Searching for HES6 Dimerization Partners

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ABSTRACT

HES6 is a novel member of the basic helix-loop-helix (bHLH) family of transcription factors that is involved in the activation of the myoblast differentiation program. Studies demonstrated that HES6 can regulate transcription from gene promoters containing an N box (CACNAG), a DNA motif located in the regulatory regions of many muscle-specific genes involved in myoblast differentiation. This suggested that a HES6 heterodimer could bind to the N box. The purpose of this study was to identify putative HES6 dimerization partners that would allow it's binding to the N box and then repress transcription. A genome-wide screen isolated several clones. Potential positive clones, the heat shock proteins HSP169 and HSP70 were identified. The functional significance of this interaction remains to be determined. A candidate gene approach allowed detection of an interaction *in vivo* between HES6 and a novel hairy-related bHLH protein, CHF2. This interaction was detected by co-immunoprecipitation using the first specific anti-HES6 antibody synthesized. Electrophoretic mobility shift assays revealed that the heterodimer was unable to bind to any of the N-boxes present in the 5'-flanking region of CHF2, which was confirmed by assessing the specificity of the controls used in the experiments. It was suggested that HES6 sequesters CHF2 by the formation of an inactive heterodimer. Although a band shift was observed when incubating each of the N-boxes in the presence of total cell extract from differentiated C2C12 cells, it was not confirmed whether HES6 was one of the proteins binding to the oligonucleotides. This work has identified a novel interaction between HES6 and CHF2 and our data suggests that this interaction lead to formation of inactive heterodimers.

RÉSUMÉ

HES6 est un facteur de transcription de la famille des hélice-boucle-hélices possédant un domaine basique impliqué dans l'activation de la différenciation des myoblastes. Des études ont démontré que HES6 peut réguler la transcription d'un gène contrôlé par une boîte N, une séquence liant des facteurs de transcription correspondant au consensus CACNAG. Ceci suggère qu'un hétérodimère de HES6 puisse lier la boite N. L'objectif de cette étude était d'identifier un partenaire potentiel de dimérization de HES6 lui permettant de lier l'ADN par le biais de la boite N et ainsi réprimer la transcription. Un criblage par interaction protéine-protéine a identifié plusieurs faux positifs. Des clones potentiellement positifs faisant parti des protéines à induction à la chaleur, HSP196 et HSP70, ont été isolées. La pertinence physiologique de cette interaction n'a cependant pas été testée. Par une approche plus spécifique, une interaction entre HES6 et un nouveau membre de la famille des hélice-boucle-hélices, CHF2, a été détectée. Cette interaction observée par co-immunoprecipitation a été détectée grâce à l'utilisation du premier anticorps spécifique à HES6. Des expériences ont démontré que l'hétérodimère était incapable de lier chacune des boîtes N présente dans la région non-transcrite du gène de CHF2. Ce résultat a été confirmé par l'évaluation de la spécificité des contrôles utilises durant l'expérimentation. Finalement, malgré que certains résultats aient démontré que des protéines présentes dans les extraits totaux de cellules C2C12 pouvaient lier chacune des boites N, il n'a pas été possible de déterminer si HES6 étaient l'une d'elles. Cette étude a donc permis d'identifier une interaction entre CHF2 et HES6 et les résultats suggèrent que HES6 séquestre CHF2 en formant un dimère inactif.

Abbreviations

AHR:	Aromatic Hydrocarbon Receptor
bHLH:	basic Helix-Loop-Helix
CHF:	Cardiac Helix-Loop-Helix Factor
DM:	Differentiating Medium
DTT :	1,4-DiThioThreitol
EMSA:	Electrophoretic Mobility Shift Assay
ESE:	Enhancer of Split E
E(spl):	Enhancer of Split complex
GTFs :	General Transcription initiation Factors
HAT :	Histone Acetyltransferase
HDAC:	Histone Deacetylase
HSP:	Heat Shock Protein
HERP:	HES-related Repressor Protein
HES:	Hairy Enhancer of Split
HEY:	Hairy/E(spl)-related with YRPW
HID:	Histone Deacetylase Interaction Domain
HLH:	Helix-Loop-Helix
HRT:	Hairy-Related Transcription factor
KLH:	Keyhole Limpet Hemocyanin
MRF:	Muscle Regulatory Factors
MyoR:	Myogenic Repressor

NICD: Notch Intracellular Domain

Stra13: Stimulated with Retinoic Acid

TAF: TBP-Associated Factors

TBP: TATA-Binding Protein

TFIIX: Transcription Factor II A, B, D, E, F, H, J

Vezf1: Vascular Endothelial Zinc Finger 1

INTRODUCTION

I. Transcriptional Regulation

Eucaryotic RNA polymerases cannot initiate transcription by themselves. Initiation of transcription by RNA polymerase II is dependent on General Transcription Factors (GTFs) [49]. RNA polymerase II associates with a wide variety of auxiliary GTFs: TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and TFIIJ before initiation of transcription. A combination of short sequence elements act as recognition signals for transcription factors to bind to the DNA in order to guide and activate the polymerase [49]. A major group of such short sequence element is often located in the immediate upstream region of the coding sequence of the gene, where they collectively constitute the promoter. In case of most genes, that are actively transcribed, the promoter region includes a conserved sequence named the TATA box (consensus TATA (A/T) A (A/T)) at a position about 25-35 base pairs (bp) upstream from the transcription start site [73]. The first general transcription factor to bind to the TATA box is TFIID through one of its subunit, the TATA-binding protein (TBP). TBP-associated factors (TAFs), the second subunit of TFIID, interact with TBP. TFIIA joins the complex followed by TFIIB and finally TFIIF associated with RNA polymerase II [49]. The polymerase would not start transcription until TFIIE, TFIIH and TFIIJ are associated to the complex. After initiation of transcription, elongation and termination of transcription take place [49].

TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIJ initiate transcription, but a number of different mechanisms have evolved to ensure that the expression of the genome is a highly regulated process [49]. A fundamental mechanism controlling transcription is the

limited ability of many transcription factors to access the genome. In eucaryotic cells, the DNA is packaged with histones in the form of chromatin, which impedes the binding of many proteins to their target DNA sequence. The primary unit of chromatin is the nucleosome, composed of 146 bp of DNA wrapped around an octamer of histone proteins [49]. Chromatin, in its normal state, is transcriptionally repressed and blocks association of TFIID and RNA polymerase. Chromatin structure can be altered through ATP-dependent nucleosome remodelling and covalent modifications of core histones, particularly acetylation-deacetylation of lysine residues and methylation of lysine and arginine residues [45]. These covalent modifications occur primarily within the N-terminal histone tails. When N-terminal lysine residues of the histones are acetylated, it results in neutralization of their basic charge, thus altering DNA binding [32]. Histone acetyltransferase (HAT) enzymatic activity, which can add acetyl groups to specific lysine residues to facilitate TFIID binding and transcription, have been identified in several transcriptional coactivator molecules [25]. Similar to acetylation, phosphorylation of histone disrupts DNA-nucleosome interactions and increases transcription factor accessibility to DNA. Activation of transcription can also occurs by simultaneous stimulation of TFIID and TFIIA to bind the TATA box.

If acetylation of histones at specific sites can induce transcription, deacetylation can make DNA bind to histones more tightly and ultimately repress transcription. The enzyme Histone Deacetylase (HDAC) is a repressor of transcription, which forms a complex with the corepressor mSin3 [18,49]. Studies demonstrated that mSin3 functioned to tether sequence-specific DNA-binding transcriptional repressors to histone deacetylase

activity and that mSin3-associated activity requires physical interaction between HDAC1 and its C-terminal region [18]. The C-terminal region of mSin3 required for interaction is designated the histone deacetylase interaction domain (HID) and plays an essential role in Sin3-mediated repression of gene expression. The mSin3-HDAC complexes are believed to deacetylate nucleosomes in the vicinity of Sin3-regulated promoters, resulting in a repressed chromatin structure [4].

DNA-binding transcription factors regulate transcription. Some transcription factors are tissue-specific. They bind a short specific nucleotide sequence, to either activate or repress transcription [49]. Two distinct functions can often be identified and located in different parts of the transcription factor protein: the activation domain and the DNA-binding domain. As the name suggests, the activation domain activates transcription of the target genes once the transcription factor has bound to the promoter [49,73]. Some activation domains are enriched in aspartic acid and glutamic acids residues, while others are rich in proline or glutamine residues. The DNA-binding domain is necessary to permit specific binding of the transcription factor to its target genes [49,73]. DNA-binding domains are categorized in several different families according to the structural motif that contacts the DNA.

Homeodomain proteins form a large family of sequence-specific DNA-binding transcription factors involved in the regulation of diverse genetic programs, including those that underlie embryonic development of animals and plants [68]. The homeodomain,

or homeobox, responsible for DNA binding is the structural element common to the family. The homeodomains is a 60 amino acids consensus sequence that was first identified in homeotic genes, a set of gene involved in embryonic development. It consists of three helices separated by short loops and preceded by a flexible arm [68]. Helix III (the C-terminal helix) contacts DNA bases in the major groove, and the flexible arm contacts bases in the minor groove [68]. DNA binding sites of homeodomain proteins are A/T-rich, but the proteins differ in affinity for specific A/T-rich sequences. Cooperative binding of homeodomain proteins to multiple DNA binding sites is thought to confer additional specificity in the regulation of target genes [68].

Another family of transcription factors is characterized by its zinc finger motif, in which a zinc atom tetrahedrally coordinated by four residues participates in the formation of a 12-amino acid loop [52]. Zinc coordinated fingers are one of the most common DNA binding motifs among eucaryotic transcription factors and are classified based on amino acid sequence of the zinc fingers [40]. The Cys-Cys, His-His class contains the largest number of members. These proteins contain two or more fingers in a tandem repeat. In contrast, steroid receptors, such as the glucocorticoid receptor, contain only two zinc coordinated structures with four (C_4) and five (C_5) conserved cysteines. The third class of zinc fingers, which also binds to single-stranded nucleic acids, has a consensus sequence of Cys-X_2-Cys-X_4-His-X_4-Cys. In accordance with their structural diversity, zinc finger proteins play a variety of important roles in cell growth, differentiation, and development [41].

The winged helix structural motif for DNA binding was defined only 7 years ago [72]. These proteins have recently been renamed "Fox" transcription factors, for Forkhead box. Winged helix transcription factors contain two polypeptide loops, or "wings," that make minor groove contacts with DNA from either side of a three-helix bundle that binds the DNA major groove. While wing 1 is stabilized by a β-sheet, parameters that stabilize wing 2 are unknown winged-helix motifs [72].

The Leucine-Zipper family of proteins is defined by a pattern of at least four leucine (L) residues repeated every seventh amino acid that mediates protein dimerization through the formation of parallel alpha-helical dimers. Sometimes the zipper is incorporated into a helix-loop-helix conformation called the basic helix-loop-helix-leucine zipper (bHLH/Zip) [47].

Basic helix-loop-helix (bHLH) factors are transcription factors that bind DNA as homo- or heterodimers and regulate transcription of target genes containing E-boxes (CANNTG), E-box-like sequences or the related N-box (CACNAG) in their promoters. Dimerization occurs through interactions of the HLH domains, while binding to DNA is mediated by the basic domain [7,15]. These factors have been shown to regulate the expression of tissue-specific genes in a number of mammalian and non-mammalian organisms [7,15]. Members of the basic helix-loop-helix (bHLH) superfamily of transcription factors are expressed in a wide range of tissues during development and play a major role in cell specification and differentiation [7,15]. Transcription factors belonging to the helix-loop-helix family are important regulatory components in transcriptional

networks of many developmental pathways[7]. They are involved in regulation of neurogenesis, myogenesis, cell proliferation and differentiation, cell lineage determination, sex determination, and other essential processes in organisms ranging from yeast to mammals.

II. The muscle differentiation program

Cell differentiation and response to signalling pathways require certain genes to be activated and others to be repressed. Regulated gene transcription is a key aspect of the differentiation programs. This has been particularly well described for the myogenic program.

Under the influence of several extracellular signals, either repressing or activating, mesodermal progenitor cells become committed to a skeletal fate. The muscle precursor cells named myoblasts proliferate and sometimes migrate but do not differentiate until expression of muscle regulatory factors (MRFs) is initiated [60,83]. Upon expression of MRFs, myoblasts exit the cell cycle and finally differentiate to produce mature muscle cells called myocytes [60,83]. Muscle specific regulatory factors are known to be members of the bHLH (basic domain Helix-Loop-Helix) family of transcription factors and include MyoD, Myogenin, Myf-5 and MRF-4. Each of these proteins share an homology of about 70 amino acids that includes a basic region and adjacent helix-loop-helix motif that mediate DNA binding and dimerization, respectively [8,69]. They each act at multiple points in the myogenic program and are exclusively expressed in skeletal muscle. Forcing their

expression in a wide range of cultured cells induces the skeletal muscle differentiation program.

To activate transcription, the myogenic bHLH factors must heterodimerize with the ubiquitous bHLH proteins, known as E proteins, E12, E47 or HEB [46,56]. The bHLH-E protein complex then binds to the consensus sequence known as the E box (CANNTG), which is located in the regulatory regions of many muscle-specific genes [60,69]. Their bHLH region can also associate with member of the myocyte enhancer binding factor-2 (MEF2) family of transcription factors, which lack myogenic activity alone, but potentiate muscle-inducing activity of myogenic bHLH proteins and are required for the activation of muscle differentiation genes [53].

According to different studies, Myf5 and MyoD are expressed in undifferentiated myogenic cells prior to myogenin and may perform similar functions in committed myoblast suggesting redundancy in MRF function [51,65]. The presence of either Myf5 or MyoD is required for the determination of myoblast identity early in the development program [65]. Furthermore, MyoD and Myf5 regulate myoblast specific genes in proliferating myoblast, autoactivate their expression but negatively regulate one another. Upon depletion of exogenous growth factors, MyoD and Myf5 activate expression of myogenin, which activates myotube-specific genes. Myogenin is a nuclear phosphoprotein that is not expressed until myoblasts exit the cell cycle. During maturation, myogenin expression declines and MRF4 is up-regulated to control

myofiber-specific genes [60]. Myogenin and MRF5 may be required for activation and maintenance of muscle gene expression. (Figure 1).

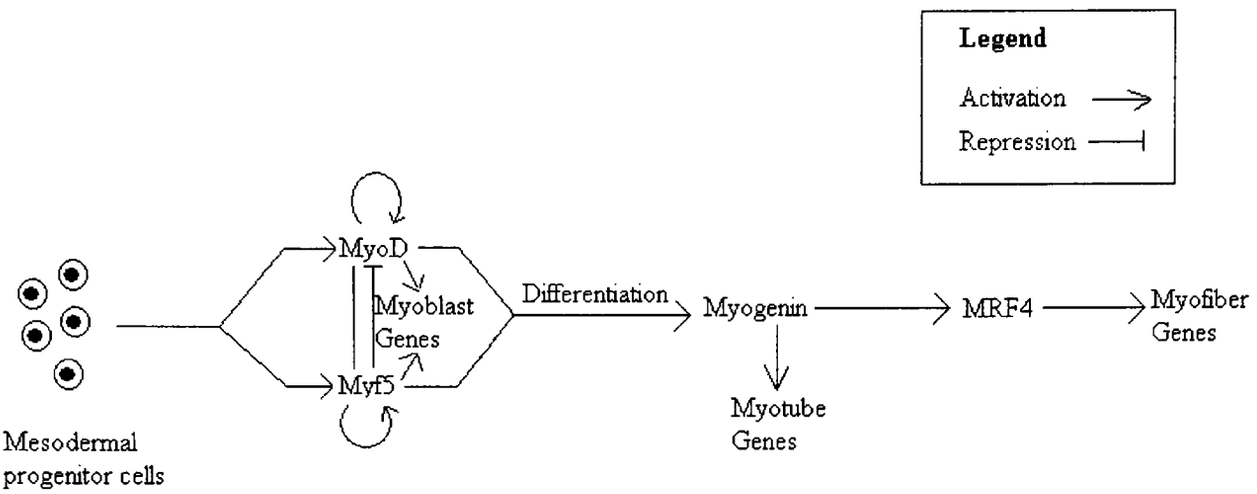


Figure 1. Hypothetical regulatory pathway for muscle determination and differentiation. Either MyoD or Myf5 becomes activated and activate myoblast genes. MyoD and Myf5 autoactivate their expression and negatively regulate one another. MyoD and Myf5 activate expression of myogenin, which activates myotube genes. Myogenin expression declines and MRF4 expression increases to regulate myofiber genes. (Adapted from Olson [60])

A number of proteins have been reported to regulate myogenic transcription either positively or negatively, by physical association with muscle regulatory proteins. Thus, several HLH proteins have been isolated and further characterized as repressors of myogenesis. The inhibitors of myogenic transcription exert their activity by distinct mechanisms including direct physical association with myogenic bHLH proteins, or by indirect mechanism such as sequestration of heterodimeric partners (Id-E12/47), competition for DNA binding (MyoR, Mist) or cytoplasmic retention of myogenic bHLH proteins [64].

Id proteins (for "inhibitors of differentiation or DNA binding"), members of the HLH family, are dominant negative regulators of basic helix-loop-helix transcription factors

and they inhibit myogenesis through passive repression [7]. Id proteins contain an HLH domain, allowing them to form dimers with bHLH proteins, but they lack the basic domain, and therefore such dimers, Id/bHLH, do not bind DNA [7]. Therefore, Id proteins do not regulate transcription directly, but indirectly, by preventing bHLH proteins from interacting with the promoter of various target genes. Id-1 has been found to inhibit differentiation in myoblast, trophoblast, erythroid, B-lymphocyte, and myeloid cells [7,51]. As discussed previously, myogenic bHLH proteins activate muscle transcription by binding to the E proteins as heterodimers. Thus, in undifferentiated myoblast, Id proteins sequester E12 and E47, which make them unable to interact with myogenic transcription factors and initiate the differentiation program [51]. A number of studies have reported that Id overexpression not only inhibits myogenic differentiation but also interfere with cell cycle arrest [51].

Twist is a bHLH protein that also plays a role in negative regulation of transcription. Twist can repress muscle transcription by sequestering E proteins in inactive complexes similarly to Id proteins [70]. In addition, Twist can inhibit the function of MEF2 proteins and MyoD by direct interactions. In the case of MEF2, the C-terminus of Twist inhibits the transcriptional activity of MEF2 factors [70]. Twist-mediated repression of Myo-D occurs via the physical interaction between the basic regions of the two proteins, thus blocking the DNA binding site of MyoD [30].

A muscle-specific bHLH protein that antagonizes the action of MyoD has also been recently identified. MyoR (myogenic repressor) is a negative regulator of myogenesis.

Its expression is elevated in undifferentiated myoblasts and absent in differentiated myotubes [50]. MyoR is known to interact with E proteins and form heterodimers that are competent for binding DNA at E box sites. However, MyoR behaves as a transcriptional repressor and inhibit myogenic transcription by interfering with the ability of myogenin and MyoD to activate myogenesis [50]. MyoR appears as a lineage-restricted transcriptional repressor of myogenesis. It remains to be established whether MyoR selectively inhibits E box-dependent transcription of bHLH proteins [64].

Signalling through the transmembrane receptor Notch has also been shown to prevent myogenesis. Upon ligand binding, cleavage of the intracellular domain of Notch occurs and Notch Intracellular Domain (NICD) is translocated into the nucleus. It then forms a complex with the DNA-binding protein RPB-J and the culmination of the signalling cascade is the activation of the expression of the genes of the HES (Hairy Enhancer of Split) family. The HES family will be further described in section IV. Since members of the HES family are transcriptional repressors, activation of the Notch pathway ultimately leads to repression of transcription [42]. Thus, HES acts as a primary effector for Notch signalling and prevents cell differentiation. While the link between Notch and HES family members has been demonstrated in neuronal differentiation [61], this link remains hypothetical in muscle differentiation. Since HES1 inhibits MyoD in cell culture system [65], Notch might negatively regulate myogenesis through this mechanism.

Recently, two novel hairy-related bHLH proteins, CHF1 and CHF2, have been described as transcriptional repressors that regulate terminal differentiation [38,39,40,74]. CHF2, cardiac

helix-loop-helix factor 2, is highly expressed in myoblast and dramatically reduced after induction of terminal differentiation, as myogenin expression rises. Its high sequence similarity with the HES family raised the possibility that CHF gene family might be a new target of Notch. CHF2 will be described in further details in section VI. Overexpression of NICD stimulates expression of CHF2 and HES family members. Notch effectors, HES and its novel partner CHF2, synergistically repress downstream target genes by forming preferentially a heterodimer. The complex recruits the mSin3 complex containing HDAC and an additional co-repressor, N-CoR, to mediate repression [39,74].

III. The Helix-Loop-Helix family of transcription factors

The helix-loop-helix (HLH) proteins are a super family of DNA-binding transcription factors that regulate numerous biological processes such as lineage commitment, segmentation, sex determination, neurogenesis, haematopoiesis, pancreatic development and myogenesis [23,60]. Because of the large number of HLH proteins described, a classification system was devised based on tissue distribution, dimerization capabilities and DNA-binding specificities. Class I HLH proteins include the E proteins, E12, E47, HEB, E2-2 and Daughterless. These proteins are expressed in many tissues, can form either homodimers or heterodimers and can only bind to the E box consensus sequence [56] Class II HLH proteins show a tissue-restricted pattern of expression. It includes members such as MyoD and Myogenin. This class is able to form homodimers but preferentially heterodimerize with E proteins [56]. Class III HLH proteins include the Myc family of transcription factors, TFE3 and SREBP-1 [85]. Class IV define a family of molecules that

are able to dimerize with the Myc proteins or with one another and include Mad, Max and Mxi [12]. Proteins that lack a basic region are members of the class V of HLH proteins. Id and Emc proteins define this class [7]. Class VI HLH proteins are distinguished by the presence of a conserved proline residue in their basic region. This group include the *Drosophila* proteins Hairy and Enhancer of Split (HES) and the hairy-related basic helix-loop-helix proteins such as CHF1 and CHF2 [22,60]. Finally, the class VII HLH proteins are categorized by the presence of the bHLH-PAS domain and include members such as the aromatic hydrocarbon receptors (AHR) [19]. HLH transcription factors are key regulators of transcription. As described previously, E proteins are characterized as activators of transcription and Id proteins, Twist and HES are defined as repressors.

IV. **The Hairy and Enhancer of Split family**

As described above, class VI HLH proteins include the hairy and enhancer of split proteins (HES) and the hairy related enhancer of split proteins. This class can be further divided into four subclasses: hairy, E(spl) (Enhancer of Split complex), Hey (Hairy/E(spl)-related with YRPW) and Stra13 (stimulated with Retinoic Acid) [22]. The E(spl) subclass includes the Hairy and Enhancer of split (HES) family of transcription factors. This family includes 7 members originally identified in *Drosophila* [67]. Members of the HES family share structural features including a basic domain with a conserved proline residue in the basic domain and a helix-loop-helix region (Figure 2). They respectively facilitate DNA binding and protein dimerization [22]. HES factors can form homodimers that preferentially bind to the consensus sequence called the N box (CACNAG) or bind to the E box but with a reduced affinity [67,75]. Some HES proteins can

heterodimerize with the E proteins but this interaction appears to titrate the E proteins and produce inactive dimers [27]. The orange domain which is always located C-terminal to the bHLH domain, is an important functional domain that confers specificity among members of the family [23]. The proline-rich region, which follows the orange domain, is necessary for stability of the protein. Finally, a WRPW motif located at the C-terminus is necessary to recruit the corepressor TLE (Transducine-Like Enhancer of split), the mammalian homologue of *Drosophila* Groucho gene [14,23,26]. TLE binds to the tetrapeptide sequence and mediate transcriptional repression by interacting with a Histone Deacetylase. (HDAC) which makes DNA bind tightly to the histones [14].

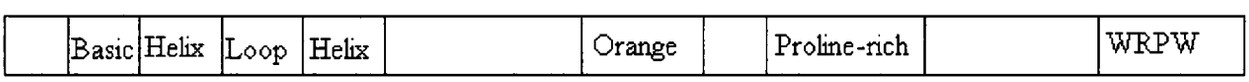


Figure 2. HES member general features of the sequence. The basic region is located next to the helix-loop-helix domain. Located C-terminal to the HLH is the orange domain, followed by the proline-rich region. In carboxyl terminus, is the WRPW motif that recruits TLE, the mammalian homologue of *Drosophila* Groucho gene.

