studies *in vivo* and *in vitro* suggest that high MEF2 activity, results in low hDREF expression, suggesting that MEF2 may be a negative regulator of hDREF.

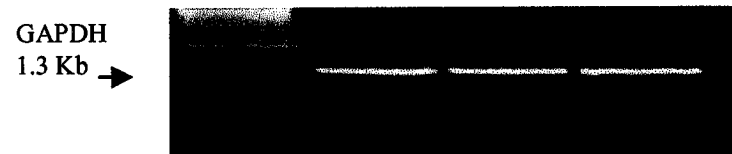
3.4 Chromatin Immunoprecipitation Assay: MEF2 proteins bind to the MEF2 consensus site in the 5'UTR of hDREF during growth and differentiation conditions.

Chromatin immunoprecipitation assays are used to demonstrate a direct interaction between a protein of interest and its DNA binding site. The purpose of this experiment was to determine if the MEF2 binding site found in the 5'UTR of hDREF could indeed bind the MEF2 protein. DNA was immunoprecipitated out of solution through the addition of a MEF2 specific antibody. The immunoprecipitated DNA was fixed to a membrane that was then was hybridized with a hDREF sequence specific probe. The 110 bp probe used in the chromatin immunoprecipitation assay was designed to be specific for the MEF2 binding site found in the 5'UTR of hDREF (nt 590-700 Figure 1). To demonstrate specificity, the probe was hybridized to EcoRV digested, total genomic DNA. The enzyme EcoRV was chosen to digest the DNA as no EcoRV restriction sites are found within the entire 5.6 Kb hDREF DNA sequence. From Figure 9A it can be seen that the probe hybridizes specifically to one band of approximately 6.0 Kb in length. The probe also hybridizes weakly to a larger band at approximately 8.0 Kb. This band may represent another copy of hDREF which has been reported to be present in the human genome (Esposito et al.1999).

Figure 8: hDREF expression in the hearts of wild type, MEF2C DN and mdx / MyoD (-/-) 3 month old mice. 5 ug of RNA were reverse transcribed into cDNA, and treated with DNase I. Total cDNA was then used to PCR a 200 bp hDREF fragment. **AI)** In comparison to wild type hearts, hDREF is highly expressed in the hearts of MEF2 DN animals. **AII)** GAPDH primers were used as a loading control to ensure all sample were equally represented. **AIII)** A no reverse transcriptase reaction was also carried out to ensure DNA contaminants were absent. **BI)** hDREF is expressed in the wild type heart but is virtually absent from the mdx / MyoD (-/-) heart, where MEF2 activity is elevated. **BII)** RT / PCR using GAPDH specific primers was used to ensure equal. **BIII)** A no reverse transcriptase reaction was also carried out to ensure DNA contaminants were absent.



I) RT/PCR of hDREF. 3 month old mouse heart



II) GAPDH (positive loading control)



III) no RT (negative control)



I) RT/PCR of hDREF. 3 month old mouse heart



II) GAPDH (positive control)



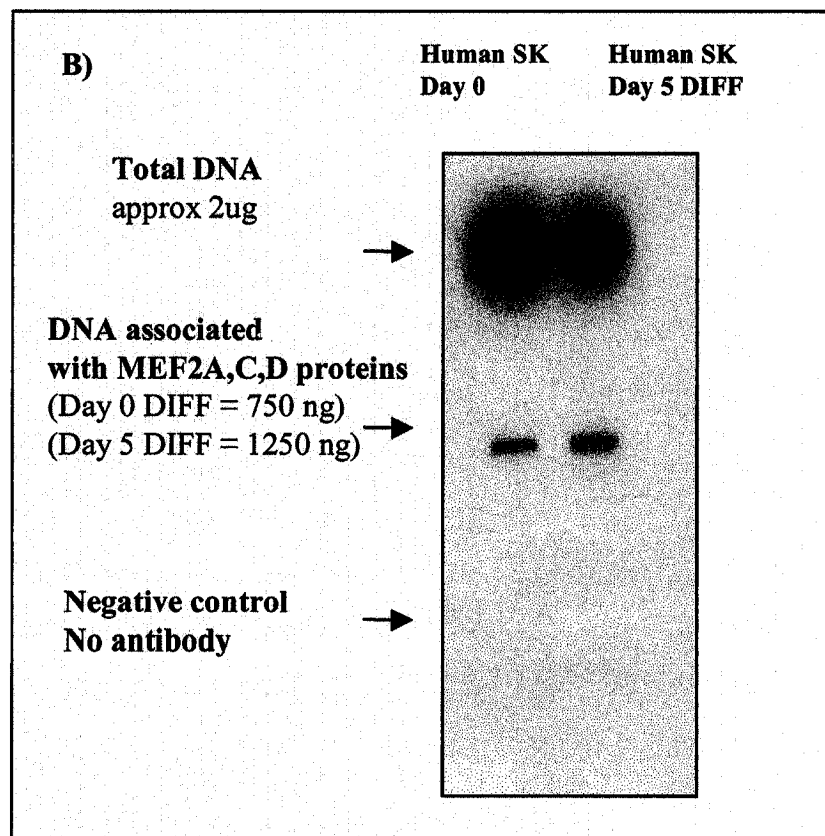
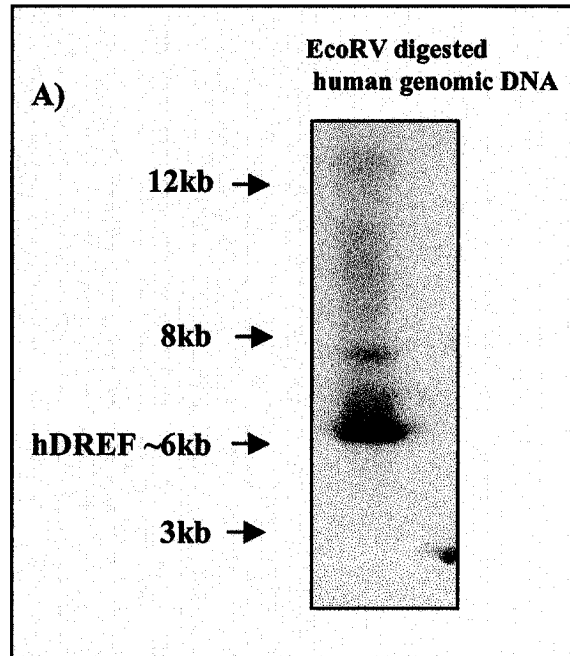
III) no RT (negative control)

Chromatin immunoprecipitation assays were used to assess the ability of the MEF2 proteins to bind to the putative MEF2 binding site found in the 5'UTR of hDREF. The ChIP assays were carried out using human skeletal muscle cells that had been cultured in growth or differentiation media for 5 days. Proteins were cross-linked to the DNA by the addition of formaldehyde to the media. Total genomic DNA was then collected from cells and incubated with MEF2 antibody (C-21 Santa Cruz), which recognized the MEF2A, C, and D proteins. DNA associated with MEF2 A, C or D proteins was then immunoprecipitated and slotted onto a membrane and hybridized with the radiolabelled probe. Variable amounts of DNA were immunoprecipitated from the cultured cells, i.e. 750 ng was obtained from proliferating cells, 1250 ng of DNA was immunoprecipitated from differentiated cells (Figure 9B). Although approximately 1.6 times more DNA was immunoprecipitated out of solution from the 5 day differentiated cell lysates, no increase in the amount of probe binding was observed. These results indicate that there is little difference in the amount of MEF2 protein which is bound to the 5'UTR MEF2 binding site during growth or differentiation. This observation suggests that a change in MEF2 activity subsequent to MEF2 binding may be the determining factor in regulating hDREF expression.

As a positive control for the ChIP assay 2 ug of total genomic DNA was removed from the lysate prior to the addition of the antibody. As the MEF2 specific binding site found in the 5'UTR of hDREF is present in the total genomic DNA regardless of growth conditions of the cells, the probe bound to the DNA from both day 0 differentiation and

Figure 9: **A)** Southern blot illustrating that the 110 bp probe used in the chromatin immunoprecipitation assay specifically hybridizes to a 6.0 Kb band of EcoRV digested genomic DNA. 10ug of human total genomic DNA was digested with EcoRV, and probed with the MEF2-specific binding sequence found in the 5'UTR of hDREF. The probe hybridizes specifically to one band of approximately 6.0 Kb, which most likely represents the genomic 6.0 Kb hDREF sequence. The probe also weakly hybridizes to a larger band at approximately 8.0 Kb, which may represent one of the other genomic copies of hDREF.

B) Chromatin Immunoprecipitation (ChIP) Assay (using MEF2 A, B, D specific antibody). DNA immunoprecipitated by anti-MEF2 (C-21) was slotted and probed with the hDREF 5'UTR / MEF2 specific probe. MEF2 associates with the hDREF sequence in growth and in differentiation conditions. Under the growth condition 750 ng of DNA was immunoprecipitated out of solution. Under the differentiation condition 1250 ng of DNA was immunoprecipitated out of solution. The pre-immune aliquots (approximately 2 ug of total genomic DNA) acted as a positive control. The no antibody aliquots acted as a negative control.



day 5 differentiation conditions (Figure 9 B). The no antibody aliquots were used as a negative control and had no antibody added at the immunoprecipitation step.

Nevertheless, these aliquots were subject to the same immunoprecipitation process using the protein A beads (Upstate) and other reagents. Due to the absence of antibody, DNA was not immunoprecipitated from the solution.

The ChIP assay was next conducted using a MEF2 antibody specific for the MEF2C protein (Cell Signaling Technology). The activity and expression level of MEF2C is highest during differentiation (Olson, et al., 1995). Therefore it was anticipated that there would also be an increase in the amount of MEF2C binding to the MEF2 consensus site in the 5'UTR of hDREF. However, no increase in the amount of MEF2C binding to the 5'UTR of hDREF was observed i.e. through comparisons of the amount of probe that hybridized to the immunoprecipitated DNA from day 0 and day 5 differentiation (Figures 10 I and 10 II). Variable amounts of DNA were immunoprecipitated from the cultured cells, i.e. 650 ng (blot I) and 800 ng (blot II) was obtained from proliferating cells, 1ug (blot I) and 1.3ug (blot II) of DNA was immunoprecipitated from differentiated cells (Figure 10 I & 10 II). These observations indicate that MEF2C binds to its consensus-binding site in the 5'UTR of hDREF during both growth and differentiation conditions. The ChIP assay was repeated twice using this antibody.

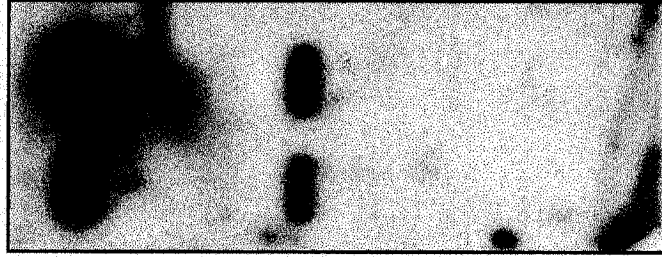
Figure 10: Chromatin Immunoprecipitation (ChIP) Assay (using a MEF2C specific antibody-Cell Signaling Technology). The MEF2C immunoprecipitated DNA was slotted and probed with the hDREF 5'UTR / MEF2 specific probe. MEF2C associates with hDREF during growth and differentiation.

I) Under growth conditions, 650 ng of DNA was immunoprecipitated out of solution from blot A. Under differentiation conditions, 1.0 ug of DNA was immunoprecipitated out of solution from blot A.

II) Under growth conditions 800 ng of DNA was immunoprecipitated out of solution from blot B. Under differentiation conditions 1.3 ug of DNA was immunoprecipitated out of solution from blot B. Blot B is a duplicate experiment. **III)** Positive control for immunoprecipitation step. As an experimental control the second MEF2C blot (blot B) was re-probed with the radiolabelled sequence for the myogenin promoter that also contains a MEF2 specific binding site. MEF2C binds and upregulates myogenin expression only during differentiation

I) Blot A

Human SK Human SK
Day 0 DIFF Day 5 DIFF



Total DNA
2ug

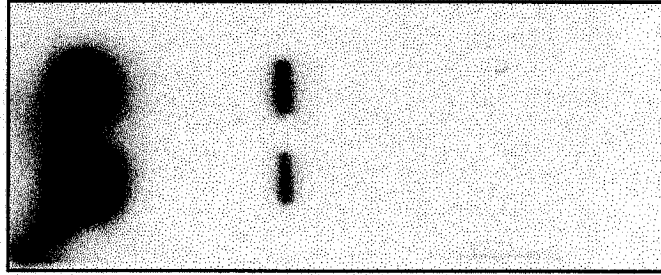
DNA associated
with MEF2C
(Day 0 DIFF= 650ng)

(Day 5 DIFF = 1ug)

No antibody
Negative control

II) Blot B

Human SK Human SK
Day 0 DIFF Day 5 DIFF



Total DNA
2ug

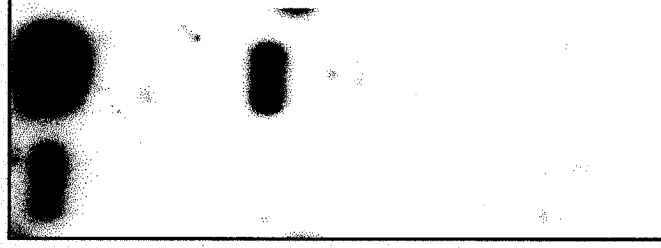
DNA associated
with MEF2C
(Day 0 DIFF= 800ng)

(Day 5 DIFF = 1.3ug)

No antibody
Negative control

III) Blot B reprobed with myogenin promoter

Human SK Human SK
Day 0 Day 5 DIFF



Total DNA
2ug

DNA associated
with MEF2C
(Day 0 DIFF= 800ng)

(Day 5 DIFF = 1.3ug)

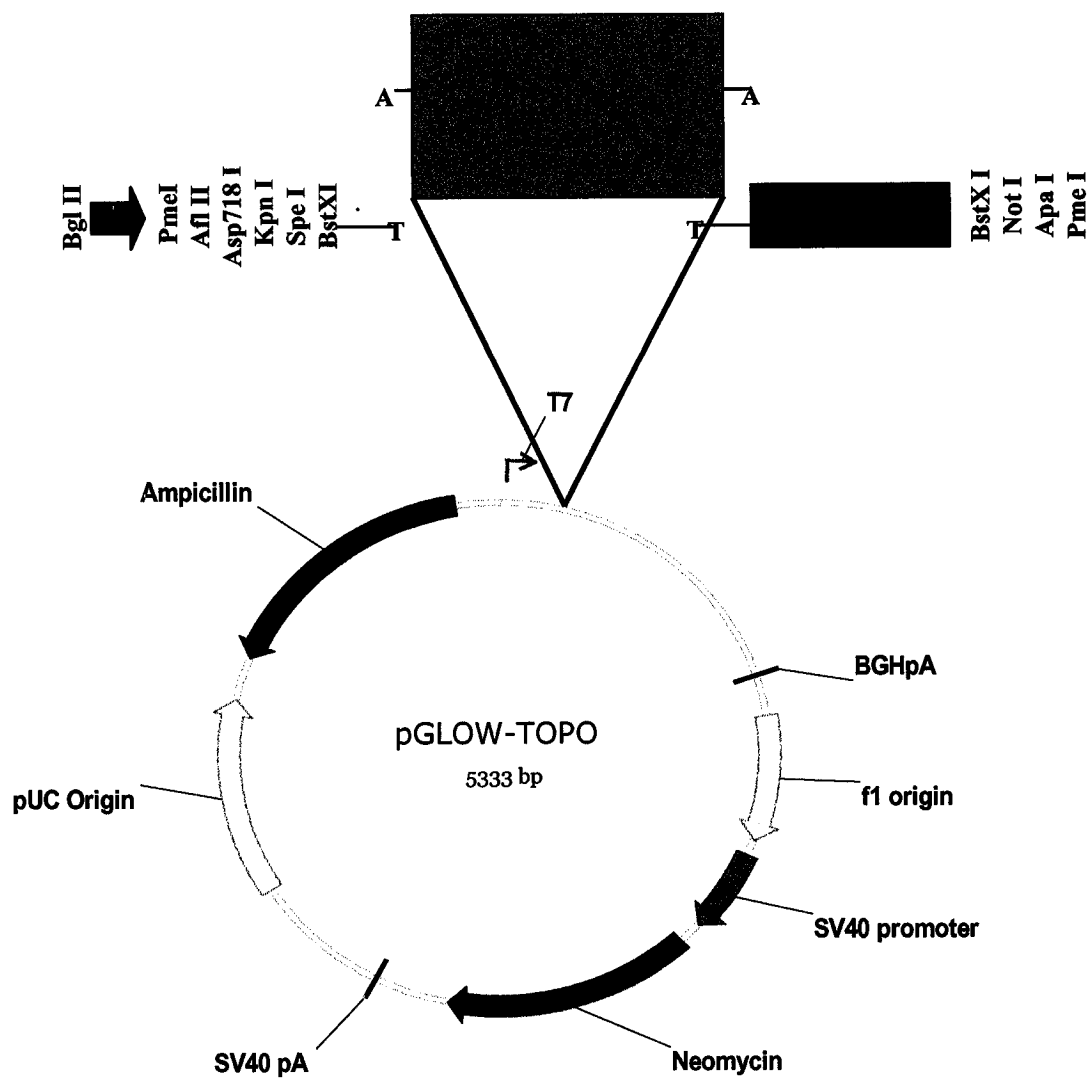
No antibody
Negative control

To confirm the specificity of the MEF2 antibody the second MEF2C blot (blot B) (Figure 10 II) was re-probed with the radiolabelled myogenin promoter sequence. The myogenin promoter also contains the MEF2 consensus binding site, which binds MEF2C during differentiation and results in a upregulation of myogenin expression (Ridgeway et al., 2000). From Figure 10 III it can be seen that in the total genomic DNA sample the myogenin probe hybridizes to both growth and differentiation conditions. However, in the MEF2C immunoprecipitated sample the myogenin promoter was accessible during differentiation conditions only. These results indicate that MEF2 associates with the myogenin promoter only during differentiation, and that MEF2 proteins bound to DNA were indeed being successfully immunoprecipitated out of solution using the ChIP assay.

3.5 Testing the hDREF UTRs in traditional reporter assays.

To determine function of the UTRs found in the hDREF sequence, the 5' UTR and 3'UTR were separately cloned in the pGLOW-TOPO reporter vector (Figure 11). The CMV promoter was also cloned into the pGLOW-TOPO reporter construct and served as a positive control. A self-ligated pGLOW-TOPO vector was used as a negative control. Transient transfections of the 5'UTR / GFP construct and the 3'UTR/GFP construct were performed in C2C12 cells. These cells were then cultured for 2 days in growth or differentiation media and GFP expression was assessed by fluorescence microscopy. The 5'UTR / GFP construct, the 3'UTR / GFP construct and the CMV promoter construct all expressed the green fluorescent protein during growth conditions (Figure 12A: A-C).

Figure 11: Cloning strategy for the 5' and 3' untranslated regions of hDREF, the CMV promoter (positive control) and self ligation (negative control) into pGLOW reporter vector (Invitrogen). The 5'UTR, 3'UTR and CMV promoter were all PCR amplified, cloned into the pGLOW vector (Invitrogen), and sequenced. The pGLOW vector was also ligated to itself as a negative control.



