IDENTIFICATION AND FUNCTIONAL ASSESSMENT OF THE FIBROBLAST GROWTH FACTOR-16 (FGF-16) PROMOTER IN CARDIAC MYOCYTES

BY

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ABSTRACT

The heparin-binding fibroblast growth factor (FGF) family plays an important role in the growth and development of the mammalian heart. FGF-16 is a relatively poorly characterized member of the FGF family whose expression has been identified as cardiac-specific, with significant induction after birth. In addition to suggesting an important role for FGF-16 in the postnatal heart, the mechanisms underlying spatial and temporal control of FGF-16 gene expression in the neonate are of interest. The induction of FGF-16 mRNA production in the postnatal period suggests that spatial control of FGF-16 gene expression in the myocardium during the early postnatal stage is regulated at the transcriptional level through cardiac-specific promoter activity.

Therefore it was ***hypothesized*** that the expression of murine FGF-16 is regulated at the transcriptional level and that the sequences that control the postnatal expression of this factor in the heart are contained within 6kb upstream of the translation initiation site. The identification of the putative transcription initiation site used by FGF-16 for its expression in the heart and the functional assessment of the identified promoter represent the major steps in testing this hypothesis.

Genomic sequences (about 6.2 kilobases) upstream of the ATG start codon of the murine FGF-16 gene were cloned. Two transcription databases were searched (1) to identify potential mFGF-16 transcription initiation sites and (2) to identify the potential binding sites of different transcription factors within the 6.2 kb of the 5'-flanking DNA sequence of mFGF-16 gene that was cloned. Within that 6.2 kb DNA cloned sequence

two potential muscle TATA boxes were identified: one 306 base pairs (bp) (TATA1) and one 1125 bp (TATA2) upstream of the FGF-16 start codon. Based on the data achieved using both RNA (Northern) blotting and (Reverse Transcriptase) Polymerase Chain Reaction (RT-PCR), the TATA1 box was eliminated as putative initiation site. Primer extension confirmed the promoter region 1125 bp upstream of the FGF-16 start codon. through the identification of the transcription initiation site 1073 bp upstream of the ATG codon. This position was subsequently designated +1.

To asses the functionality of the newly identified promoter, a series of hybrid luciferase (Luc) reporter genes were then generated with varying lengths of this promoter sequences. These genes (–4.7, –2.7, –1.2 and –0.2FGF-16p.GFP/Luc) were used to transiently transfect neonatal rat cardiac myocytes. Hybrid -4.7, –2.7 and –1.2FGF-16p.GFP/Luc genes were expressed significantly above the 'background' promoterless gene (-pGFP/Luc) levels in transfected cardiac myocytes, however, no significant activity of -0.2FGF-16p.GFP/Luc was observed.

These data suggest that the FGF-16 promoter is located 1073 bp upstream of coding sequences and that sequences located between -1.2 and -0.2 kb upstream of the transcription start site are required to target cardiac expression. The detection of a significant luciferase activity for those constructs is consistent with the fact that potential binding sites for cardiac- or muscle-related regulatory elements that might be able to facilitate the expression were identified within their sequence.

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ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per minute
C-terminal	carboxy terminal
DMEM	Dulbecco's modified eagle medium
EDTA	ethylenediaminetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGF-1	fibroblast growth factor-1
FGFR	fibroblast growth factor receptor
GFP	green fluorescent protein gene
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hFGF-16	human fibroblast growth factor 16
kb	kilobase
Luc	firefly luciferase gene
rLuc	Renilla luciferase
MEF2	myocyte enhancer factor

min	minutes
mFGF-16	mouse fibroblast growth factor 16
M-MLV	(Moloney Murine Leukemia Virus) Reverse Transcriptase
MSBP	Muscle specific binding protein
-pGFP/Luc	GFP/Luc promoterless reporter gene
PBS	phoshate buffered salt solution
PBS-CMF	phosphate buffered salt solution calcium and magnesium free
PCR	polymerase chain reaction
rFGF-16	rat fibroblast growth factor 16
mFGF-16	mouse fibroblast growth factor 16
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SE	standard error
SD	standard deviation