The HES family includes 7 members: HES1 to HES7. HES1 is expressed in a variety of cells including muscle cells, embryonic neural and mesodermal cells. HES1 is described as a repressor of neural differentiation and its role in the control of the proper timing of neural differentiation has been well characterized in different studies [8,34,67]. HES2 is described as a negative regulator of positive bHLHs and bind with a greater affinity to the E box [36]. HES3 is produced exclusively in cerebellar Purkinje cells [67]. HES4 and HES7 were recently identified and found to be similar to HES1. HES7, expressed in the presomatic mesoderm, is known to suppress E47-induced transcriptional activation and

plays a role in somite segmentation [10,11] HES5 is expressed at high level in neuronal progenitor cells and consequently plays a role in neuronal differentiation [33], but it is also expressed in muscle cells. Finally, HES6, a novel HES member identified recently [8], is expressed in several tissues such as nervous tissue, spinal cord, thymus, pancreas, epithelial cells and muscle cells. HES6 will be further described in the next section.

V. HES6, member of the hairy enhancer of split family

The recently characterized HES6 gene was shown to be located on chromosome 2q37.1-2q37.3. The sequence analysis of the cDNA predicts a protein of 224 amino acids in the mouse and 222 amino acids in humans. The mouse and human HES6 protein sequences are 85% identical and their corresponding bHLH and orange domains are about 95% identical [78]. *In situ* hybridization revealed no HES6 expression in 8.5 embryonic days (E8.5) mouse embryos [78]. In E9.5 and E10.5 embryos, mHES6 was predominantly expressed in all neurogenic placodes and their derivatives and in the brain [78]. HES6 was also expressed in the trunk, in the dorsal root ganglia and in the myotome. In limb buds, HES6 is expressed in skeletal muscle and presumptive tendons [78]. During muscle development, the expression of HES6 was described to occur during both myoblast commitment and differentiation [63].

An HES6 defining feature compared to other HES members is the truncated loop region. It is five amino acids shorter than HES1 and HES2 and four amino acids shorter than HES3 and HES5 [8]. A group from Japan generated two constructs to assess the region responsible for the functional difference between HES6 and HES1. One construct

included HES6ins which had a five amino acids insertion in the loop region and the other construct included HES1del with a five amino acids deletion in the loop region [8]. The transcriptional activity of these mutants was examined by transient transfections. The luciferase reporter gene under the control of the N-box containing promoter was repressed by HES6ins as did HES1 [8]. Thus, the insertion of those five amino acids conferred the N box-dependent repression activity on HES6. Furthermore, HES1del did not repress transcription, just as HES6 did [8]. The deletion of the five amino acid residues from the HES1 loop region abolished the N box-dependent repression activity of HES1. They concluded that the loop region was critical for the specific activities of HES6 and HES1 and that some of theses activities were interchangeable by the alteration of the loop [8]. However, the insertion of just five amino acids was not sufficient to confer full activities, additional changes are required to full inhibitory activity and it hasn't been determined yet which amino acid residues are required or if just the length is important [8].

Comparison of DNA-binding activity of HES6 and HES1 was performed by electrophoretic mobility-shift assay (EMSA) [8]. The basic helix-loop-helix proteins expressed in *E.coli* were incubated in presence of the double stranded oligonucleotides fragments containing either the N box or the E box [8,58]. Although an HES1 homodimer was able to bind to the N box with high affinity and to the E box with low affinity, the HES6 homodimer was not able to bind either to the N box or to the E box [8,67]. However, the HES6-HES1 heterodimer was capable of binding the E box albeit with low affinity and results indicated that HES6 influenced both the N box and E box binding of HES1 [8].

Because HES6-HES1 heterodimers were shown to bind DNA, it demonstrated that HES6 could heterodimerize with other bHLH molecules to bind the N box.

That hypothesis was confirmed in transient transfection of C2C12 myoblasts [27]. Myoblasts were transfected with a full length HE6 construct or a HES6 construct lacking the WRPW motif along with a luciferase reporter gene construct including or lacking 6 N boxes. The expression of the reporter gene was suppressed by HES6 in templates containing N boxes [27]. Furthermore, this effect required the WRPW motif since the truncated form of HES6 did not affect transcription from reporter templates containing N boxes [27]. It was concluded that HES6 repress transcription in myoblasts in a N box-dependent manner [27]. To further demonstrate that hypothesis, HES6 was fused to the VP16 activation domain of the herpes simplex virion. This construct transformed HES6 from a repressor to an activator. Transient transfection demonstrated that HES6-VP16 specifically activated transcription of the N box-containing luciferase reporter gene in C2C12 myoblasts whereas the full-length VP16 alone had no effect [27]. Those results clearly demonstrated that HES6 binds to the N box in myoblasts.

As previously described, HES transcription factors interact with TLE (Transducine-like enhancer) repressors through mediation of the WRPW motif at the extreme COOH-terminal [14]. A Yeast Two-Hybrid interaction assay showed that yeast cells co-transformed with TLE1 and HES6 were able to grow on selection plates while yeast cells transfected with HES6 or TLE alone were not [27] demonstrating the capacity of HES6 to interact with TLE. Furthermore, yeast cells co-transformed with HES6 lacking the WRPW motif and

TLE were unable to grow on the selective plates meaning that protein-protein interaction between TLE and HES6 requires the WRPW motif [27]. Transient transfection of the truncated HES6 (lacking the WRPW motif) showed that this motif was necessary for repression of the reporter gene so the WRPW motif is then necessary to recruit TLE. Upon deletion of that motif, inhibition of transcription was dramatically reduced because HES6 was unable to recruit TLE. Consequently, repression of transcription is specific for the N box and requires the WRPW COOH-terminal peptide [27].

Transcriptional analysis revealed different behaviour for HES6 when expressed in neuronal cells or in muscle cells [8, 27]. Transient transfections of HES6 and HES1 along with a luciferase reporter gene under the control of the N-box promoter into NIH3T3 cell were performed [8]. HES1 repressed transcription from the N-box containing promoter while HES6 did not repress or activate transcription [8]. Thus, in cells co-expressing HES6 and HES1, HES6 suppressed the N box-dependent transcriptional repression by HES1 [8]. In neural differentiation HES6 suppresses HES1 and promotes cell differentiation [8]. However, when the same type of experiment was performed in muscle cells, HES6 did not antagonize HES1-mediated repression but led to additive inhibition [27]. In myoblasts HES6 cooperates with HES1 to further inhibit transcription from N box-containing promoters [27].

Examination of the pattern of expression of HES6 in myoblast demonstrated that HES6 expression is induced during differentiation [27]. Cells were grown in growth medium and then switched to differentiating medium. RNA was extracted after 0, 3 and 7 days of

differentiation and analyzed by Northern blot [27]. HES6 mRNA was induced in differentiating medium and was maximal after 7 days. Furthermore, when placed in differentiating medium, C2C12 cells expressing HES6 differentiated to form fused myotubes [27]. Moreover, cells transfected with the truncated form of HES6 (lacking WRPW) never fused suggesting that the WRPW motif is essential to the induction of differentiation.

As previously reported, HES6 homodimers did not show any affinity for the E box or the N box recognized by other HES proteins. However a recent publication demonstrated an interaction of HES6 homodimers to the Enhancer of Split E box (ESE box) by electrophoretic mobility shift assay [17]. The ESE box is a 12 nucleotides motif containing an E box and referred to as the preferred binding site of *Drosophila* EoS proteins [17]. The *Drosophila* Enhancer of Split (EoS) proteins are a group of the basic helix-loop-helix family of transcription factors. Similarly to HES6, they act as repressors of transcription and regulate differentiation and cell fate. This study was then the first to demonstrate the capacity of HES6 to bind DNA as a homodimer. They confirmed the specificity of the results by competing the binding with unlabelled ESE box-containing oligonucleotides. Moreover, excess of unlabelled E box or N box containing oligonucleotides had no effect on the binding, even in 200 fold molar excess [17].

Since HES6 previously showed transcriptional repressor activity in models containing an N-box, they tested the ability of HES6 to alter transcription in a model containing an ESE-box. Transient transfection demonstrated that HES6 homodimer had no effect on the

luciferase activity in absence of the ESE box in the promoter region [17]. However, when two ESE box motifs were introduced, a decrease of the luciferase activity was observed [17]. The HES6 homodimer is then able to bind DNA through the ESE box and repress transcription.

This study presented further evidence that HES6 may have a role in regulating myogenic differentiation and somite formation [17]. They analysed the overexpression of HES6 on C2C12 myoblast differentiation by fluorescence and showed that myotubes formed in cells transformed with HES6. Furthermore, cells stained with anti-troponine-T antibody, a marker of terminal differentiation, revealed that myotubes expressing the marker were present in HES6 over-expressing cultures [17]. To support the *in vitro* results, they tested *in vivo* the expression of HES6 in the embryonic myotome of mouse and *Xenopus*. In situ hybridization revealed that HES6 mRNA was present in developing somites but undetectable in adult skeletal muscle [17]. To further test the effect of HES6 overexpression on myogenesis in vivo, they performed *in situ* hybridization on *Xenopus* embryos and showed that HES6 expression matches expression of MyoD data. Undifferentiated myotomes expanded in embryos over-expressing HES6 demonstrating the implication of HES6 in early differentiation [17]. However, when embryos where stained with an antibody of terminal differentiation, a decrease of 63% of the expression of the marker was observed in cells overexpressing HES6. Thus, HES6 is expressed during muscle differentiation *in vivo* and *in vitro* but its expression decrease at terminal differentiation. Although HES6 overexpression inhibited terminal differentiation, it promoted the

formation of early tissue-expressing markers of muscle commitment and early differentiation [17].

Although HES6 is expressed in both muscles and nerves, it shows some differences in its function and regulation of the molecules involved. In neurons, HES6 was never shown to be able to bind the N box nor the E box as opposed to muscles where HES6 was shown to potentially bind as an heterodimer to the N box and to bind to the ESE box as a homodimer [8,17,27]. Furthermore, HES6 N-box dependent repression is specific to muscle cells and was never observed in neuroblasts or fibroblasts. Finally, in muscle cells HES6 cooperates with HES1 to fully repress transcription while in neuroblasts HES6 suppresses HES1 from repressing transcription [8,27]. Because of those differences, HES6 function appears distinct in muscle cells.

Published studies on HES6 allow assessing that HES6 is able to bind DNA as an heterodimer to the N box and as an homodimer to the ESE box [8,17,27]. HES6 induce muscle differentiation but its expression decreases at terminal differentiation [17,27]. HES6 interacts with TLE through the WRPW motif and deletion of that motif makes repression of transcription impossible [27]. HES6 behaves differently in muscle cells compared to nervous cells [8,27]. While HES6 antagonize HES1 in nerronal cells, it has an additive effect in muscle cells.

Because HES6 was shown to repress transcription in myoblasts and to induce early differentiation, it was proposed that HES6 inhibits a repressor of myogenesis and

ultimately induces the differentiation program. The hypothesized mechanism proposes formation of a heterodimer between HES6 and an unknown partner and binding to the N box of a target gene, likely an inhibitor of myogenesis. It will ultimately recruit TLE through the WRPW motif and repress transcription of the target gene (Figure 3).

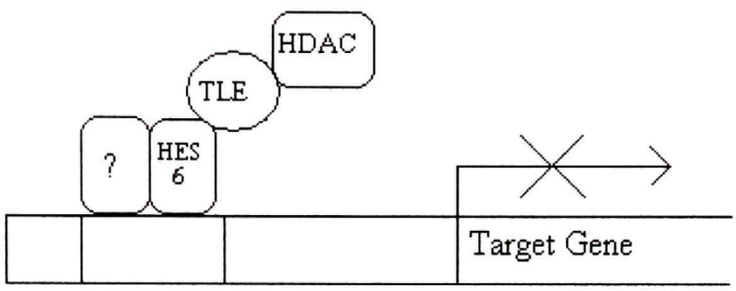


Figure 3. Potential mechanism of repression of HES6 in muscle differentiation. HES6 interact with an unidentified partner, potentially member of the bHLH family. The complex recruit TLE and a HDAC, which will repress transcription of a target gene and ultimately repress a repressor of myogenesis.

VI. CHF2, a novel hairy-related bHLH protein

As discussed previously, basic helix-loop-helix transcription factors can be divided into 4 categories: Hairy, E(spl), Hey and Stra13 [22]. The Hey proteins (Hairy/E(spl)-related with YRPW) include Hey1, Hey2 and HeyL. The Hey proteins are also referred as HERP (HES-related repressor protein), HRT (Hairy-related transcription factor), gridlock or CHF (Cardiovascular helix-loop-helix factor) because several groups have identified them and named them differently. CHF transcription factors present in the cardiovascular system were identified by a yeast two-hybrid screen using the bHLH/PAS domain of ARNT as a bait to probe a human heart cDNA library [15]. They isolated two novel cDNAs that interact with ARNT in yeast. Their patterns of expression was predominantly

cardiovascular during development, consequently they named them "cardiovascular helix-loop-helix Factor 1 and 2" (CHF1 and CHF2) [15] HRT transcription factor were identified by a search in the DNA sequence databases for novel bHLH proteins with homology to the bHLH region of dHAND and eHAND [57]. Hey genes were isolated and characterized from mouse and human (hairy and Enhancer-of-split related with YRPW motif; HEY1, HEY2, and HEYL) [71]. HERP genes were isolated and described by a group who screened the GenBank DNA sequence database using the sequence corresponding to the basic helix-loop-helix region of mouse HES1. Three expressed sequence tag clones were identified and named human HERP1, human HERP2, and mouse HERP2 [38] respectively. Finally, when the gene responsible for the gridlock mutation in zebrafish was cloned, it was named gridlock and was later shown to be identical to Hey-2 (Hrt-2, CHF-1) [31]. Hey2 is then known under the name of HERP1, HRT2, gridlock or CHF1. HeyL is sometimes called HRT3 and Hey1 can be referred as HERP2, HRT1 or CHF2 [22]. This thesis will refer to this transcription factor as CHF2.

CHF proteins are structurally similar to the HES family. Both families share common domains including the basic helix-loop-helix domain, the orange domain and the tetrapeptide motif [37,38,39,48]. Although the invariant proline residue in the basic domain and the WRPW tetrapeptide at the carboxyl terminus are replaced in CHF by a glycine and by YRPW respectively, the overall structure of the protein is similar to each other [37,38,39,48]. CHF basic helix-loop-helix proteins form dimers through their HLH domain that properly positions and orients the basic domain for specific DNA sequences such as the E box. As described previously, transcriptional repression by HES proteins requires

recruitment of TLE/Groucho via the conserved WRPW motif. Repression of transcription by CHF transcription factors occurs via recruitment by the bHLH domain of the mSin3 complex containing HDAC1 and an additional corepressor, N-CoR [39]. A recent study demonstrated that CHF1 associates with HES1 in solution and formed a stable heterodimer upon DNA binding that repressed transcription [39]. Although HES1 and CHF1 homodimers showed distinct DNA preferences for E boxes, and individually repressed target genes, their co-expression demonstrated more than additive repression [39]. To address the role of CHF proteins in the regulation of myogenesis, their pattern of expression was analyzed [74]. CHF proteins exhibited a transient expression pattern in developing somites and limb buds, suggesting a potential role in skeletal muscle development [74]. It was found that CHF1 was not expressed in proliferating myoblast while CHF2 expression was high in myoblast, but was reduced significantly after induction of terminal differentiation, as myogenin expression increased [74]. Comparison between mRNA expression of HES6 and CHF2 actually showed inverse patterns of expression. While CHF2 mRNA was detectable in proliferating myoblasts, it became undetectable after 3 days in DM [74], as opposed to HES6 mRNA, which was expressed at high level after 3 days in DM. Semi quantitative RT-PCR data confirmed that CHF2 expression is inhibited by HES6 gain of function [13].

CHF2 repression of myogenic differentiation *in vitro* was assessed by cotransfection of 10T1/2 cells with a MyoD expression plasmid and CHF2, which resulted in a dramatic decrease of myosin heavy chain-positives cells. The mechanism of repression might involve formation of an inactive heterodimer with MyoD [74]. CHF2-MyoD heterodimer

cannot bind to the E box which results in sequestration of MyoD and ultimately repression of myogenesis.

VII. Outline of the research project

Although HES6 homodimers have been shown to bind the ESE box [17], they cannot bind to the N box [8]. As reported by Bae *et al*, the length of the loop is responsible for the incapacity of HES6 to bind DNA as a homodimer. However, since HES6 can repress transcription specifically from N box-containing promoters in muscle cells, HES6 could potentially dimerize with a bHLH partner in myoblasts to bind the N box, recruit TLE and repress transcription. This hypothesis applied specifically to muscle cells because of the unique functions of HES6 in myoblasts. As presented in section V., HES-6 N box dependent repression is specific to myoblasts and was not observed in fibroblasts and neuroblasts. Furthermore, HES6 does not exhibit the same properties in myoblasts than neuroblasts: HES6 antagonizes HES1 in neuroblasts instead of cooperating with it as in myoblasts.

The aim of this research project was to identify an HES6 partner of dimerization in myoblasts using both a genome-wide screen and a candidate gene approach. Obvious potential partners of dimerization could not be hypothesized. E proteins, which are partners of dimerization for other myogenic bHLH such as MyoD, did not show any interaction with HES6 in electrophoretic mobility shift assays (EMSA) [27]. Other HES family members appear mostly expressed in neural tissues except for HES1, which doesn't dimerize efficiently with HES6. Finally, members of the CHF family were

defined to have an inverse pattern of expression with HES6. Because no potential partner could be identified, search started with a genome wide screen using the DupLEX-A Yeast-Two-Hybrid System from Origene. This system is a method for detecting protein-protein interaction *in vivo*. It relies on the fact that transcription factors have separable DNA binding domains and transcriptional activation domains. Neither domain can activate transcription on its own; they have to be tethered to one another to activate transcription. Interaction between two proteins can be tested by fusing one to the DNA binding domain and the other to the activation domain. If the two proteins interact, the transcriptional activator is reconstituted and can activate transcription of a reported gene (Figure 2). In this case, HES6 was fused to the DNA binding domain, *E.coli* LexA protein, and a muscle cDNA library was fused to the activation domain, acid blob domain B42. This system then allowed searching for potential partner of dimerization.

A candidate gene approach was used to test the potential interaction between HES6 and CHF2. Although HES6 and CHF2 have reciprocal temporal expression [27,74], a recent publication demonstrated that HES1 formed a stable heterodimer with CHF1 to ultimately bind DNA through the E box or the N box [37,38]. Althought it was demonstrated that HES6 can bind DNA through an ESE box [17], none were identified in the CHF2 flanking region. Based on this data, we tested the hypothesis of an interaction between HES6 and CHF2 to the N-boxes present in CHF2 5'-flanking region. A potential interaction could explain the inverse temporal pattern of expression of the two proteins either via formation of an inactive dimer (which would ultimately lead to sequestration) or by formation of an active dimer that would repress transcription of a target gene,

CHF2 itself. Interaction between two bHLH transcription factors was tested by co-immunoprecipitation, a method that allows detection of specific protein-protein interactions *in vivo.*

Aims:

1. **Identify HES6 putative dimerization partner using a genome-wide screen**
2. **Detect a protein-protein interaction between HES6 and CHF2**
3. **Asses the capacity of the dimer to bind DNA at an N-box**

MATERIALS AND METHODS

A. Yeast Two-Hydrid

I. Sequencing of the bait

The clone pT7T3-HES6 was obtained from I.M.A.G.E consortium Clone I.D. 385069, GeneBank Accession: W62881. The clone was completely sequenced using the thermosequenase kit (Amersham Pharmacia; Baie d'Urfé, QC). Sequencing reactions were amplified in PCR machine (Omnigene, TR3 SM3) and thermal cycling conditions were 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min with a final extension cycle of 72°C. Sequencing reactions were run on a 6% acrylamide/Bisacrylamide gel (Gibco BRL; Burlington, ON) containing 7mM Urea (Invitrogen; Burlington ON), 0.8X TTE (appendix I) using a BioMax STSi sequencing apparatus from Kodak (Rochester, NY). Sequencing gel was dried (BioRad Gel Dryer, 165-1745) and exposed on a Kodak X-OMAT AR film overnight at room temperature.

II. Construction of the bait

For the two-hybrid interaction assay, the bait plasmid was constructed using the vector pEG202 encoding the DNA binding protein LexA from *E. coli* provided in the DupLEX-A Yeast Two-Hybrid System (Origene Technologies; Rockville, MD) The clone pT7T3-HES6 was amplified by PCR using primers that would mutate a stop codon to a glutamine in the N-Terminus and introduce restriction sites at both ends allowing subcloning into pEG202. The primers used: 5'CCCGAATTCATGGCTCCGTCTCA3' and 5'GGCACCGAGCTCTCCACACACT3' respectively introduced a XhoI and an EcoRI restriction sites. Each PCR reaction was performed using 10ng of DNA, 100pmol of each primer, 10mM of NH_4SO_4 (Merk, Mississauga, ON), 20mM dNTP (Amersham Pharmacia), 1 µl of pfu enzyme (Stratagene, LaJolla CA) and 1X of pfu buffer in a total volume of 100µl with a mineral oil overlay (Fisher Scientific; Ottawa, ON). PCR reaction was amplified in the PCR machine (Omnigene, TR3 SM3) and thermal cycling conditions were 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 50°C for 45 sec, 72°C for 2 min with a final extension cycle of 72°C for 10 min. PCR products were resolved by an agarose gel 0.9% (Gibco BRL) containing ethidium bromide (Sigma, Oakville, ON) in 1X Tris-Boric Acid EDTA buffer (appendix I) run in a gel box (BRL life Technologie, Model H5) at 100 volts. The band was excised and purified using the Gel Purification kit from Qiagen Inc. (Mississauga, Ont). The product was subsequently sequenced to ensure that the mutations were introduced. The insert was then digested overnight at 37°C using XhoI and EcoRI restriction enzymes (New England Biolabs-NEB-, Mississauga, ON) in presence of 1X BSA (NEB) then purified using the Nucleotide Removal Kit (Qiagen Inc.).

The vector pEG202 (Origene technologies) was digested overnight at 37°C using XhoI and EcoRI restriction enzymes in presence of 1X BSA. Removal of 5' phosphate group from the digested vector was done with Calf Intestinal alkaline Phosphatase (NEB) and the vector was purified using the Nucleotide Removal kit from Qiagen. Insert and vector were ligated at 14°C overnight using 1µl of T4 DNA ligase (NEB), 1X T4 DNA ligase buffer and 1mM of ATP (Amersham Pharmacia) in a final volume of 20µl. XL10 Gold Ultracompetent cells (Stratagene) were transformed according to the manufacturer procedure. Transformed bacteria were plated on Luria Bertoni, LB (Appendix I) plate supplemented with ampicillin 100mg/ml (Merk) and left at 37°C overnight. Colonies were tested for the presence of the construct by XhoI and EcoRI digest. Positives colonies were sequenced to ensure that HES6 was cloned in frame with the binding domain. A large DNA preparation of the positive construct was made using Midi-Prep Kit from Qiagen.

III. Components of the DupLEX-A Yeast Two-Hybrid kit

The DupLEX-A Yeast Two-Hybrid kit provided several yeast strains, reporter genes and control plasmid as well as a bait plasmid and a target plasmid. They are described in the table bellow for better understanding.

TABLE 1: Description and characteristics of the different components provided in The DupLEX-A Yeast Two-Hybrid kit.

Yeast Strains	Description	Characteristic
EGY48	MATα trp1 his3 ura3 leu2, 6 LexAop-LEU2	Yeast Strain with LEU2 reporter gene of a High sensitivity
EGY194	MATa trp1 his3 ura3 leu2, 4 LexAop-LEU2	Yeast Strain with LEU2 reporter gene of a Medium sensitivity
EGY188	MATa trp1 his3 ura3 leu2, 2 LexAop-LEU2	Yeast Strain with LEU2 reporter gene of a Low sensitivity
EGY40	MATα trp1 his3 ura3 leu2, 0 LexAop-LEU2	Negative control Yeast Strain
Reporter Gene plasmids		
pSH18-34	URA3, Amp^r, 8 op.-LacZ	LacZ reporter gene of a High sensitivity
pJK103	URA3, Amp^r, 2 op.-LacZ	LacZ reporter gene of a Medium sensitivity
pRB1840	URA3, Amp^r, 1 op.-LacZ	LacZ reporter gene of a Low sensitivity
pJK101	URA3, Amp^r, GAL1-2 op.-LacZ	LacZ reporter gene whose expression is driven by the yeast GAL1 promoter. Used in the repression assay
Control plasmids		
pRHFM1	HIS3, Amp^r, ADH promoter expressing Lex-A homoedomain	Used as a positive control in testing for autoactivation (no activation)
pSH17-4	HIS3, Amp^r, ADH promoter expressing Lex-A-GAL4 activation domain	Used as a negative control in testing for autoactivation (strong activation)
pEG202-Max	HIS3, Amp^r, expresses Lex-A-Max fusion	Used as a positive control in the repression assay
Bait plasmid		
pEG202	HIS3, Amp^r, ADH promoter expressing Lex-A followed with a polylinker	Used to make the bait fusion protein
Target plasmid		
pJG4-5	TRP1, Amp^r, Inducible GAL1 promoter expressing B42-HA tag followed by target protein expressing muscle cDNA library	Used for the screen in the large scale transformation

IV. Testing the autoactivation potential of the bait

Some bait proteins can activate reporter genes on their own which prevents their use in a yeast two-hybrid screen. To test for autoactivation of LacZ reporter gene by the bait fusion, yeast strains provided in the DupLEX-A Yeast Two-Hybrid system (Origene) EGY48, EGY194, EGY188 and EGY40 (negative control) were transformed with the following combinations of vectors (Origene):

1. pEG202-HES6 + pSH18-34 (The bait + High sensitivity LacZ reporter gene)
2. pSH17-4 + pSH18-34 (Negative control + High sensitivity LacZ reporter gene)
3. pRFHM1 + pSH18-34 (Positive control + High sensitivity LacZ reporter gene)

Small-scale transformation was performed according to manufacturer protocol (Origene). Transformed yeast cells were plated on YNB (gal)-His-Ura (Appendix I) + X-Gal (Invitrogen) plates. A Whatman paper (Fisher Scientific) was put in the petri dish with 2ml of Z buffer (appendix I) containing 1mg/ml X-Gal in N,N-dimethyl formamide (Merk). A similarly cut Hybond-P nitrocellulose filter (Amersham Pharmacia) was put on the surface of the plate containing transformed yeast cells, pulled off and frozen twice at –70°C for 5 min. It was then placed side up on the Whatman filter paper and incubated for 2 hours at 30°C.