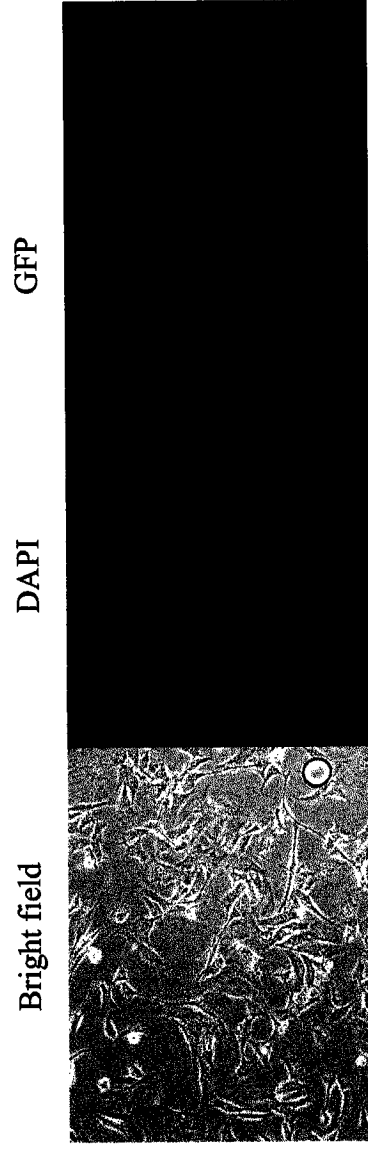
Adapted from Invitrogen pGlow –TOPO vector

However in differentiation (low serum) conditions, GFP expression was eliminated in both the 5'UTR / GFP and the 3'UTR / GFP transfected cells (Figure 12B: A-C). GFP expression from the CMV promoter construct did not vary between growth and differentiation conditions. The self-ligation construct of pGLOW did not express GFP under any conditions, indicating that circularization of the GLOW plasmid alone is insufficient to promote GFP expression.

3.6 Mutagenesis of the hDREF UTR reporter constructs.

The presence of the MEF2 binding sites in the 5' and 3'UTRs of hDREF initially lead us to the hypothesis that hDREF may represent a novel MEF2 dependent gene. The UTR / reporter assays indicated that during times of high MEF2 activity, the GFP reporter was down regulated. To determine if the MEF2 binding sites found in the UTRs of hDREF were responsible for mediating the repression of the reporter seen during high MEF2 activity, a series of mutagenesis assays were undertaken. The MEF2 binding site found in the 5'UTR / pGLOW construct was mutated using the Quick Change Site Directed Mutagenesis Kit (Qiagen). In the 3'UTR, the proximal MEF2 binding site was also mutated using the Quick Change Site Directed Mutagenesis Kit (Qiagen). This construct contained the 3'UTR, with one functional MEF2 binding site and one mutated MEF2 binding site. The mutated 5'UTR / pGLOW and the 3'UTR MEF2 (1 site) / pGLOW construct were separately transfected transiently into C2C12 cells and cultured in growth or differentiation media for 2 days. GFP expression was then assessed by qualitative

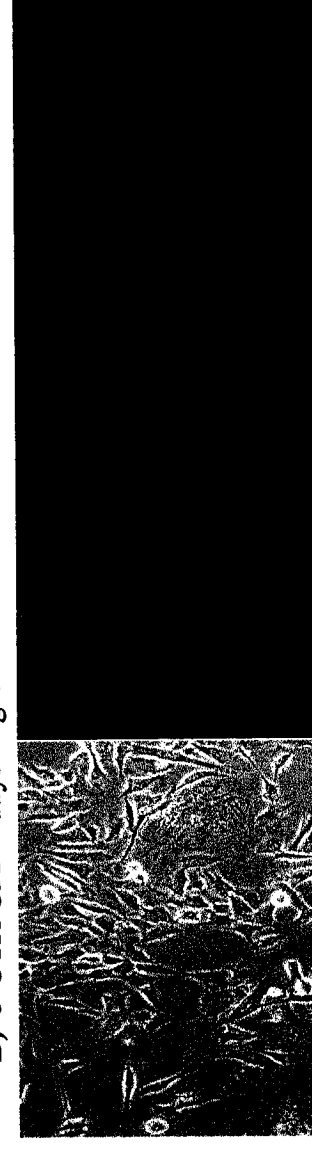
Figure 12A: Transient transfections of 5'UTR / pGLOW and 3'UTR / pGLOW in C2C12 cells cultured in growth media. GFP expression under the control of the 5'UTR (A), the 3'UTR (B), and the CMV promoter (C). Transfected cells were cultured for 2 days in growth media after which time GFP expression was assessed. All three constructs readily expressed GFP when cultured in growth media.



A) 5'UTR/GFP 2 days in growth media 20 X

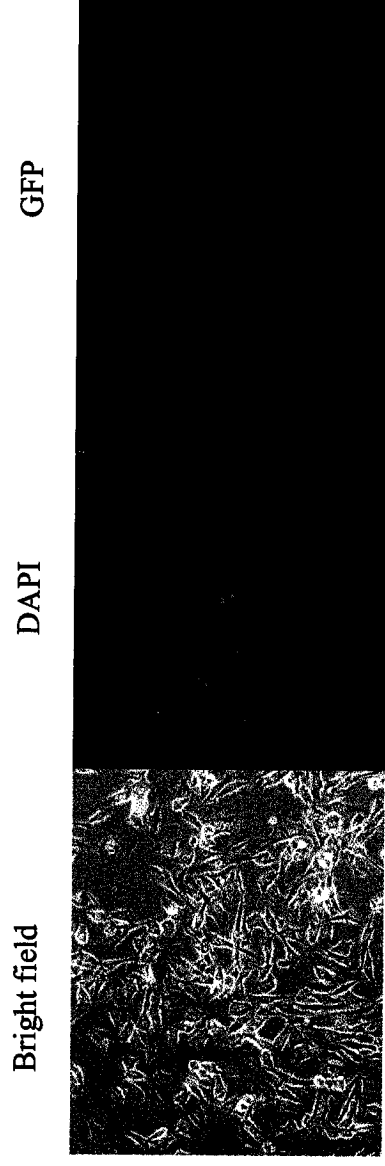


B) 3'UTR/GFP 2 days in growth media 20 X

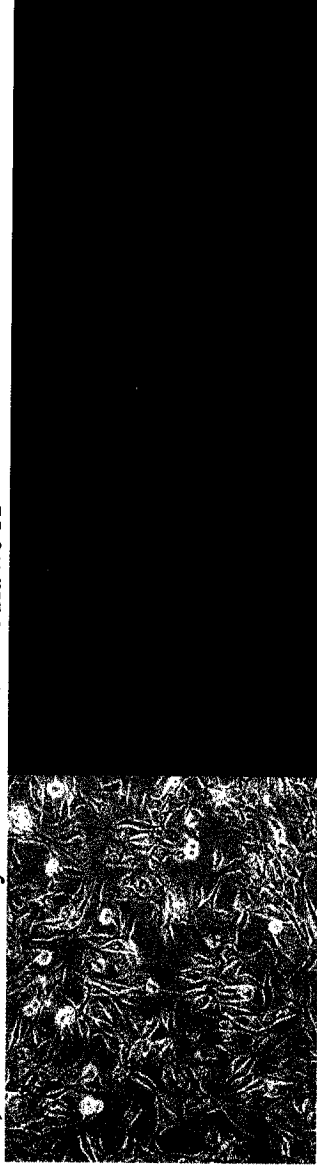


C) CMV/GFP 2 days in growth media 20 X

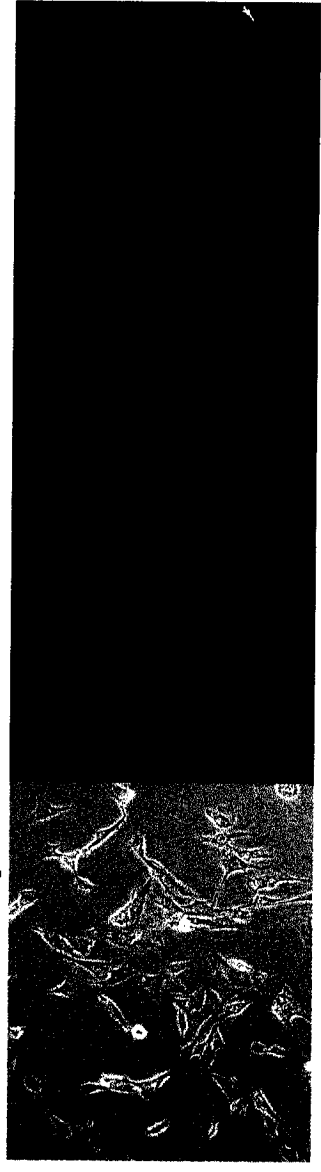
Figure 12B: Transient transfections of 5'UTR / pGLOW and 3'UTR / pGLOW in C2C12 cells cultured in differentiation media. GFP expression under the control of the 5'UTR (A), the 3'UTR (B), and the CMV promoter (C). Transfected cells were cultured for 2 days in differentiation media after which time GFP expression was assessed. Under differentiation conditions GFP expression was found to be absent in both the 5'UTR / pGLOW and 3'UTR / pGLOW constructs, but was unaffected in the CMV construct.



A) 5'UTR/GFP 2 days differentiation media 20 X



B) 3'UTR/GFP 2 days in differentiation media 20 X



C) CMV/GFP 2 days in differentiation media 20 X

fluorescence microscopy. Results from the mutated 5'UTR / pGLOW construct indicated that GFP expression was unchanged in both growth and differentiation (Figure 13A panel A & Figure 13B panel A). These data suggest the loss of the functional MEF2 binding site results in a loss of transcriptional control and the continual expression of GFP. Results from the mutation of the (proximal) MEF2 site found in the 3'UTR/ pGLOW construct indicated that GFP was expressed during growth (Figure 13A panel B) and was weakly expressed during differentiation conditions (Figure 13B panel B). These results suggest that the one functional MEF2 site present in the 3'UTR was capable of weakly downregulating GFP expression during times of high MEF2 activity (differentiation) but lacked the ability to fully inhibit expression. These results suggest that both MEF2 binding sites are required for complete transcriptional regulation of the UTR reporter constructs.

3.7 Overexpression of hDREF in H9C2 cardiomyocytes.

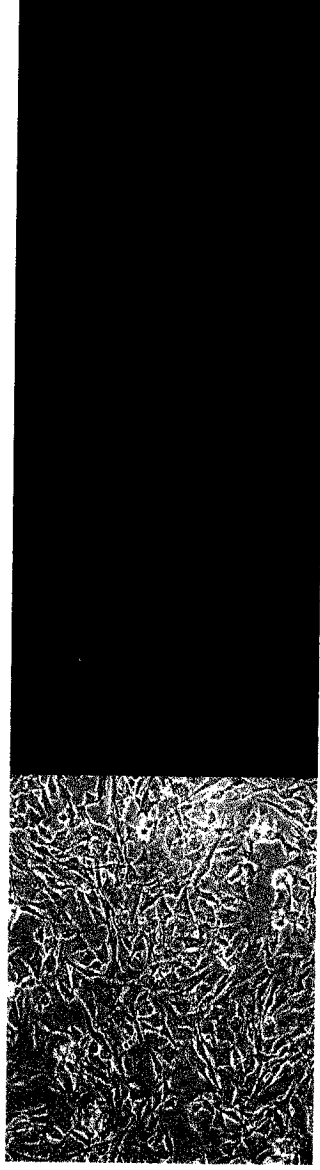
In *drosophila*, DREF is a key regulator of many genes involved in DNA synthesis, and has been shown to be required for the regulation of both the mitotic cell cycle and endocycle (Hirose et al., 1999). For example PCNA, E2F and the DNA polymerase α subunit have all been shown to be DREF responsive genes (Hirose et al., 1999). Based on the known function of DREF in *drosophila*, we chose to examine the effects of hDREF overexpression in the rat cardiomyocyte cell line H9C2. Cells were stably transfected with the hDREF- HIS- myc / pcDNA3.1 construct or the empty vector myc-

Figure 13A: Transient transfections of 5' UTR/ pGLOW (with mutated MEF2 binding site) and 3'UTR / pGLOW (with one functional and one mutated MEF2 site) in C2C12 cells cultured in growth media. GFP expression under the control of the mutated 5'UTR (**A**), the mutated 3'UTR (**B**), and the CMV promoter (**C**). The mutated 5'UTR and 3'UTR / pGLOW constructs both readily expressed GFP when cultured in growth media. The CMV construct also readily expressed GFP.

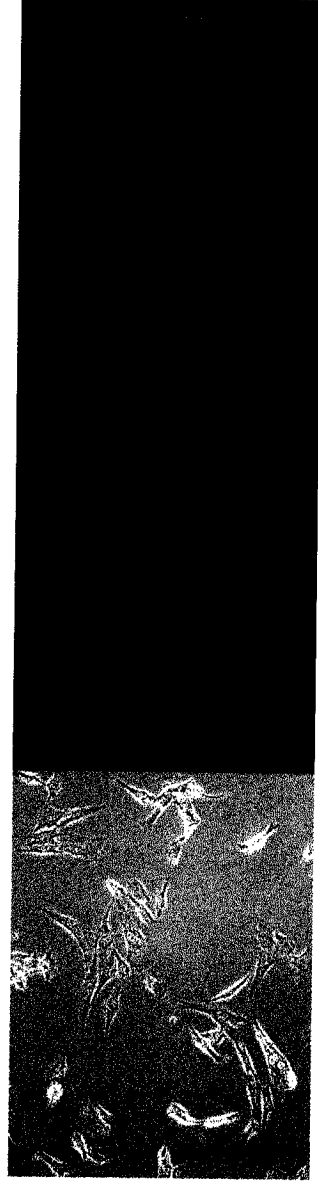
Bright field

DAPI

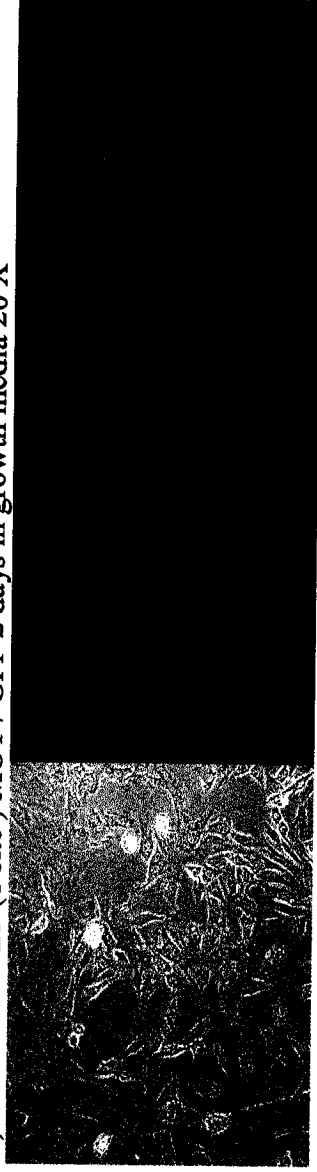
GFP



A) 5' UTR MEF-MUT / GFP 2 days in growth media 20 X

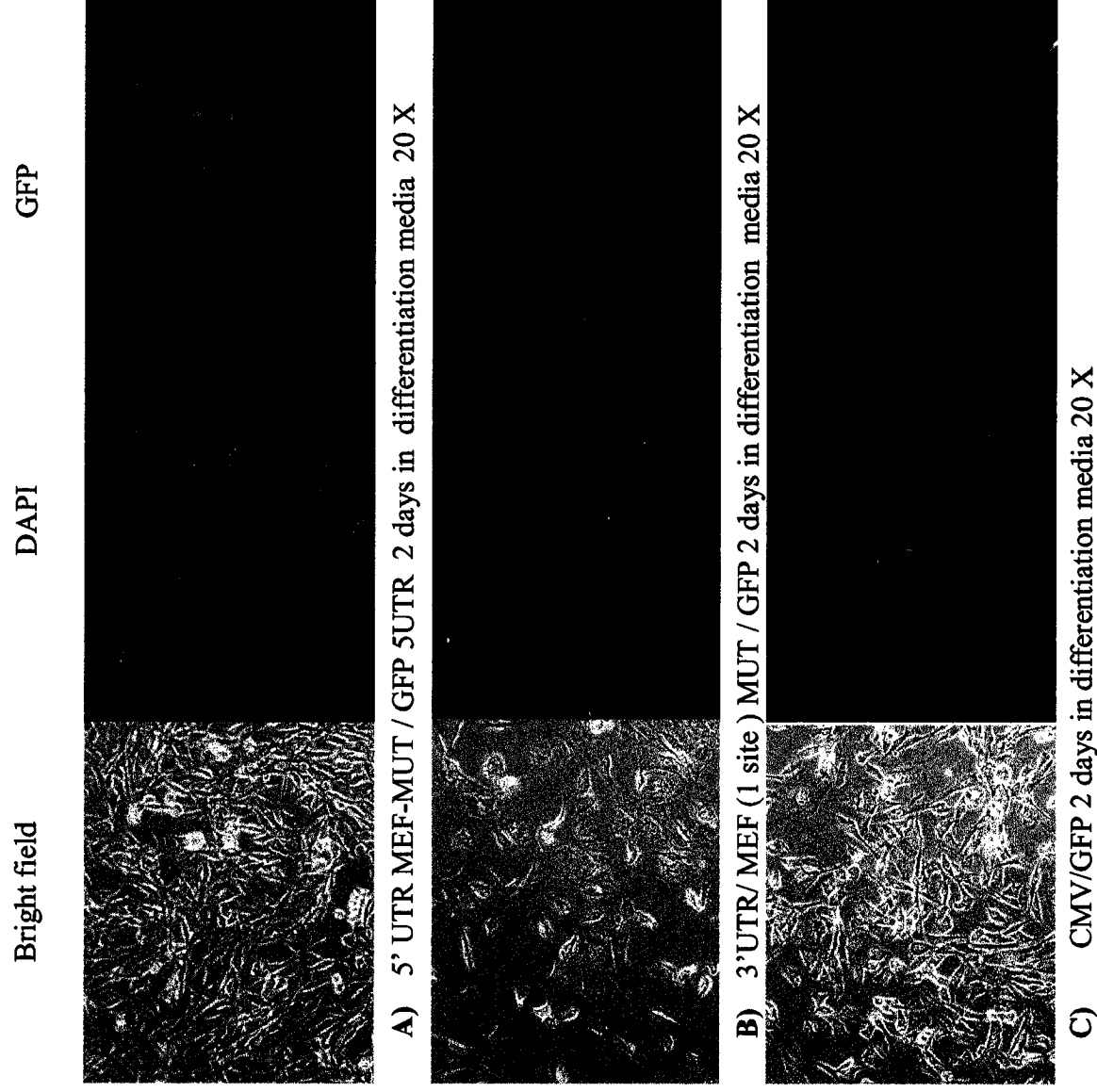


B) 3'UTR/MEF (1 site) MUT / GFP 2 days in growth media 20 X



C) CMV/GFP 2 days in growth media 20 X

Figure 13B: Transient transfections of 5'UTR/ pGLOW (with mutated MEF2 binding site), and 3'UTR / pGLOW (with one functional and one mutated MEF2 site) in C2C12 cells cultured in differentiation media. GFP expression under the control of the mutated 5'UTR (**A**), the mutated 3'UTR (**B**), and the CMV promoter (**C**). The mutated 5'UTR / pGLOW construct exhibited unrestricted GFP expression under differentiation conditions. The mutated 3'UTR / pGLOW construct weakly expressed GFP under differentiation conditions. The CMV construct readily expressed GFP regardless of culture conditions.