CHAPTER 1: INTRODUCTION

1.1 FGF-16 and the Fibroblast Growth Factors Family

1.1.1 General characteristics of the fibroblast growth factors family

The FGF family counts now 23 members. All vertebrates express members of the FGF family. The origin of this family has been traced back as far as *Caenorhabditis elegans* (Birnbaum *et al*, 2005) and no FGFs are expressed in unicellular organisms (bacteria or yeast) (Itoh and Ornitz, 2004). FGF-1 and FGF-2, also called acidic FGF and basic FGF, respectively, were the first members identified and are considered the prototypes of this family. All the related proteins identified subsequently were "named" in their numerical order of identification.

Many of the FGFs are expressed during development in different tissues. Some of them, such as FGF-3 and FGF-4 are reported to be expressed almost exclusively during embryonic development and they have not been identified in adult tissues (Ornitz, 2001). Other FGFs, such as FGF-1, FGF-2, FGF-8, FGF-9, and FGF-20 are expressed in the embryo as well as in the adult, but a distinct spatial pattern of

expression is maintained at both of these stages. However, for the same FGF, some isoforms, such as FGF-1.A (the transcript isoform resulting from FGF-1.A promoter) are expressed mainly in heart postnatally to adulthood, and less specifically during embryogenesis, while the expression of the transcript isoforms resulting from the promoter B, C and G, for example, is not modified in terms of tissue and level of expression in adult compared with embryo (Madiai and Hackshaw, 2002).

Structural studies of FGF-1 and FGF-2, showed that their secondary structure consists of a triangular (trefoil) array of β-sheets, forming a loop between strands 8 and 9 (β8-β9 loop), and strands 10 and 11 (β10-β11 loop) (Faham *et al*, 1998; Ornitz 2000). The amino acid sequence within this loop is preserved in all FGF family members and this is considered to be the determining feature of this family. The β8-β9 loop plays a central role in heparin binding, in the interaction with high affinity FGF receptors (FGFR) (β8-β9 loop), and also stabilization of FGF molecules against thermal denaturation (Ornitz 2000).

It is well established for most of the FGF family members that the heparin/ heparan sulphate binding region is a requirement in order for them to interact with the FGFR and generate specific signals. The affinity for heparin/ heparan sulphate limits the ability of these molecules to diffuse in the interstitial space and therefore it is widely accepted that all FGFs exert their effects at the site of their synthesis or very close to it (Ornitz, 2000). Furthermore, studies showed that the amino acid sequence at the

heparin/ heparan sulphate binding region is not completely conserved among the FGF family members.

The core of the heparin/ heparan sulphate binding region, consisting of 140 amino acids, has only 28 highly conserved residues, while the rest of the amino acids sequence is different. However, these residues differ more among members of different subfamilies of FGFs and less among the members of the same subfamily and may affect properties such as receptor affinity. The variation in amino acid sequence also suggests that different FGFs have different affinities for heparin/heparan sulphate and this can offer some specificity for certain heparan sulphate sequences and/or for heparin/heparan sulphate concentrations that further modulate the FGF-FGFR interaction. Indeed, a model of tissue-specific regulation of FGF activity based on heparin/ heparan sulphate local concentration, that vary from a tissue from another, has been proposed (Ornitz, 2000).

1.1.2 FGFs promoter, genes structure and transcripts

The FGF genes are scattered throughout the genome and based on their similarity in sequence, it is considered that they were generated by gene duplication followed by translocation during evolution (Itoh and Ornitz, 2004). Several FGF genes

are grouped in clusters in the human genome, for example human FGF-3, FGF-4 and FGF-19 are all located on chromosome 11 and separated by only 40 kb and 10 kb, respectively.

The regulation of FGFs gene expression occurs at different levels, such as transcription, mRNA stability and translation. (Shibata et al, 1991; Murphy, 1990; Kardami *et al*, 1995). The promoter region of many, but not all FGFs has been identified, cloned and functionally assessed using different cell types.