Ability of the bait to autoactivate LEU2 reporter gene was tested by picking colonies from the yeast cells transformed with the bait and plated on YNB(glu)-His-Ura (appendix I) plate. Those colonies were diluted 1:100, 1:1000 and 1:10,000 in sterile water and

plated on YNB(gal)-His plate and on YNB(gal)-His-Leu plate and were incubated at 30°C for 1-2 days. Only selective plates lacking histine should have presented colonies.

V. Repression Assay

The ability of the bait to enter the nucleus and bind to LEX-A operator was tested by transforming the EGY48 yeast strain with the following combination of vectors provided by Origene DUPLEX-A Yeast Two-Hybrid System:

1. pEG202-HES6 + pJK10 (test)
2. pEG202-Max + pJK101 (repression)
3. pJK101 alone (no repression)

Transformation 1 and 2 were plated on a YNB(glu)-His-Ura plate and transformation 3 was plated on a YNB(glu)-Ura plate and incubated for 1-2 days at 30°C. Colonies from plate 1 and 2 were streaked on YNB(gal)-His-Ura + X-Gal and colonies from plate 3 were plated on a YNB(gal)-Ura + X-Gal plate and incubated for 12-24 hours at 30°C.

VI. Large scale library transformation

Large-scale transformation was performed as described in the DupLEX-A Yeast Two-Hybrid System protocol (Origene). EGY94 yeast strain was transformed along with the reporter gene plasmid pRB1840, the bait plasmid pEG202-HES6 and the library plasmid pJG4-5. Transformants were plated on YNB(glu)-His-Ura-Trp plates and incubated for 2-3 days at 30°C. Colonies were scraped off using sterile water and the edge of a

microscope slide (VWR Scientific, Mississauga ON). The slurry was harvested 5 min. at 1500Xg in a Sorval RC5B centrifuge at room temperature. The pellet was resuspended in distilled water and spun as previously. The pellet was then suspended in 1 volume of sterile water and half a volume of 50% glycerol (Merk) was added. Cells were aliquoted and frozen at –70°C.

VII. Screening for potential positive transformants

YNB(gal)-His-Ura-Leu-Trp media was inoculated with 1 x 10^7 transformed cells/ml and incubated at 30°C for 4 hours with shaking (New Brunswick Scientific Shaker). Aliquots of 100µl were plated onto 100mm YNB(gal)-His-Ura-Leu-Trp plates and incubated at 30°C. Colonies were picked after 2 and 3 days and streaked again on a YNB(gal)-His-Ura-Leu-Trp plate, which were labelled as Master plate Day 2 and Master plate Day 3

Colonies from both master plates were streaked on the following plates and incubated for 1-2 days at 30°C:

1. YNB(glu)-His-Ura-Leu-Trp
2. YNB(gal)-His-Ura-Leu-Trp
3. YNB(glu)-His-Ura-Trp + X-Gal
4. YNB(gal)-His-Ura-Trp + X-Gal

VIII. Recovering plasmids from yeast

Potential positives transformants that grew on the YNB(gal)-His-Ura-Leu-Trp but not on the YNB(glu)-His-Ura-Leu-Trp and that did turn blue on the YNB(gal)-His-Ura-Trp + X-Gal but not on the YNB(glu)-His-Ura-Trp + X-Gal were recovered. The first 50 colonies that arose were grown in YNB(glu)-Trp medium at 30°C overnight. Plasmids were recovered from yeast cells according to DupLEX-A Yeast-Two-Hybrid System protocol (Origene).

Recovered DNA was transformed into electrocompetent KC8 cells (Origene) using a BioRad electroporator. Cells were plated onto LB plates containing 50mg/ml ampicilin and were incubated at 37°C overnight. Colonies from that plate were re-streaked on a minimal (-trp) plate (according to DupLEX-A Yeast Two-Hybrid System protocol). DNA was isolated from bacteria by the alkaline lyses method and digested with EcoRI and XhoI. The digested products were resolved on an agarose 0.9% gel containing ethidium bromide and 1X TBE.

IX. The Mating Test

EGY40 yeast cells (Origene) were transformed with the pEG202-Hes6 bait and the reporter gene plasmid pRB1840 (Origene). The EGY194 yeast cells (Origene) were transformed with pJG4-5 (Origene) library plasmid isolated from the screen. Transformants were mated by streaking the two in a + pattern on YPD plate (Appendix I) and incubated at 30°C overnight. A replica was performed the next day on YNB(gal)-His-Ura-Trp-Leu.

X. Sequencing of the potential positives

Positives clones that passed all the specificity tests were sequenced using the primers provided in the DupLEX-A Yeast Two-Hybrid system kit (Origene) and the thermosequenase kit. Sequencing reactions were amplified and thermal cycling conditions were 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min with a final extension cycle of 72°C for 10 min. Clone sequences were compared to clones from the Genebank database for identification.

B. Synthesis of HES6 antibody

I. Anti-HES6 antibody production

A peptide from an antigenic region located in the C-terminus region unique to HES6 was synthesized: GSGRSSWPPGGSPESGGC. The hapten was coupled to the carrier protein, keyhole limpet hemocyanin (KLH), following the instruction of the Imject Antibody Production and Purification kit (Pierce; Rockford, Illinois). A New Zealand White rabbit of 12 weeks was used for the injection. All animal protocols were approved by the Institutional Animal Care and Use Committee. A pre-immune blood sample was taken prior to injections. The coupled-hapten was mixed with Freund's complete adjuvant (Gibco BRL) 1:1 for the first injection. The emulsion was injected intramuscularly and rabbits were boosted 3 times with KLH conjugated to HES6 peptide in Freud's incomplete adjuvant (Gibco BRL). A blood sample of 5 ml was taken prior to each following injection. After the third boost, the rabbit was bled. Blood samples were centrifuged (Sorvall RT7) for 10 min at 2000rpm. Serum was aliquoted and Sodium

Azide (Kodak, Rochester, NY) was added to a final concentration of 0.02%. Samples were stored at –20°C

II. Anti-HES6 Antibody Purification

Antibody was purified from the rabbit serum following manufacturer procedure described in the Imject Antibody Production and Purification kit (Pierce). The purified antibody fractions were pooled and dialysed against PBS (appendix II) using a Slide-A-Lyzer cassette (Pierce).

III. Cell Culture

Cos7 cell were obtained from the American Tissue Culture Collection (Rockville, MD). They were maintained in Dulbecco's Modified Eagle Media (DMEM) low glucose (Gibco BRL) supplemented with 10% fetal bovine serum (Cansera, Rexdale, Ont) at 37°C with 5% CO_2. Cells were seeded at 300,000 cells per P60 plates (Fisher Scientific) for transfection. The following day, 0.5ml of serum free DMEM (Gibco BRL)was mixed with 30µl of GenePorter transfection reagent (Gene Therapy Systems, San Diego, CA) and 0.5ml of serum free DMEM was mixed with 6µg of pCMV-HA (Invitrogen) or 6µg of pCMV-HA-HES6 provided by Dr. S. Stifani (Montreal Neurological Institute, McGill University). DNA and transfection reagent were mixed together and incubated for 30 min at room temperature. Following incubation, 1ml of serum-free DMEM was added and then the mix was added drop-wise to the cells. Cells were incubated 5 hours at 37°C. Following incubation, 2ml of DMEM low glucose supplemented with 20% FBS was

added to the cells. The next day, the media was changed for 3 ml of DMEM low glucose supplemented with 10% FBS.

IV. Western Blot

Cellular extracts were prepared 48 hour post-transfection by washing cells twice with PBS and 400µl of lysis buffer 2X (Appendix II) was added. Cells were incubated 15 min on ice, then scraped (Canpar, St-Léonard, QC) and 400µl of water was added. Samples were centrifuged at 13,000rpm (Eppendorf Centrifuge 5915C) for 15 minutes. The supernatant was used for the western blot. Aliquots of 60µl were taken and 30µl of SDS 3X buffer (Appendix II) was added. Samples were boiled for 4 minutes and then resolved on a 14% Sodium Dodecyl Sulfate PolyAcrylamide gel electrophoresis (SDS-PAGE). Gel consisted of a 14% resolving gel (14% acrylamide/Bisacrylamide (Gibco BRL), 1X Tris-Cl/SDS pH 8.8, 0.1% Ammonium Persulfate-APS- (Sigma), 0.1% TEMED (BioRad)) and a 4% stacking gel (4% acrylamide/Bisacrylamide, 1X Tris-Cl/SDS pH 6.8, 0.1% APS, 0.1% TEMED). The rainbow ladder (Amersham Pharmacia) was used as a molecular weight maker. Gel was run initially at 100V and then at 150V in an electrophoresis gel box, BioRad, mini 2-D.

Proteins from the gel were transferred on a nitrocellulose membrane (Amersham Pharmacia) at 100V for 45 min in transfer buffer (Appendix II) with agitation at 4°C. Membrane was blocked using 5% skimmed milk (Nestley; North York, Ont) in 1XTBS with 0.2% Tween-20 (Merk), (TBS-T) overnight at 4°C. After 3 washes of 15 min with TBS-T, primary antibody, pre-immune serum from the rabbit or the serum after 2 boosts,

was diluted 1:100 in TBS-T and the membrane was incubated 1 hour with agitation at room temperature. The membrane was washed 3 times for 15 minutes with TBS-T followed with addition of the secondary antibody, Anti-Rabbit IgG conjugated to Horseradish peroxydase (Amersham Pharmacia) diluted 1:50,000 in TBS-T. After an incubation of 1 hour, the membrane was washed. Detection was performed using the ECL Western Blotting Detection Reagents (Amersham Pharmacia). The membrane was stripped by immersing it in stripping buffer (Appendix II) and incubated at 50°C for 30 min. The membrane was washed 2X15 min in TBS-T 0.2% and then blocked as described previously. Detection was performed as described before but instead of using serum as the primary antibody, Anti-HA Antibody (Clonetech Laboratories, Palo Alto, CA) diluted 1:1000 was used.

A final Western blot was performed using the purified Anti-HES6 antibody as a primary antibody to a dilution 1:1000.

C. Co-Immunoprecipitation

I. Transfection conditions

Cos7 cells (American Tissue Culture Collection) were transfected as described in section B. III with the following combinations of plasmids:

1. Not Transfected
2. 3µg pCHF2-Flag (Dr. Chin, Harvard Medical School) + 3µg pBlueScript (Stratagene)
3. 3µg pCMV-HA-HES6 (Dr.Stifani, Montreal Neurological Institute, McGill University) + 3µg pBluescript

4. 3μg pCMV-HA-HES6 + 3μg pCHF2-Flag
5. 3μg pCMV-HA-HES6 + 3μg pBlueScript
6. 3μg pCMV-HA-HES6 + 3μg pCHF2-Flag

The day following transfection, the cell culture media was changed for 3 ml of DMEM low glucose supplemented with 10% FBS.

II. Immunoprecipitation

EZview Red Anti-Flag M2 Beads (Sigma) and anti-Myc Beads (Santa Cruz Biotechnology, Santa Cruz CA) were washed and equilibrated by adding 1ml of TBS (Appendix II) and vortexed (Vortex Genie, Sci 37494). Beads were incubated 15 min at 4°C with agitation and centrifuged 1 min at 10,000rpm (Centrifuge, Eppendorf 5415C). Supernatant was taken out carefully and the wash was repeated twice.

A total cell extract of the transfected cells was prepared as described in section B.IV. An aliquot of 80μl of each total cell extract was taken out and used to test the efficiency of transfection. Cell extract of transfections 1 to 4 were incubated with 60μl of equilibrated EZview Red Anti-Flag M2 affinity gel and total cell extract of transfections 5 and 6 were incubated with 20μl of equilibrated anti-Myc Beads. Incubation was at 4°Co overnight. The next day, tubes were centrifuged for 30 sec at 10,000rpm and the supernatant was aspirated carefully. Beads were washed with 1ml of TBS (Appendix II), mixed at 4°C for 15 min and centrifuged at 10,000rpm for 30sec. The wash was repeated 3 times. Proteins were eluted from the beads by adding 25μl of 1X SDS Buffer, vortexing, and then incubating 5 min at room temperature. Tubes were boiled 5 min and then centrifuged 1 min at 10,000rpm. Supernatant was loaded on a 14% SDS poly-acrylamide gel. Proteins

were transferred to a PVDF membrane (Amersham Pharmacia) as described in section B.IV. PVDF membranes were soaked in 100% methanol (Merk) for 10sec, washed 5min in water and equilibrated with transfer buffer prior to blotting. Non-specific binding sites were blocked with 5% milk in TBS-T. Membrane was then washed 3 times for 15 minutes using TBS-T. Primary antibody, Anti-HES6 antibody, was diluted 1:100 in TBS-T and membrane was incubated 1 hour with agitation at room temperature. Thus, three washes of 15 minutes with TBS-T were performed, followed by addition of the secondary antibody, Anti-Rabbit IgG Antibody coupled to HRP (Amersham Pharmacia) diluted 1:50,000 in TBS-T. After an incubation of 1 hour, the membrane was washed. Bands were detected with the ECL+ Western Blotting Detection Reagents (Amersham Pharmacia) and membrane was exposed on ECL+ autoradiography film (Amersham Pharmacia) for 1 min.

To test efficiency of transfection, aliquots of the total cell extract of the six transfections were mixed with 40µl of SDS 3X buffer. The samples were boiled 4 minutes and then 40µl of each cell extract was loaded on 2 different 14% SDS poly-acrylamide gels. Proteins were transferred on PVDF membrane as described in section B.IV and the Western Blot was performed as described previously using Anti-HES6 rabbit antibody 1:1000 for one membrane and using Anti-Flag M2 mouse antibody (Santa Cruz Biotechnology) 1:1000 for the other as a primary antibody and Anti-Rabbit IgG Antibody coupled to HRP (Amersham Pharmacia) 1:50,000 was used as a secondary antibody for one membrane and Anti-Mouse IgG Antibody coupled to HRP (Amersham Pharmacia)

1:50,000 for the other. Detection was performed with the ECL+ Western Blotting Detection Reagents (Amersham Pharmacia).

D. Electrophoretic Mobility Shift Assay (EMSA)

I. Oligonucleotides

The following double stranded oligonucleotides of the N-Boxes located in the 5'-flanking region of CHF2 as well as an ESE box [17] containing two Enhancer of Spit E box and an E box Class B [39] were synthesized and used for EMSA.

N-Box 269-274 coding: 5'GATCGCTGCACCAGTGGG3'
N-Box 269-274 non-coding: 5'CTAGCGACGTGGTCACCC3'
N-Box 2229-2234 coding: 5'GATCAGAGCACAAGCATT3'
N-Box 2229-2234 non-coding: 5'CTAGTCTCGTGTTCGTAA3'
N-Box 2283-2288 coding: 5'GATCAGTCCACCAGAGAA3'
N-Box 2283-2288 non-coding: 5'CTAGTCTGGTGGTCTCTT3'
N-Box 10318-10323 coding: 5'GATCATAACACCAGGGTT3'
N-Box 10318-10323 non-coding: 5'CTAGTATTGTGGTCCCAA3'
N-Box 12631-12636 coding: 5'GATCTCCCCACCAGGTC3'
N-Box 12631-12636 non-coding: 5'CTAGAGGGGTGGTCCA3'

ESE-Box coding: 5'GATCGGTGGCACGTGCCATTTGGCACGTGCCATG3'
ESE-Box non-coding: 5'CTAGCCACCGTGCACGGTAAACCGTGCACGGTAC3'
E-Box Class B coding: 5'GATCAATGGCACGTGCCACCCTCGA3'
E-Box Class B non-coding: 5'CTAGTTACCGTGCACGGTGGGAGCT3'

II. Preparation of the probes

Double stranded oligonucleotides were prepared by mixing 50µg of the coding oligonucleotides and 50µg of the non-coding oligonucleotides with 50µl of 50mM NaCl (Merk) and incubating the samples for 2 min at 88°C, 10 min at 65°C and 10 min at 37°C. Oligomers (4µl) were labelled by filling in with 2 units of Klenow enzyme (NEB)

in presence of ^{32}P (Amersham Pharmacia), of 2,5µl of 10X Nick Translation Buffer (appendix III) and 1µl of 5mM dNTP (-dCTP).

Samples were incubated for 90min at room temperature and then 50µl of TE (10mM Tris-HCl pH 7.4, 1mM EDTA) was added. Labelled probes were separated from unincorporated nucleotides by fractionation. G-50 Sephadex column were prepared by putting a small volume of sterile siliconized glass wool (Chromatographic specialities Inc) into a 1 ml Syringe (Becton Dickinson) and adding 0.7ml of Sephadex fine G-50 (Amersham Pharmacia). Labelled oligonucleotides were added to the column and spun 2 min at setting 3 in an IEC clinical centrifuge (IC international equipment company). 1µl of each of the purified labelled oligonucleotides was used to count the number of cpm/µl using a liquid scintillation analyzer (Canberra Packard Canada 1600CA).

III. In vitro-translated proteins

In vitro-translated HES6 and CHF2 proteins were prepared using the TnT Quick T7 Coupled Reticulocyte Lysate Systems (Promega). The clone pT7T3-HES6 obtained from I.M.A.G.E consortium Clone I.D. 385069 and pcDNA-hCHF2 (Dr. Chin, Harvard Medical School) were used for in vitro translation. The presence of full-length protein was confirmed by autoradiography with S^{35}-methionine (ICN Bioscientifics, Aurora, OH) labelled translation performed in parallel. Proteins were mixed with 1X SDS buffer and run on a 14 % SDS poly-acrylamide gel. The gel was dried on a Whatman (Fisher Scientific) and exposed to a Kodak X-OMAT AR film for 15 hours at room temperature.

IV. Total and Nuclear Cell extract

C2C12 cells (American Tissue Culture Collection) were maintained in DMEM High Glucose (Gibco BRL) supplemented with 10% fetal bovine serum until they reached confluency, then they were switched to DM, DMEM with 2% Horse serum (Vector Labs, LaJolla, CA) to induce myotube fusion.

In one experiment, cells were maintained for 4 days in DM and a total cell extract was prepared from those as described in section B.IV. In another experiment, C2C12 cells were maintained in DM for 7 days and a nuclear extract was prepared from 25 P100 plates. Nuclear Extracts were prepared using the standard procedure for nuclear extract preparation adapted from Dignam et al [24]. Cells were harvested at 2000rpm (Sorval RC 5B plus) for 10 min in cell culture medium at room temperature and then washed twice with PBS. The pellet was resuspended in 5 volume of cold PBS and centrifuged 20 min at 2000rpm at 4°C, then was resuspended in 5 volumes of buffer A (Appendix III) and incubated 10 min on ice. The cells were then centrifuged 15 min at 2000rpm at 4°C and resuspended in 2 volumes of buffer A. The cell lyses was performed with 10 strokes of a glass Wheaton MicroTissue homogeniser (B type pestle) (Wheaton Scientific, Millville, NJ). Homogenate was transferred to an eppendorf tube and centrifuged 10 min at 2000rpm at 4°C. Pellet was centrifuged again at 14,500rpm 20 min. and resuspended in 0.3ml of Buffer C/10^8 cells (Appendix III). Cells were homogenized with 20 strokes of a Wheaton MicroTissue homogeniser (A type pestle), transferred to a Corex tube and stirred for 30 min on ice. The sample was centrifuged 30 min at 12,500rpm (Centrifuge Eppendorf 5415C) at 4°C and dialysed as described in section B.II. with Buffer D

(Appendix III) for 2 hours with agitation at 4°C. Dialysis product was centrifuged at 12,500rpm for 20min at 4°C. Supernatant was aliquoted and stored at –80°C.

V. Electrophoretic Mobility Shift Assay conditions

Protein-DNA complexes were formed by incubation of 15ul of in vitro translated proteins described above with 5000cpm of radio labelled probe on ice for 30 minutes in a buffer containing 20mM HEPES pH 7.5, 100mM KCl, 10% Glycerol, 10μM $ZnSO_4$, 2mM β-Mercaptoethanol, 0.1% Nonidet P-40, 4μg/ul of BSA and 0.25ng/ul of dI/dC (Sigma), a non-specific competitor. Complexes were resolved by electrophoresis on a 5% acrylamide gel 30:0.8 (Sigma) containing TBE buffer 0.5X (Appendix III), 50μl TEMED (BioRad) and 150μl APS 10% (Sigma) at 200Volts using a BioRad Protean II. Dried gel was exposed to Kodak X-OMAT AR X-Ray film at –80°C overnight.

In some experiments, 15 μl of total cell extracts or 5μg of nuclear cell extracts were used to form the protein-DNA complex instead of IVT proteins. Some binding reactions were carried in the presence of 10, 25, 50, 100 fold molar excess of unlabelled ESE box probe or E box Class B probe or 0.1, 1, 10, 25 fold molar excess of unlabelled ESE box probe.

Supershift was carried with 4μl of antibody as described above with either 15μl of total cell extract from C2C12 cells differentiated for 4 days or with 5ug of nuclear extract from C2C12 cells differentiated for 7 days. Antibodies used were either Rabbit Anti-HES6 antibody or α-NAC 3081 vs 939 antibody (Dr. St-Arnaud, Shriners Hospital).

RESULTS

A. The Genome Wide Screen

I. Identification of HES6 dimerization partner by Yeast Two-Hybrid

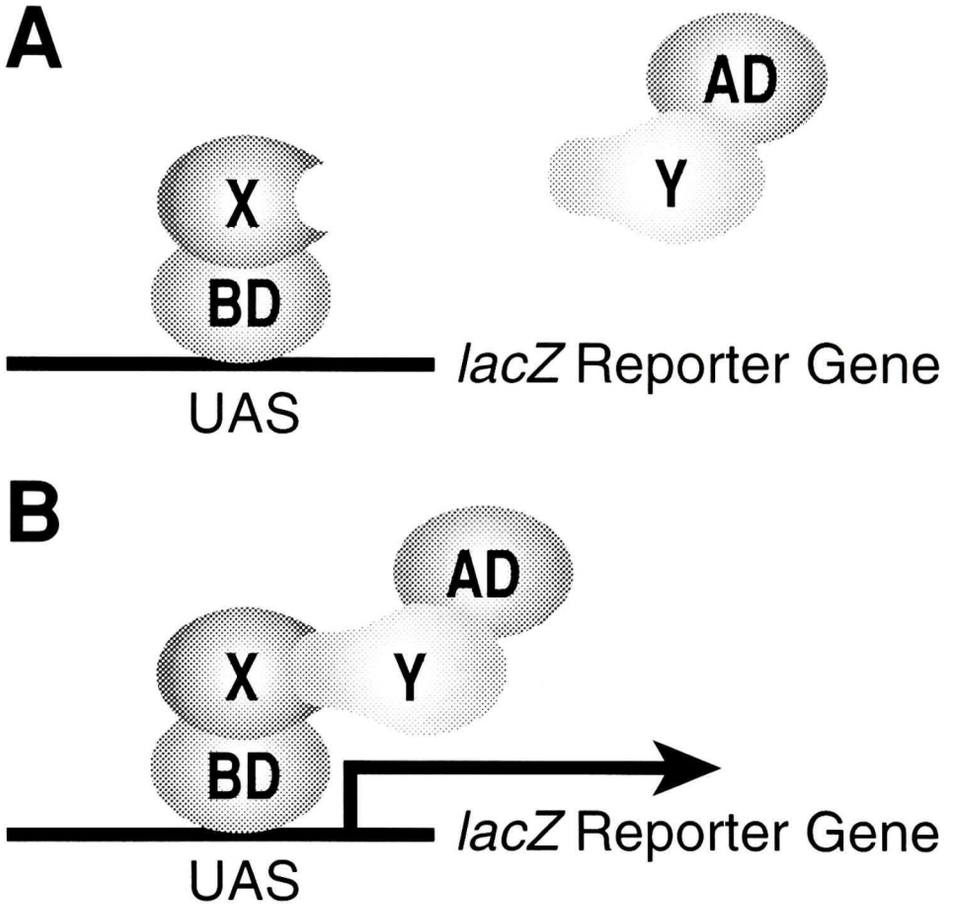
Recent publications demonstrated that HES6 could not bind to the N box or the E box as a homodimer although it was able to repress transcription in an N box dependent manner [6,24]. It was thus hypothesized that HES6 could bind to DNA as a heterodimer, potentially with another bHLH protein. As a first approach to identify this putative dimerization partner, a genome wide screen approach was used. The DupLEX-A Yeast Two-Hybrid system was used to detect a protein-protein interaction *in vivo* with HES6. This system exploits the fact that transcription factors have a separable DNA binding and activation domain; neither can activate transcription on their own. Interaction between two proteins was detected by fusing HES6 to the DNA-binding protein and a muscle cDNA library to the activation domain. Upon interaction, an active transcription factor will be reconstituted and activation of the reporter gene will occur (Figure 4).