HIS / pcDNA3.1. Transfection of the hDREF- HIS- myc / pcDNA3.1 construct into the H9C2 cells resulted in overexpression of the hDREF-HIS-myc fusion protein. Stable clones were assessed for hDREF expression by Western blot analysis and by quantitative analysis of band intensity using the NIH image software (available for download at: <http://rsb.info.nih.gov/nih-image/>). To determine levels of hDREF expression, all bands were normalized using tubulin as a loading control (Western blots were reprobed with tubulin antibody). The lowest band was set at 1.00 and all other bands, of various intensities, were normalized to that of that standard band. From the Western blots and the NIH image software it was determined that in Figure 14 I & II band B, was the lowest expressing clone and as such its level of expression was set as 1.00. Band A was also a low expressing clone and its level was found to be 1.17. Bands C and D were found to be high expressers and their level of expression was found to be 4.16 and 4.70 respectively. Finally bands E and F were determined to be a medium expressing clones and their level of expression was calculated to be 3.50 and 2.00 respectively.

In order to qualitatively ensure that 100 % of the cell populations were expressing the hDREF-HIS-myc fusion protein, each clone was grown on a 10 cm plate, fixed and then stained with the myc antibody (Invitrogen) and DAB. It was found that in all clones, 100% of the population expressed the myc tagged hDREF. Figure 14 III illustrates high, medium, and low expressing clones stained with the myc antibody and DAB. These results indicate that the stable clones were uniformly expressing the hDREF-myc fusion protein.

To determine what effect overexpression of hDREF would have on post-mitotic cells, the hDREF clones were cultured in differentiation media for 5 days. The extent of

differentiation was determined using the fusion index. The fusion index was calculated as the number of nuclei in myotubes divided by the number of total nuclei (three trials were used to determine the fusion indexes in this experiment). A myotube was defined as a cell with multiple nuclei (2 or >) and expressing myosin heavy chain. Each bar in Figure 15A represents the average of the three trials obtained from the 2 high-expressing clones, the 2 medium-expressing clones and the two low-expressing clones.

Untransfected H9C2 and empty vector transfected H9C2 cells were used as positive controls. After 5 days in differentiation media, approximately 60.0 ± 6.0 % of the cell population of both untransfected H9C2 cells and empty vector-transfected H9C2 cells had differentiated. Clones that expressed hDREF at low, medium and high levels were 45.0 ± 3.0 %, 35.0 ± 3.0 % and 25.0 ± 3.0 % differentiated respectively (Figure 15).

Student T-test results indicated a statistically significant difference between H9C2 cells and hDREF low expressing cells, H9C2 cells and hDREF medium expressing cells and H9C2 cells and hDREF high expressing cells. Table 1 in the appendix summarizes the P-values found for these data. These data suggest that increased hDREF expression leads to a decrease in the formation of H9C2 myofibers.

Interestingly the clones expressing hDREF at medium and high levels displayed a unique sub-population of single cells that did not fuse to form myotubes and instead became polyploidy, multinucleated cells (Figure 15C). In the clones that expressed hDREF to a medium and a high level, 1.0 ± 0.1 % and 2.0 ± 0.2 % (respectively) of the total cell population contained these unorthodox multinucleated cell types. Cells were considered

multinucleated if they were myosin heavy chain positive, had 3 or more nuclei, and retained the rounded cell morphology typical of proliferating myoblasts (Figure 15 A & C). P-values of medium versus high expressing clones (P value = 0.02) indicated a significant statistical difference between the two populations. These results indicated that hDREF overexpression in H9C2 cells inhibited normal differentiation and in a minority of cells, induced an irregular nuclear phenotype.

3.8 hDREF/ H9C2 cells continue to replicate their DNA under differentiation conditions.

As previously mentioned, the *drosophila* protein DREF has been shown to direct DNA replication. Therefore, to determine if hDREF was also capable of directing DNA synthesis, BrdU incorporation assays were carried out using the high expressing hDREF / H9C2 clones. Cells were incubated for 3 days in low serum media; on the third day the media was replaced with fresh low serum media that contained BrdU. Cells were then cultured for an additional 2-day period. After five days in differentiation media, the hDREF overexpressing cell line continued incorporating BrdU, whereas the control untransfected H9C2 cells had incorporated minimal amounts of BrdU (Figure 16). These findings suggest that the overexpression of hDREF results in continued DNA synthesis during a time when cells normally would have exited the cell cycle.

Figure 14: **I & II)** Western blots of stable clones expressing hDREF-myc at various levels. Blots were probed with the myc antibody (Invitrogen) and secondary goat anti-mouse conjugated to HRP (Sigma). The same blots were then reprobed with tubulin (Developmental Studies Hybridoma Bank) as a loading control. One medium expressing clone from blot I was used as a positive control on blot II. Two high expressing clones (C&D) two medium expressing clones (E&F) and two low expressing clones (A&B) were identified. X1- X10 denote non-expressing clones. **III)** Myc expression in stable cell lines. Stable clones were grown in a 10 cm plate, fixed and stained with a myc antibody conjugated to HRP (Invitrogen) and DAB. One hundred percent of the cells in all of the stable cell lines express hDREF-HIS-myc.

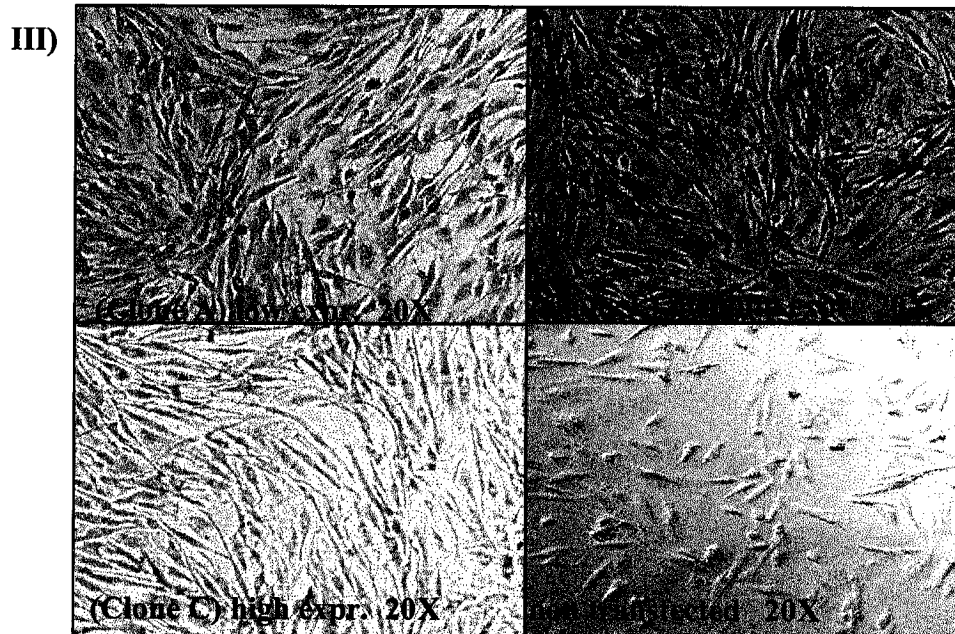
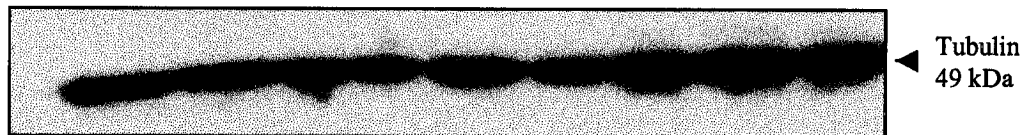
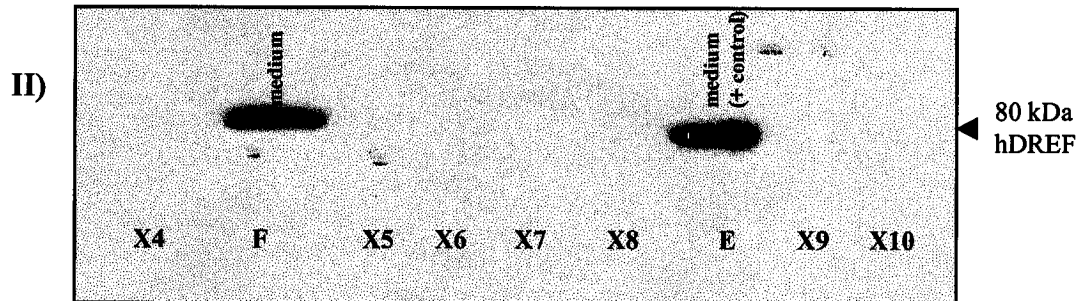
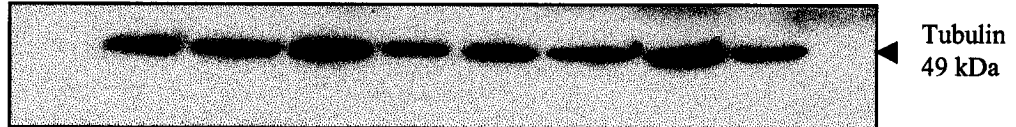
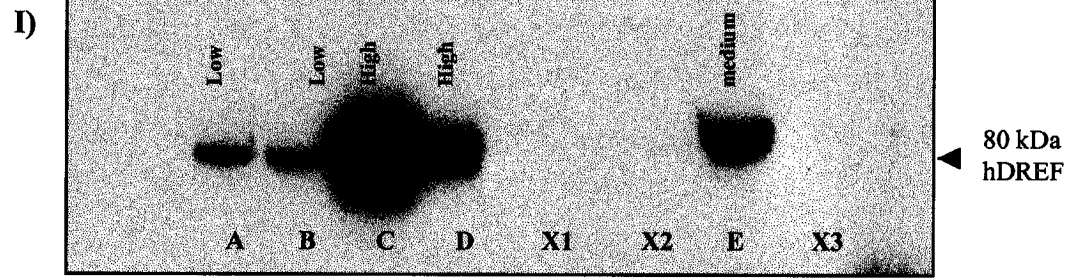
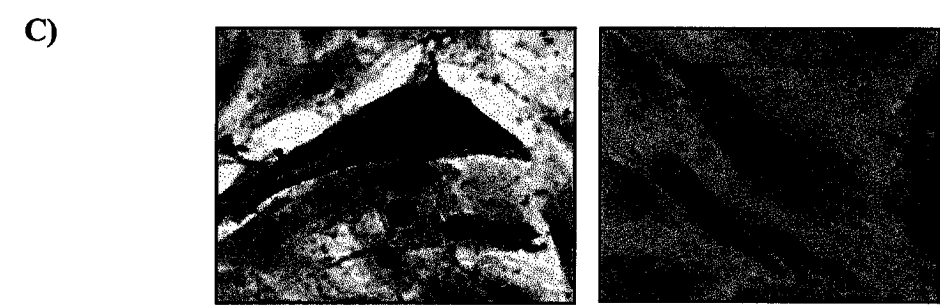
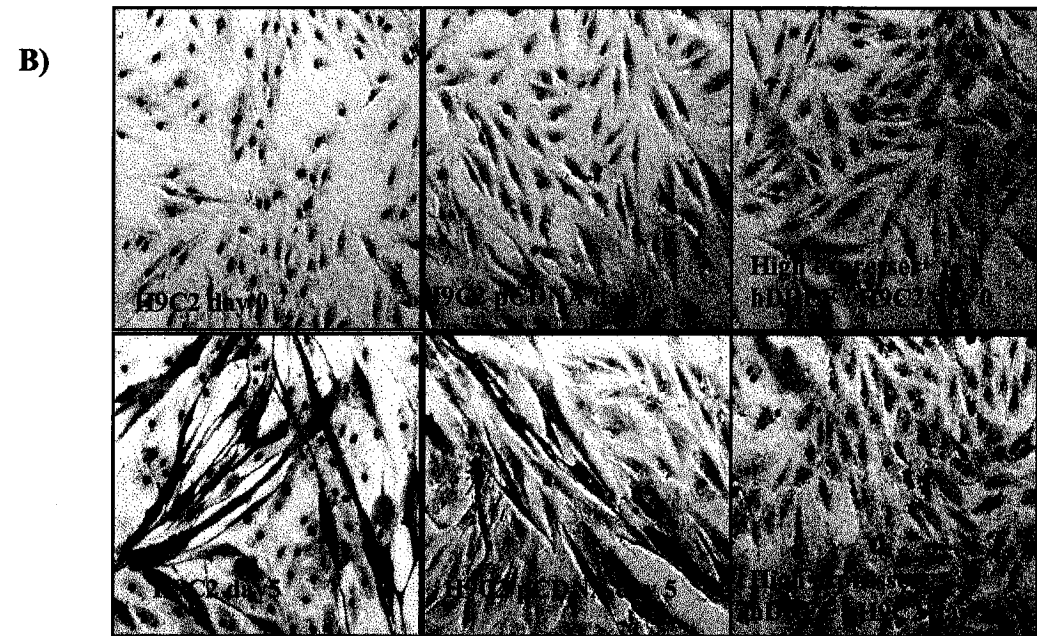
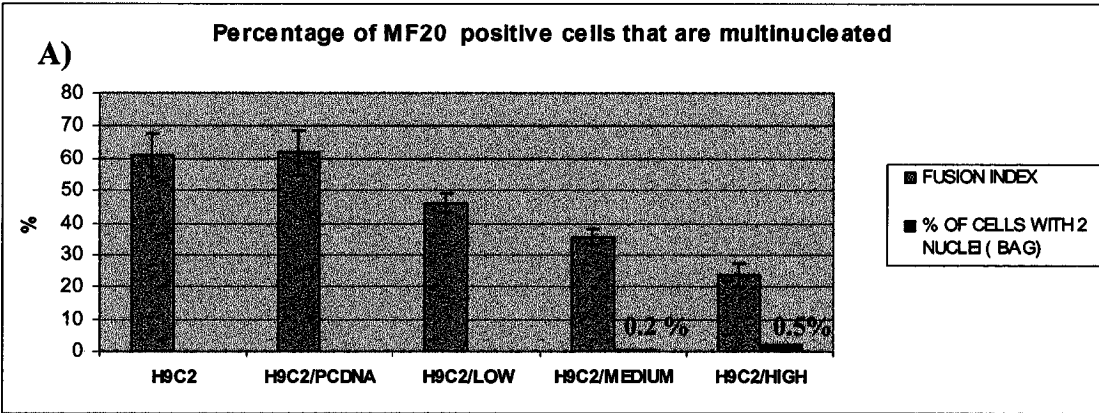


Figure 15: Differentiation of hDREF / H9C2 clones. **A)** There is a significant decrease in differentiation in the high and medium expressing hDREF / H9C2 cell lines and a low occurrence of multinucleated bag like cells (0.2 % in the medium and 0.5 % in the high expressers). Each bar represents the average of 3 different cells counts taken using the hDREF / H9C2 clones and untransfected cells. **B)** Comparison view of cells at day 0 and day 5 of differentiation, stained with hematoxylin and MF20 Mag. 20X. **C)** hDREF /H9C2 high expressing cell lines have a low occurrence of the bag-like phenotype. Cells stained with hematoxylin and MF20 (Mag.40X).



3.9 Overexpression of hDREF in H9C2 cells does not result in an increased growth or death rate.

The continued incorporation of BrdU suggested that hDREF was involved in promoting mitosis. Therefore, cell growth was directly measured in the hDREF / H9C2 cell lines. The 2 hDREF / H9C2 high expressing cell lines and untransfected H9C2 cells, were cultured over a 5-day time course in growth or differentiation media. Cell counts of the viable and non-viable cell populations revealed that the hDREF / H9C2 cells and untransfected H9C2 cells grew and died at the same rate in both growth and differentiation media (Figure 17 A-D). The P values for the growth and death rates are summarized in Tables 2, 3, 4, 5 (see appendix) and indicate that the differences in death and growth rates between hDREF / H9C2 cells and untransfected H9C2 were not significant. These results, combined with those from the BrdU assay, suggest that hDREF does promote DNA synthesis but does not promote or enhance completion of the entire mitotic cell cycle.

In order to ensure the reliability of the cell count results, Fluorescent Activated Cell Sorting analyses (FACS) was performed using day five time point cells. Cells being used for FACS analysis cultured for 5 days in growth or differentiation media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts.

As expected, FACS analyses revealed similar levels of cell viability. Analysis of

Figure 16: After 5 days in differentiation media hDREF / H9C2 cells continue to incorporate BrdU. Cells were incubated for a total of 5 days in low serum media. On the third day of culture the media was replaced with fresh media containing BrdU. Nuclei were stained with DAPI. After 5 days in differentiation media hDREF / H9C2 clones continued incorporating BrdU and replicate DNA (middle panel), whereas untransfected H9C2 cells did not (-control: lower panel). In contrast, untransfected H9C2 myoblasts cultured in growth media readily incorporate BrdU (+ control: upper panel). BrdU incorporation was visualized through detection with an anti-BrdU antibody (Amersham) conjugated to FITC and the fluorescence Ziess microscope.