In the case of FGF-1, four different transcripts were identified as result of transcription initiation from four distinct promoters, only one of which has a TATAA box (Chatoni and Chiu, 1997).

The control of FGF-2 expression also occurs at the transcriptional level and involves different factors that can act within the promoter region (Pasumarthi, 1997). It also can be controlled at the post-transcriptional level by synthesis of an endogenous anti-sense (AS) FGF-2 mRNA, resulting from transcription of the FGF-2 gene in the opposite direction. The resulting transcripts (sense and anti-sense) are overlapping at the 3'-end region and are also expressed in a tissue and developmental specific manner (Gagnon, et al, 1999). The promoters for both FGF-2 and AS-FGF-2 are, however, similar in that that they are both TATA-less and contain Sp1 binding sequences (Pasumarthi, 1997; Gagnon, et al, 1999) as well as binding sites for factors that are active in different tissues (eg Nkx2.5 in heart and Myo-D in heart and skeletal muscle)

(Gagnon, et al, 1999).

FGF-3 transcription is initiated from multiple promoters that are also characterized by a lack of TATA box (Djenabi *et al*, 1999). The transcription initiation site of the longest transcript was identified approximately 6kb upstream of the ATG codon (Djenabi *et al*, 1999).

FGF-4 expression is tightly regulated and it is restricted to embryonic period and carcinoma cells (Bryans *et al*, 1995). FGF-4 expression is controlled by elements in the promoter (Lucas *et al*, 1994), but it seems that the enhancer domain located in the untranslated region of the third exon is absolutely required for FGF-4 expression (Lamb, et al, 1997; Bryans *et al*, 1995). Furthermore, the promoter regions involved in FGF-4 expression are highly conserved between human and mouse (Lucas *et al*, 1994).

FGF-5 is one of the less FGFs that has a TATA-box consensus sequence within its promoter. Furthermore, the elements located very close to the transcription initiation site of FGF-5 (-314/+48) are required for FGF-5 expression (Gelfman *et al*, 1998). The FGF-23 promoter also has a TATA-box / TFIID binding site and its expression is regulated by vitamin D (Ito *et al*, 2005).

The FGF family is a very diverse one. This results mostly from the differential splicing of FGF mRNA (Ornitz and Itoh, 2001), the alternative promoter usage for FGFs transcription (Pasumarthi, 1997) and from the usage of an unconventional alternative translation start (eg. CTG in the case of FGF-2 translation in the heart) in

addition to the conventional one (ATG) (Kardami *et al*, 1995). Multiple protein isoforms resulting from alternative splicing have been reported, including FGF-1 (Myers *et al* 1993; Madiai and Hackshaw, 2002), FGF-5 (Ornitz and Itoh, 2001), FGF-8 (MacArthur *et al*, 1995), FGF-12 and FGF-13 (Hartung *et al*, 1997). Furthermore, the diversity is also extended to the RNA level. Multiple transcripts were identified in both physiological and experimental or pathological conditions as result of usage of alternative transcription initiation sites for many of the FGFs, such as rFGF-2 (Pasumarthi, 1997, Cattini *et al*, 1998), human (h) and mouse (m) FGF-1 (Madiai *et al*, 1999, Zhang *et al*, 2001). Furthermore, while multiple transcripts were identified for FGF-9 (Cinaroglu *et al*, 2005) which is the closest related FGF member to FGF-16 in terms of amino acid sequence, only one transcript was identified in the case of FGF-16 (Sontag, 2003).