II. Constructing the bait

The first step of the yeast two-hybrid assay required cloning of HES6 into the bait plasmid pEG202 provided in the Origene kit (Figure 5). The bait protein gene, HES6, had to be fused in-frame with LexA, the binding domain, to ensure that a correct LexA-HES6 fusion protein would be transcribed. Since no restriction sites present in the HES6 sequenced allowed an in-frame subcloning into pEG202, they had to be introduced in both the amino and the carboxyl terminus. Primers that would introduce an EcoRI and a XhoI restriction site were used for PCR. Furthermore, the amino terminus primer had to

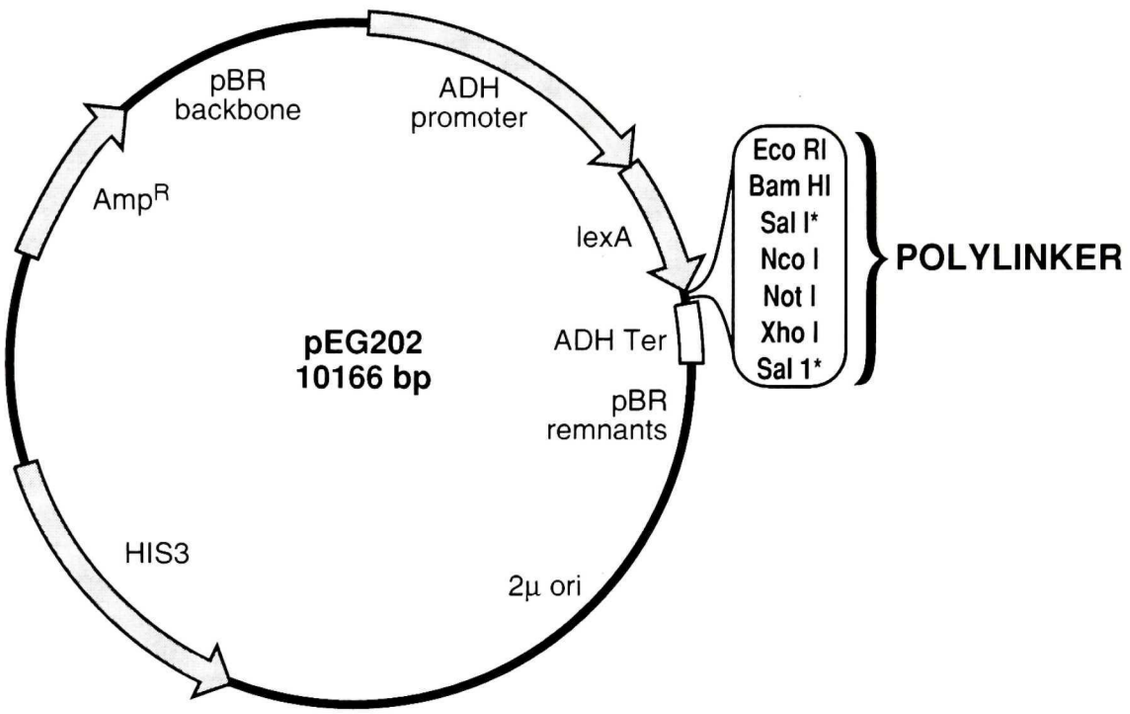
Figure 4: The Yeast Two Hybrid protein interaction assay. (A) Because X, potentially the bait, isn't interacting with Y, an active transcription factor comprised of a DNA binding domain (BD) and an activation domain (AD) is not reconstituted. There is then no activation of the reporter gene. (B) Protein-protein interaction between X and Y reconstitute an active transcription factor which ultimately activates transcription of the lacZ reporter gene. UAS, upstream activiation sequence.

METHODS 1:
Yeast Two Hybrid System



Ref.: Strategies in Molec. Bio. Vol. 8, No. 1, p. 3-5

Figure 5: pEG202 plasmid vector map. Bait plasmid used for constitutive expression of LexA-bait fusion protein in yeast. The polylinker contains restriction sites not found anywhere else in the vector. The plasmid includes a yeast selectable marker (HIS3) and a bacteria selectable marker (Amp R), and the ADH (alcool dehydrogenase) promoter and terminator (ter) site. ori, bacterial origin of replication; pBR, pBR322 plasmid backbone and remnants.


pBR
backbone
ADH
promoter
Amp^R
lexA
pEG202
10166 bp
ADH Ter
pBR
remnants
HIS3
2μ ori
Eco RI
Bam HI
Sal I*
Nco I
Not I
Xho I
Sal 1*
POLYLINKER

mutate a stop codon located 2 amino acids upstream from the HES6 translation start site to a glutamine residue otherwise only the LexA binding domain would have been translated. Since the HES6 sequence is G-C rich, NH_4SO_4 was added to enhance denaturation during the PCR reaction. Amplification of HES6 occurred at an annealing temperature of 50°C and with addition of 10mM of NH_4SO_4 (Figure 6, lane 2). An amplimer of approximately 650 base pairs was produced. No amplification was detected without NH_4SO_4 or with 50mM or 100mM of NH_4SO_4 (Figure 6, respectively lane 1-3-4). Sequencing of the PCR product revealed that the restriction sites had been introduced properly and that no spurious mutations occurred during the amplification. The digested PCR product and bait plasmid (pEG202) were ligated and sequencing of the construct showed that HES6 was subcloned in frame with LexA and that the stop codon was properly mutated to a glutamine residue.

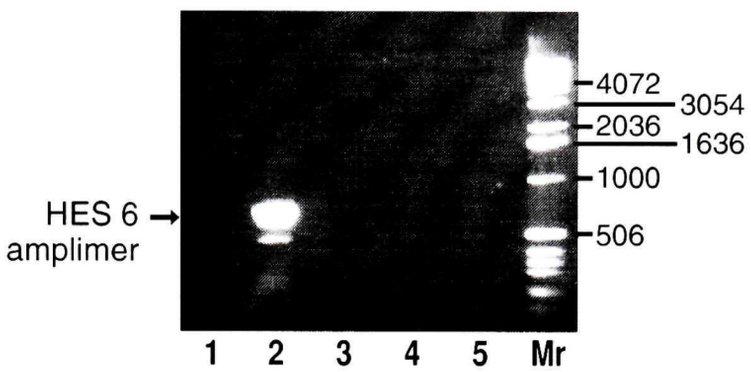
III. Optimization of the yeast two-hybrid assay

A. Testing for autoactivation of the bait

In some cases, the bait protein contains cryptic activation domains. In those cases, the bait proteins can activate the reporter gene on its own, without protein-protein interaction, which makes the screen very difficult and increases the number of false positives. Many false positives occur because of this, which is why it's important to validate results with a complementary experiment [86].

The kit from Origene allows selection using two reporter genes, LEU2 and LacZ reporter genes. Activation of the LEU2 reporter gene allows growth on a medium lacking leucine

Figure 6: Agarose gel electrophoresis of the HES6 amplification PCR products. Amplifications were held as described in Materials and Methods. No amplification occurred without NH_4SO_4 (1) or with addition of 50mM (3) or 100mM (4) of NH_4SO_4. Amplification of HES6 with addition of 10mM of NH_4SO_4 (2) showed an amplimer of about 650bp while amplification without DNA showed no band (5). Mr, molecular size markers in bp.


HES 6
amplimer
4072
3054
2036
1636
1000
506
1
2
3
4
5
Mr

while the LacZ reporter gene allows expression of the β-galactosidase protein, which can be detected using a colorimetric assay (colonies will stain blue on a medium containing X-gal). The kit also provides different yeast strains and reporter gene plasmids (described in Table 1) with different levels of sensitivity so that baits that activate transcription on their own can still be assayed by using a less sensitive reporter. The bait was transformed in each of the yeast strains provided in combination with each of the reporter gene plasmids. The positive control plate was covered of yeast cell colonies as opposed to the negative control plate, which only had around 50 colonies. Although all yeast strains showed some autoactivation of the LEU2 reporter gene, the plates containing transformed EGY194 yeast cells, a medium sensitivity strain, had significantly less colonies than the other strains. Furthermore, the number of colonies present was closer to the number observed on the negative control plate than any other yeast strains used. LacZ assay revealed no autoactivation of the lac-Z reporter in any of the combinations. Since the combination of EGY194 and pRB1840 gave less autoactivation of the LEU2 reporter gene compared to yeast cells of the same strain transformed with any of the other reporter gene plasmids, pRB1840 was the reporter gene plasmid chosen for the large scale transformation.

B. Testing the capacity of the bait protein to enter the nucleus

Protein-protein interaction is detected through activation of the reporter gene. Because reporter genes are activated upon binding of the complex to a LexA operator located in the nucleus, the bait has to be able to enter the nucleus. Transforming yeast cells with the bait along with pJK101 allowed testing of the bait's capacity to enter the nucleus. The

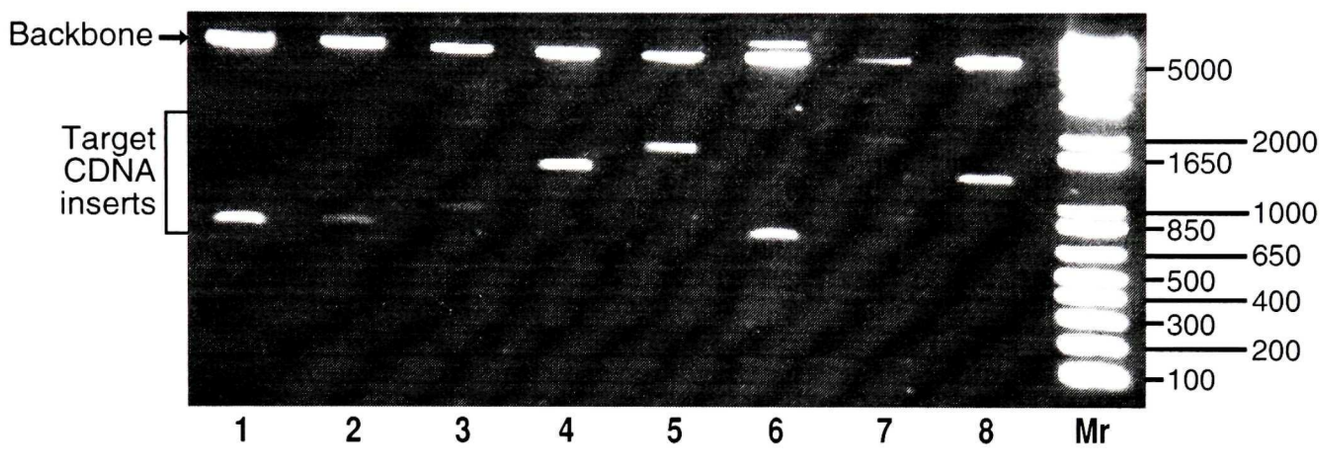
plasmid pJK101 contains a LacZ reporter gene whose expression is driven by the yeast GAL1 promoter and contains two LexA operators, which have been placed between the promoter and the reporter gene. Activation of the LacZ reporter gene will occur upon binding of the GAL4 protein to the promoter. If the bait enters the nucleus, it will bind to the LexA operators and repress activation of the reporter gene. In case of repression, colonies will remain white, while if there is no repression, colonies will stain blue. Yeast cells transformed with pJK101 and the bait were white (data not shown). This result shows that the LexA-HES6 fusion bait proteins entered the nucleus and bound to the LexA operator sequence to repress transcription from the GAL1 promoter.

IV. Results from the large scale transformation

Large scale transformation was performed twice. The yeast strain EGY194 was transformed with the reporter gene plasmid pRB1840 along with the bait plasmid and the target plasmid, the library-containing plasmid pJG4-5. Transformation allowed yeast cells to grow on selective plates. The plasmid pRB1840 contains a URA3 gene, which allows transformed cells to grow on a medium lacking uracil. The plasmid also includes a LacZ operator, which allows expression of β-galactosidase on plate containing X-gal upon protein-protein interaction, thus colonies will stain blue. The bait plasmid includes a HIS3 gene, which would give the capacity of transformed cells to grow on a selective plate lacking histidine. Finally, the target plasmid provides the capacity to grow on a medium lacking tryptophane. Thus transformed yeast cells were selected based on their capacity to grow on a medium lacking histidine, uracil and tryptophane. Furthermore, the yeast strain EGY194 provides a LEU2 gene, which allows growth on a medium lacking

leucine upon protein-protein interaction. The colonies would then be selected based on their capacity to activate the LEU2 and LacZ reporter gene. Moreover, because of the GAL 1 promoter, the expression of the target genes is dependent on galactose, activation of the reporter genes of potential positives would only occur in presence of galactose as a sugar source rather than glucose. Comparing the number of colonies that grew on the medium lacking leucine and containing glucose with the number of colonies present on the plate lacking also leucine but containing galactose allowed detection of a potential interaction between HES6 and a partner of dimerization. The first screen revealed 5 plates where there were more colonies on the medium containing galactose than on the plates containing glucose. Colonies from all those potential positive plates activated the LacZ reporter gene only on the plate with galactose. The second screen revealed the same type of results on 12 different plates. In both screens, plates that tested positive for both reporter genes had over 250 colonies. DNA from the first 50 colonies to arise from each potential positive plate was isolated to be further used for transformation into a –trp *E.coli* strain, a strain which is unable to grow on a medium lacking tryptophane. This transformation enabled selection of the library plasmid since this plasmid gave the capacity to grow on a medium lacking tryptophane to transformed cells. Library plasmids then isolated from the bacteria were digested with EcoRI and XhoI to release the insert of the target plasmid. Figure 7 shows a typical digestion result. The insert size varied between different plasmid preparations to another, which meant that different potential partners had been isolated. On this figure, some clones appear to have the same size so they might potentially be identical (lanes 1-2-6 and lanes 4-8). Thus, although 50 plasmids were isolated from each positive plate, only inserts of different sizes were

Figure 7: Agarose gel electrophoresis of the digested plasmids isolated from the yeast Two-Hybrid Screen. Plasmids from the screen were previously digested with EcoRI and XhoI restriction enzymes, which released the cDNA insert from the plasmid vector. A band of 850 is observed in lane 1,2 and 6 suggesting that identical clones were isolated. Lane 4 showed a band of 1650 pb, lane 5 a band of 2000 pb and lane 8 a band of 1500 pb. A band of approximately 6.4kb appeared in each lane, representing the plasmid backbone.


Backbone
Target CDNA inserts
5000
2000
1650
1000
850
650
500
400
300
200
100
1
2
3
4
5
6
7
8
Mr

sequenced. Results from the sequencing are described in Table 2. Sequencing results showed that many different clones were isolated from the two screens and that each of them was obtained more than once

TABLE 2.: Sequencing results from the large scale transformation screen.

Clone identification	**I. Number of times isolated**
Mus Musculus Tumor Necrosis Factor	3
Mus Musculus Aldolase 1	4
Vascular Endothelial Zinc Finger 1	5
Mus Musculus Creatine Kinase	6
Heat Shock 70kD Protein 5	9
Mus Musculus ATP Synthase 1	5
Mus Musculus Actin	7
Mus Musculus Glyceraldehyde-3-Phosphate dehydrogenase	3
Mus Musculus Voltage Dependant Anion Channel encoding Mitochondrial Proteins	6
Mus Musculus Ribulose 1,5 Biphosphatecarboxylase/ Oxygenase	4
Mus Musculus Phosphorylase Kinase	2
Troponin C	5
Mus Musculus 16S Ribosomal RNA	4
Mus Musculus Titin Immunoglobulin	9
Mus Musculus 0 Day Neonate skin cDNA	7
Heat Shock protein C 169	6
Mus Musculus Acetyl-CoA acyltransferase	8

Although the yeast two-hybrid assay from Origene includes a thorough selection, it appears that only false positives were isolated. The genome-wide screen identified many clones, althought only the Heat Shock Protein HSP70 and HSP169 could potentially interact with HES6 in a relevant fashion. However, the heat shock proteins are unlikely to be putative dimerization partners. The result of the genome-wide screen will be analyzed further in the dicsussion.

B. The Candidate Gene Approach

I. CHF2 as a potential partner of dimerization for HES6

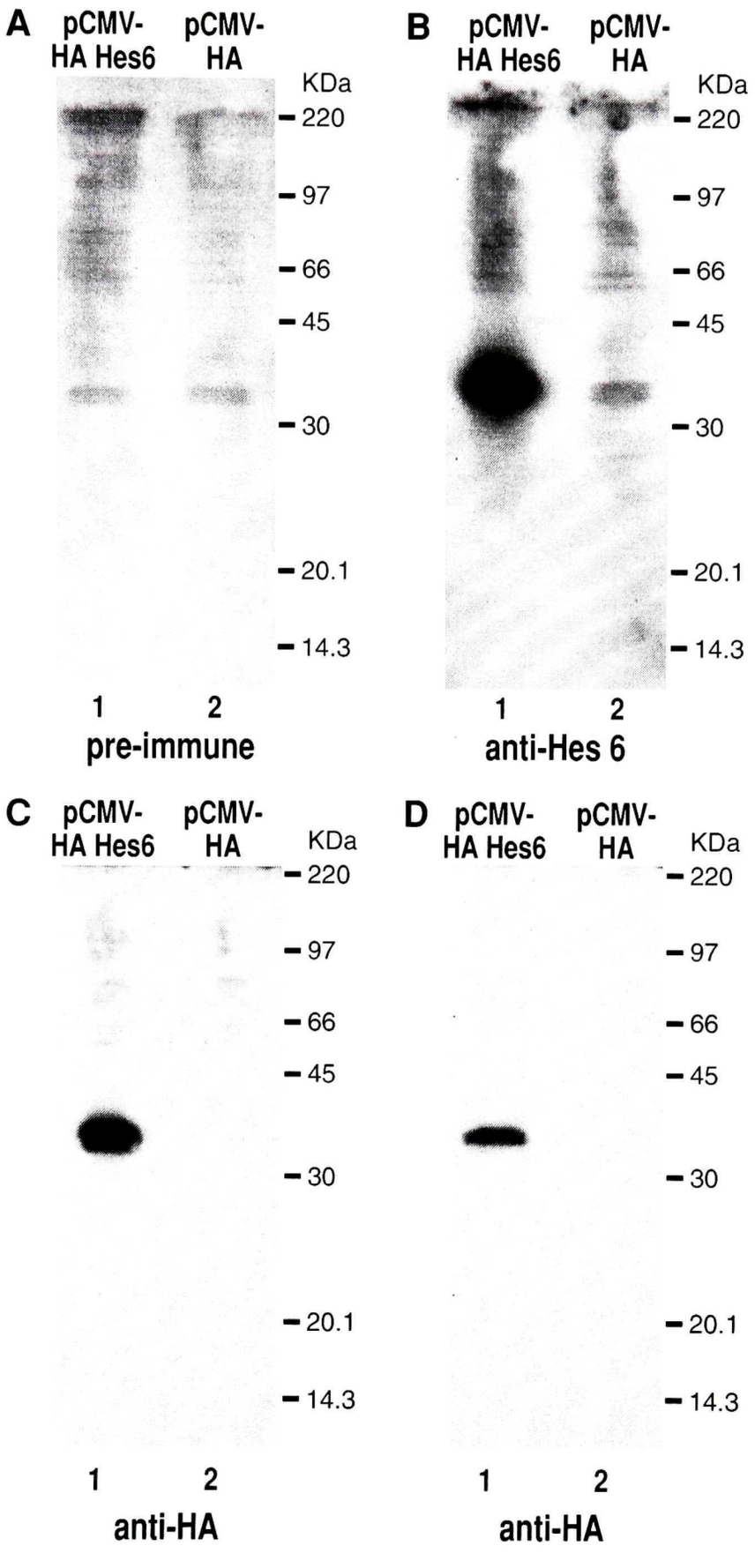
Because of the number of false positives that arose from the screens, a candidate gene approach was initiated. Recent publications described a novel basic helix-loop-helix transcription factor, CHF2, and characterized it as a repressor of myogenesis [37,38,39,74]. Furthermore, studies demonstrated that HES1 formed a stable heterodimer with CHF1 and that the complex bound DNA through the E box or the N box [36]. Based on this data, it was hypothesized that HES6 might interact with CHF2 to further repress its expression. This interaction between CHF2 and HES6 was tested *in vivo* .

II. Synthesis of an Anti-HES6 antibody

To observe the formation of a dimer *in vivo*, a co-immunoprecipitation assay was performed. In order to increase the specificity, an antibody specific to HES6 was produced for further use in the co-immunoprecipitation experiment. In a first step, the most antigenic regions of HES6 were analyzed using the software Protean from DNAstar. This software helps to predict and display patterns, secondary structural characteristics and physicochemical properties of protein sequences via its comprehensive suite of protein analysis tools. Comparison between all HES protein sequences allowed identification of regions unique to HES6. A peptide from a unique antigenic region of HES6 was synthesized, GSGRSSWPPGGSPESGGC, coupled to KLH and injected into rabbits. Total cell extracts of Cos7 cells transfected with an HA-tagged HES6 expression vector were used for Western blot analysis, which would allow for testing of the specificity of the antibody produced. Immunoblotting with the pre-immune serum

revealed only faint background band around 33 kDa (Figure 8 a). However, blotting with the serum taken after the third boost revealed a strong band in cells expressing HA-tagged HES6 (Figure 8b, lane 1) and only background staining in cells expressing the tag alone (Figure 8b, lane 2). This suggested that the injection of the HES6 peptide induced the synthesis of anti-HES6 antibody molecules. The expression vector transfected into the cells expressed a HES6 protein fused to an HA-tag. Blotting the membrane with an antibody against that tag would confirm whether HES6 was expressed or not by the cells. If not, the band observed when blotting with the anti-HES6 antibody would not be specific to HES6. The membranes were thus subsequently blotted with an anti-HA antibody. A band of the correct size appeared on the membrane previously blotted with the pre-immune serum (Figure 8c, lane 1). This result confirmed that the HA-tagged HES6 was expressed although not detected by the pre-immune serum confirming that the rabbit was not expressing any anti-HES6 antibody prior to the immunizations. No band was observed in cells expressing the tag alone because the size of the HA tag is too small to be seen on this membrane (Figure 8c, lane 2) as opposed to the tag fused to HES6. The presence of a band of the correct size on the membrane previously blotted with the anti-HES6 serum confirmed the presence of antibodies against HES6 in the serum (Figure 8d, lane 1). Moreover, the tag alone did not reveal a band of 33 KDa (Figure 8d, lane 2). Those results taken together confirmed that the immunized rabbit produced anti-HES6 antibodies. Furthermore, the results confirmed the specificity of the antibody produced. Because the intensity of the signal from the Western blot performed with the serum was strong enough; the rabbit was bled after the third boost. The purified serum was tested for

Figure 8: Western Blot performed with total cell extracts from Cos7 cells using the non-purified serum of rabbits prior and after injection of the coupled-hapten as described in Materials and Methods. (A) Western blot using serum pre-immune serum of total cell extracts from Cos7 cells expressing pCMV-HA-HES6 (1) or pCMV-HA (2). Only faint background staining is observed. (B) was blotted with the non-purified rabbit serum after the third boost. (1) Total cell extract of Cos7 cells transfected with pCMV-HA-HES6 revealed a strong band around 33KDa, which is not observed with total cell extracts of cells transfected with the vector alone (2). (C) is the stripped membrane from panel A, subsequently blotted with an Anti-HA antibody (1) Cos7 cell extracts expressing pCMV-HA-HES6 revealed a strong band at the expected size for the fusion protein confirming the specificity of the anti-HES6 antiserum. (2) Cos7 cell extracts expressing the vector alone showed no band. (D) is the stripped membrane of panel B subsequently blotted with an Anti-HA antibody. (1) Cos7 cell extracts expressing pCMV-HA-HES6 revealed a strong band confirming that the band previously observed is the HES6-HA protein. (2) Cos7 cell extracts expressing the vector alone showed no band. Mr, molecular size markers in kDa.