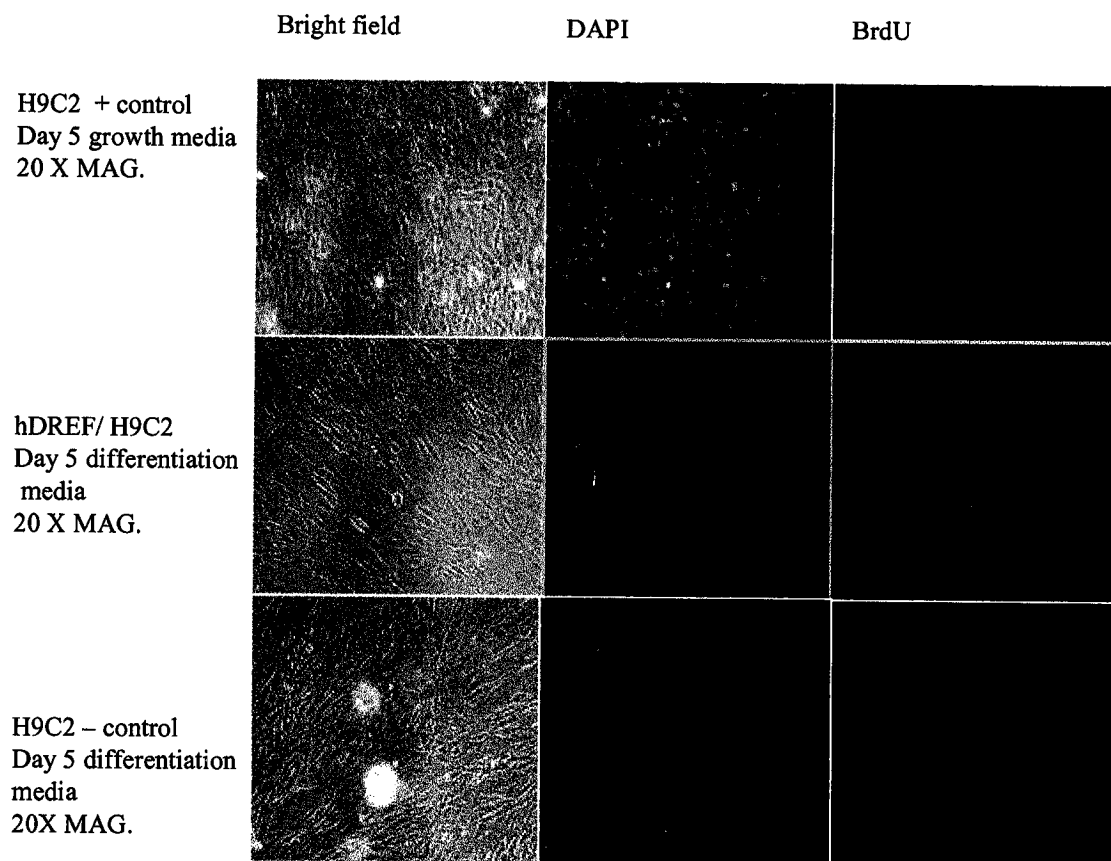
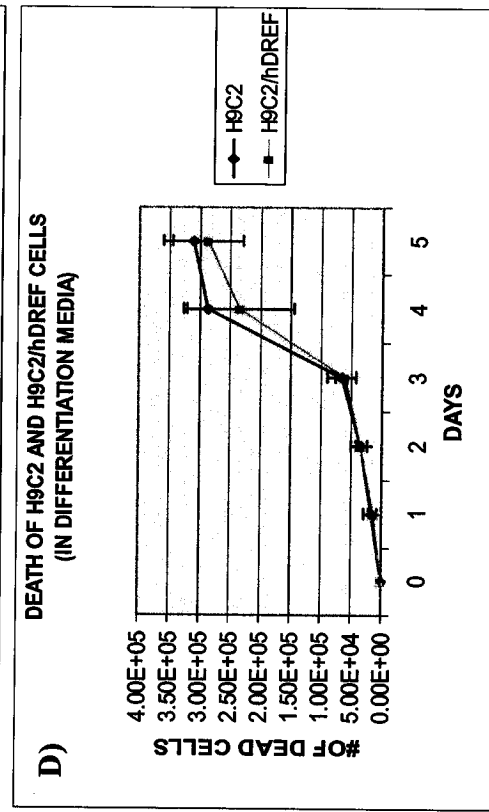
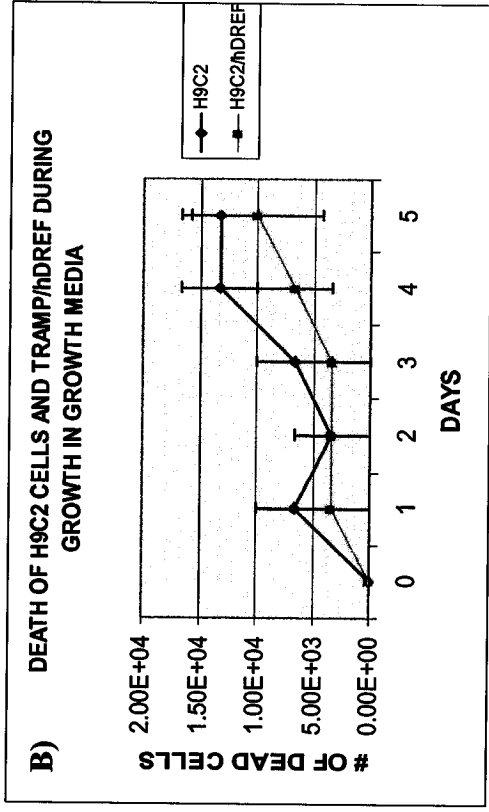
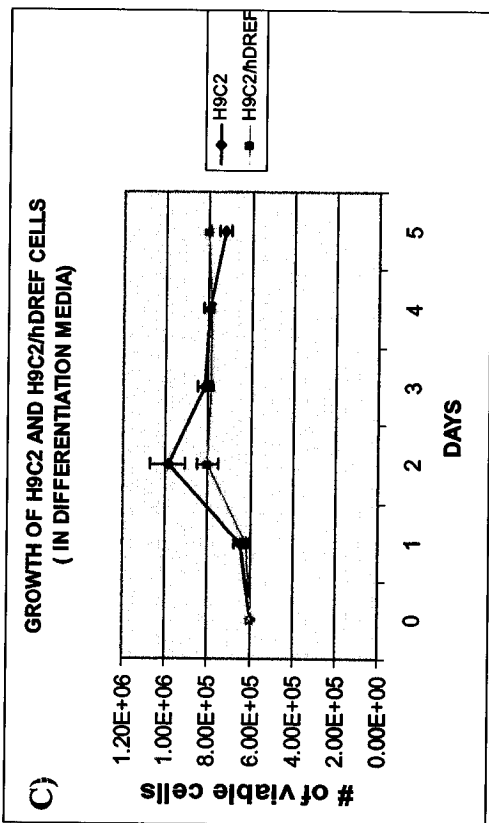
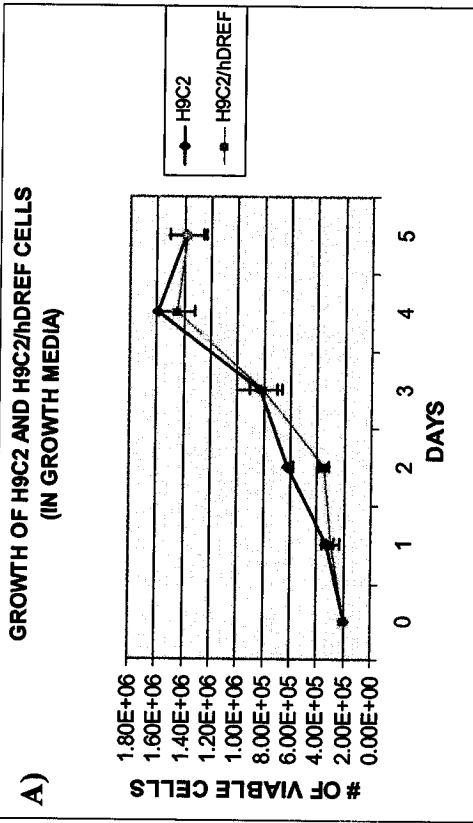


Figure 17: H9C2 and hDREF /H9C2 cells grow and die at the same rate in growth and in differentiation media. 2.0×10^5 cells were plated on day 0 of growth and 6.0×10^5 cells were plated on day 0 of differentiation. Three separate plates of cells were counted for each time point with a hemacytometer, and the results averaged. Error bars indicate the degree of variation from the mean from each of the three trials. **A)** Growth of H9C2 and hDREF / H9C2 cells over 5 days in growth media. **B)** Death rate of H9C2 and hDREF / H9C2 cells over 5 days in growth media. **C)** Growth of H9C2 and hDREF / H9C2 over 5 days in differentiation media. **D)** Death rate of H9C2 and hDREF / H9C2 cells over 5 days in differentiation media.



hDREF / H9C2 and H9C2 cells cultured in growth media revealed that 89 % and 87 % (respectively) of the population were viable (Figure 18 A& B and table 6).

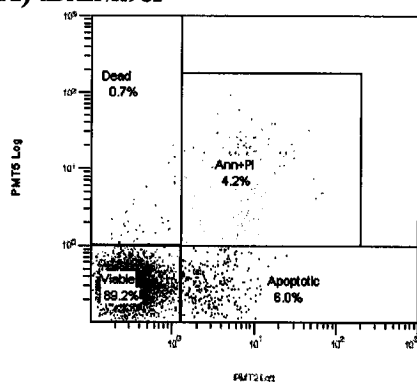
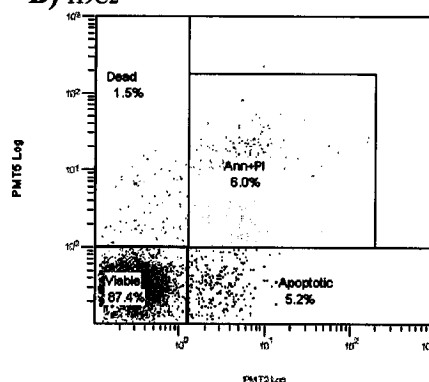
Flow cytometry was also performed on cells cultured in growth media that had been found floating in suspension and counted as non-viable. Previously in the cell count data, cells which were non-adherent were counted as non viable. However flow cytometer analysis of these cells revealed that 65 % of the hDREF / H9C2 population and 67 % of the H9C2 cell population were indeed apoptotic or dead (Figure 18 C& D and table 7).

These results suggest while the actual cell count data was slightly skewed, due to inaccuracies in the counting method, the trends observed in the cells counts were accurate, and that hDREF / H9C2 cells grow and die at the same rate as untransfected cells under growth conditions. Flow cytometry was also performed on hDREF / H9C2 cells and untransfected H9C2 cells, cultured in differentiation media for 5 days. From the population of hDREF / H9C2 and H9C2 cells, which had been counted as viable cells, it was determined that 92 % and 90 % (respectively) of the populations were indeed viable (Figure 19 A & B and table 8). These data support the finding that the trends observed in the cell count analyses were accurate, and that hDREF /H9C2 cells grow at the same rate as untransfected cells in differentiation media. Flow cytometry was also conducted on the cells that had been cultured under differentiation conditions and counted as non-viable. Analysis of hDREF / H9C2 and H9C2 cells revealed that 39 % and 35 % (respectively) of the population were apoptotic or dead (Figure 19 C & D and table 9). These results indicate that in the non-viable cell counts many of the cells that were counted as dead cells were actually non-adherent viable cells. The number of dead or

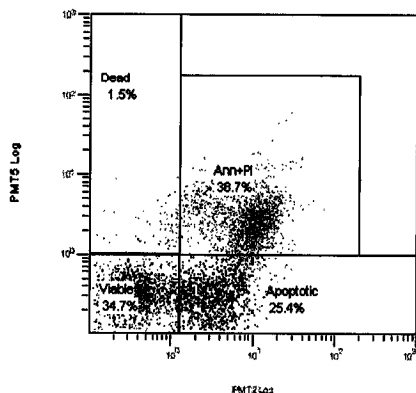
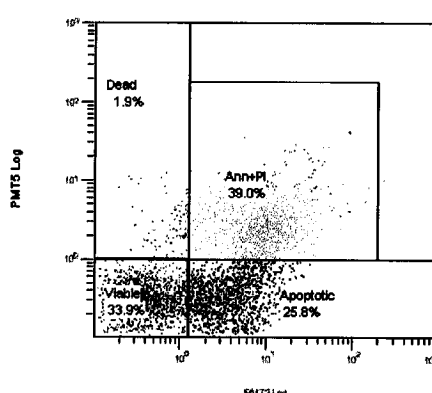
Figure 18: A-D) The hDREF / H9C2 cells do not have increased growth or death rate when cultured in growth media. Cell count data was used to determine the number of cells that were viable or dead, in a given population. Flow cytometry ensured that cells which had been counted as living were viable, and those which had been counted as dead were indeed non-viable. Cells were cultured for 5 days in growth media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts.

Table 6 & Figure 18: A) In the hDREF / H9C2 cells cultured in growth media and counted as living, 89% were viable. B) In the H9C2 cells cultured in growth media and counted as living, 87 % were viable.

Table 7 & Figure 18: C) Of the hDREF /H9C2 cells cultured in growth media and counted as dead, 65 % were dead or apoptotic. D) In the H9C2 cells cultured in growth media and counted as dead, 67 %were dead or apoptotic.

A) hDREF/H9C2**B) H9C2****Table #6: FACS analysis of the cells counted as living, cultured in growth media.**

5 days in growth media	Dead = annexin + propidium + annexin and propidium)	Viable
A) hDREF/H9C2	0.7 % + 4.2 % + 6.0 % = 11 %	89.9%
B) H9C2	1.5 % + 6.0 % + 5.2 % = 13 %	87%

C) hDREF/H9C2**D) H9C2****Table # 7: FACS analysis of the cells counted as dead, cultured in growth media.**

5 days in growth media	Dead = annexin + propidium + annexin and propidium)	Viable
C) hDREF/H9C2	1.5 % + 38.7 % + 25.4 % = 65%	35 %
D) H9C2	1.9 % + 39.0 % + 25.8 % = 67 %	34 %

apoptotic cells for both hDREF/ H9C2 and untransfected H9C2 cells was therefore greatly over estimated which resulted in an exaggerated death rate. However, because the trend in the cell counts/flow cytometry analysis between both populations was the same, the data remains valid and confirms that the hDREF / H9C2 and untransfected H9C2 cell populations die at a similar rate under differentiation conditions. One possible explanation for the subpopulation of viable cells found in the non-adherent / non viable cell population is, “mitotic shake off”. Mitotic shake-off occurs as cells that are actively undergoing mitosis become temporarily dislodged from their adherent surface.

3.10 Overexpression of hDREF in C2C12 skeletal muscle myoblasts.

In order to examine the effects of hDREF overexpression in skeletal muscle, C2C12 mouse myoblasts were stably transfected with the hDREF-HIS-myc / pcDNA3.1 construct or the empty vector myc HIS / pcDNA3.1. Stable clones were assessed for hDREF expression by Western blot and by quantitative analysis of band intensity using the NIH Image software. To determine levels of expression, all bands were normalized as in section 3.7 (Figure 20 I). It was determined that clone C had the lowest intensity band and as such its level of expression was set as 1.00. Band B was a high expressing hDREF / H9C2 clone which was used as a positive control, and was set as 5.69. Band A was a medium expressing hDREF / H9C2 clone which was used as another positive control, and its level of expression was found to be approximately 1.28. Despite repeated attempts at creating stable hDREF / C2C12 cell lines, only one clone was successfully obtained. The fact the only one low expressing clone was obtained from transfection

Figure 19: A-D) The hDREF / H9C2 cells do not have an increased growth or death rate when cultured in differentiation media. Cell count data was used to determine the number of cells that were viable or dead, in a given population. Flow cytometry ensured that cells which had been counted as living were viable, and those which had been counted as dead were non viable. Cells were cultured for 5 days in differentiation media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts.

Table 8 & Figure 19: A) hDREF /H9C2 cells cultured in differentiation media and counted as living, 92 % were viable. B) In the H9C2 cells cultured in growth media and counted as living, 90 % were viable.

Table 9 & Figure 19: C) Of the hDREF / H9C2 cells cultured in differentiation media and counted as dead, 38 % were dead or apoptotic. D) In the H9C2 cells cultured in growth media and counted as dead, 35 % were dead or apoptotic.

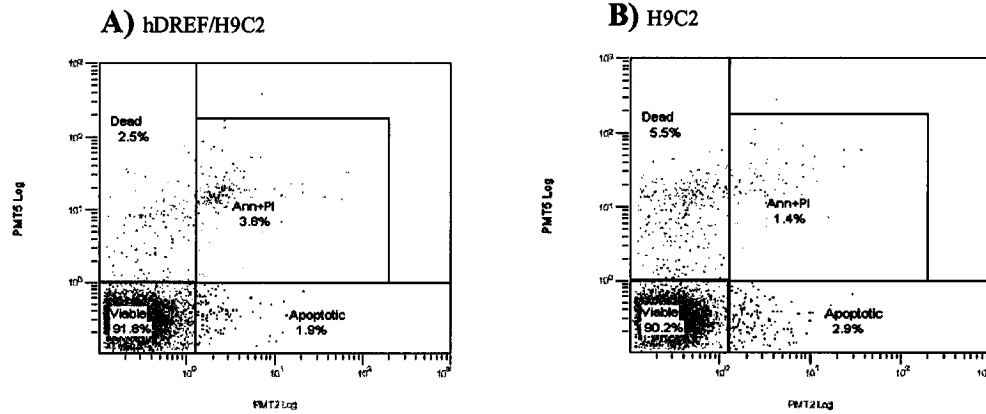


Table # 8: FACS analysis of the cells counted as living, cultured in differentiation media.

5 days in differentiation media	Dead = annexin +propidium + annexin and propidium)	Viable
hDREF/H9C2	2.5 % + 3.8 % +1.9 % = 8.2 %	92 %
H9C2	5.5 % +1.4 % + 2.9 % = 9.8 %	90 %

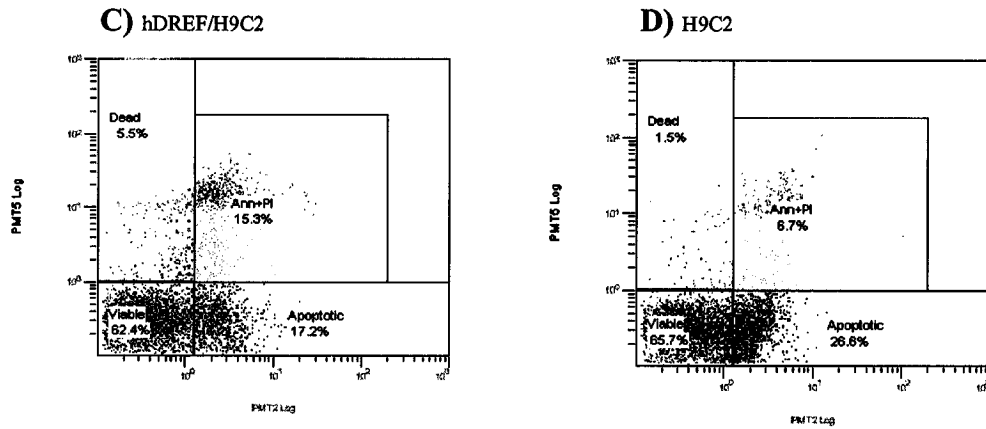


Table # 9: FACS analysis of the cells counted as dead, cultured in differentiation media.