The biological significance of the alternative promoter usage is not completely understood, but this mechanism has often been associated with genes containing no "classical" TATA box in their promoters. Many of the FGFs lack the conventional elements of the promoter region, such as a TATA box and CCAT box. However, for all the "unconventional" FGF promoters, multiple GC box sequence motifs were identified as transcription start sites where the Sp1 transcription factor binds. (Alam *et al*, 1996; Shibata *el al*, 1991; Pasumarthi, 1997). Interestingly, even in these cases, for some FGFs isoforms, functional TATA and CAAT consensus sequences were identified in the promoter region, while other transcripts from the same gene do not make use of any

of these elements within the identified promoter region (Madiai and Hackshaw, 2002). Furthermore, each of these transcripts is expressed in a tissue and/or developmentally specific manner, suggesting that this mechanism of alternative promoter usage might play a role in the fine regulation of tissue and temporal expression of FGFs (Chatoni and Chiu, 1997).

1.1.3 The "FGF-9" subfamily

Based on the phylogenetic studies, the hFGF gene family was divided in to seven subfamilies, FGF-16 belonging to "the FGF-9 subfamily", together with FGF-9 and FGF-20 (Itoh and Ornitz, 2001). Members of the same subfamily are closely related in structure as well as in biochemical and developmental functions. FGF-16 shares an amino acid sequence similarity of 62% with FGF-20 and 73% with FGF-9 (Kirikoshi *et al.*, 2000). In addition to the structure, the members of the same FGF subfamily also have a similar pattern of expression and in many cases a similar role (Ornitz and Itoh, 2001). All FGF-9 subfamily members (FGF-9, FGF-16 and FGF-20) are expressed in heart during mouse development, although each of them appears to have a unique site of expression. All three subfamily members are expressed in both epicardium and endocardium during embryogenesis (Lavine *et al*, 2005). In addition, both FGF-9 and FGF-20 expression was found in both ventricles (Lavine *et al*, 2005, Colvin *et al*,

1999). FGF-9 RNA was also detected during mouse embryogenesis in several tissues, such as lung pleura, skeletal myoblasts in the early limb bud, spinal cord motor neurons, olfactory bulb, and gut lumenal epithelium (Colvin *et al*, 1999) and it seems that it plays key roles in organ formation and organization (Colvin *et al* 2001) since null FGF-9 mice die during embryogenesis because of heart defects (Lavine *et al*, 2005) as well as of major lung and airways defects (Colvin *et al* 2001). There are also reports that FGF-9 and FGF-20 play roles in cell proliferation, cell survival and cell locomotion (Granerus and Engstrom, 2003; Tsai *et al*, 2002; Houchen, 2003; Jeffers *et al*, 2001). FGF-9, together with FGF-4, FGF-7 and FGF-8, was also reported to be expressed in lens fibers during development and seems to play important roles in normal eye development, maintaining lens transparency and preventing cataract induction (Lovicu and McAvoy, 2005). In retina, FGF-9 expression has a biphasic pattern: it is elevated at birth and adulthood, but relatively decreased during terminal retinal differentiation (4-14 days postnatal) (Cinaroglu *et al*, 2005). Possible FGF-16 expression was also identified in adult mouse retina (Wilson *et al*, 2003), but the fact that this was assessed by microarray using cDNA oligofragments raises questions about specificity of hybridization, especially taking into account that the FGF-16 cDNA sequence is very closely related (73%) to that of FGF-9 whose expression was already identified in retina. Both FGF-9 and especially FGF-20 are also expressed in different parts of the CNS and demonstrated that their expression within the nervous tissue is restricted (Ohmachi *et al*, 2000; Nakamura *et al*, 1999; Kanda *et al*, 1999). FGF-16 expression in

nervous tissue has not been reported.

Many FGFs exert their effects via specific FGF receptors at the site of their production or very close to it (Ornitz, 2000). Most of them are secreted and contain within their sequence a hydrophobic N-terminal signal peptide that serves as signal sequence in the process of secretion. By contrast, the members of the FGF-9 subfamily (FGF-9, FGF-16 and FGF-20) lack that cleavable signal required for secretion (Revest *et al*, 2000). However, they are some of the rare proteins that despite this, they are secreted via a bipartite, uncleaved signal sequence (Miyakawa and Imamura, 2003, Revest *et al*, 2000). FGF-1 and FGF-2 also lack the cleavable signal peptide, but they are not secreted, but released from cells using an ATP-dependent exocytotic mechanism that is independent of the endoplasmic reticulum pathway (Mignatt, 1992, 1991, Forkiewicz *et al*, 1995). In addition, FGF-2 is released by transient membrane disruption in contracting cardiac myocytes (Kaye *et al*, 1996; Clarke *et al*, 1995).