A
pCMV-HA Hes6
pCMV-HA
KDa
220
97
66
45
30
20.1
14.3
1
2
pre-immune
B
pCMV-HA Hes6
pCMV-HA
KDa
220
97
66
45
30
20.1
14.3
1
2
anti-Hes 6
C
pCMV-HA Hes6
pCMV-HA
KDa
220
97
66
45
30
20.1
14.3
1
2
anti-HA
D
pCMV-HA Hes6
pCMV-HA
KDa
220
97
66
45
30
20.1
14.3
1
2
anti-HA

immunoblotting on a western blot and gave a clear single band at a dilution of 1:1000 (Figure 9)

III. HES6 forms a heterodimer with CHF2 *in vivo*

Cos7 cells were transfected with a Flag-tagged CHF2 and/or a HA-tagged HES6 and the efficiency of transfection was evaluated by Western blot analysis. Total cell extracts were resolved on a SDS-PAGE gel and transferred on a nitrocellulose membrane. Bands were detected by immunoblotting with either the anti-HES6 antibody or an anti-Flag antibody to detect respectively HES6 or CHF2. On figure 10, when anti-HES6 antibodies were used, no bands were observed in untransfected cells or cells transfected with CHF2-Flag alone (lanes 1-2) while all cell extracts from cells transfected with HES6-HA showed a single band around the expected size (Figure 10, lanes 3 to 6). Cells transfected with a Flag-tagged CHF2 revealed a band on Western blot when blotted with an anti-Flag antibody (Figure 11, lanes 2-4-6) as opposed to cells that weren't transfected with CHF2-Flag (lanes 1-3-5). Transfected COS7 cells expressed the expected proteins at a detectable level.

A co-immunoprecipitation allows detection of protein-protein interactions *in vivo*. This method requires cells expressing both of the proteins of interest (A and B). The cell extract from those cells is then incubated in presence of beads coupled to an antibody that will specifically interact with one of the protein of interest (protein A in this case). Incubation of those beads with the cell extract will allow a specific interaction between them and protein A. A centrifugation will then bring down the beads along with protein

Figure 9: Western Blot of Cos7 cell extract transfected with pCMV-HA-HES6 using the purified serum from the rabbit injected four times with the coupled-hapten. The Anti-HES6 antibodies gave a signal up to a dilution of 1:1000. Mr, molecular size markers in kDa.

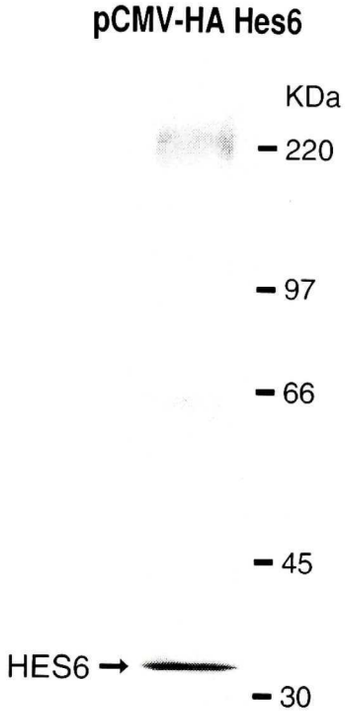

pCMV-HA Hes6
KDa
220
97
66
45
HES6
30

Figure 10: Western Blot of Cos7 cell extracts blotted with the Anti-HES6 antibodies to assess the efficiency of transfection. (1) Non-transfected Cos7 cells revealed no HES6 expression as well as cells transfected with flag-tagged CHF2 alone (2). Cos7 cells transfected with either pCMV-HA-HES6 alone (3 and 5) or with pCMV-HA-HES6 along with flag-tagged CHF2 (4 and 6) revealed a band, assessing the expression of HES6 protein in those cell extracts. Mr, molecular size markers in kDa.

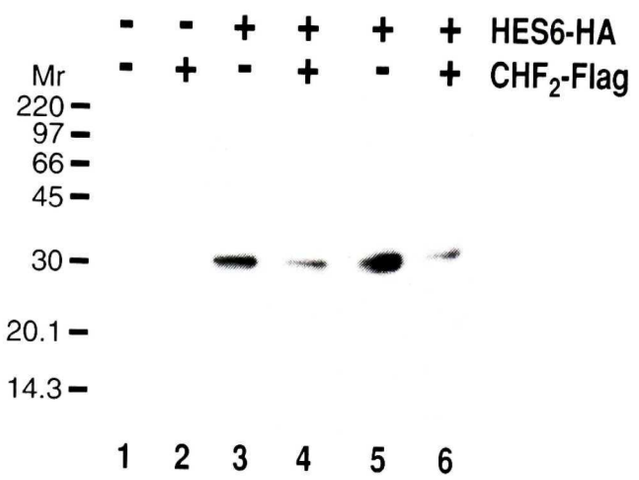
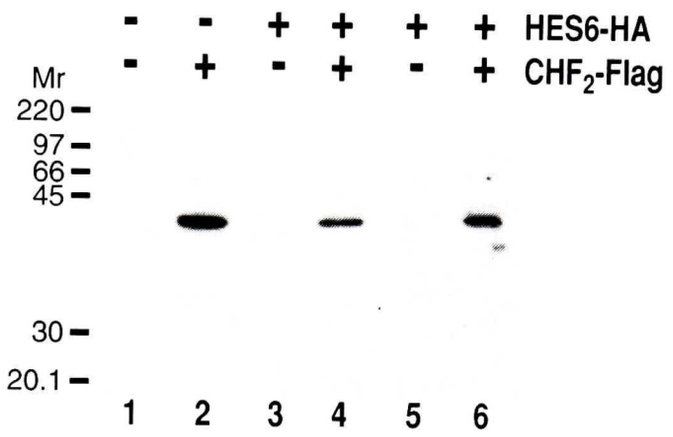

- - + + + + HES6-HA
- + - + - + CHF2-Flag
Mr
220
97
66
45
30
20.1
14.3
1 2 3 4 5 6

Figure 11: Western Blot of Cos7 cell extracts blotted with the Anti-Flag M2 mouse antibodies to verify the expression of CHF2-Flag in transfected cells. (1) Untransfected Cos7 cells revealed no CHF2-Flag expression as well as cells transfected with pCMV-HA-HES6 alone (3 and 5). Cos7 cells transfected with either flag-tagged CHF2 alone (2) or with flag-tagged CHF2 along with pCMV-HA-HES6 (4 and 6) revealed a band, assessing the expression of CHF2-flag protein in those cell extracts. Mr, molecular size markers in kDa.


- - + + + + HES6-HA
- + - + - + CHF2-Flag
Mr
220
97
66
45
30
20.1
1 2 3 4 5 6

A. If there is a protein-protein interaction between A and B, both proteins will be brought down. Interaction between the beads and the proteins will be broken with SDS and heating and the proteins isolated will then be run on an acrylamide gel and analysed by Western Blot using an antibody against protein B. If protein A doesn't interact with protein B, no band should be observed since protein B wouldn't have been precipitated with the beads.

In this experiment, Cos7 cells were transfected with a vector expressing HES6 and a vector expressing CHF2 in order to detect protein-protein interactions. Several control cell extracts were also subjected to a co-immunoprecipitation to establish to specificity of the interaction. Cos7 cells were transfected with either a Flag-tagged CHF2 expression vector, an HA-tagged HES6 expression vector or both. Then, total cell extracts from non-transfected cells and cells expressing a Flag-tagged CHF2 and/or HES6 were subjected to co-immunoprecipitation. Cell extracts were incubated with either anti-Flag beads, which would specifically interact with the Flag-tagged CHF2 protein, or they were incubated with anti-Myc beads, which should not interact with either HES6 nor CHF2. The immunoprecipitation was followed by a Western blot analysis using the anti-HES6 antibody produced previously. A specific *in vivo* interaction was detected between HES6 and CHF2 when immunoprecipitated with anti-Flag beads (Figure 12, lane 4). Specificity of the interaction was confirmed by the controls. Non-transfected cells did not show any sign of co-immunoprecipitation when precipitated with anti-Flag beads (Figure 12, lane 1), as well as cells transfected with either CHF2 or HES6 alone (Figure 12, lane 2-3). This result confirmed that HES6 did not bind unspecifically to the beads and that CHF2

Figure 12: Western Blot of the Co-Immunoprecipitation experiment performed as described in Materials and Methods. Total Cos7 cell extracts were incubated in presence of EZview Red Anti-Flag M2 beads (1-4) or Myc beads (5-6) and the precipitated proteins were detected with the anti-HES6 antibody (1) Non-transfected cells showed no band assessing that no unspecific band can be observed from Cos7 cell extracts. (2) Although CHF2-Flag protein was precipitated from cell extracts of Cos7 cells expressing CHF2-Flag alone, the anti-HES6 antibody did not reveal a band confirming that no unspecific band can be observed from CHF2 alone. (3) Cos7 cells expressing HES6-HA alone revealed no band when blotted with its specific antibody assessing that the beads cannot precipitated HES6 in a unspecific manner. (4) A co-immunoprecipitation is observed, CHF2-Flag was precipitated and because of a protein-protein interaction between CHF2 and HES6, a band was revealed with the anti-HES6 antibody. (5) Cell extracts from Cos7 cells expressing HES6-Ha alone or (6) HES6-HA along with CHF2-Flag precipitated with anti-Myc beads revealed no interaction confirming the binding specificity of the anti-Flag beads and the specificity of the interaction between HES6 and CHF2 observed in lane 4. Mr, molecular size markers in kDa.

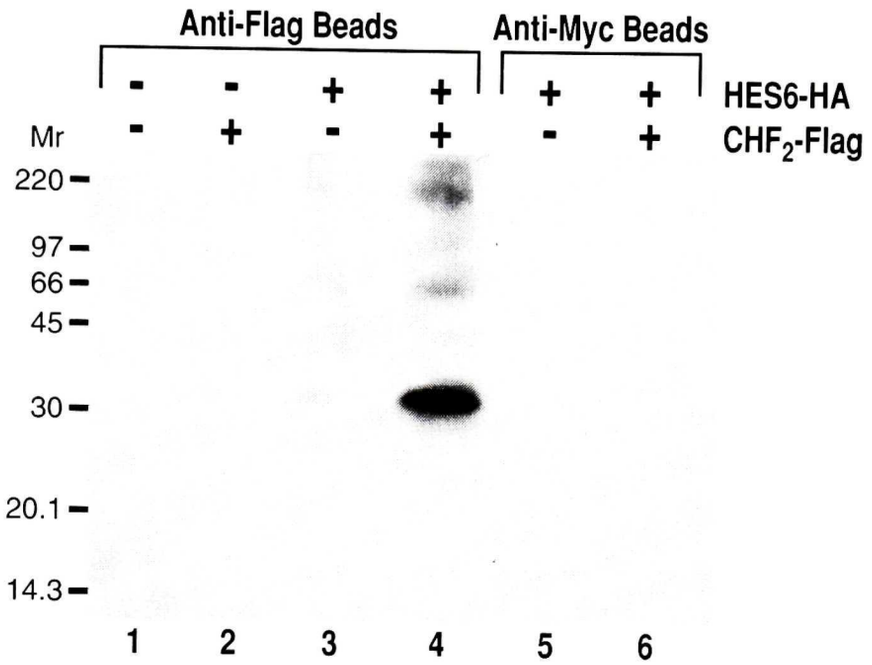
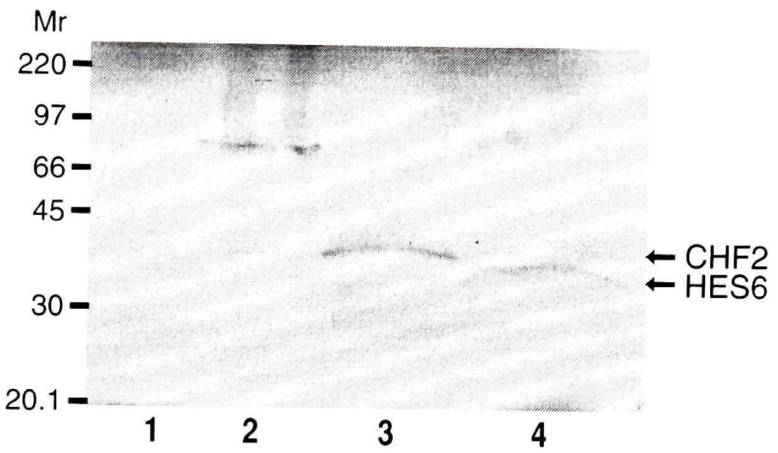

Anti-Flag Beads
Anti-Myc Beads
- - + + + + HES6-HA
Mr - + - + - + CHF2-Flag
220
97
66
45
30
20.1
14.3
1 2 3 4 5 6

Figure 15: *In vitro* translated proteins prepared in parallel using ^{35}S methionine as described in Material and Methods were used to verify equal protein production. The negative control (lane 1) revealed no protein production as opposed to the positive control (lane 2), which revealed bands as expected. (lane 3) *In vitro* translation with pCDN3-CHF2 plasmids reveals a single band as well as the vitro translation of pT7T3-HES6 (lane 4). Mr, molecular size markers in kDa.

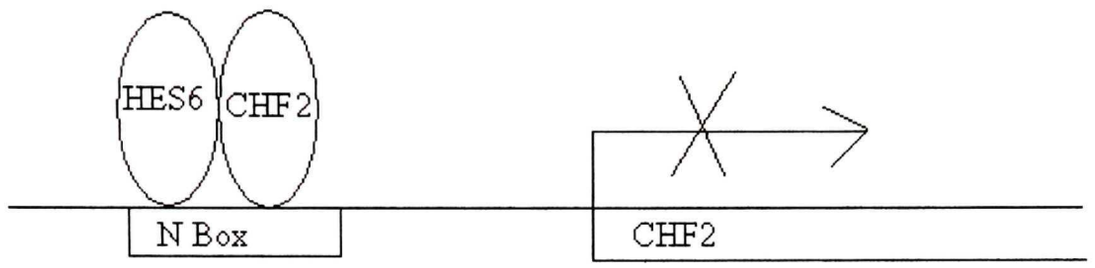

Mr
220
97
66
45
30
20.1
1
2
3
4
CHF2
HES6

could not bind to the anti-HES6 antibody unspecifically. Furthermore, the results confirm that Cos7 cells did not yield an unspecific signal and that the signal is dependent on the presence of both HES6 and CHF2. Immunoprecipitation performed with unspecific beads (anti-Myc beads), did not reveal precipitation either (Figure 12, lanes 5-6), even in the presence of both CHF2 and HES6 (Figure 12, lane 6). This result demonstrated the specificity of the binding of the anti-Flag beads to the CHF2-Flag protein and of the immunoprecipitation. Thus, co-immunoprecipitation was observed only in total cell extract precipitated with beads specific to flag-tagged CHF2 proteins and all the controls were negatives. We conclude that HES6 intereacts with CHF2 in mamalian cells.

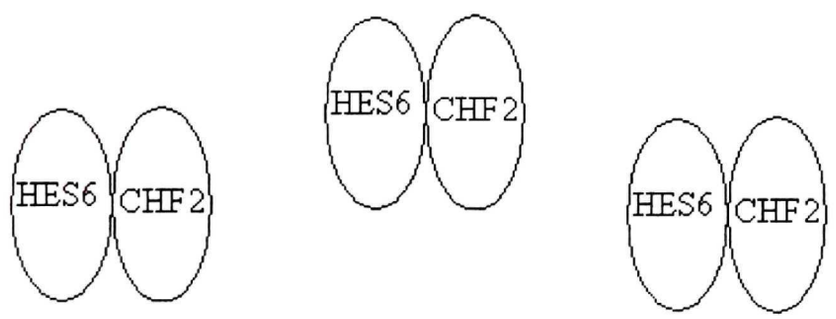
IV. HES6-CHF2 heterodimer doesn't bind any of the N boxes of the 5'-flanking region of CHF2

Recent data demonstrated that HES6 and CHF2 had reciprocal temporal expression patterns [13,27,74]. The formation of the HES6/CHF2 heterodimer could potentially repress CHF2 and explain why CHF2 expression decreases when HES6 expression increases. Repression of CHF2 can occur either by formation of an active or an inactive dimer. An active dimer would bind DNA and directly repress transcription of a target gene, as opposed to an inactive dimer, which only sequesters the protein. It then remained to be determined whether the HES6/CHF2 interaction formed an active or inactive dimer. Although the HES6 homodimer is unable to bind the N box [8], previous data had demonstrated that HES6 could repress transcription in an N box-dependent fashion [27]. It was then hypothesized that HES6 could form a heterodimer to bind DNA and ultimately repress transcription. Because HES6 can heterodimerize with CHF2, it could form an active dimer that would potentially bind to an N box and repress transcription of a target

gene, potentially CHF2 itself, which would explain the reciprocal temporal pattern of expression. This hypothesized model is illustrated on figure 13. HES6 could also form an inactive dimer that would sequester CHF2 away from its targets, thus initiating myogenesis [74].



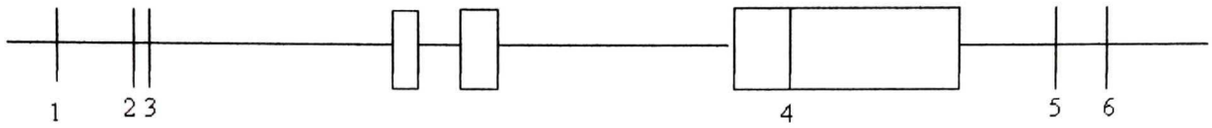
CHF2-HES6 Active Dimer



CHF2-HES6 Inactive Dimer

FIGURE 13: The heterodimer HES6-CHF2 could either form an active dimer or an inactive dimer. As an active dimer, it would potentially bind DNA through the N box of the 5'-flanking region of CHF2 and ultimately repress transcription. As an inactive dimer, HES6 would sequester CHF2.

To test the hypothesis of an active dimer, an electrophoretic mobility shift assay (EMSA) was performed to test the binding capacity of the heterodimer CHF2-HES6 to N boxes located in the 5'flanking region of CHF2 (figure 14)



Legend:
1: N box 269-274
2: N box 2229-2234
3: N box 2283-2288
4: N box 7323-7328
5: N box 10318-10323
6: N box 12631-12636

FIGURE 14: Representation of the location of the N boxes present in the CHF2 sequence. N boxes 1, 2, 3, 5, 6 are located in the flanking regions while N box 4 is located in an exon

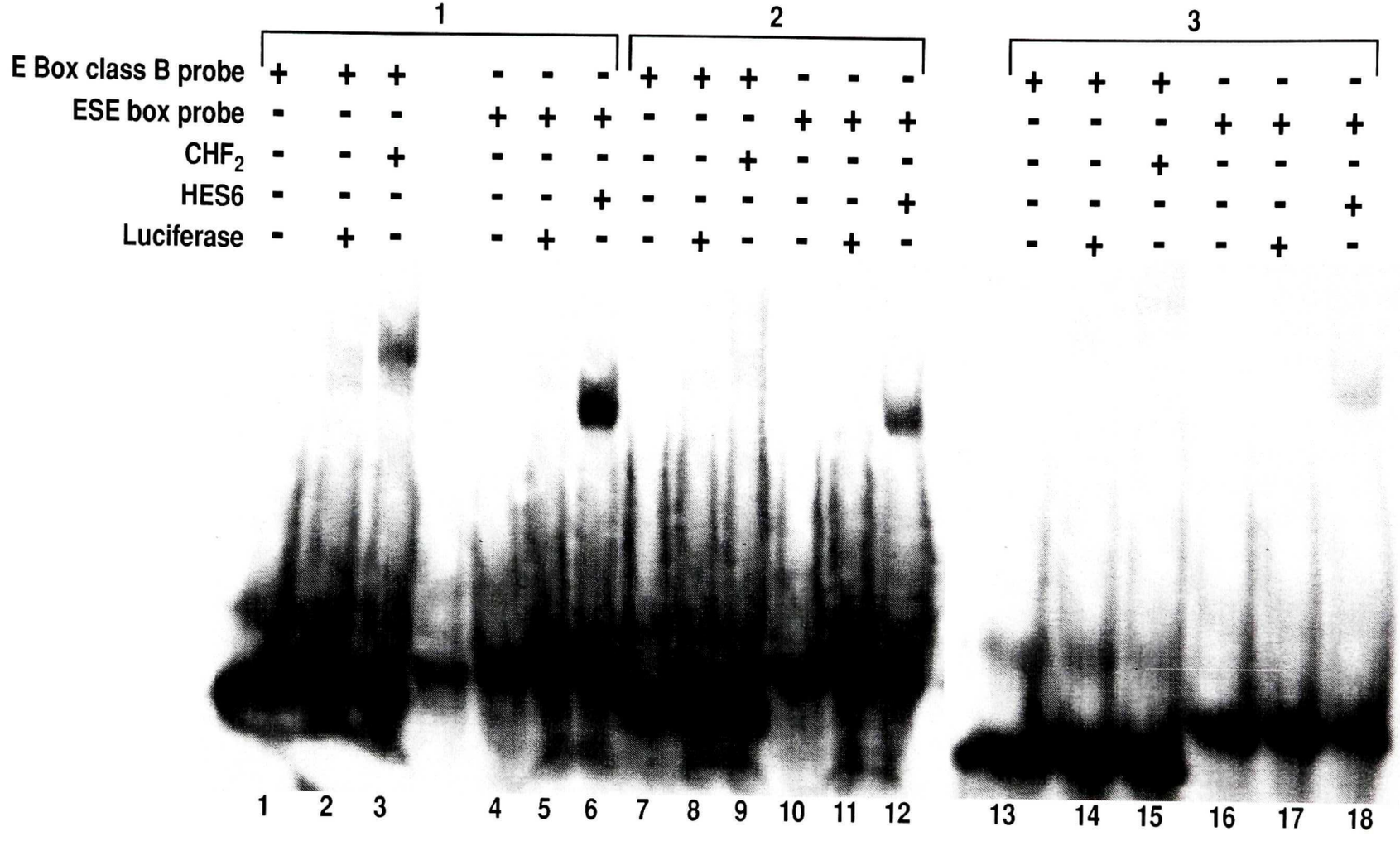
HES6 and CHF2 were synthesized by *in vitro* translation and prepared in parallel with ^{35}S methionine to verify the protein production. As shown on figure 15, CHF2 and HES6 were synthesized properly since a band of the expected size was observed (respectively lane 3 and 4). Furthermore, the negative control showed no band as opposed to the positive control provided in the kit (Figure 15, lanes 1 and 2).

It was demonstrated that HES6 could bind DNA as a homodimer through an ESE box [17] and that CHF2 could bind as a homodimer to an E box [74]. In this case, a binding reaction between HES6 and an ESE box-containing probe and between CHF2 and an E box class B [39] probe were used as positive controls in EMSA reaction to assess the DNA binding capacity of the *in vitro* translated proteins. Furthermore, each probes used were incubated in presence of an unrelated protein, the luciferase protein, to evaluate the background given by *in vitro* translated protein. Finally, each probe was incubated without any proteins to ensure that the probes alone did not give any unspecific bands.

The conditions used for EMSA reactions were based on conditions used for the binding of HES6 to the ESE box [17] and the binding of CHF1 to class B E box (CACGTG) [39]. Addition of a divalent ion ($MgCl_2$) gave no binding reaction while the presence of a monovalent ion ($ZnSO_4$) did (data not shown). EMSA reactions were subsequently held in presence of $ZnSO_4$. The amount of dI/dC, a non-specific competitor, and of BSA necessary for an EMSA reaction was evaluated. EMSA reactions where both CHF2 and HES6 demonstrated a good DNA binding to their respective probes were observed when 0.25ng/µl of dI/dC and 4µg/µl of BSA was added (Figure 16, 1-6). The *in vitro* translation protein gave some background as observed in lane 2 and 5 but CHF2 clearly bound to the E box class B probe and HES6 to the ESE box probe as observed by the band shifts (lane 3 and 6). When binding was performed without BSA and more dI/dC (50ng/µl) the evidence of binding of CHF2 to the E box class B disappeared (Figure 16, lane 9) while barely no effect was observed the binding reaction of HES6 (Figure 16, lane 12). EMSA reactions incubated with no BSA and with 0.25ng/µl of dI/dC showed barely any DNA binding activity for CHF2 (Figure 16 lane 15) and an important decrease in the band intensity for HES6 binding reaction (Figure 16, lane 18). The optimal EMSA binding conditions were then defined as 0.25ng/µl of dI/dC, 4µg/µl of BSA and 10µM $ZnSO_4$.