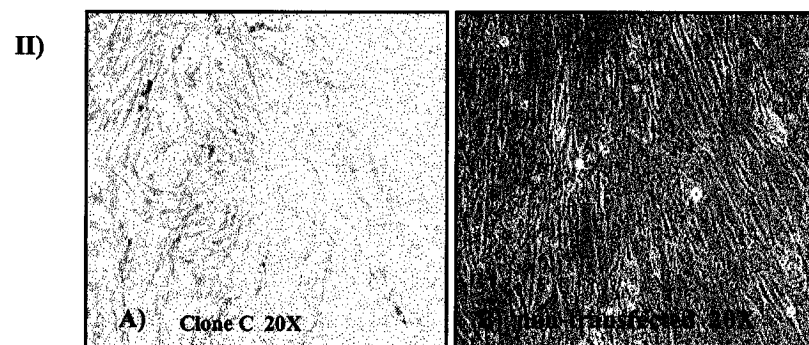
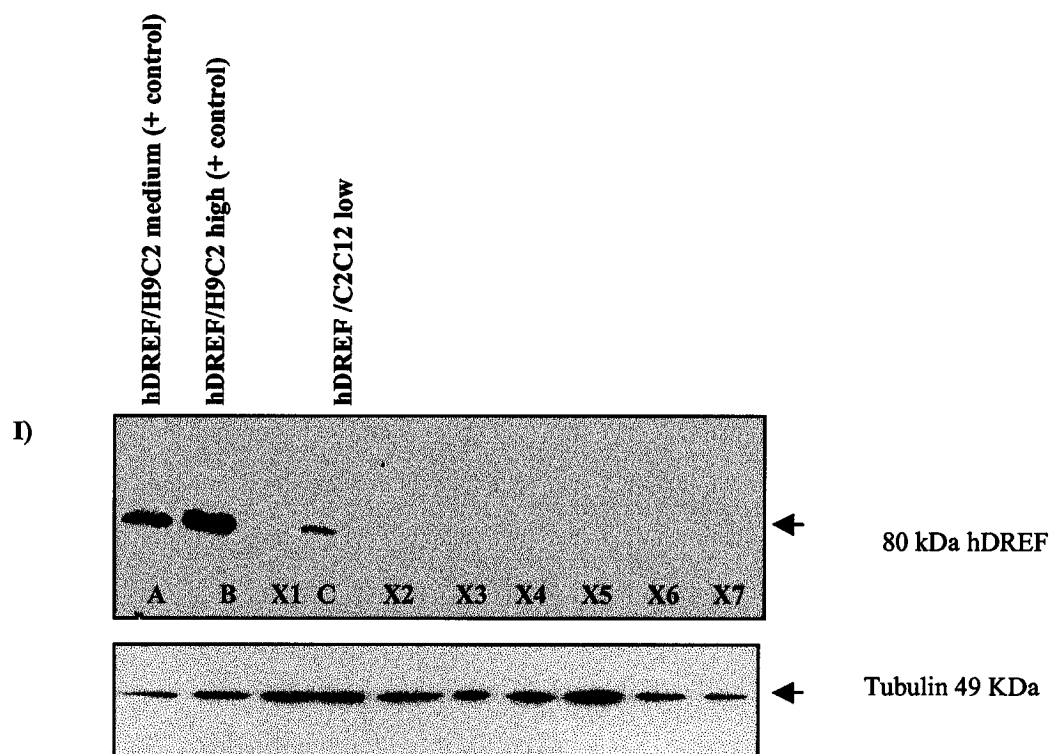
5 days in differentiation media	Dead = annexin +propidium + annexin and propidium)	Viable
hDREF/H9C2	5.5 % + 15.3 % + 17.2 % = 38 %	62 %
H9C2	1.5 % + 6.7 % + 26.6 % = 34 %	66 %

experiments in the myoblast cell line suggests that high levels of hDREF expression may be lethal in skeletal muscle cells. In order to ensure that 100 % of the cell population was expressing the hDREF-HIS-myc fusion protein, the hDREF / C2C12 clone was grown on a 10 cm plate, fixed and then stained with the myc antibody and DAB. It was found that 100% of the hDREF / C2C12 cell population expressed the myc tagged hDREF (Figure 20 II). This result indicates that these cells are uniformly expressing the hDREF-HIS-myc fusion protein.

To determine what effect overexpression of hDREF would have on post-mitotic skeletal muscle cells, the hDREF / C2C12 clone and an empty vector pcDNA /C2C12 clone were cultured in differentiation media for 5 days. The extent of differentiation was determined using an index of myoblast fusion. After five days in low serum media approximately 80 ± 6 % of the untransfected and empty vector transfected C2C12 cells had differentiated and formed MF20 positive myotubes. However, only 10 ± 3 % of the hDREF / C2C12 cell population had differentiated and formed MF20 positive myotubes (Figure 21 A). Student T-test results indicated that there was a statistically significant difference between the C2C12 and hDREF / C2C12 cell populations ($P = 6.0 \times 10^{-6}$).

Similar to the medium and high expressing hDREF / H9C2 clones, the hDREF / C2C12 clone formed multinucleated single cells, which expressed myosin heavy chain, yet did not form myotubes. As mentioned previously, the multinucleated bag like cells were defined as single cells that expressed the myosin heavy chain (MF20) marker, contained

Figure 20: **I)** Western blot of whole cell extracts from stable hDREF / C2C12 clone. The blot was probed with the myc antibody (Invitrogen) and secondary goat anti-mouse conjugated to HRP (Sigma). The same blot was then re-probed with tubulin (Developmental Studies Hybridoma Bank) as a loading control. One low expressing clone (C) was obtained from the transfection experiments. X1- X7 denote non-expressing clones. hDREF / H9C2 high (A) and medium (B) expressers were used as positive controls. **II)** Myc expression in stable cell line. The hDREF/ C2C12 stable clone was grown in a 10 cm plate, fixed and stained with a myc antibody conjugated to HRP (Invitrogen) and DAB. 100% of the cells in the hDREF /C2C12 stable cell line expressed hDREF-HIS-myc.

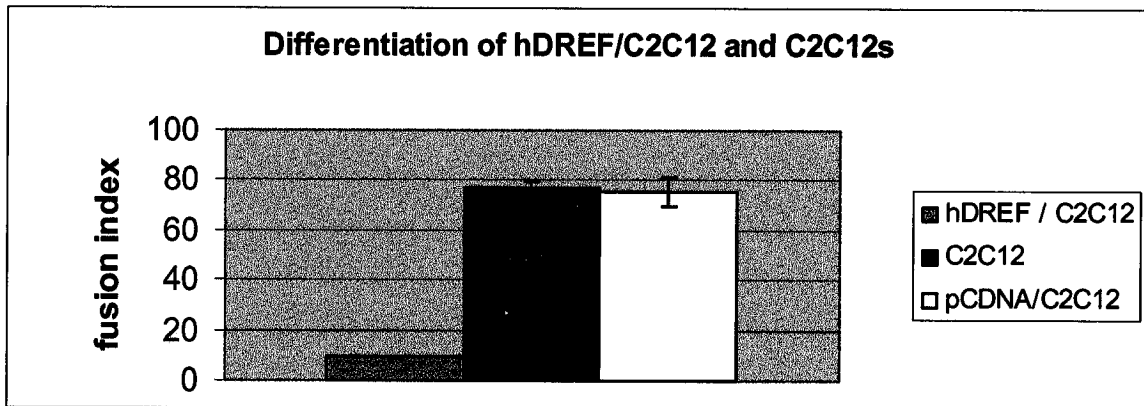


multiple nuclei (>2), and did not form typical elongated myotubes. Further analysis of the hDREF / C2C12 clone revealed that after five days in low serum media, 41 ± 1 % of the population remained undifferentiated and did not express MF20. Ten percent, plus or minus, one percent of the cell population had formed myotubes which expressed MF20. However a large proportion of the hDREF / C2C12 population displayed the unusual multinucleated phenotype. 22 ± 1 % of the hDREF / C2C12 cell population expressed MF20 and had become multinucleated with 2-10 nuclei per cell, 21 ± 1 % expressed MF20 and had become multinucleated with 11-15 nuclei per cell and finally 6 ± 1 % expressed MF20 and had become multinucleated with 16-20 nuclei per cell (Figures 21 B).

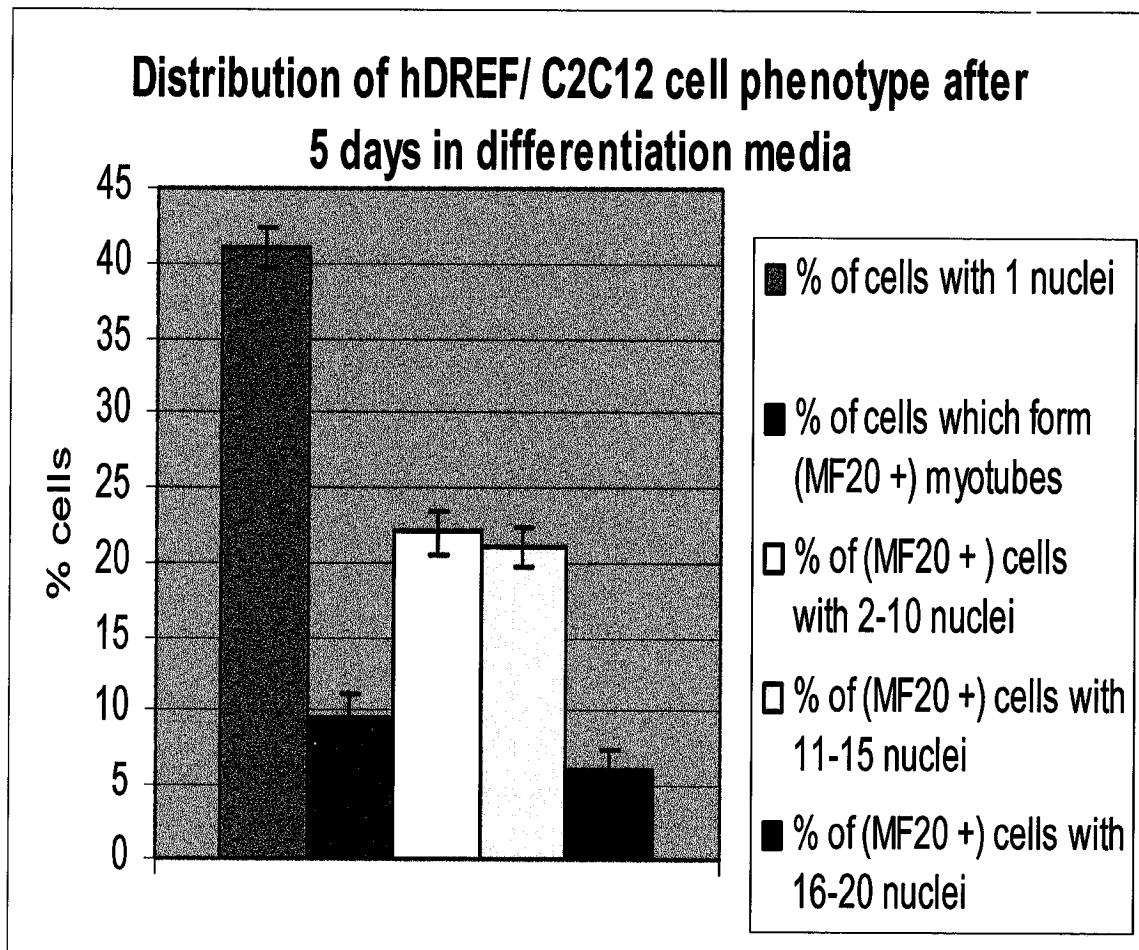
To further characterize the development of the multinucleated cells, the hDREF/ C2C12 cells were grown in differentiation media and photographed over a 5-day time course (Figure 22). Hematoxylin and DAPI nuclear staining of the hDREF / C2C12 cells revealed that several cells were multinucleated. Nevertheless immunocytochemistry indicated that these cells were also expressing markers of differentiation i.e. myosin heavy chain (Figure 22). These observations suggest that hDREF overexpression in C2C12 myoblasts leads to continued DNA replication in cells that also express markers of differentiation.

Figure 21: hDREF /C2C12 cells form multinucleated bag like cells after 5 days in differentiation media **A)** Percentage of hDREF / C2C12 cells and C2C12 cells forming myotubes after 5 days in low serum media. Each bar represents the average of three different cell counts **B)** Distribution of cell phenotypes seen in the hDREF / C2C12 population after 5 days in differentiation media.

A)



B)



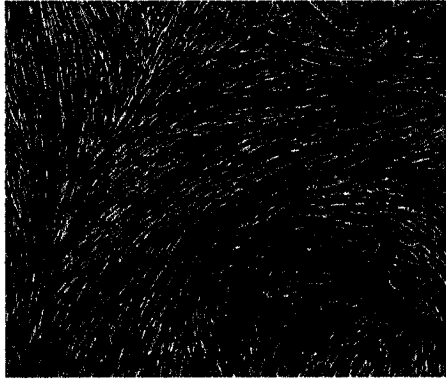
3.11 Continued DNA replication in differentiated hDREF / C2C12 myoblasts.

To determine if hDREF / C2C12 cells were capable of continued DNA synthesis under differentiation conditions, BrdU incorporation assays were carried out. Cells were incubated for 3 days in low serum media, and on the third day the media was replaced with fresh low serum media that contained BrdU. Cells were then cultured for an additional 2 days. The hDREF / C2C12 overexpressing cell line continued incorporating BrdU, whereas the control C2C12 cells had incorporated minimal amounts of BrdU (Figure 23). These results suggest overexpression of hDREF leads to aberrant and continued DNA replication conditions that would normally prevent growth of muscle cells.

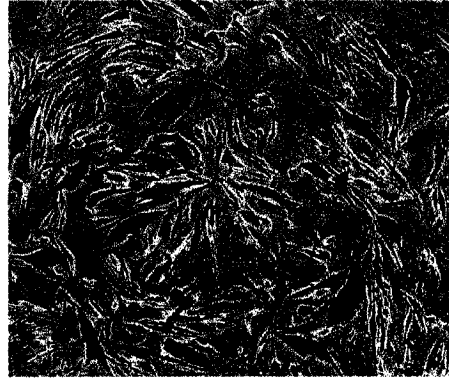
3.12 hDREF / C2C12 cells have a growth advantage and an accelerated death rate in differentiation media.

To determine if hDREF overexpression also affected the rate at which cells completed the mitotic cycle, the growth rates of the hDREF / C2C12 and untransfected C2C12 cell lines were assayed under growth and differentiation conditions. It was found that untransfected and transfected cells appear to grow and die at similar rates under growth conditions (Figure 24 A& B). However in differentiation media, where the effects of overexpressing hDREF seem to be most profound, hDREF / C2C12 cells not only had a slight growth advantage, but also died at an accelerated rate. The growth advantage seen with the hDREF / C2C12 cells suggests that overexpression of hDREF in myoblasts results in the cells completing successively more rounds of mitosis. Interestingly, the rate at which cells were dying was also increased. These results were in contrast to those

Figure 22: hDREF / C2C12 cells form multinucleated bag like cells after 5 days in differentiation media. C2C12 cells (A) and hDREF / C2C12 cells (B-E) were cultured for 5 days in differentiation media, fixed and stained with hematoxylin and MF20. F) hDREF / C2C12 were cultured for 5 days in differentiation media, fixed and stained with DAPI.



A) C2C12 DAY 5 100X Mag.



B) C2C12/ hDREF DAY 5 100X Mag.



C) C2C12/ hDREF DAY 5 1000X Mag.



D) C2C12/ hDREF DAY 5 1000X Mag.



E) C2C12/ hDREF DAY 5 1000X Mag.



F) C2C12/ hDREF DAY 5 1000X Mag.

Figure 23: After 5 days in differentiation media hDREF / C2C12 cells continue to incorporate BrdU. Cells were incubated for a total of 5 days in low serum media. On the third day of culture the media was replaced with fresh media containing BrdU. Nuclei were stained with DAPI. After 5 days in differentiation media hDREF / C2C12 clone continued incorporating BrdU and replicate DNA (middle panel), whereas untransfected C2C12 cells did not (-control: lower panel). In contrast, untransfected C2C12 myoblasts cultured in growth media readily incorporate BrdU (+ control: upper panel). BrdU incorporation was visualized through detection with an anti-BrdU antibody (Amersham) conjugated to FITC and the fluorescence Ziess microscope.

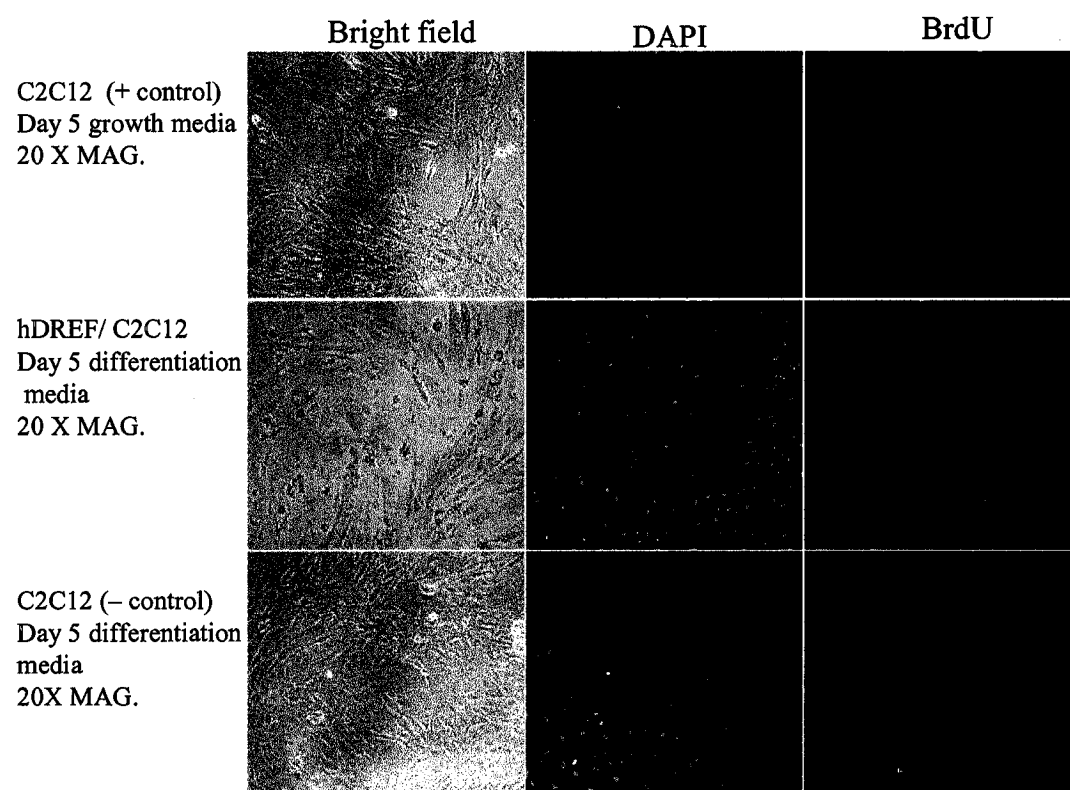
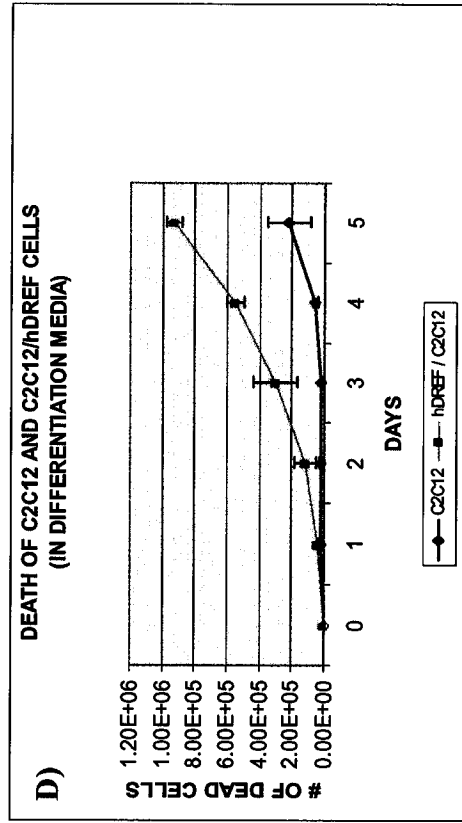
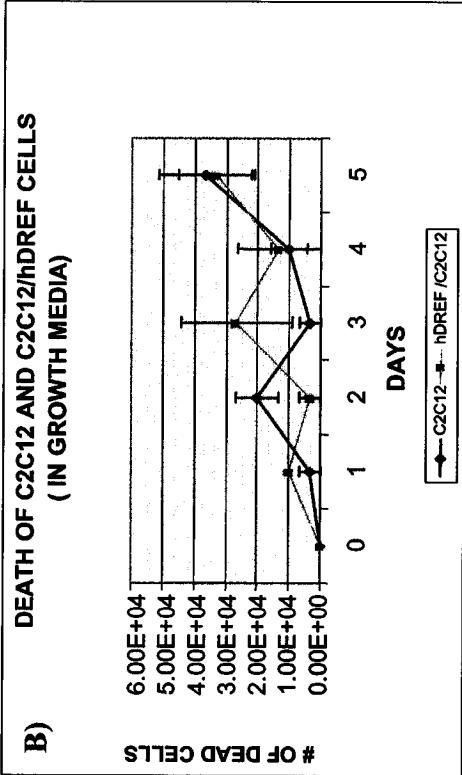
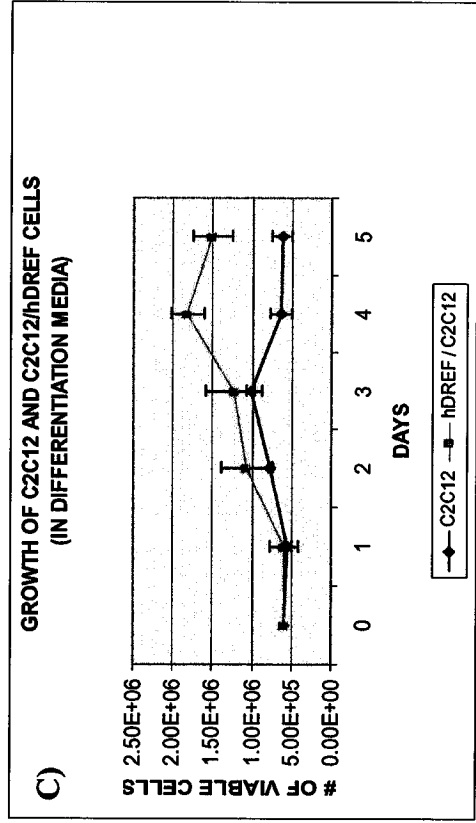
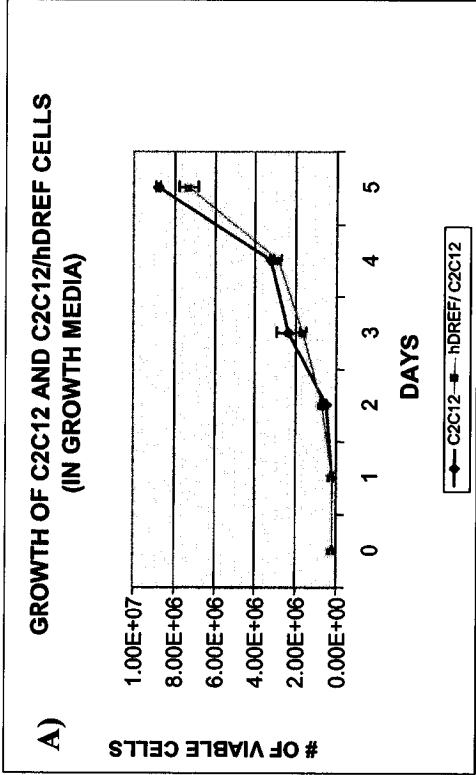