FGF-16 gene and transcript

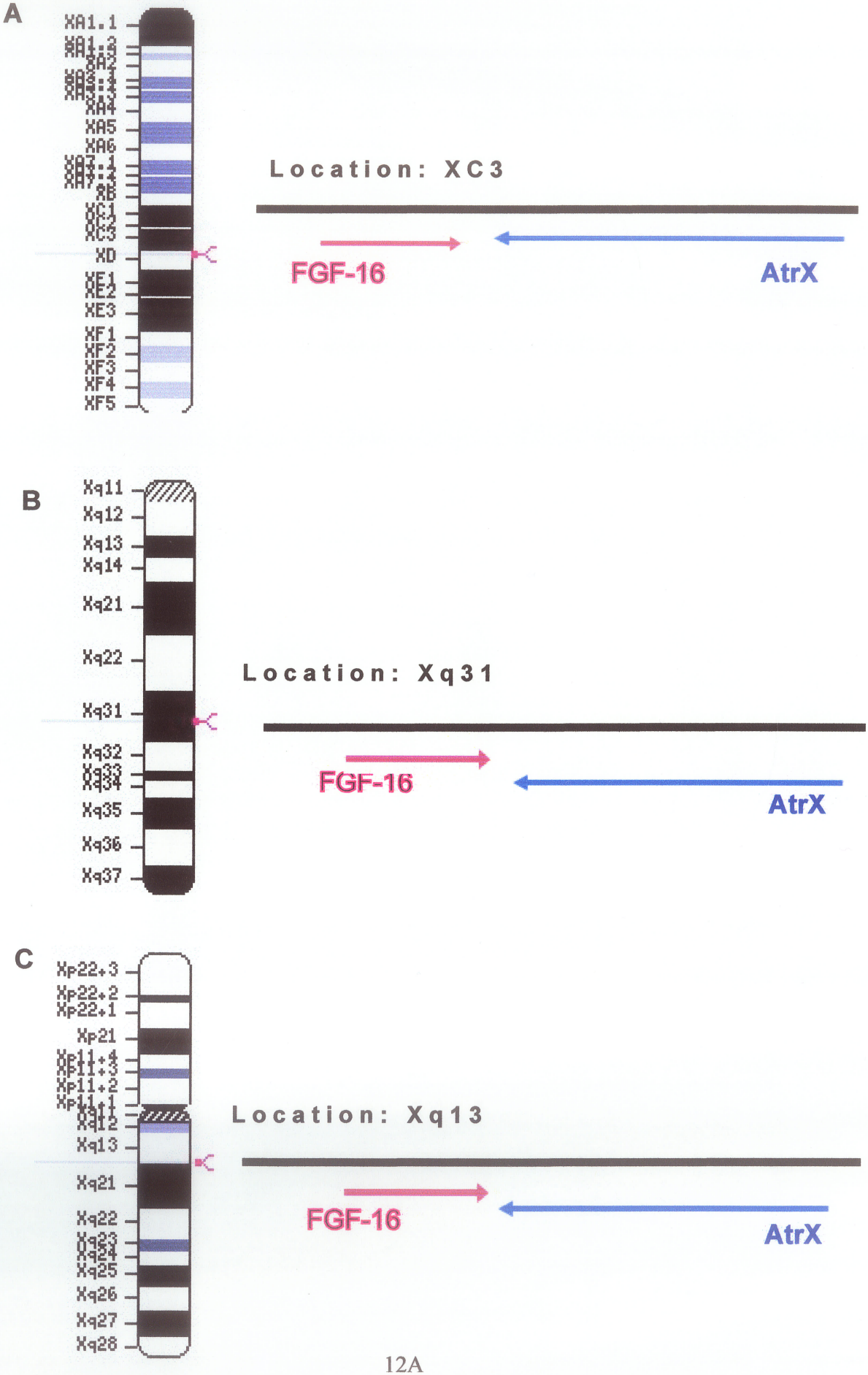
Fibroblast growth factor-16 was first identified from rat heart by homology-based polymerase chain reaction (Miyake *et al*, 1998). Unlike other family members, for which multiple transcripts were detected, only one transcript of approximately 1.8 kb was identified in the case of this factor, at least in the mouse adult heart (Sontag and Cattini, 2003). The FGF-16 cDNA that was cloned from adult mouse adult heart RNA showed a product of 624 bp coding sequence and a 26 kDa protein (Sontag and Cattini, 2003). Mouse FGF-16 protein has 207 amino acids and shares a high similarity in sequence (about 99%) with both human and rat FGF-16. The only differences between mouse and human are a Q^{16} versus H^{16} and R^{205} versus H^{205}, while between mouse and rat the differences are a F^{124} versus Y^{124} and T^{150} versus A^{150}.