To assess the capacity of the heterodimer to bind any of the N boxes present in the 5'-flanking region of CHF2, some controls were added in addition to the ones previously described. Each N box was incubated with CHF2 or HES6 *in vitro* translated protein

Figure 16: DNA binding properties of *in vitro* translated CHF2 to the E box Class B and of i*n vitro* translated HES6 to the ESE box. In experiment 1 (1-6), electrophoretic mobility shift assays were performed in presence of 0.25ng/µl of dI/dC and 4µg/µl of BSA. Both HES6 and CHF2 demonstrated binding, respectively to the ESE box (6) or the E box class B (3). *In vitro* translated proteins gave a small background as observed in lane 2 and 5. In experiment 2 (7-12), electrophoretic mobility shift assays were held without BSA and with 50ng/µl dI/dC. CHF2 showed a dramatic decrease of binding activity (9) while HES6 binding activity barely changed (12). Background from the *in vitro* translated proteins is still observed (8 and 11). In experiment 3 (13-18), the reactions were performed without BSA and with 0.25ng/µl of dI/dC. The DNA binding activity of CHF2 to E box Class B is barely observable (15) and the binding activity of HES6 to ESE box probe decreased compared to experiment 1 and 2.


1
2
3
E Box class B probe
ESE box probe
CHF2
HES6
Luciferase
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

alone to ensure that the protein cannot bind to DNA as a homodimer. For each of the 5 N-boxes tested, no binding was observed when incubated with both HES6 and CHF2 proteins (figures 17-21, lane 11). The only band observed appeared to be caused by background from the amount of proteins present in the *in vitro* translated reaction (figure 17-21, lanes 8-13) since the intensity of the band is constant from one reaction to another and was not observed in reaction lacking lysate. The band shift observed in the positive controls supported the result observed by assessing the capacity of the *in vitro* translated proteins to bind DNA (Figure 17-21, lanes 3 and 6). In each case, the probe alone gave no band shift (Figure 17-21, lane 7).

V. Control experiments

The results from the EMSA experiments suggested that the heterodimer is unable to bind to any of the N boxes tested. To confirm that result, several control experiments were performed. Because our conclusion is supported by the fact that the positive controls did bind DNA, the first step was to evaluate the specificity of the positive controls used. To do so, the specificity of the interaction of either HES6 or CHF2 to its probe was competed by its unlabelled oligonucleotide. The proteins will then be able to bind to either the unlabelled or the labelled probe. Binding to unlabelled probe will reduced the signal observed, so the more unlabelled probe there will be, the more the signal will be reduced. Moreover, competition between the unlabelled and the labelled probe should be specific to the probe used; meaning that addition of an unrelated unlabelled probe should not have any effect on the binding. Each of the controls was tested with 10- to 100- fold molar excess of the unlabelled probe. Specificity of the interaction between CHF2 and

Figure 17: Electrophoretic mobility shift assays performed in presence of the N-box 269-274 from the 5'-flanking region of CHF2. The binding capacity of *in vitro* translated CHF2 to the E box class B and of i*n vitro* translated HES6 to the ESE probes was confirmed in lanes 3 and 6 respectively. *In vitro* translated luciferase protein incubated with each of the DNA probes assessed the background coming from the IVT reaction (3, 5 and 8). CHF2 or HES6 proteins alone showed no binding activity to the N-box (9,10) nor did the heterodimer, HES6-CHF2 (11).

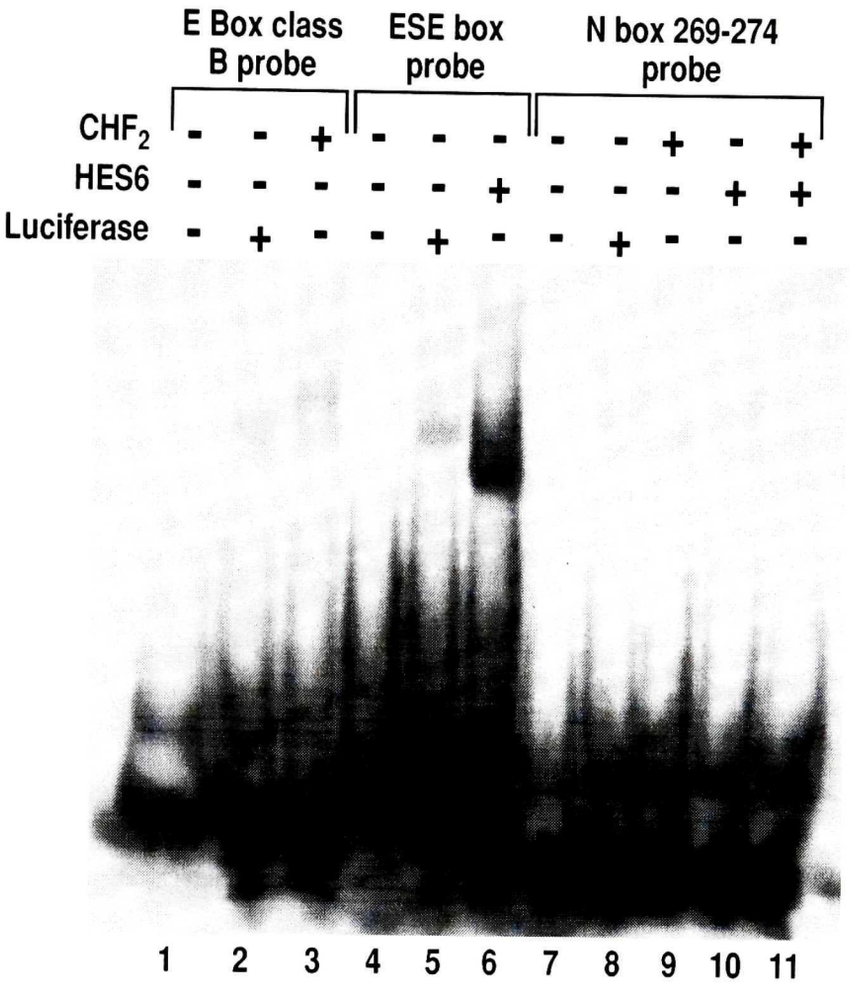

E Box class B probe
ESE box probe
N box 269-274 probe
CHF2 - - + - - - - - + - +
HES6 - - - - - + - - - + +
Luciferase - + - - + - - + - - -
1 2 3 4 5 6 7 8 9 10 11

Figure 18: Electrophoretic mobility shift assays performed in presence of the N-box 2229-2234 from the 5'-flanking region of CHF2. The binding capacity of *in vitro* translated CHF2 to the E box class B and of i*n vitro* translated HES6 to the ESE probes was confirmed in lanes 3 and 6 respectively. *In vitro* translated luciferase protein incubated with each of the DNA probes assessed the background coming from the IVT reaction (2,5 and 8). CHF2 or HES6 proteins alone showed no binding activity to the N-box (9,10) nor did the heterodimer, HES-CHF2 (11).

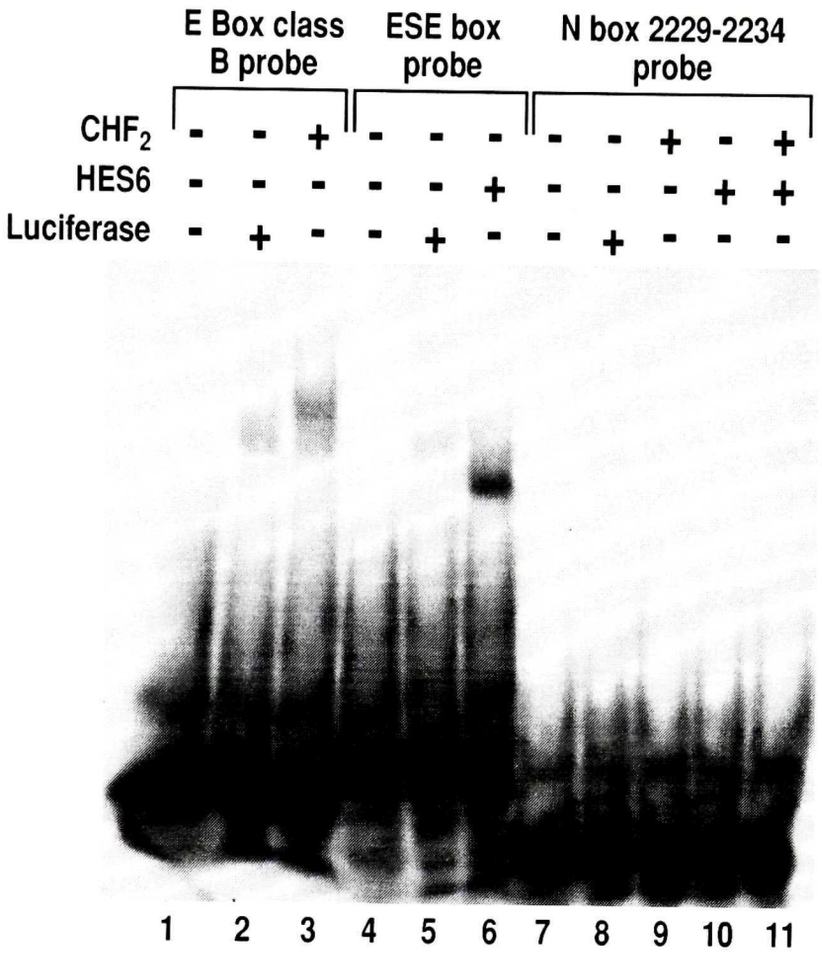

E Box class B probe
ESE box probe
N box 2229-2234 probe
CHF2 - - + - - - - - + - +
HES6 - - - - - + - - - + +
Luciferase - + - - + - - + - - -
1 2 3 4 5 6 7 8 9 10 11

Figure 19: Electrophoretic mobility shift assays performed in presence of the N-box 2283-2288 from the 5'-flanking region of CHF2. The binding capacity of *in vitro* translated CHF2 to the E box class B and of i*n vitro* translated HES6 to the ESE probes was confirmed in lanes 3 and 6 respectively. *In vitro* translated luciferase protein incubated with each of the DNA probes assessed the background coming from the IVT reaction (2,5 and 8). CHF2 or HES6 proteins alone showed no binding activity to the N-box (9, 10) nor did the heterodimer, HES-CHF2 (11).

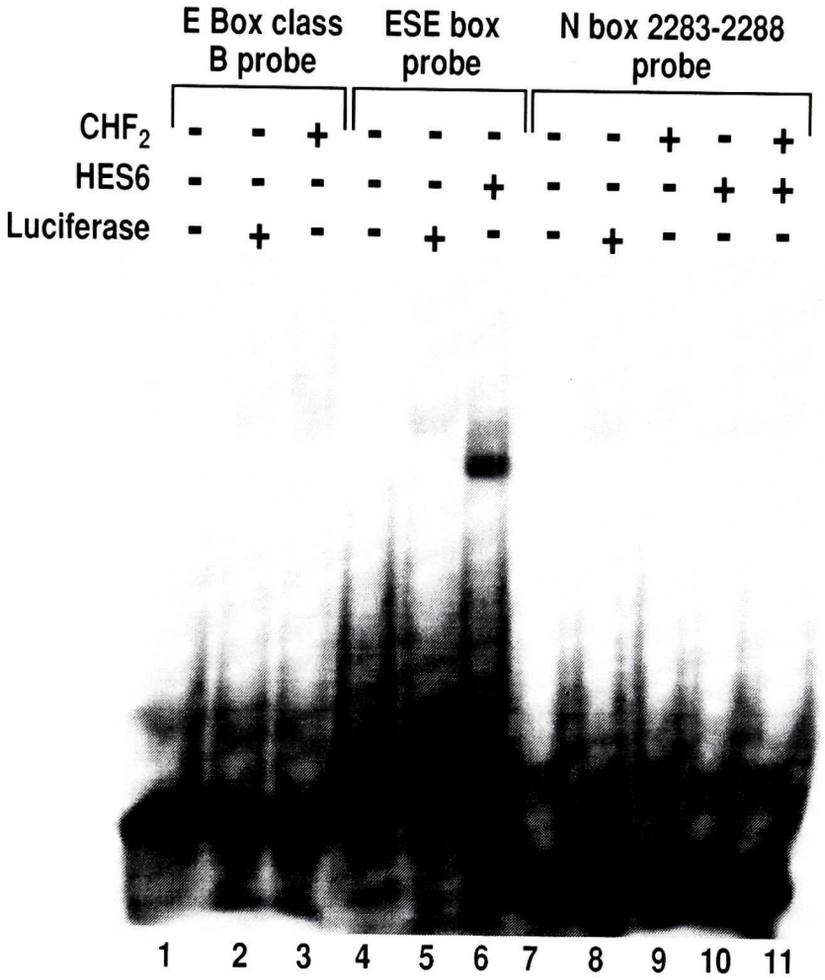

E Box class B probe
ESE box probe
N box 2283-2288 probe
CHF2
HES6
Luciferase
- - + - - - - - + - +
- - - - - + - - - + +
- + - - + - - + - - -
1 2 3 4 5 6 7 8 9 10 11

Figure 20: Electrophoretic mobility shift assays performed in presence of the N-box 10,318-10,323 from the 5'-flanking region of CHF2. The binding capacity of *in vitro* translated CHF2 to the E box class B and of i*n vitro* translated HES6 to the ESE probes was confirmed in lanes 3 and 6 respectively. *In vitro* translated luciferase protein incubated with each of the DNA probes assessed the background coming from the IVT reaction (2,5 and 8). CHF2 or HES6 proteins alone showed no binding activity to the N-box (9, 10) nor did the heterodimer, HES-CHF2 (11).

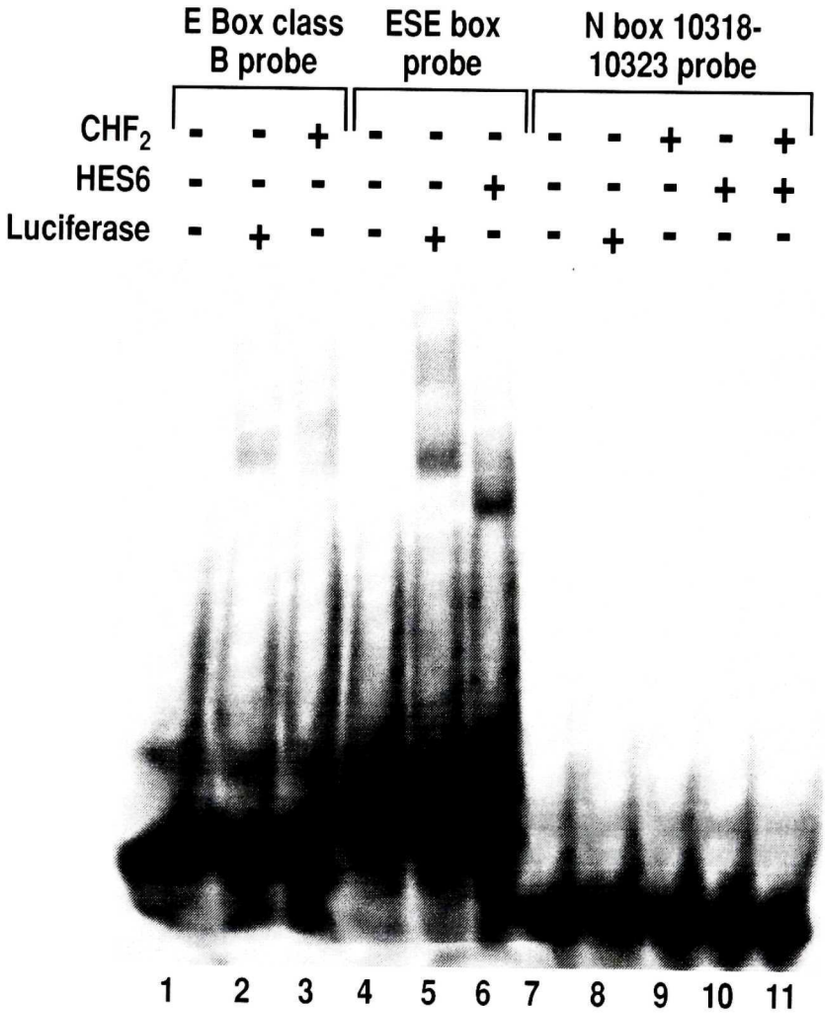
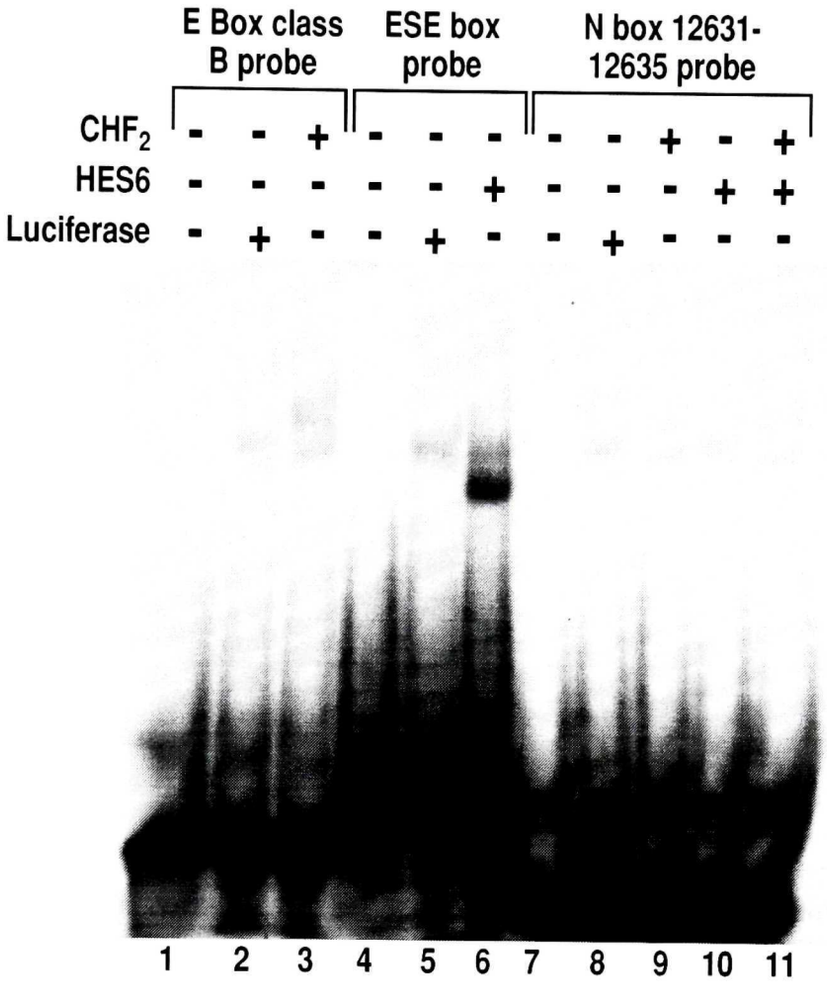

E Box class B probe
ESE box probe
N box 10318-10323 probe
CHF2 - - + - - - - - + - +
HES6 - - - - - + - - - + +
Luciferase - + - - + - - + - - -
1 2 3 4 5 6 7 8 9 10 11

Figure 21: Electrophoretic mobility shift assays performed in presence of the N-box 12,631-12635 from the 5'-flanking region of CHF2. The binding capacity of *in vitro* translated CHF2 to the E box class B and of i*n vitro* translated HES6 to the ESE probes was confirmed in lanes 3 and 6 respectively. *In vitro* translated luciferase protein incubated with each of the DNA probes assessed the background coming from the IVT reaction (2,5 and 8). CHF2 or HES6 proteins alone showed no binding activity to the N-box (9, 10) nor did the heterodimer, HES-CHF2 (11).


E Box class B probe
ESE box probe
N box 12631-12635 probe
CHF2
HES6
Luciferase
1 2 3 4 5 6 7 8 9 10 11

the E box class B was confirmed (Figure 22). Indeed, an excess of unlabelled N box 269-274 or ESE box had no effect even in 100 fold molar excess (Figure 22, lanes 2-5 and lanes 10-13) as opposed to excess of unlabelled E box (Figure 22, lanes 6-9). Only 10 molar fold excess of cold E box class B oligonucleotide competed enough to make the band disappear almost completely (Figure 22, lane 6) while 100 fold molar excess competed completely (Figure 22, lane 9). Binding of HES6 to the ESE box-containing oligonucleotide was also competed by the presence of unlabelled ESE box-containing probe (Figure 23, lane 10-13). Specificity of the interaction was confirmed by the observation that up to 100 molar fold excess of either unlabelled N box 269-274 or E box class B had no effect (Figure 23, lanes 2-5 and lanes 6-9)

Experiments have demonstrated that the *in vitro* translated proteins were able to bind to DNA, and that their binding was specific. Thus, the proteins produced can bind specifically to DNA. In this case, if no binding occurred between the heterodimer and the probes, it may be because of the probes. It was then important to assess the binding capacity of each N-box. They were incubated in presence of total cell extract, which would allow many different proteins expressed, including potentially HES6 and its putative dimerization partner, to bind to the probes. The probe alone gave no band shift as expected (Figure 24, 1,3,5,7,9) while binding was observed for each of the N boxes upon addition of differentiated C2C12 total cell extract (Figure 24, lane 2,4,6,8,10). This binding could result from the interaction of proteins present in the total extract with the probe. It confirmed that the probes used were capable of binding protein, which meant that the negative results observed were true.

Figure 22: Electrophoretic mobility shift assays were performed in presence of *in vitro* translated CHF2 protein and ^{32}P labelled E box class B probes. The binding reactions were competed by addition of unlabelled N box (2-5) or unlabelled ESE box (10-13) in 10, 25, 50, 100-fold excess. Neither the unlabelled N-box probe nor the unlabelled ESE box probe competed with the DNA binding activity of CHF2 to the E box class B. However, addition of unlabelled E boxes class B competed with the binding (6-9), confirming the specificity of the interaction.