Figure 24: hDREF/C2C12 cells and untransfected C2C12 cells grow and die at the same rate in growth media. However, hDREF / C2C12 have an increased rate of growth and an increased death rate in differentiation media. 2.0×10^5 cells were plated on day 0 of growth and 6.0×10^5 cells were plated on day 0 of differentiation media. Three separate plates of cells were counted with a hemacytometer for each time point, and the results averaged. Error bars indicate the degree of variation from the mean from each of the three trials. **A)** Growth of C2C12 and hDREF / C2C12 cells over 5 days in growth media. **B)** Death rate of C2C12 and hDREF / C2C12 cells over 5 days in growth media. **C)** Growth of C2C12 and hDREF / C2C12 over 5 days in differentiation media. hDREF / C2C12 cells have an increased growth rate over untransfected cells **D)** Death rate of C2C12 and hDREF / C2C12 cells over 5 days in differentiation media. hDREF / C2C12 cells have an increased rate of death, over untransfected cells.



found in the hDREF / H9C2 cell lines where hDREF overexpression did not lead to any observable increase in the growth rate of cells. These data indicate that while hDREF is involved in regulating the S-phase of the cell cycle, it may also be involved in regulating the mitotic cycle in specific cell lineages. The P values for the C2C12 vs hDREF / C2C12 cells are summarized in tables 10,11,12,13 of the appendix. These results were also confirmed using flow cytometry analyses. Cells were cultured for 5 days in growth or differentiation media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts. Flow cytometry results from the hDREF / C2C12 and C2C12 cells cultured in growth media and counted as living revealed, 69 % and 69 % (respectively) of the population were viable , while approximately 30 % of both population were dead (Figure 25 A &B and table 14). Due to the fact that the Coulter Counter FACS machine produces sorts which can have as much as 30 % variability, a reading were 30% of the population, which had been counted as viable was actually dead, was considered acceptable. These results therefore suggest that in the hDREF / C2C12 and C2C12 cell counts the majority of cells that were counted as living were indeed viable cells. Therefore based on the cell count and flow cytometry data, the hDREF / C2C12 cells have no growth advantage over untransfected C2C12 cells when cultured in growth media. Flow cytometry was also performed on the non-viable cell populations that had been culture in growth media. From the hDREF / C2C12 and C2C12 cell populations that had been counted as dead, flow analysis revealed that 53 % and 51 % (respectively) of the cells were apoptotic or dead (Figure 25 C & D and table 15). Thus flow cytometry analysis of the non-viable cell populations indicated that the rates of cell death were

overestimated, due to non adherent living cells being counted as non-viable. Despite this overestimation, the combined analyses of cell counts and flow cytometry did reveal similar trends i.e. that hDREF / C2C12 and untransfected C2C12 cell populations die at a similar rate in growth media.

Flow cytometry was next conducted on hDREF / C2C12 cells and untransfected C2C12 cells, cultured in differentiation media for 5 days. From the population of hDREF / C2C12 and C2C12 cells counted as living it was found that 82.3 % and 78.0 % (respectively) were indeed viable (Figure 26 and table 16). These results indicate that the trends observed in the cells counts were accurate, and that hDREF /C2C12 myoblasts have a growth advantage over untransfected cells when cultured in differentiation media. Flow cytometry was also conducted on the non-viable cell population that had been cultured in differentiation media. From the population of hDREF / C2C12 and C2C12 cells counted as dead it was found that 80 % and 27 % (respectively) of those populations were apoptotic or dead (Figure 26 and table 17). The results indicate that the majority of hDREF / C2C12 cells that were counted as non-viable were indeed dead or apoptotic. However, flow cytometry analysis of the untransfected C2C12 cell population, indicated that the majority of the cells in that population were in fact non-adherent viable cells, indicating that the death rate was greatly over estimated. Taking into account both cell count data and FACS analysis data it can be suggested that hDREF / C2C12 myoblasts display an increased degree of apoptosis compared to wild type myoblasts when cultured under differentiating conditions

Figure 25: A-D) The hDREF / C2C12 cells do not have increased growth or death rates when cultured in growth conditions. Cell count data was used to determine the number of cells that were viable or dead, in a given population. Flow cytometry ensured that cells which had been counted as living were viable, and those which had been counted as dead were non viable. Cells were cultured for 5 days in differentiation media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts.

Table 14 & Figure 25: hDREF / C2C12 myoblasts have no growth advantage over untransfected C2C12 cells when cultured in growth media. A) 69 % of the hDREF / C2C12 cells cultured in growth media and counted as living, were found to be viable cells, by flow analysis. B) 69 % of the C2C12 cells cultured in growth media and counted as living were found to be viable cells by flow cytometry.

Table 15 & Figure 25: hDREF / C2C12 cells did not have an increased death rate over untransfected C2C1 cells cultured in growth media. C) Of the hDREF / C2C12 cells cultured in growth media and counted as dead, 53 % were dead or apoptotic. D) In the C2C12 cells cultured in growth media and counted as dead, 51% were dead or apoptotic.

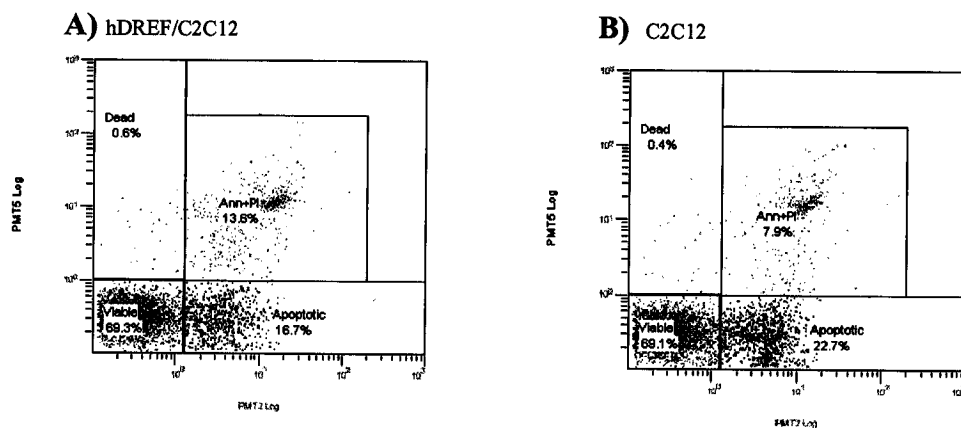


Table # 14: FACS analysis of the cells counted as alive, cultured in growth media.

5 days growth media	Dead = annexin + propidium + annexin and propidium)	Viable
A) hDREF/C2C12	0.6 % + 13.6 % + 16.7 % = 30 %	69 %
B) C2C12	0.4 % + 7.9 % + 22.7 % = 31 %	69 %

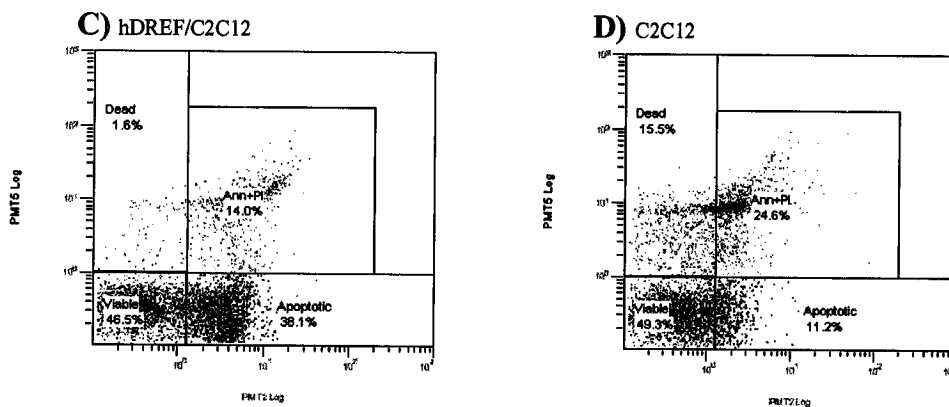


Table # 15: FACS analysis of the cells counted as dead, cultured in growth media.

5 days in growth media	Dead = annexin + propidium + annexin and propidium)	Viable
C) hDREF/C2C12	1.6 % + 14.0 % + 38.1 % = 54 %	47 %
D) C2C12	15.5 % + 24.6 % + 11.2 % = 51 %	49 %

Figure 26: A-D) hDREF / C2C12 cells have growth advantage, and increased death rate over that of untransfected C2C12 cells in differentiation media. Cell count data was used to determine the number of cells that were viable or dead, in a given population. Flow cytometry ensured that cells which had been counted as living were viable, and those which had been counted as dead were non viable. Cells were cultured for 5 days in differentiation media, stained with annexin V-FITC / propidium iodide (PI) and sorted using flow cytometry. The day 5-time point was used as an indicator of accuracy for all other cell counts.

Table 16 & Figure 26: A) Of the hDREF /C2C12 cells cultured in differentiation media and counted as living, 82 % were viable. B) In the C2C12 cells cultured in differentiation media and counted as living, 78 % were viable.

Table 17 & Figure 26: C) Of the hDREF /C2C12cells cultured in differentiation media and counted as dead, 80 % were dead. D) In the C2C12 cells cultured in differentiation media and counted as dead, only 27 % were in fact dead or apoptotic cells.

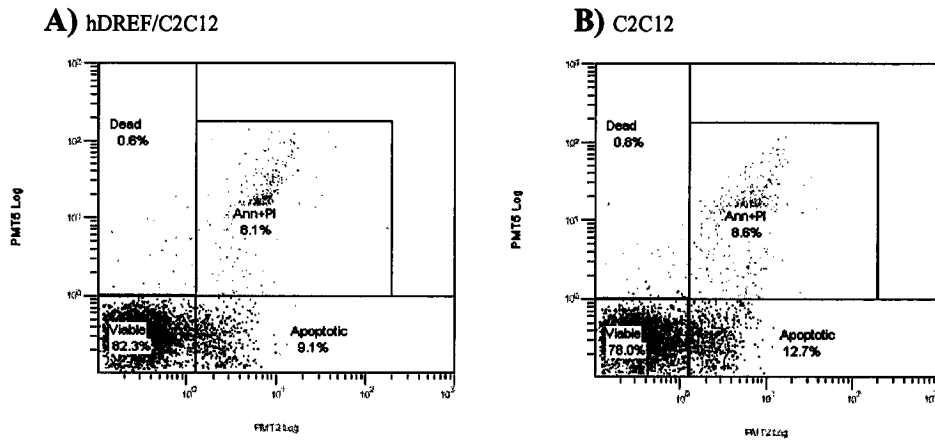


Table # 16: FACS analysis of the cells counted as alive, cultured in differentiation media.

5 days in differentiation media	Dead = annexin +propidium + annexin and propidium)	Viable
A) hDREF/C2C12	0.6 % + 8.1 % + 9.1 % = 18 %	82 %
B) C2C12	0.8 % + 8.6 % + 12.7 % = 22 %	78%

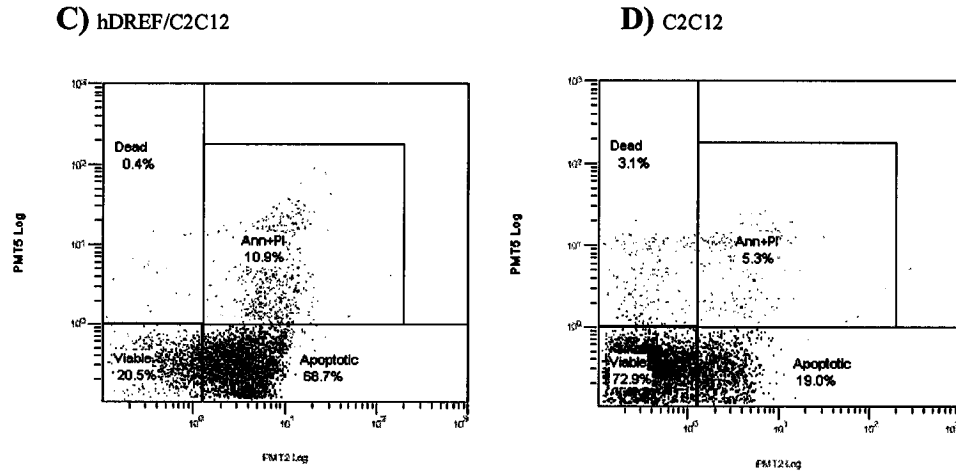


Table # 17: FACS analysis of the cells counted as dead cultured in differentiation media.

5 days in differentiation media	Dead = annexin +propidium + annexin and propidium)	Viable
C) hDREF/C2C12	0.4 % + 10.9 % + 68.7 % = 80%	21 %
D) C2C12	3.1 % + 5.3 % + 19.0 % = 27%	73 %

3.13 Single hDREF/ C2C12 exhibit endoreduplication and become multinucleated.

The unusual phenomenon observed in the multinucleated bag like cells appeared to be endoreduplication, i.e. the replication of DNA in the absence of cell division. To further examine this phenomenon, serial dilution experiments were conducted. The hDREF / C2C12 cells, which had been previously been found to replicate their DNA and become multinucleated during differentiation, were serially diluted to low density culture conditions (approximately 20 cells / 10 cm petri plate). This low density resulted in an effective isolation of single cells. Over 100 individual cells were tracked over a 5-day differentiation time course. Of those cells that were tracked, 4 became multinucleated. Figure 27 A illustrates one cell over 5 days in differentiation media becoming multinucleated. hDREF / C2C12 cells were also plated at a density of one cell/well. Tracking the development of 100 single cells (one cell / well) over 5 days in differentiation media revealed one cell that initially had one nuclei, but after 5 days had replicated its DNA such that 5 individual nuclei were present in the cell (Figure 27 B). These results suggest that hDREF overexpression results in a propensity for continued DNA replication and does not simply result in cells that are incapable of normal fusion to form myotubes.

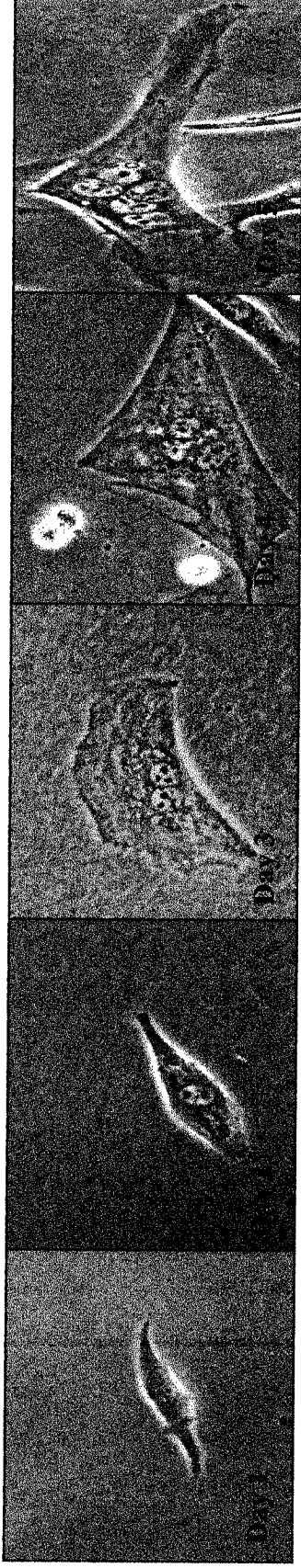
3.14 hDREF/ C2C12 multinucleated cells express markers of differentiation.

In order to access the degree to which the hDREF / C2C12 cells differentiate, the cells were grown in differentiation media for 5 days and stained for markers of muscle differentiation including myogenin, MEF2C and myosin heavy chain (Figures 28 & 29).