The hFGF-16 gene was mapped initially to chromosome 8, locus 8p21.3 (Kim, 2001), probably because of its similarity to FGF-20, another "FGF-9" subfamily member located at 8p22-p21.3 locus. Subsequently, dog, rat, mouse and human FGF-16 genes were all localized to chromosome X. Indeed, FGF-16 is the only FGF family member that is encoded on the same homologous chromosome for all these species (NCBI Gene Database: http://www.ncbi.nlm.nih.gov). Furthermore, the chromosomal domain where this factor is encoded seems to be very conserved for human, mouse and rat. FGF-16 is located on chromosome X near AtrX gene (α

thalassemia/mental retardation syndrome X-linked homolog) (NCBI Gene Database: http://www.ncbi.nlm.nih.gov). Human FGF-13 is also encoded on chromosome X, however no FGF cluster was identified on this chromosome (Fig.1).

Fig.1: Chromosomal localization and the chromosomal domain of FGF-16 in mouse (A), rat (B) and human (C).

For all three species, FGF-16 gene is encoded on chromosome X, being the only FGF family member that is encoded on the same homologous chromosome for all these species (NCBI Gene Database: http://www.ncbi.nlm.nih.gov). The chromosomal domain of this factor is also very much conserved; for all these species FGF-16 gene is located near AtrX gene (α thalassemia / mental retardation syndrome X-linked homolog) (NCBI Gene Database: http://www.ncbi.nlm.nih.gov).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FGF-16
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Xq13
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Location: Xq31
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Xq27
Xq28
Location: Xq13
FGF-16
AtrX

Pattern of FGF-16 gene expression

FGF-16 is expressed in all developmental stages in different tissues.

In rat, during embryogenesis, FGF-16 is expressed at high level in brown adipose tissue (Konishi *et al.*, 2000) and at low level in heart at the end of the gestational period (Miyake, 1998). Postnatal, FGF-16 expression decrease totally in brown adipose tissue and it starts to be expressed in heart (Miyake, 1998) (Fig.2).

In mouse, during embryogenesis, FGF-16 is expressed in inner ear (Wright, 2003) and in specific cell types from heart, namely endocardium and pericardium (Lavine *et al*, 2005,). FGF-16 expression in inner ear cases at the end of the gestational period (Wright, 2003) and in adult mice, FGF-16 expression was identified only in the retina (Wilson, 2003) and in the cardiac myocytes (Miyake, 1998, Sontag and Cattini, 2003; Sontag 2005) (Fig.2).