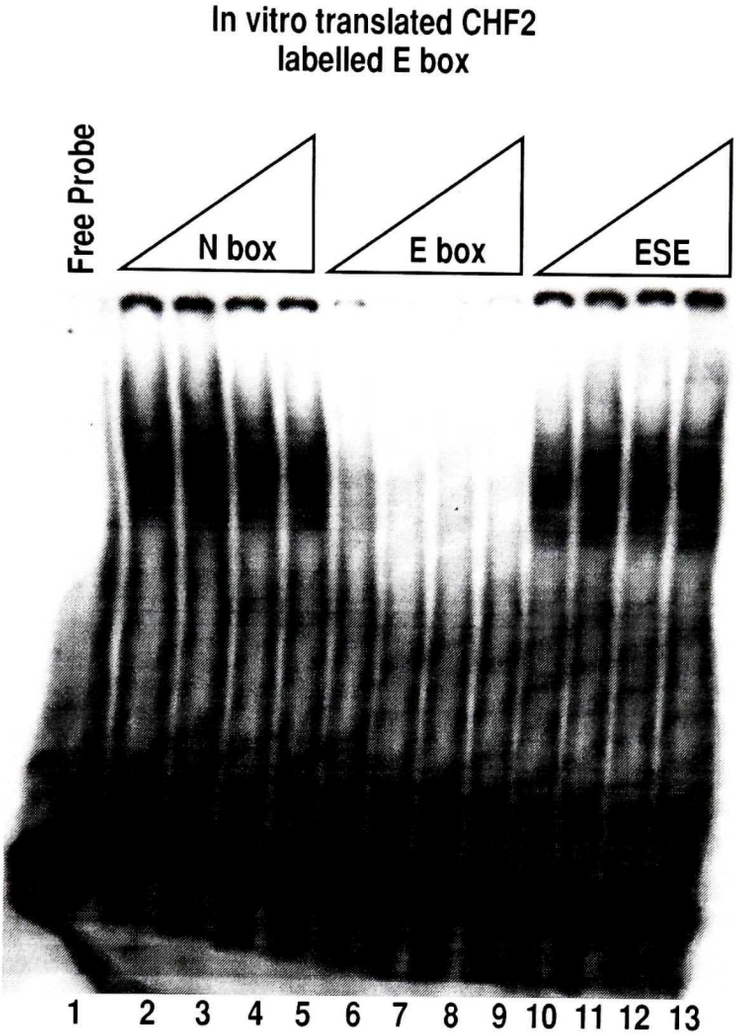

In vitro translated CHF2
labelled E box
Free Probe
N box
E box
ESE
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 23: Electrophoretic mobility shift assays were performed in presence of *in vitro* translated HES6 protein and ^{32}P labelled ESE box probe. Reactions were carried in presence of unlabelled N box (2-5) or unlabelled E box class B (6-9) in 10, 25, 50, 100-fold excess. Neither the unlabelled N-box probe nor the unlabelled E box probe competed with the DNA binding activity of HES6 to the ESE box probe. However, addition of unlabelled ESE box probe competed with the binding (10-13) confirming the specificity of the interaction

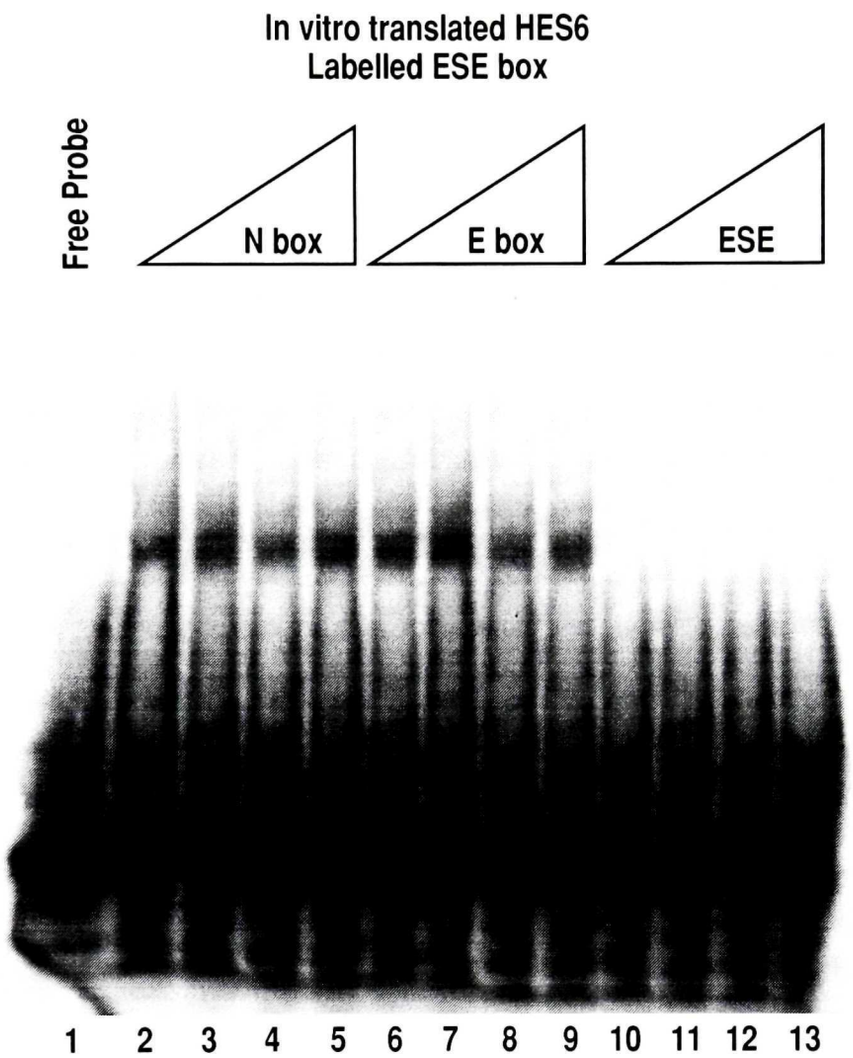
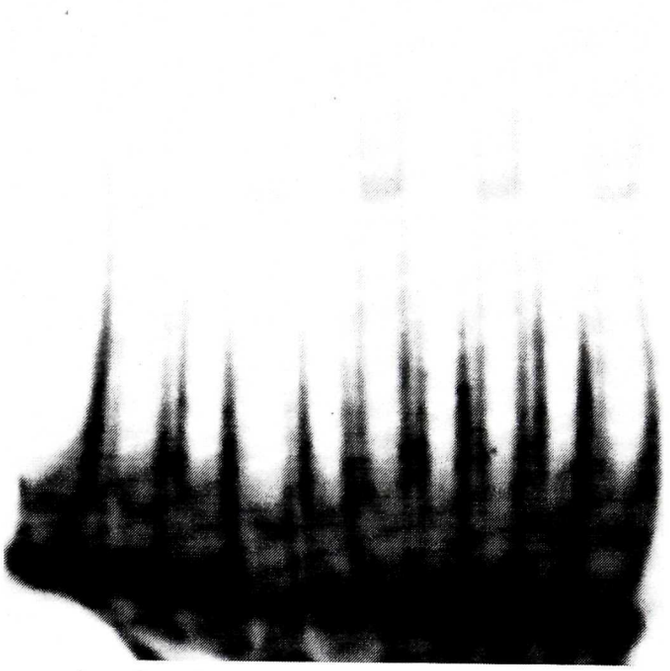

In vitro translated HES6
Labelled ESE box
Free Probe
N box
E box
ESE
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 24: Electrophoretic mobility shift assays held in presence of total cell extracts from C2C12 cells differentiated 4 days and each of the N boxes. The probe alone revealed no band (1,3,5,7,9) while the addition of total cell extracts to the reaction allowed a binding reaction for N-box 269-274 (2), N-box 2229-2234 (4), N-box 2283-2288 (6), N-box 10318-10323 (8) and N-box 12631-12635 (10).

N box 269-274	+	+	-	-	-	-	-	-	-	-
N box 2229-2234	-	-	+	+	-	-	-	-	-	-
N box 2283-2288	-	-	-	-	+	+	-	-	-	-
N box 10318-10323	-	-	-	-	-	-	+	+	-	-
N box 12631-12635	-	-	-	-	-	-	-	-	+	+
Total cell extracts of cell C_2C_{12} cells	-	+	-	+	-	+	-	+	-	+

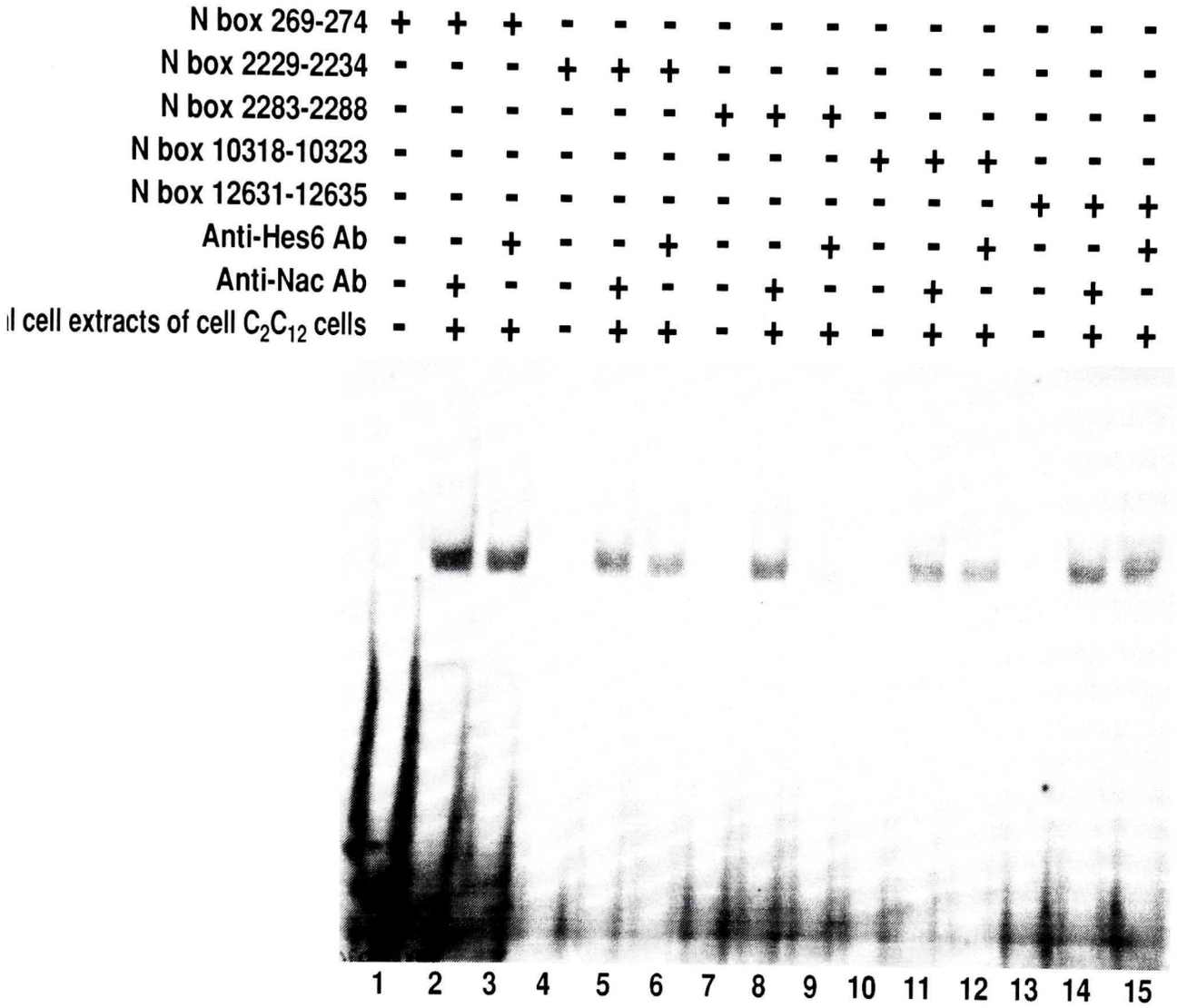


1 2 3 4 5 6 7 8 9 10

Since HES6 expression increases in differentiated myoblasts [27], the binding of HES6 and an unknown partner, but not CHF2, could potentially be responsible for the band observed in figure 24. To test whether HES6 did bind any of the N boxes tested, a supershift using the anti-HES6 antibody was performed. In this type of experiment, the probes are incubated in presence of proteins and an antibody against to protein of interest. The antibody will bind to the protein of interest, and if this protein is binding to the probe, it will form a larger complex. Because of the presence of the antibody the complex will be larger and would not migrate as far in the gel as the complex without the antibody. A supershift will then be observed. In some cases, the antibody will bind to the DNA binding site of the protein, which will result in the incapacity of the protein to bind to the probe. The band will then simply disappear. In both cases, it will demonstrate that the protein of interest is binding to the probe. The supershift experiment was then carried in presence of either an unspecific antibody or the antibody specific to HES6. Results revealed no change for any of the 5 N boxes (Figure 25). The reduction in signal observed in lane 9 was not reproduced in subsequent experiements. Thus, no supershift occurred with this specific anti-HES6 antibody.

From this result, two conclusions could be made; either HES6 is not binding to the N boxes tested or this specific antibody is unable to interact with HES6 when bound to DNA. Because it was already demonstrated that HES6 interacts with the ESE DNA probe, addition of the anti-HES6 antibody to the binding reaction should reveal a supershift unless the complex blocks the antibody-binding site. Cellular extracts where

Figure 25: DNA binding reactions of the N boxes were carried in presence of total cell extracts of C2C12 cell differentiated 4 days, unrelated anti-Nac antibody (2, 5, 8, 11, 14) or anti-HES6 antibody (3, 6, 9, 12, 15). Each N-box demonstrated binding activity in presence of the unspecific antibody, anti-Nac (2, 5, 8, 11, 14) and no supershift appeared with the addition of the anti-HES6 antibody (3, 6, 9, 12, 15).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l cell extracts of cell C2C12 cells - + + - + + - + + - + + - + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

incubated in presence of the ESE box-containing oligonucleotide. On figure 26, the binding reaction between the ESE box-containing probe and the differentiated C2C12 total cell extract (lane 2) demonstrating that proteins from the extract are binding. Because literature demonstrated that HES6 bound to the ESE box, addition of the anti-HES6 antibody should give a supershift. However, a band of the same size as the one observed without antibody was observed with the addition of the unspecific antibody (Figure 26, lane 3) or the anti-HES6 antibody (Figure 26, lane 4) Since transcription factors are expressed in the nucleus, nuclear extracts should present a greater concentration of HES6 protein compared to total extracts, which would ultimately increase the chances of a supershift. However, no supershift appeared upon addition of either the anti-HES6 antibody (Figure 26, lane 7) or the unspecific antibody (Figure 26, lane 6). From those results, we concluded that the anti-HES6 antibody used in those experiments was inappropriate for a supershift.

Furthermore, to evaluate the specificity of the binding reaction between the nuclear extracts and the ESE box-containing oligonucleotides, binding was competed out by unlabelled oligonucleotides as done previously. Binding reactions between nuclear extracts and the ESE probe was not altered by the addition of up to 25 molar fold excess of unlabelled E box class B and N box 269-274 (Figure 27, lanes 7-10 or lanes 11-14). Competition with up to 100-fold molar excess of unlabelled N-box or E-box probes were performed and revealed no alteration of the DNA binding activity either (data not shown). However, addition of 1 fold molar excess of cold ESE box-containing oligonucleotides competed with the binding reaction (Figure 27, 4). This result confirmed

Figure 26: DNA binding reactions carried in presence of the ESE box probe, total cell extract of differentiated C2C12 cells (2-4) or nuclear cell extract of differentiated C2C12 cells (5-7). Addition of anti-Nac antibodies did not interfere with the binding activity of the ESE box probe with both total cell extracts (3) or nuclear cell extracts (6). No supershift is observed upon addition of anti-HES6 antibody with either the total cell extract (4) or the nuclear cell extract (7).

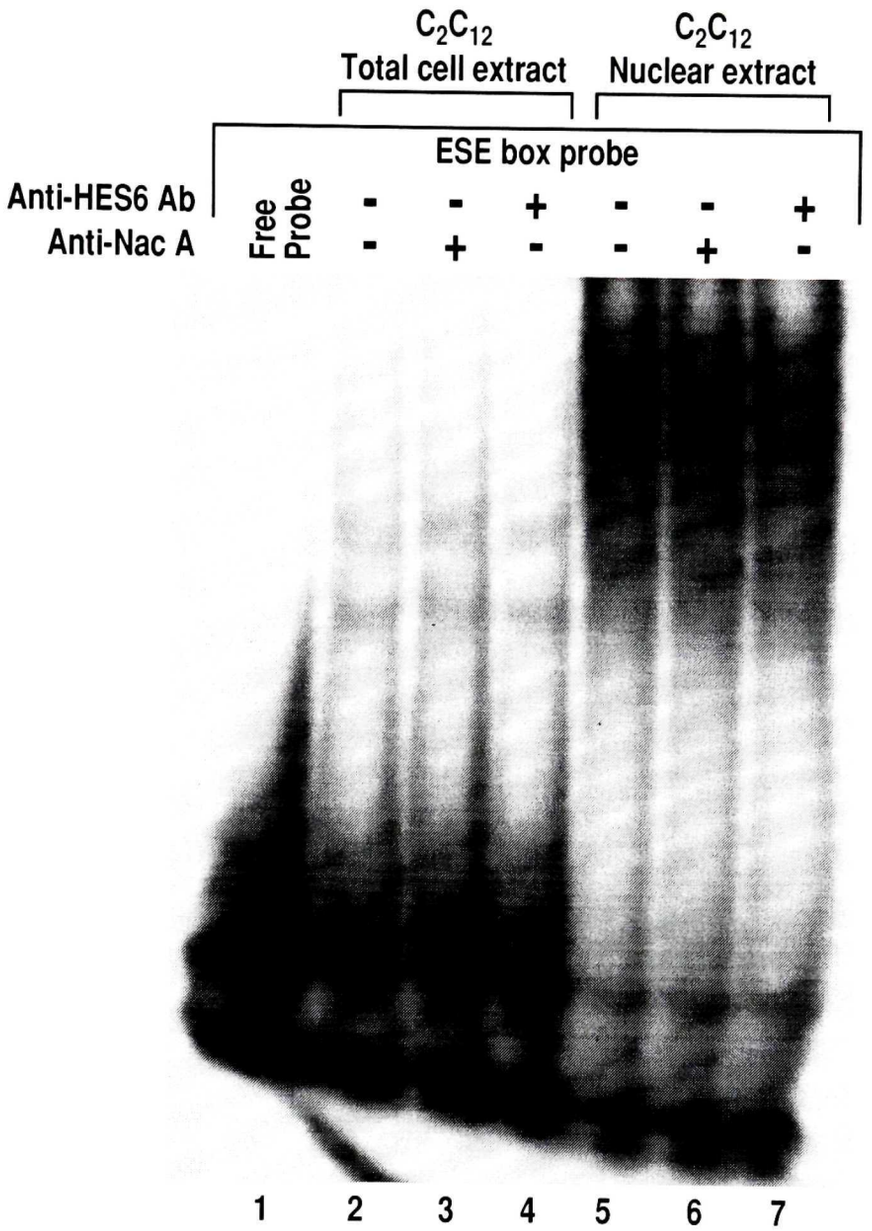
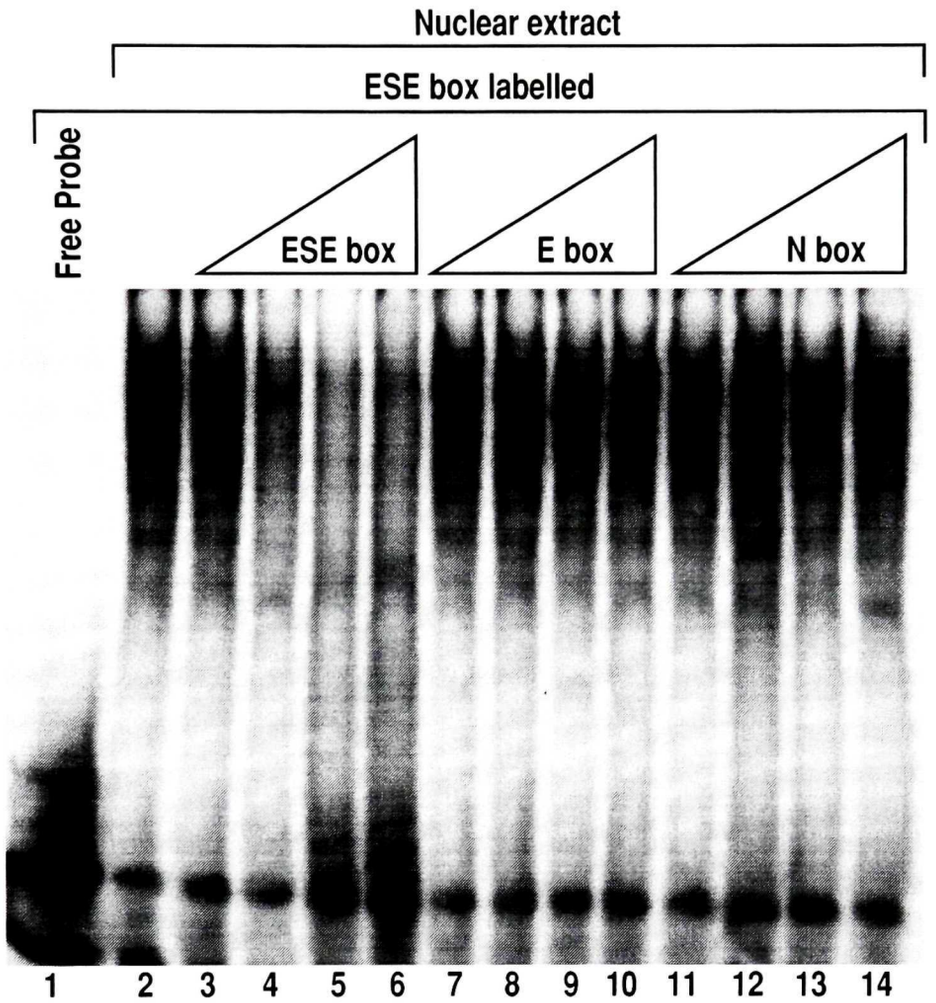

C2C12
Total cell extract
C2C12
Nuclear extract
ESE box probe
Anti-HES6 Ab
Anti-Nac A
Free Probe
- - + - - +
- + - - + -
1 2 3 4 5 6 7

Figure 27: Electrophoretic mobility shift assays carried in presence of nuclear extract and labelled ESE box oligonucleotides as described in Material and Methods. (2 The labelled probe incubated with nuclear extract showed a band shift which was competed with 0.1 (3), 1 (4), 10 (5) or 25 (6) fold excess of unlabelled ESE box oligonucleotide. 0.1, 1, 10, 25-fold excess of unlabelled E box probe (7-10) or N-box probe (11-14) did not compete with the binding reaction.


Nuclear extract
ESE box labelled
Free Probe
ESE box
E box
N box
1
2
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14

the specificity of the binding between the nuclear extract and the ESE box-containing oligonucleotide.

DISCUSSION

A. Highlight of the research project

The aim of this research project was to identify a HES6 dimerization partner for myoblast differentiation using a genome-wide screen and a candidate gene approach. The genome-wide screen using the Yeast two-hybrid system mostly isolated false positives. However, a co-immunoprecipitation experiment demonstrated that HES6 and CHF2 interact with one another in solution and that this interaction is specific. This assay was performed with specific anti-HES6 antibody. This antibody is the first specific antibody against HES6 to be produced. It is designed to recognized HES6 only and no other member of the HES family. Electrophoretic mobility shift assays did not show any DNA binding between the HES6-CHF2 dimer and any of the N box present in the flanking region of the CHF2 gene. This result was supported with various controls.

B. False positives were identified by the Genome Wide Screen

The Origene yeast two-hybrid kit was selected specifically because it was the only one to include a muscle cDNA library. The use of that type of library would increase chances to identify the putative dimerization partner of HES6 for myoblast differentiation. Clones isolated from this assay had to have several attributes to be considered as a potential partner for HES6. They would have to be able to enter the nucleus, or more specifically they would have to be a transcription factor expressed in skeletal muscle cells. Furthermore, they would have to have the same expression pattern as HES6, meaning that their expression should not be down regulated when HES6 expression is up-regulated.

Analysis of the results from the genome wide screen revealed that most of the clones identified were false positives; they weren't potential partners of dimerization of HES6 in the myogenic differentiation program although they activated both reporter genes. Table 3 describes the identified clones and the reasons why they were excluded as dimerization partner. Some of the clones were described as key molecules in the metabolism of sugars or synthesis of ATP and others were defined as key regulators of muscle contraction. Even though the clones isolated all play a key role in muscle cells, it is unlikely that those proteins act as dimerization partner of HES6. None of them are transcription factors and their main localization is in the cytoplasm rather then the nucleus. However, two of the identified clones could have been potential dimerization partner: the vascular endothelial zinc finger 1, Vezf1, and the heat shock proteins HSP70 and HSP169.

Vezf1 is described as a transcription factor that activates transcription mediated by the human endothelin-1 promoter and was also detected in muscle cells [2]. Because Vezf1 was a transcription factor and that it was located in the nucleus of muscle cells it was likely that it could be a potential partner of dimerization for HES6. Further analysis revealed however that what did activate the reporter gene during the yeast two-hybrid screen was Vezf1 5' flanking region. Indeed, sequence of the isolated clone showed a very long 5'-flanking region and a stop codon located a few base pairs before the translation start site. The target vector is constructed with its own start site before the clone's sequence, which is followed by the activation domain. Translation is terminated after the activation domain at the stop codon included in the vector. Thus, it produces a clone fused to the

activation domain. For this particular clone, translation of the target plasmid began at the vector start site and translated the 5' flanking region of Vezf1. However, translation was stopped at the stop codon located prior to the actual start site of the transcription factor. Thus, what actually activated the reporter gene was not Vezf1 since the coding region of the protein was never translated, but the 5' flanking region. The identified clone is then another false positive. This hypothesis is reinforced by the fact that the activation domain was not even fused to the clone because translation was stopped, which means that it is impossible for an active transcription factor to be reconstituted in the absence of that domain.

The heat shock proteins HSP70 and HSP169, although they are not transcription factors, could potentially interact with HES6. Heat shock proteins are constitutively expressed in the cytoplasm and migrate in the nucleus under stress [62]. Several examples in the literature have demonstrated interaction between HSP proteins and transcription factors. A well described example is the interaction between HSP90 and steroid hormone receptors. HSP90 is an ATP dependent molecular chaperone involved in the folding and activation of an unknown number of substrate proteins [86]. These substrate proteins include protein kinases and transcription factors [62].Within the target cells steroid hormones bind to steroid hormone receptors, which are complexed to chaperones, heat shock protein 90 (HSP90). Steroid hormone receptors are intracellular transcription factors that can be activated, among other possibilities, by the specific and high affinity binding of ligand to exert positive or negative effects on the expression of target genes. Binding of HSP90 leads to different allosteric changes of steroid hormone receptors

making them competent to exert positive or negative effects on the expression of target genes by different mechanisms [86]. After dissociation of chaperones, steroid hormone receptor complexes can bind to chromatin organized DNA sequences in the vicinity of target genes, termed hormone response elements. Those complexes are then able to initiate chromatin remodelling and to relay activating or repressing signals to the target gene transcription machinery [86]. Steroid hormone receptor complexes can also initiate protein-protein interactions with other sequence-specific transcription factors. Another report also demonstrated interaction between heat shock proteins and transcription factors. HSP90 was shown to physically interact with the bHLH motif of the dioxin receptor, an event that appears important for the conversion of the bHLH domain into a DNA-binding conformation but not for dimerization processes [5]. This data demonstrates that heat shock proteins can associate with transcription factors, and specifically to bHLH motifs. Because HSP70 and HSP169 are expressed in muscle cells, it is likely that they do interact with HES6. An immunohistochemistry experiment on myoblast would assess if heat shock proteins are expressed in the nucleus at the same time as HES6 is. The interaction between HES6 and HSP could be tested by co-immunoprecipitation. The impact of co-expression of HSP70 or HSP169 on HES6-mediated transcriptional repression could be tested in transient transfection assays.