Staining of the cells revealed that the multinucleated hDREF / C2C12 cells expressed all three markers of differentiation. These results indicate the overexpressing hDREF / C2C12 cells continue to replicate DNA, yet simultaneously express markers of differentiation. Moreover, these observations suggest that overexpression of hDREF does not inhibit the activity of muscle transcription factors. Nevertheless, myotube formation was impaired during periods of elevated hDREF expression indicating that hDREF is capable of regulating certain stages in the differentiation process.

Figure 27: A) hDREF/C212 cells become multinucleated over 5 days in differentiation media. Cells were plated at a low density and an individual hDREF / C2C12 cell was tracked over 5 days in differentiation media. B) Cells were plated at a density of one cell per well. An individual hDREF / C2C12 cell was tracked over 5 days in differentiation media becomes multinucleated. After 5 days the multinucleated cell was fixed and stained with hematoxlin

A)



B)

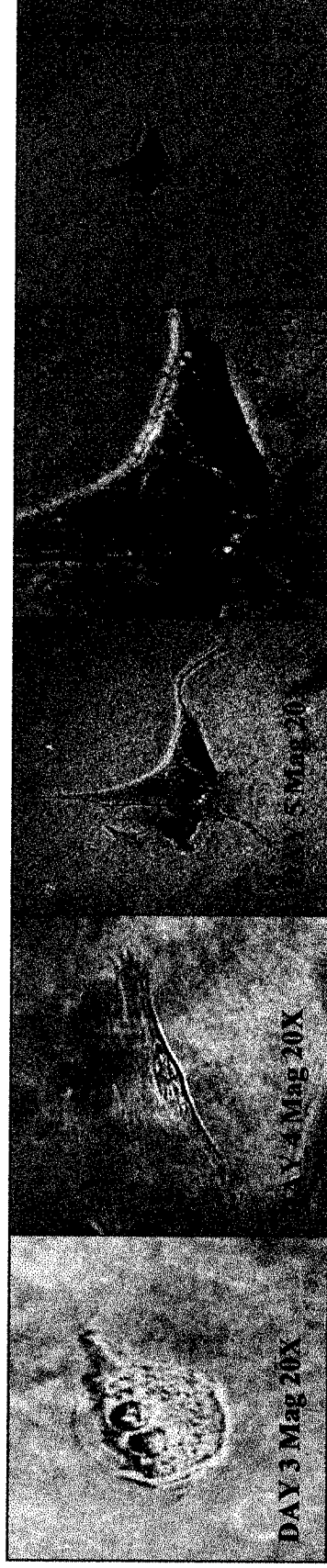
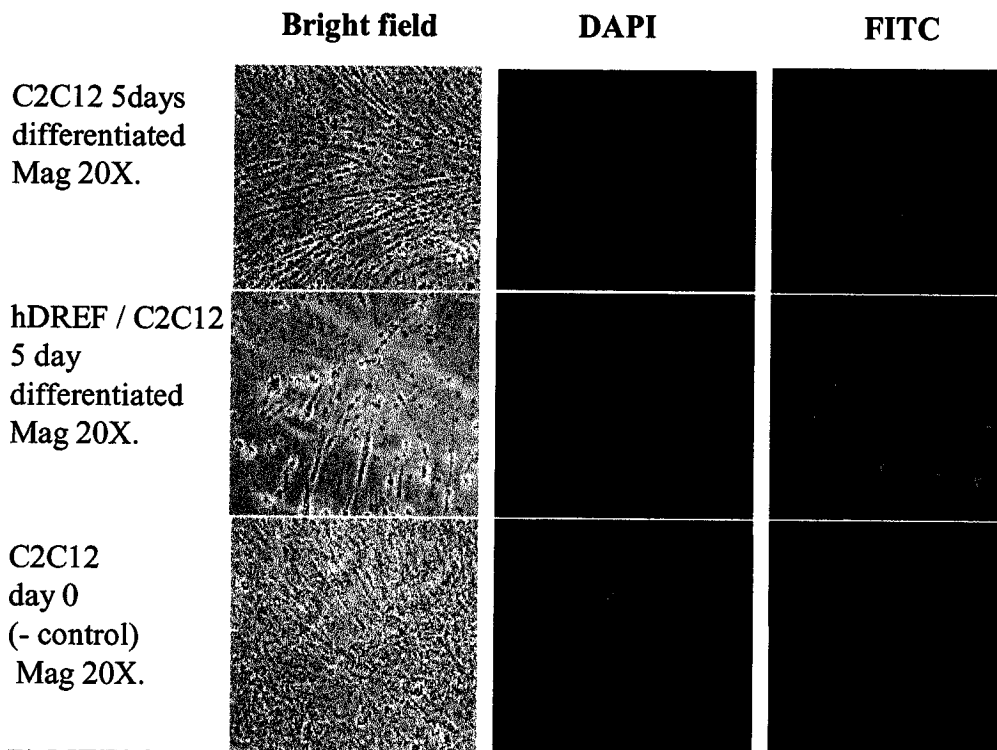


Figure 28: hDREF/ C2C12 multinucleated cells express markers of differentiation.

hDREF / C2C12 cells cultured in differentiation media for 5 days express myosin heavy chain and MEF2C. hDREF /C2C12 cells were fixed and stained with an anti myosin heavy chain (Developmental Studies Hybridoma Bank) or MEF2C (Cell Signaling Technology) and a sheep anti mouse conjugated to FITC (Stressgen) secondary antibody. DAPI was added to the cells to stain nuclei. Mag 20X.

A) Myosin Heavy Chain



B) MEF2C

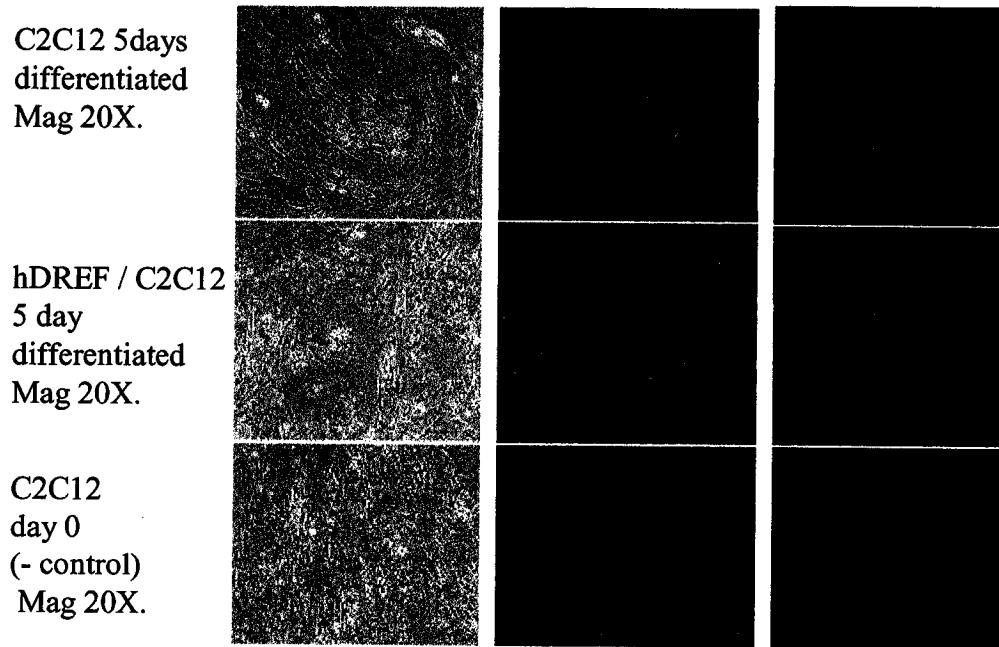
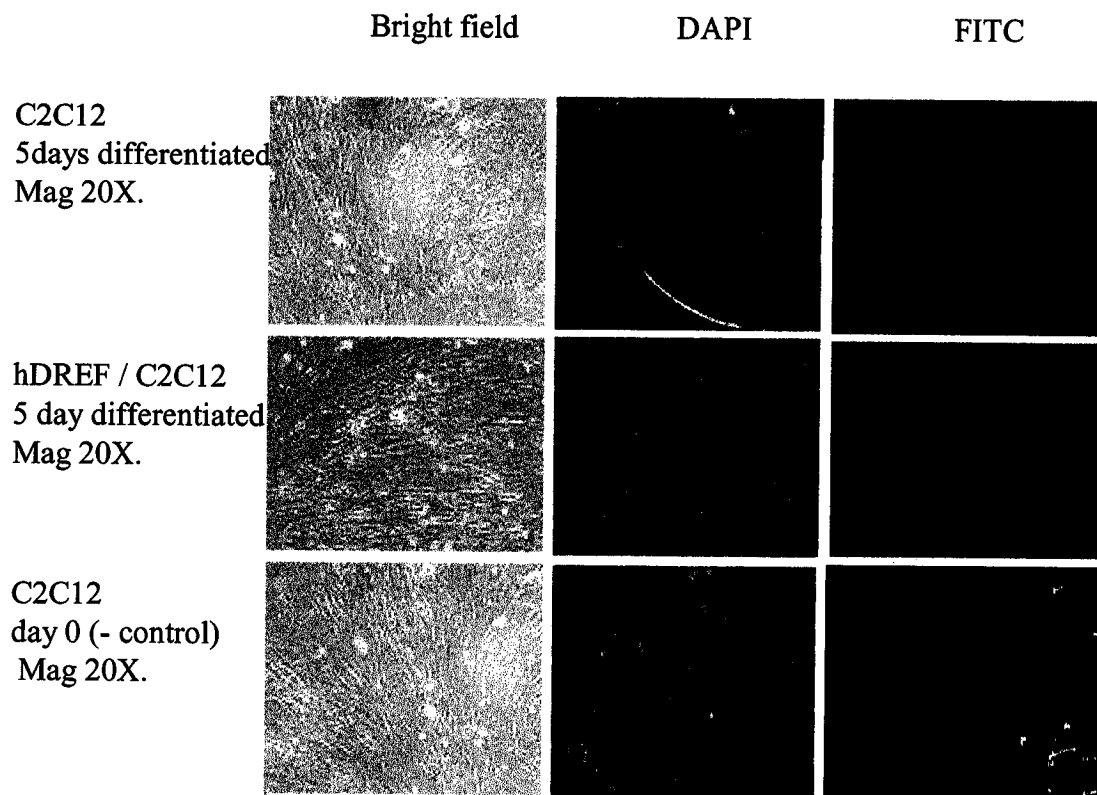


Figure 29: hDREF/ C2C12 multinucleated cells express markers of differentiation.

hDREF / C2C12 cells cultured in differentiation media for 5 days express myogenin. hDREF /C2C12 cells were fixed and stained with an anti myogenin (Developmental Studies Hybridoma Bank) and a sheep anti mouse conjugated to FITC (Stressgen) secondary antibody. DAPI was added to the cells to stain nuclei.

Myogenin



Chapter 4

4.0 Discussion

4.1 Summary of observations.

Experimental observations suggest that hDREF expression is negatively regulated by MEF2 during differentiation. Endogenous expression studies indicated that during times of high MEF2 activity / expression, hDREF expression was downregulated. Results from the chromatin immunoprecipitation assay indicated that the MEF2 proteins bind to their consensus binding site in the 5'UTR of hDREF, regardless of culture conditions.

Expression studies with the 5' and 3'UTR/GFP reporter constructs indicated that GFP expression was significantly downregulated during differentiation. The observed downregulation was mediated through the MEF2 binding sites which, when mutated resulted in unregulated hDREF expression irrespective of culture conditions. Taken together, these data suggest that hDREF is a MEF2-regulated gene, and that MEF2 acts to negatively regulate hDREF expression during differentiation. Functional studies revealed that overexpression of hDREF in skeletal and cardiac cells led to a propensity for continued DNA replication, resulting in polyploidy. Overexpression of hDREF in skeletal muscle myoblasts, but not cardiomyocytes, resulted in an increased growth and death rate indicating that hDREF may have tissue specific function. Interestingly, it was also found that the multinucleated hDREF/C2C12 cells expressed markers of differentiation when cultured in differentiation media. Data from functional studies suggests that hDREF is intimately involved in promoting DNA synthesis.

4.2 MEF2: a global regulator of cell fate.

The idea that one transcription factor (MEF2) is capable of inducing cellular differentiation by upregulating genes involved in differentiation, while at the same time acting to suppress genes involved in the mitotic cell cycle and DNA synthesis is an attractive theory. The results from the expression studies indicated that during periods of elevated MEF2 activity, hDREF was downregulated. Results from the ChIP assay indicated that the MEF2 protein is associated with its binding site found in the 5'UTR of hDREF during both growth and differentiation. These observations suggest that a change in MEF2 activity is responsible for the repression on hDREF expression during differentiation. While the ChIP assay was not conducted on the MEF2 binding sites found in the 3'UTR of hDREF, I predict that these sites would also bind the MEF2 proteins under both culture conditions. The UTR / pGLOW reporter assay experiments confirmed that hDREF repression was mediated through the MEF2 binding site found in the UTRs of hDREF, as mutations in the MEF2 binding sites resulted in a loss of repression. Taken together these results indicate that the MEF2 is capable of acting as a negative regulator and acts to repress the expression of a putative regulator of DNA synthesis (hDREF).

4.2.1 MEF2 as a negative regulator of hDREF expression.

Although it is apparent that MEF2 is capable of negatively regulating hDREF expression *in vitro*, what remains unclear is the mechanism by which this is achieved. One probable co-repressor of hDREF expression are the class II HDACs. These proteins have been shown to associate with DNA bound-MEF2 proteins and elicit a decrease in MEF2-

dependent gene expression (Lu et al., 2000a; Lu et al., 2000b; Zhang et al., 2002). Interestingly class II HDACs are most highly expressed in the same tissues as MEF2, that including neurons, skeletal muscle and cardiac muscle (Lu et al., 2000b). HDAC-mediated suppression of MEF2 is relieved by CaMKI and CaMKIV induced phosphorylation of HDAC proteins (McKinsey et al., 2002). This directed phosphorylation event allows for HDAC disassociation from MEF2, followed by 14-3-3 mediated nuclear exclusion (Lu et al., 2000a; Zhang et al., 2002; Zhang et al., 2001a).

4.2.2 MEF2-associated HDACs. A transcriptional repressor complex for hDREF?

If the class II HDAC proteins are indeed the cofactors responsible for repression of hDREF expression, then inhibition of HDAC activity should relieve this repression. To test this hypothesis, HDAC sensitive drugs such as Trichostatin A or Leptomycin B could be added to the cells transfected with the 3' and 5' UTR / GFP reporter constructs. Addition of Trichostatin A to cells results in an inhibition of HDAC deacetylase activity, which permits chromatin to remain in a transcriptionally active form (Yoshida and Horinouchi, 1999). Continued treatment of cells with Trichostatin A results in cell cycle arrest and subsequent apoptotic death (Yoshida and Horinouchi, 1999). Leptomycin B binds to the nuclear export protein CRM1 and results in an inhibition of cargo loading and subsequent nuclear export, leading to HDAC retention (Yoshida and Horinouchi, 1999). The inability to shuttle HDACs out of the nucleus results in constitutively deacetylated chromatin, which remains transcriptionally inactive. Through the addition

of these compounds one could monitor any putative repressive effects of the HDAC / MEF2 complex on hDREF expression.

While the HDAC/ MEF2 mediated interaction does represent a possible mechanism of repression, this theory is not without limitations. For example, HDACs have been shown to dissociate from MEF2 and are mostly cytoplasmic during skeletal muscle differentiation (Zhang et al., 2001a). Lu et al. (2000) demonstrated that HDACS remain associated with MEF2 and specifically repress myoblast differentiation during growth. However, during differentiation HDACs are shuttled out of the nucleus and chromatin associated with the muscle specific genes becomes re-acetylated and transcriptionally active. Therefore, it is unlikely that HDACs are responsible for the repression of hDREF expression at a time when their activity is low (i.e. during differentiation). However, we cannot entirely discount a role for HDAC proteins prior to conducting the appropriate experiments. Indeed HDACs remain the only proteins identified to date that have been shown to physically interact with MEF2 and inhibit MEF2-dependent transcription.

4.2.3 MEF2-associated proteins and transcriptional repressor complexes.

Results from the ChIP assay suggest that the repressive effects that MEF2 proteins exert on hDREF expression is primarily mediated through the MEF2C protein as it 1) readily binds to the MEF2 binding site found in the 5'UTR of hDREF and 2) is the predominant isoform expressed during skeletal muscle differentiation. This, however, does not

exclude other MEF2 family members from heterodimerizing with MEF2C and forming the basis of a repressive complex. Ornatsky and McDermott (1996) showed that transcriptional activity of MEF2C, and A are dependent on their heterodimerization with MEF2D. In this model, hDREF suppression may be mediated through the assembly or disassembly of monomeric and heterodimeric forms of MEF2. One way to test this hypothesis would be to differentially express the MEF2 proteins in hDREF expressing stable cell lines. Briefly, stable clones, which express the 5' or 3' UTR reporter constructs could be generated using cells which do not express MEF2 (e.g. COS cells). These stable cell lines could then be transiently transfected with various combinations of MEF2A, B, C or D and the GFP reporter expression monitored. The results from this assay would permit an evaluation of which MEF2 family members are required in combination, to mediate hDREF repression.

One other possibility is that hDREF expression is regulated through steric hindrance of the putative promoter regions. In this model, the hDREF sequence is kept in a transcriptionally accessible form during growth. Subsequently, the binding of MEF2 with the appropriate co-factors leads to physical or structural hindrance for the complex (es) which normally engage hDREF expression. This theory fits with the finding that the MEF2 protein binds to its consensus binding sequence in the 5'UTR of hDREF irrespective of the culture conditions, and that it is only during differentiation that MEF2 mediates a repressive effect on hDREF expression.

It is also possible that MEF2 acts as an “anchor – like protein” which is capable of binding both co-activators and co-repressors thereby bring them into proximity with hDREF. In this scenario the associated MEF2 binding partner would be dependent on the temporal status of the cell cycle, on the culture conditions and signals that the cell received from its environment. The subsequent heterodimer formation with DNA would facilitate the ability of a cofactor(s) to repress / enhance hDREF expression.

4.2.4 MEF2 as a positive regulator of hDREF expression.

Although the MEF2 protein is bound to its binding site found in the 5'UTR of hDREF during growth and differentiation, my observations suggest that it is only during differentiation that MEF2 exerts a repressive effect on hDREF expression. The question that remains is; what purpose does MEF2 binding serve during cell growth? It may be that MEF2 acts alone or in concert with co-activators to upregulate hDREF expression. While the identity of putative co-factors remains unknown, it has previously been shown that MEF2 transcriptional activity is upregulated by phosphorylation of the transactivation domain by mitogen activated protein kinases (MAPKs) ERK5 and p38 (Lu et al., 2000b). MEF2 has also been shown to physically interact with GATA4 and MyoD in cardiac and skeletal muscle respectively, resulting in an upregulation of MEF2 dependent gene expression (Molkentin et al., 1995; Morin et al., 2000). Histone acetyl transferases (HATs) have also been shown to increase MEF2 activity by direct physical association with MEF2. Once bound to MEF2 they act to re-acetylate the surrounding histones and relax chromatin structure (Sartorelli et al., 1997).