FGF-16 was first described as a factor predominately expressed in brown adipose tissue during rat embryogenesis (Miyake, 1998; Konishi *et al.*, 1999), likely involved in adipocyte proliferation and thermogenesis. The FGF-16 mRNA level in brown adipose tissue decreases after differentiation of this tissue and it diminishes even more after birth (Konishi *et al.*, 2000). During mouse development, FGF-16 expression, together with FGF-4, was identified in inner ear beginning in the very early stages of

development (Wright, 2003). FGF-16 expression in ear does not overlap with that of FGF-4, and is closely regulated during different stages. Close to the end of the embryonic stage, FGF-16 expression in ears ceases. In the adult mouse, FGF-16 expression was also identified in retina where it may have an angiogenic effect on retina blood vessels, but the way in which this was assessed is subject to debate (Wilson, 2003).

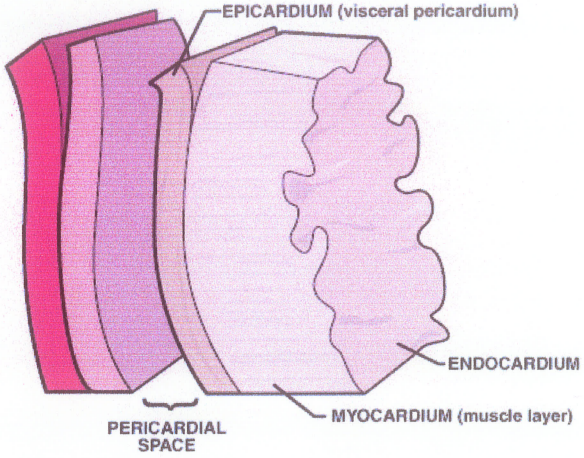
The pattern of FGF-16 expression in heart is especially intriguing. In the rat embryo, FGF-16 expression in heart was identified at a very low level and only at the end of the gestational period (Miyake *et al,* 1998). At birth, FGF-16 expression in cardiac myocytes increase very much. The induction of FGF-16 expression in neonatal rat cardiac myocytes (Miyake *et al.*, 1998) coincides with a multitude of changes that take place in the mammalian heart after birth and these events are likely influenced by a complex series of signals affecting growth and development.

In the mouse, FGF-16 expression was identified in cardiac myocytes in adult animals (Sontag and Cattini, 2003), while during embryogenesis, FGF-16 expression is restricted to the endocardium and pericardium (Lavine *et al*, 2005) (Fig.2). However, the expression of FGF-16 in mouse cardiac myocytes appears to be induced at birth (Sontag, 2005). These data suggest that FGF-16 is expressed in non-myocytes/non-contractile heart cells during development, while after birth FGF-16 is expressed in cardiac myocytes.

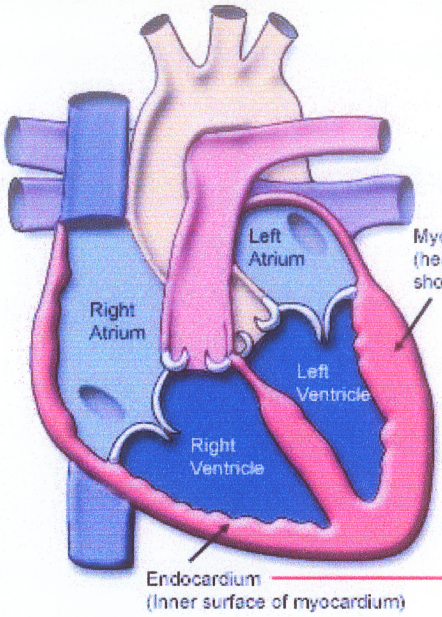
Fig. 2: FGF-16 expression in mouse and rat heart during development, postnatal and in adult.

Pictures were modified from their original obtained from: "Freshman: Human Body, Chapter 2 - The Cardiovascular System"- http://www.mswatch.com/Education (A) and Texas Heart Institute- http://www.texasheartinstitute.org/ myocard.html (B and C).