Although the yeast two-hybrid system had proven useful or detecting protein-protein interaction *in vivo,* all versions of it have been described to give many false positives. Dr. Erica Golemis and Dr. Ilya Serebriiskii of the Fox Chace Cancer Center did a survey on 100 yeast two-hybrid library screens that were performed

(http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html) [87]. Of the 100 screens performed, 54 gave real interactors, 30 are still in progress, 13 gave only false positives and 3 were aborted. Even in the screens that produced real interactors, false positives were a big problem, more then 90% of the screens that gave real interactors had a large number of false positives. The most common false positives obtained where the heat shock proteins, ribosomal proteins, ferritin and Ubiquitin. Other false positives reported where cytochrome oxydase, mitochondrial proteins, tRNA, zinc fingers, cytoskeletal proteins and inorganic pyrophosphatase. The false positives that came out of the screen for this project are mostly among the most common false positives listed.

One disadvantage of the yeast two-hybrid system is that the fusion of the DNA binding domain or the activation domain might change the conformation of the bait or prey such that it impairs their regular function. For instance, no interaction between X and Y occurs when they should. This is related to using *S. cerevisiae* as the host cell. The bait protein must fold correctly and be stable in the cell to be able to interact with some other protein. Protein interactions are also dependent on posttranslational modifications. The yeast system may be too simple to process higher eucaryotic proteins. New yeast two-hybrid systems have been developed that co-express the enzyme responsible for modification [76,77]. Another major problem of the screen is the possibility that a third protein Z, is bridging the two test proteins allowing the domains to be close enough to activate transcription of the reporter. Many false positives occur because of this[13]. Truncations may be used to lower the false negative rate. In certain circumstances, a subunit or portion of a protein may make better bait than the whole protein. One example

of this case is the removal of a membrane anchoring domain from a protein by truncation to accelerate nuclear localization. Therefore, in some cases use of a truncated form of the protein of interest should be tested as the bait. Finally because only two rounds of screen of the library have been performed, the whole muscle cDNA library hasn't been screened. It would then be important to screen the whole library before concluding that the method can only isolate false positives

TABLE 3.: Table of the description of the clones identified through the Yeast- Two-Hybrid screen for identification of HES6 dimerization partner and the reason why it is described as a false positives

Clone identification	II. Description of the clone	III. False positive: Reason
Tumor Necrosis Factor	Involved in inhibition of tumour growth and modulation of the inflammatory function [79]	Not a transcription factor, involve in cell death
Mus Musculus Aldolase 1	Catalyzes a key reaction in glycolysis and energy production [28]	Not a transcription factor, main localization is in the cytoplasm, Involved in the glucose metabolism
Vascular Endothelial Zinc Finger 1 (Vezf1)	Zinc Finger transcription factor that activates transcription mediated by the human endothelin-1 promoter [2]	Stop codon located before the start site of the protein. Activation domain is not translated
Mus Musculus Creatine Kinase	Catalyzes the reversible exchange of a phosphoryl group between phosphocreatine and ATP [66]	Not a transcription factor, main localization is in the cytoplasm
Heat Shock 70kD Protein 5	Acts like chaperones, involved in mediating protein folding and transport, maintaining proteins in an inactive form and preventing protein degradation [62].	Interact with HES6 probably only for its proper folding
Mus Musculus ATP Synthase	Synthesizes ATP [84]	Not a transcription factor, main localization is not the nucleus
Mus Musculus Actin	Dynamic polymers whose ATP-driven assembly in the cell cytoplasm drives shape changes, cell locomotion. Actin filaments also participate in muscle contraction [1].	Not a transcription factor, protein whose main function is to participate in muscle contraction
Mus Musculus Glyceraldehyde-3-Phosphate dehydrogenase	Key enzyme in intermediary metabolism catalyzing the oxidation and subsequent phosphorylation of substrate aldehydes to acyl phosphate [58]	Not a transcription factor, main localization is in the cytoplasm
Mus Musculus Ribulose 1,5 Biphosphatecarboxy lase/ Oxygenase	Bifunctional enzyme that can fix carbon dioxide or molecular oxygen, which leads to photosynthesis [58]	Not a transcription factor, main localization is in the cytoplasm
Mus Musculus Phosphorylase Kinase	Regulate Glycogen metabolism and catalyzes Ca2+ Mg2+ dependant phosphorylation and activation of glycogen phophorylase [58]	Not a transcription factor, main localization is in the cytoplasm
Troponin C	Ca^{2+} receptor that controls regulation and skeletal muscle contraction [55]	Not a transcription factor, protein whose main function is to participate in muscle contraction
Mus Musculus 16S Ribosomal RNA	Single strand of RNA from the 30s subunit of the ribosome [58]	Not a transcription factor, subunit of the ribosome
Mus Musculus Titin Immunoglobulin	Plays a number of important roles in muscle contraction and elasticity [43]	Not a transcription factor, protein whose main function is to participate in muscle contraction
0 Day Neonate skin cDNA	Thyroid stimulating hormone [21]	Not a transcription factor, main localization is in the cytoplasm
Heat Shock protein C 169	Acts like chaperones, involved in mediating protein folding and transport, maintaining proteins in an inactive form and preventing protein degradation [62].	Interact with HES6 probably only for its proper folding
Mus Musculus Acetyl-CoA acyltransferase	Plays a role in intermediary metabolism, cell synthesis, break down, energy production and growth [58]	Not a transcription factor, main localization is in the cytoplasm

B. HES6 forms an inactive dimer with CHF2

The candidate gene approach necessitated a specific anti-HES6 antibody, which was synthesized in rabbits. This is the first specific antibody for HES6 to be produced and western blots revealed its specificity. A co-immunoprecipitation performed with this antibody revealed that HES6 interacted with CHF2 *in vivo* (Figure 15). The formation of this heterodimer could potentially repress CHF2 and explain why CHF2 expression decreases when HES6 expression increases. Repression of CHF2 can occur by formation of an active or an inactive dimer. However, because the dimer was not found to be able to bind to any of the N boxes located in the 5'-flanking region of CHF2, we interpret this data to mean that the complex formed an inactive dimer (Figure 17-21). However, the validity of electrophoretic mobility shift assays was confirmed by assessing the specificity of the different controls. HES6 would then sequester CHF2 whose expression would decrease in time because CHF2 would potentially be unable to autoactivate its own expression. This hypothesis could explain the reciprocal temporal expression pattern of HES6 and CHF2. Furthermore, recent data demonstrated that CHF2 formed a heterodimer with MyoD that did not bind to the E-box [74]. Sequestration of MyoD makes it unavailable to form a dimer with the E47 proteins to bind the E-box and it ultimately leads to repression of transcription of myogenin, which inhibits the differentiation program (Figure 28, A). Expression of HES6 would then sequester CHF2, which would allow MyoD to interact with E47 proteins, bind DNA and ultimately activate transcription of myogenin (Figure 28, B). Because HES6 is described as an activator of myogenesis and CHF2 as a repressor of myogenesis, this model would explain why those proteins with opposed function would interact with one another. The temporal reciprocal

pattern of expression of CHF2 and HES6 in muscle cells could potentially be explained by this interaction. This model hypothesizes that CHF2 potentially autoactivates its expression which is inhibited by its sequestration leading to a decrease of CHF2 expression. To further support this hypothesis, it would have been interesting to confirm that *in vitro* translated HES6 and CHF2 also form a dimer in solution. Potentially, in vitro translated proteins do not go throught the same post-translation modifications as proteins from the nuclear extract of C2C12 cells, which could modify their binding site and alter protein-protein interactions. The model would would have been fortified by a gel retardation assay demonstrating that *in vitro* translated CHF2 can bind the E-box only in absence of HES6.

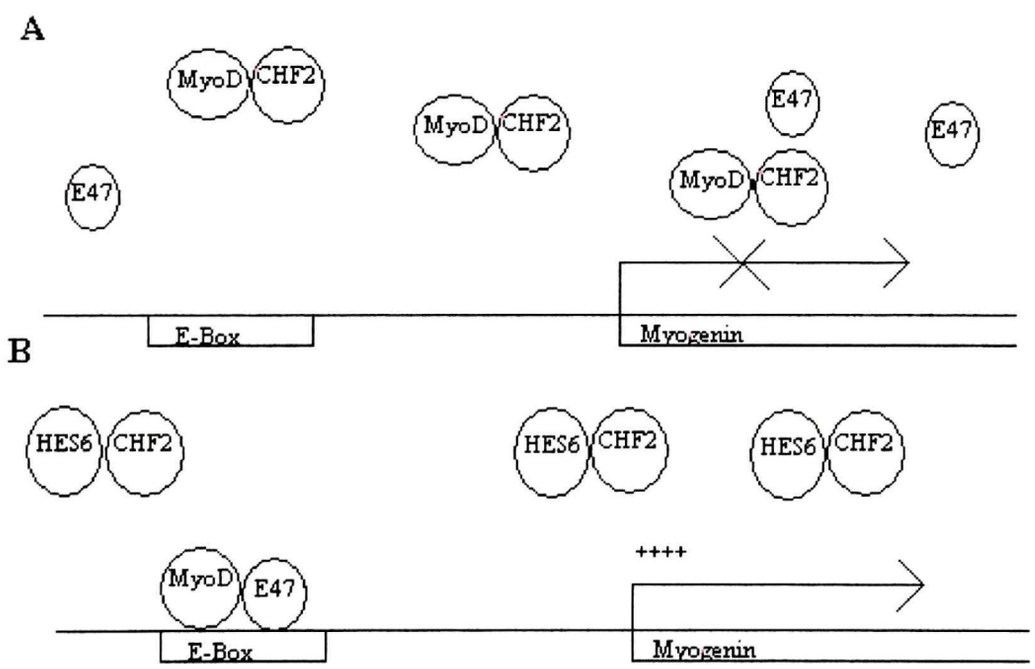

Figure 28. Potential model that would explain the activation or repression of the myogenin gene based on recent data from Sun *et al* [74]. (A) Sequestration of MyoD by CHF2 makes MyoD unable to interact with the E47 protein and bind the E-box to activate transcription of the myogenin gene. (B) HES6 sequesters CHF2, which then allows MyoD to bind to the E47 protein and activate transcription of myogenin.

Results from the electrophoretic mobility shift assays revealed that even though the HES6-CHF2 heterodimer was unable to bind any of the N boxes located in the 5'-flanking region of CHF2, proteins in the total cell extract of differentiated cells was able to (Figure 24). However, our experiments did not demonstrate whether HES6 was one of the proteins involved in the band shift. Results revealed that the specific anti-HES6 antibody available was unable to interact with HES6 when bound to DNA (Figure 25-26). The following experiment would have also been informative: another gel retardation assay could have been performed in presence of the N-boxes oligonucleotides and nuclear extract of C2C12 cells transfected with a HA tagged HES6 construct. Addition of an anti-HA antibody to the reaction then would allow to determine wether HES6 was part or not of the band shift observed in figure 24. It is then still possible that HES6 forms a heterodimer with some unknown dimerization partner and bind to the N box to actively repress CHF2 expression or the expression of a different target gene.

C. HES6 dimerization partner haven't been identified

Using both a genome wide screen and a candidate gene approach, the HES6 potential partner of dimerization in myoblast was not identified. The Yeast Two-Hybrid allowed isolation of several clones where only one could potentially interact with HES6, the Heat Shock Proteins. However, their interaction is unlikely to be responsible of the induction of differentiation in myoblast. We hypothesized that the role of heat shocks proteins was limited to chaperones. The candidate gene approach revealed an interaction between CHF2 and HES6, where HES6 sequesters CHF2, which ultimately leads to activation of

myogenin. Because the complex is unable to bind DNA, it remains to identify the protein, which would form a dimer with HES6 and allow binding to the N-box to ultimately repress transcription.

CONCLUSION

This research project brought novel knowledge at two levels. The first specific antibody against HES6 was produced. This will allow specific detection of HES6 without the use of tags. Moreover, this antibody is specific to HES6 alone, and should not be able to recognize any other of the HES members. Finally, the antibody gave a clear and specific signal up to a dilution of 1:1000, which demonstrated how specific it was.

The candidate gene approach clearly demonstrated that HES6 and CHF2 formed a heterodimer in mammalian cells. Moreover, the co-immunoprecipitation experiment was strongly supported with controls. The assay did not only demonstrate that HES6 and CHF2 interact with one another, but it also demonstrated that the result was specific. Electrophoretic mobility shift assays revealed no interaction between the heterodimer and any of the N boxes located in the flanking region of the CHF2 gene. This result was again supported by various controls. Thus, the data suggest that the CHF2-HES6 dimer is non-functional to bind DNA.

FUTURE EXPERIMENTS

Since the heat shock proteins were identified from the yeast two-hybrid assay, it would be interesting to test if they do interact with HES6 *in vivo*. A co-immunoprecipitation performed in a way similar to the one performed with CHF2 would allow testing of the interaction.

An alternative to the Yeast Two-Hybrid method to identify a potential partner of dimerization was proposed and also allowed a genome wide screen. Anti-HES6 antibodies would have to be coupled to beads and would be used for an immuno-affinity chromatography assay. Cell extract of differentiated C2C12 cells would be applied on a column containing those beads. HES6 and his partner should be able to bind the beads. A salt gradient applied on the column would elute the column and release HES6 and its partner. The eluate would then be run on a SDS-PAGE gel, which would separate proteins by size. Bands could be cut out and eluted to be further sequenced. Proteins would be cut in pieces with trypsin and the product would be used for mass spectrometry analysis. Initiation of this project was performed before the end of the thesis. Because of the concentration of the antibody available was not high enough to allow coupling to beads an alternative was proposed. Anti HA-beads were proposed as an alternative to the anti-HES6 beads. C2C12 cell differentiated 4 days would then be transfected with the pCMV-HA-HES6 vector and the nuclear extract would be used for immunoprecipitation. However, transfection of differentiated C2C12 cells never succeeded although different

kits of transfection and different ratio of transfection mixture and DNA were tested. It would then remain to achieve transfection in order to perform that experiment.

Electrophoretic mobility shift assays revealed that the HES6-CHF2 heterodimer did not interact with any of the N boxes present in the 5'-flanking region of CHF2. However, a control demonstrating that CHF2 and HES6 *in vitro* translated proteins can also form a dimer would strengthen our conclusion. This interaction can be easily demonstrated with a protein pull-down assay using *in vitro* translated HES6 and CHF2 proteins. Moreover, the heterodimer might be able to bind to different N boxes or it could bind to totally different consensus sequences. The N-box sequence, CACNAG, allows 4 different combinations and only two were tested (CACCAG and CACAAG) in the EMSA reactions performed. It would then remain to identify potential DNA binding sites and test the DNA binding activity of the heterodimer by electrophoretic mobility shift assays.

The model proposed previously suggested that HES6 sequesters CHF2, which ultimately allows MyoD to form a dimer with E47 bind the E box and activate myogenin expression (Figure 28). The following experiment would strengthen the sequestration model: an electrophoretic mobility shift assay with recombinant CHF2 binding to the E box could be performed in the presence of increasing amounts of recombinant HES6. If HES6 truly sequesters CHF2, the increasing amounts of recombinant HES6 protein added to the EMSA reaction would progressively inhibit CHF2 from binding to its cognate binding element. It would also be important to validate that model using a luciferase assay. According to the model, C2C12 cells transfected with a luciferase reporter plasmid

including several E boxes should see their luciferase expression decrease with the increase of CHF2 expression. CHF2 expression would sequester MyoD, which would not be able to form a heterodimer to bind to the E-box and activate expression of luciferase. However, increasing expression of HES6 should sequester CHF2, and allow an increase of the luciferase expression.

Because it was impossible to detect HES6 when bound to DNA, it would be important to synthesize an antibody that would allow its detection in EMSA. Using that antibody, it would be essential to confirm that HES6 binds to the ESE box-containing oligonucleotides in a supershift held in presence of the *in vitro* translated protein, total and nuclear cell extracts. This will assess the validity of the control. Furthermore, a supershift performed in presence of total cell extracts of differentiated cells and each of the N boxes 5'-flanking region of CHF2 tested would allow to evaluate if HES6 was partly responsible for the band shift observed in figure 24.

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APPENDIX I

Luria Bertoni medium (LB) pH 7.0:

10g Peptone (Merk, Burlington ON)
5g Yeast Extract (Merk)
10g NaCl (Merk)
7.5g Agar (Merk)

TBE 10X:

108g Tris Base (Merk)
9.3g EDTA (Merk)
55g Boric Acid (Merk)
H_2O to 1000ml

TTE 20X:

216g Tris Base
72g Taurine (ICN, Aurora, ON)
4g Na_2EDTA (Merk)
H_2O to 1000ml

YPD rich medium:

20g Peptone (Merk)
10g Yeast Extract (Merk)
20g Glucose (Merk)
0.1 NaOH (Merk)
20g Agar (Merk)
H_2O to 1000ml

YNB-ura-his-leu-trp selective medium:

1.7g Yeast nitrogen base w/o amino acids (Difco, Mississauga, ON)
5g Ammonium Sulfate (Merk)
0.6g -his-leu-ura-leu dropout mix (Bio 101, Carlsbad, CA)
20g Glucose (Merk)
Or 20g Galactose (Sigma, Oakville, ON)
10g Raffinose (Sigma)

10g Agar (Merk)
H_2O to 1000ml

Add 4mg/ml Leucine (Sigma), 4mg/ml Tryptophane (Sigma), 4mg/ml Histidine (Sigma) or 4mg/ml Uracil (Sigma) to make the appropriate medium

Z Buffer:

16g Sodium Phosphate dibasic (Merk)
5.5g Sodium Phosphate monobasic (Barker, Piscataway, NJ)
0.75g Potassium chloride (Merk)
0.246g Magnesium chloride (Merk)
2.7ml β-mercaptoethanol (Fisher Scientific, Ottawa, ON)
H_2O to 1000ml

APPENDIX II

PBS:

2.68mM KCl (Merk)
8.06mM Na2HPO4 (Merk)
1.47mM KH2PO4 (Barker Chemical, Piscataway, NJ)
137mM NaCl (Merk)

Lysis Buffer 2X:

100mM Tris-HCl (Merk)
300mM NaCl (Merk)
2mM EDTA (Merk)
2mM EGTA (Sigma)
2% Triton (Merk)
5μg/ml Leupeptine (Roche, Laval, QC)
5μg/ml Pepstatin (Roche)
5μg/ml A-protinin (Roche)
1mM PMSF (Sigma)

SDS buffer 3X:

0.175M Tris-Hcl
30% Glycerol (Merk)
6% SDS (Merk)
1mg/ml Bromophenol blue (BioRad, Misssissauga, ON)

Transfer Buffer:

39mM Glycine (Invitrogene)
48mM Tris (Merk)
0.037% SDS
20% Methanol (Merk)

TBS 10X (Western)

50mM Tris-HCl
150mM NaCl

Stripping Buffer:

100mM β-mercaptoethanol
2% SDS
62.5mM Tris-HCl pH6.7

TBS (Co-Immunoprecipitation):

500mM NaCl
50mM Tris-HCl pH 7.4

APPENDIX III

10X Nick Translation Buffer:

- 0.5M Tris-HCl pH 7.2
- 0.1M $MgSO_4$ (Merk)
- 1mM 1,4-dithiothreitol (....)
- 500µg/ml BSA

Buffer A:

- 10mM Hepes pH 7.9 (Merk)
- 1.5mM $MgCl_2$ (Merk)
- 10mM KCl (Merk)
- 0.5mM DTT

Buffer C:

- 20mM Hepes pH 7.9
- 25% Glycerol
- 0.42mM NaCl
- 1.5mM $MgCl_2$
- 0.2mM EDTA
- 0.5mM DTT
- 0.5mM PMSF
- 5µg/ml Leupeptine
- 5µg/ml Pepstatin
- 5µm/ml A-protinin

Buffer D

- 20mM Hepes pH 7.9
- 20% glycerol
- 0.1M KCl
- 0.2mM EDTA
- 0.5mM DTT
- 0.5mM PMSF
- 5µg/ml Leupeptine
- 5µg/ml Pepstatin
- 5µg/ml A-protinin

TBE 10Xfor EMSA:

- 900mM Tris
- 900mM Boric Acid
- 2mM EDTA

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05439-2-08.0

EAR SUBSTANCES AND ATION DEVICES NCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT | Licence Number / Numéro de permis

CENSEE

Pursuant to section 24(2) of the Nuclear Safety and Control Act, this licence is issued to:

Hôpital Shriners pour l'Enfant/Shriners
Hospitals for Children
1529 Cedar Avenue
Montréal, QC
H3G 1A6
Canada

hereinafter «the licensee».

ERIOD

This licence is valid from: June 1 2003 to May 31 2008.

CENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances listed in section IV) of this licence.

This licence is issued for: laboratory studies: 10 or more laboratories where radioisotopes are used or handled (836)

IUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Hydrogen 3	10 GBq	n/a	n/a
2	Carbon 14	200 MBq	n/a	n/a
3	Phosphorus 32	1 GBq	n/a	n/a
4	Sulfur 35	1 GBq	n/a	n/a
5	Calcium 45	200 MBq	n/a	n/a
6	Chromium 51	200 MBq	n/a	n/a
7	Iodine 125	1 GBq	n/a	n/a
8	Phosphorus 33	200 MBq	n/a	n/a
9	Technetium 99m	100 MBq	n/a	n/a

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

OCATION(S) OF LICENSED ACTIVITIES

1529 Cedar Avenue
Montréal, QC

ONDITIONS

1. Prohibition of Human Use
This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

2. Area Classification
The licensee shall classify each room, area or enclosure where more than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-2)

10. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

11. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

12. Disposal (Laboratories)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems, the licensee shall ensure that the following limits are not exceeded:

COLUMN 1 - Nuclear Substance - - -	COLUMN 2(a) LIMITS solids to municipal garbage system (quantity per kilogram)	COLUMN 3(b) LIMITS liquids(water soluble)to municipal sewer system (quantity per year)
Carbon 14	3.7 MBq	10 000 MBq
Chromium 51	3.7 MBq	100 MBq
Cobalt 57	0.37 MBq	1000 MBq

Cobalt 58	0.37 MBq	100 MBq
Hydrogen 3	37 MBq	1 000 000 MBq
Iodine 125	0.037 MBq	100 MBq
Iodine 131	0.037 MBq	10 MBq
Phosphorus 32	0.37 MBq	1 MBq
Phosphorus 33	1 MBq	10 MBq
Sulfur 35	0.37 MBq	1000 MBq
Technetium 99m	3.7 MBq	1000 MBq

(a) The limits in column 2 apply to quantities of solid waste of less than three tonnes per year. Nuclear substances released to the municipal garbage system must be in solid form and uniformly distributed in the waste with a concentration that is less than the limits in column 2. Where more than one nuclear substance is disposed of at one time, the quotients obtained by dividing the quantity of each substance by its corresponding limit in column 2 shall not exceed one.

(b) The limits in Column 3 apply to the water soluble liquid form of each nuclear substance which may be disposed of per year per building.
(2161-3)

13. Contamination Meter Requirements
The licensee shall make available to workers at all times at the site of the licensed activity a properly functioning portable contamination meter.
(2572-1)

14. Export Restrictions
This licence does not authorize the licensee to export for the valid period of this licence:
(a) any quantity in any form of:
    (i) Deuterium;
    (ii) Plutonium;
    (iii) Thorium;
    (iv) Tritium;
    (v) Uranium; or
(b) any quantity of the elemental form, or any quantity of a compound or mixture greater than or equal to 37 GBq/kg, or any sealed source or device greater than or equal to 3.7 GBq of:
    (i) Actinium 225, 227;
    (ii) Californium 248, 250, 252, 253, 254;
    (iii) Curium 240, 241, 242, 243, 244;
    (iv) Einsteinium 252, 253, 254, 255;
    (v) Fermium 257;
    (vi) Gadolinium 148;
    (vii) Mendelevium 258;
    (iix) Neptunium 235;
    (ix) Polonium 208, 209, 210; or
    (x) Radium 223;
(c) any quantity of Americium 241 or 243, except in a compound or mixture, or any sealed source or device;
(d) any quantity of Neptunium 237, except in a compound or mixture, or any sealed source or device;
(e) any quantity of Radium 226, or any quantity of a compound or mixture of Radium 226;
(f) any sealed source or device (except for medical applicators) of more than 0.37 GBq of Radium 226; and
(g) any neutron generator system (including tubes) designed for operation without an external vacuum system and utilizing electrostatic acceleration to induce a tritium-deuterium nuclear reaction.
(2403-2)

15. Annual Compliance Report

The licensee shall submit, two months prior to the anniversary of the expiry date in Section II of the licence, to the Commission or to a person authorized by the Commission, a written annual compliance report of the licensed activity containing operational information in quantity and form acceptable to the Commission.
(2916-4)

Designated Officer pursuant to subsection 37(2)(c) of the Nuclear Safety and Control Act