However, results from the 3' and 5'UTR / GFP mutagenesis assays suggest that while MEF2 is bound to its binding site during growth, it is not required to facilitate hDREF expression, as mutation of this site did not result in a decrease in expression. Mutation of the MEF2 binding sites did however lead to a loss of hDREF repression under differentiation conditions.

4.3 Endoreduplication and hDREF expression.

It is somewhat paradoxical that as the maturing heart begins to grow using hypertrophic growth strategies (which include endoreduplication) there is a subsequent decrease in hDREF expression, which we have shown induces endoreduplication when overexpressed. One possible explanation is that the endoreduplication seen during cardiac hypertrophy is the result of a hDREF independent pathway. It may be that hDREF is involved in regulating DNA synthesis solely within the confines of the cell cycle, and it is only when hDREF is overexpressed does it result in aberrant DNA synthesis in post-mitotic cells. Alternatively, the decrease in hDREF expression may be the result of some regulatory mechanism which allows for the low levels of hDREF to signal for continued DNA synthesis in differentiated cells. The third possibility is that at the onset of the endocycle the high levels of hDREF present in the cell at that time are sufficient to signal for several rounds of DNA synthesis, in the absence of any further hDREF expression. Despite the numerous possible explanations for this phenomenon, the mechanisms that regulate this complex cascade of events remain unclear.

4.4 hDREF a regulator of DNA synthesis.

Data from functional experiments indicated that overexpression of hDREF in skeletal and cardiac muscle led to aberrant DNA synthesis during differentiation. Interestingly overexpression of hDREF led to no observable phenotype when cells were cultured in growth media. The absence of an observable phenotype when cells were cultured in growth media may be due to the fact that during growth hDREF is already highly expressed, and further expression has no effect on an already saturated cell cycle. However, during differentiation, when endogenous hDREF expression is normally downregulated, forced expression of hDREF results in the observed DNA replication and endoreduplication.

The experimental data suggest that hDREF functionally parallels the *drosophila* protein DREF. In *drosophila* the DRE binding site has been found in the promoter regions of many DNA replication related genes, including PCNA, E2F, Raf, DNA polymerase, cyclin A and TBP (Hirose et al., 1996; Hirose et al., 1994; Hirose et al., 1999). Ectopic expression of DREF results in continued DNA synthesis, cell cycling and endoreduplication resulting in the development of polyploidy (Hirose et al., 2001; Hirose et al., 1996; Hirose et al., 1999). Inhibition of DREF in developing *drosophila* resulted in a greater than 50 % mortality rate, a decrease in DNA polymerase and PCNA expression as well as a decrease in DNA synthesis (Hirose et al., 2001; Hirose et al., 1996; Hirose et al., 1999). Furthermore, DREF has also been found to act as an antagonist to the BEAF (boundary element associated factor), which is involved in the boundary activity of the

special chromatin structure (scs) region of the *drosophila* hsp70 gene (Hart et al., 1997; Hirose et al., 2001). This research suggests that DREF may also be involved in regulating chromatin structure and may represent a putative chromatin remodeling protein.

I propose that hDREF is likely the human ortholog of DREF. This supposition is based on the known function of DREF as a regulator of DNA synthesis in both the cell cycle and endocycle and the results obtained from the hDREF overexpression experiments. hDREF has most likely diverged in sequence from DREF in regions of limited functional significance and remained homologous in the regions necessary for its activity. Defining the human consensus-binding site and target genes of hDREF will be of the utmost importance for providing the necessary evidence for this theory.

4.5 The regulation of DREF in *Drosophila*.

Although little is known about the mechanisms which control DREF, some candidate genes do exist which are capable of regulating DREF expression or the ability of the DREF protein to bind to its target sequence. Hirose et al. (1994) found that the *zerknult* (*Zen*) protein, a homeobox gene that regulates the differentiation of the dorsal root of the *drosophila* embryo and the optic lobe, was also capable of regulating DREF activity (Hirose et al., 1994). Although *Zen* does not bind to the DRE sequence itself, overexpression of the *Zen* protein results in a reduction of DREF activity (Hirose et al.,

1994). Based on these observations, it was hypothesized that the Zen protein binds to some regulatory element in the promoter region of DREF and downregulates expression at the transcriptional level (Hirose et al., 1994). More recently, DREF has been shown to interact with the *drosophila* Mi-2 protein (Hirose et al., 2002). The Mi-2 protein belongs to the chromatin helicase DNA binding family and forms part of a chromatin remodeling complex which has histone deacetylase activity (Hirose et al., 2002). Gel shift assays showed the dMi-2 protein inhibits DREF DNA binding activity, but does not dislodge DRE bound DREF (Hirose et al., 2002). Ectopic expression of DREF in the eye imaginal disc results in a rough eye phenotype that could be reversed by the expression of dMi-2 (Hirose et al., 2002). These results suggest that DREF activity is negatively modified by this protein-protein interaction.

4.6 Future work on hDREF.

Based on the functional similarities observed between hDREF and DREF, I propose that these putative orthologs target similar if not identical genes. Candidate genes such as E2F, PCNA, TBP, cyclin A and DNA polymerase are all DREF sensitive and are intimately involved in regulating cell cycle progression and DNA synthesis. As such, these genes represent likely candidates for hDREF regulation. Northern blot analysis of candidate gene expression in the wild type and overexpressing cell lines would be the first step in determining if these genes are regulated by hDREF. Based on the existing research, I would predict that hDREF dependent genes would continue to be expressed during differentiation in the hDREF overexpressing cell lines. Putative targets of hDREF

may also be identified using micro-array gene-ChIP technology. Using total RNA collected from wild type and hDREF overexpressing cell lines, relevant hDREF targets could be identified simultaneously. Once putative hDREF regulated genes have been identified, Northern blot analysis would be conducted in order to verify the observed increase/ decrease in gene expression.

Presuming that hDREF maintains transcription factor activity, it will be of the utmost importance to define the human consensus binding sequence for hDREF. Using the existing DRE sequence (TATCGATA), gel shift assays could be used to determine the consensus sequence that is required for hDREF binding. Preliminary evidence suggests that the 5'promoter of human PCNA contains a DRE-like binding sequence (TACTGATA) in which two nucleotides have been inverted. Furthermore, PCNA expression was upregulated in the overexpressing hDREF / H9C2 cell lines which have been cultured in differentiation conditions. These preliminary observations are encouraging and suggest that the human PCNA gene may be a functional transcription target for the hDREF protein (data not shown).

Another approach to characterizing hDREF function would be to make use of serial deletion constructs of hDREF. Direct comparison of these deletion constructs to full length hDREF in transfected cells would effectively define the domains which are critical for hDREF activity. A similar approach could then be utilized in animal models. For

example, a dominant negative transgenic animal that expresses only the N-terminal domain of hDREF (which, in dDREF contains the homodimer and DNA binding domains) could be used as a means of assessing hDREF function in vivo. Assuming analogous function between DREF and hDREF the N-terminal domain would compete with endogenous hDREF for binding sites found in the regulatory regions of hDREF regulated genes. Using this approach, one could monitor any alteration in the phenotypic consequences that have been attributed to hDREF.

Preliminary data also suggests that the 80 kDa hDREF protein migrates at approximately 300 kDa in a non denaturing native gel. These observations suggest that the hDREF protein may associate as a heterodimer or may form part of a larger multi-protein complex. As previously mentioned, Hirose et al. (2002) found that the *drosophila* protein Mi-2 forms a complex with unbound DREF protein, and inhibits DREF dependent transcription. However, it is possible that the DREF protein has an alternative function within the Mi-2 complex, which has previously been shown to possess histone deacetylase activity. Clearly, understanding hDREF function will be dependent on the ability to identify relevant interacting proteins.

4.7 DREF, a putative transposase that mediates transposition of mobile elements.

Although the objective of this thesis was not to test the putative transposase potential that the hDREF protein harbors, the discovery of an ancient transposable element within the

human genome is of significant interest. One must consider the possibility that the hDREF phenotype may originate from the putative ability of the hDREF protein to promote transposition. The best-studied families of transposable elements are the Mariner elements and Ac (Activator dissociator) elements, which are classified based on the amino acid similarity of their transposase and the sequence similarity of their terminal inverted repeats. The majority of transposable elements found in the human genome contain intact TIRs but lack a functional transposase. Conversely, other elements such as hDREF encode a translated protein which may represent an active transposase, yet cannot bind the protein due to mutations in the TIR sequences. Over a hundred Mariner transposons have been found throughout the human genome, some of which appear to be autonomous transposable elements, (Morgan, 1995; Reiter et al., 1999). The TIRs of the human Mariner family are identical to those of the SINE family in all but one position suggesting that the SINEs, which lack a functional transposase, represent non-autonomous transposable elements dependent on Mariner type transposase for genome mobility (Morgan, 1995). Sequence comparisons of orthologous human and cattle genes revealed the presence of transposable element insertions in the human genome, that were absent in the cattle genome indicating that Mariner and SINE elements have been mobile within the human genome since the last common ancestor (Morgan et al., 1995). Smitts et al., (1996) analyzed a subgroup of human medium reiterated frequency repeats (MERs) but did not find the transposase responsible for the accumulation of the Ac-like MER1 subgroup. These results suggest that somewhere in the human genome there exists an Ac-like transposase that is responsible for the spread of the MER1 transposable elements (Smit and Riggs, 1996). The discovery of the actively transcribed hDREF protein is

significant due to the fact that other transposable elements in the same family may be able to bind this protein resulting in a trans-mediated transposition. If hDREF is an active transposase it may promote transposition into genes involved in DNA replication. Conversely, hDREF mediated transposition may not be capable of directing these mobile element and insertions may be dependent on an as of yet unidentified retrotransposon.

4.8 Conclusions.

Based on the evidence obtained from this research two substantive conclusions have been made. Firstly, that MEF2 has been shown to act as a transcriptional repressor and a negative regulator of hDREF expression during differentiation. However, in the absence of MEF2 binding to the consensus sequences in the 5' and 3'UTRs, hDREF expression remains elevated irrespective of culture conditions. As such, I conclude that MEF2 is a critical regulator of hDREF expression. Secondly, the results from functional assays suggest that hDREF is a key regulator of DNA synthesis. Overexpression of hDREF resulted in aberrant DNA synthesis in post-mitotic cells, leading to endoreplication. Based on the similarities between DREF and hDREF, I suggest that hDREF represents the human ortholog of *drosophila* DREF, and that the main function of hDREF is to ensure completion of DNA synthesis.

Appendix

cell type	Fusion index (+/- standard error)	t-test P-value
H9C2	60.0 $\pm$ 6.0	0.51
H9C2/PCDNA	60.0 $\pm$ 6.0	
H9C2	60.0 $\pm$ 6.0	2.2E-04
H9C2/LOW	45.0 $\pm$ 3.0	
H9C2	60.0 $\pm$ 6.0	3.2E-05
H9C2/MEDIUM	35.0 $\pm$ 3.0	
H9C2	60.0 $\pm$ 6.0	6.4E-06
H9C2/HIGH	25.0 $\pm$ 3.0	

Table 1: Summary of the fusion indexes and P-values found for the untransfected and transfected hDREF/H9C2 clones.

H9C2 vs. hDREF / H9C2 (Viable cells in growth media)	Day	Average of cell counts (3 trials)	t-test P- value
H9C2	1	3.22E+05 $\pm$ 4.97E+04	0.77
hDREF / H9C2		2.97E+05 $\pm$ 6.49E+04	
H9C2	2	6.19E+05 $\pm$ 4.10E+04	0.094
hDREF / H9C2		3.53E+05 $\pm$ 3.93E+04	
H9C2	3	8.33E+05 $\pm$ 1.66E+05	0.089
hDREF / H9C2		8.03E+05 $\pm$ 1.02E+05	
H9C2	4	1.59E+06 $\pm$ 6.67E+03	0.34
hDREF / H9C2		1.45E+06 $\pm$ 1.30E+05	
H9C2	5	1.39E+06 $\pm$ 1.31E+05	0.91
hDREF / H9C2		1.37E+06 $\pm$ 1.33E+05	

Table 2: Summary of the P-values found for the viable H9C2 vs. hDREF / H9C2 cells cultured in growth media for 5 days.

H9C2 vs. hDREF / H9C2 (Non viable cells in growth media)	Day	Average of cell counts (3 trials)	t-test P-value
H9C2 hDREF / H9C2	1	6.67E+03 $\pm$ 3.33E+03 3.33E+03 $\pm$ 3.33E+03	0.52
H9C2 hDREF / H9C2	2	3.33E+03 $\pm$ 3.33E+03 3.33E+03 $\pm$ 3.33E+03	1.0
H9C2 hDREF / H9C2	3	6.67E+03 $\pm$ 3.33E+03 3.33E+03 $\pm$ 3.33E+03	0.052
H9C2 hDREF / H9C2	4	1.33E+04 $\pm$ 3.33E+03 6.67E+03 $\pm$ 3.30E+03	0.23
H9C2 hDREF / H9C2	5	1.33E+04 $\pm$ 3.33E+03 1.00E+04 $\pm$ 5.77E+03	0.64

Table 3: Summary of the P-values found for the non-viable H9C2 vs. hDREF / H9C2 cells cultured in growth media for 5 days.

H9C2 vs. hDREF / H9C2 (Viable cells in differentiation media)	Day	Average of cell counts (3 trials)	t-test P-value
H9C2 hDREF / H9C2	1	6.47E+05 $\pm$ 2.91E+04 6.17E+05 $\pm$ 1.20E+04	0.73
H9C2 hDREF / H9C2	2	9.90E+05 $\pm$ 8.35E+04 8.03E+05 $\pm$ 4.98E+04	0.082
H9C2 hDREF / H9C2	3	8.20E+05 $\pm$ 3.06E+04 7.90E+05 $\pm$ 5.77E+03	0.39
H9C2 hDREF / H9C2	4	7.98E+05 $\pm$ 2.67E+04 7.90E+05 $\pm$ 5.77E+03	0.22
H9C2 hDREF / H9C2	5	7.23E+05 $\pm$ 2.73E+04 8.00E+05 $\pm$ 5.77E+03	0.052

Table 4: Summary of the P-values found for the viable H9C2 vs. hDREF / H9C2 cells cultured in differentiation media for 5 days.

H9C2 vs. hDREF / H9C2 (Non viable cells in differentiation media)	Day	Average of cell counts (3 trials)	t-test P- value
H9C2 hDREF / H9C2	1	1.67E+04 $\pm$ 1.20E+04 1.33E+04 $\pm$ 6.67E+03	0.82
H9C2 hDREF / H9C2	2	3.33E+04 $\pm$ 1.76E+04 3.33E+04 $\pm$ 8.82E+03	1.00
H9C2 hDREF / H9C2	3	6.70E+04 $\pm$ 2.33E+04 6.00E+04 $\pm$ 1.73E+04	0.83
H9C2 hDREF / H9C2	4	2.87E+05 $\pm$ 4.06E+04 2.33E+05 $\pm$ 8.82E+04	0.61
H9C2 hDREF / H9C2	5	3.10E+05 $\pm$ 4.93E+04 2.87E+05 $\pm$ 5.78E+04	0.77

Table 5: Summary of the P-values found for the non-viable H9C2 vs. hDREF / H9C2 cells cultured in differentiation media for 5 days.

C2C12 vs. hDREF / C2C12 (Viable cells in growth media)	Day	Average of cell counts (3 trials)	t-test P-value
C2C12 hDREF / C2C12	1	1.93E+05 $\pm$ 1.86E+04 2.47E+05 $\pm$ 3.38E+04	0.24
C2C12 hDREF / C2C12	2	5.37E+05 $\pm$ 1.86E+04 7.37E+05 $\pm$ 6.39E+04	0.06
C2C12 hDREF / C2C12	3	2.38E+06 $\pm$ 5.67E+05 1.67E+06 $\pm$ 1.33E+05	0.29
C2C12 hDREF / C2C12	4	3.23E+06 $\pm$ 6.67E+04 2.91E+06 $\pm$ 2.07E+05	0.21
C2C12 hDREF / C2C12	5	8.80E+06 $\pm$ 1.20E+05 6.30E+06 $\pm$ 4.51E+05	0.07

Table 10: Summary of the P-values found for the viable C2C12 vs. hDREF / C2C12 cells cultured in growth media for 5 days.

C2C12 v.s. hDREF / C2C12 (Non viable cells in growth media)	Day	Average of cell counts (3 trials)	t-test P-value
C2C12 hDREF / C2C12	1	3.33E+03 $\pm$ 3.33E+03 1.00E+04 $\pm$ 0.00E+00	0.12
C2C12 hDREF / C2C12	2	2.00E+04 $\pm$ 6.67E+03 3.33E+03 $\pm$ 3.33E+03	0.68
C2C12 hDREF / C2C12	3	3.33E+03 $\pm$ 3.33E+03 2.67E+04 $\pm$ 1.76E+04	0.26
C2C12 hDREF / C2C12	4	1.00E+04 $\pm$ 5.77E+03 1.33E+04 $\pm$ 1.33E+04	0.83
C2C12 hDREF / C2C12	5	3.67E+04 $\pm$ 1.45E+04 3.33E+04 $\pm$ 1.20E+04	0.87

Table 11: Summary of the P-values found for the non-viable C2C12 vs. hDREF /C2C12 cells cultured in growth media for 5 days.

C2C12 vs. hDREF / C2C12 (Viable cells in differentiation media)	Day	Average of cell counts (3 trials)	t-test P-value
C2C12 hDREF / C2C12	1	5.47E+05 $\pm$ 4.48E+04 6.00E+05 $\pm$ 1.76E+05	0.78
C2C12 hDREF / C2C12	2	7.70E+05 $\pm$ 3.06E+04 1.07E+06 $\pm$ 3.15E+05	0.40
C2C12 hDREF / C2C12	3	1.02E+06 $\pm$ 4.40E+04 1.23E+06 $\pm$ 3.48E+05	0.32
C2C12 hDREF / C2C12	4	6.30E+05 $\pm$ 1.39E+05 1.82E+06 $\pm$ 2.12E+05	0.009
C2C12 hDREF / C2C12	5	6.20E+05 $\pm$ 1.27E+05 1.50E+06 $\pm$ 2.52E+05	0.035

Table 12: Summary of the P-values found for the viable C2C12 vs. hDREF / C2C12 cells cultured in differentiation media for 5 days.

C2C12 vs. hDREF / C2C12 (Non viable cells in differentiation media)	Day	Average of cell counts (3 trials)	t-test P-value
C2C12 hDREF / C2C12	1	2.00E+04 ± 1.15E+04 3.67E+04 ± 6.67E+03	0.28
C2C12 hDREF / C2C12	2	2.00E+04 ± 5.77E+03 1.17E+05 ± 7.17E+04	0.25
C2C12 hDREF / C2C12	3	1.67E+04 ± 3.33E+03 3.00E+05 ± 1.35E+05	0.10
C2C12 hDREF / C2C12	4	5.33E+04 ± 1.76E+04 5.48E+05 ± 4.97E+04	0.00072
C2C12 hDREF / C2C12	5	2.20E+05 ± 1.30E+05 9.33E+05 ± 4.81E+04	0.0067

Table 13: Summary of the P-values found for the non-viable C2C12 vs. hDREF / C2C12 cells cultured in differentiation media for 5 days.

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