A. Schematic representation of heart layer (section)



B. FGF-16 expression in heart during embryogenesys

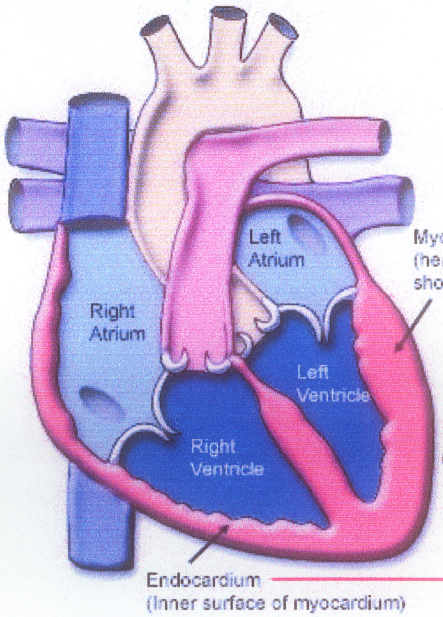


Myocardium (heart muscle) shown in red → Low level of expression at the end of the gestation (Miyake *et al*, 1998)

Epicardium (Outer surface of myocardium) → FGF-16 expression (Lavine *et al*, 2005)

Endocardium (Inner surface of myocardium) → FGF-16 expression (Lavine *et al*, 2005)

C. FGF-16 expression in postnatal and adult heart



Myocardium (heart muscle) shown in red → FGF-16 expression (Miyake *et al*, 1998; Sontag and Cattini, 2003, 2005)

Epicardium (Outer surface of myocardium) → ??? FGF-16 expression

Endocardium (Inner surface of myocardium) → ??? FGF-16 expression

It must be underlined that both the induction of rFGF-16 expression in heart at birth, as well as the shifting of mFGF-16 site of production within the same organ from the non-myocyte layer to the cardiac myocyte are unique processes among FGFs and these facts suggest that FGF-16 expression is very precisely controlled in terms of site of production (spatial control) and stage of development (temporal control).

Interestingly, all FGF-9 subfamily members (FGF-9, FGF-16 and FGF-20) are expressed in heart during development, although each of them appears to have a unique site of expression (Lavine *et al*, 2005). However, while FGF-20 and FGF-9 are expressed postnatally in many tissues, showing a certain level of preferential expression for some tissues, such as nervous system in case of FGF-20, postembryonic and adult expression of FGF-16 seems to be restricted to heart, and more specific to its contractile layer of myocytes (Sontag, 2005). Thus, although the members of the same subfamily tend to have a similar pattern of expression and in many cases a similar role (Ornitz and Itoh, 2001), FGF-16 appears to be unique in both its cardiac specificity in adult and its induction in postnatal cardiac myocytes.

1.2 Gene expression in the heart

1.2.1 The expression of fibroblast growth factors in heart

Embryonic Heart

The heart is the first fully functional organ in the developing embryo and is formed from a complex structure of cells derived from different sources, especially from the regions of dorsolateral mesoderm (Harvey and Rosenthal, 1999). There are many factors involved in the processes of myocyte and non-myocyte proliferation, chamber formation, heart polarity and axis orientation, valve development, specialization of the conduction system, to name but a few. There are also many pathways in which different molecules are signaling and play distinct roles in heart development. All these need a precise coordination. The FGF family has an important place among the molecules whose signals play different roles in the organization and function of the embryonic heart (Harvey and Rosenthal, 1999). They are expressed at all developmental stages, but this varies at the different periods of development. As discussed previously, all FGF-9 subfamily members (FGF-9, FGF-16 and FGF-20) are

expressed in heart during embryogenesis, although each of them appears to have a unique site of expression (Lavine *et al*, 2005). FGF-4 is also expressed in the heart during the embryonic period (Zhu *et al*, 1996). Its signals, together with those of FGF-8 contribute to the heart induction in early heart morphogenesis in many species, including chicken, mouse, zebrafish (Barron, 2000; Alsan and Schultheiss, 2002). Their appropriate expression is absolutely required to turn on the expression of other cardiac-specific genes, such as Nkx 2.5, GATA 4 and Mef 2 (Alsan and Schultheiss, 2002). Thus, FGF-4 is important during embryogenesis and its expression is restricted to this stage of development (Ornitz and Itoh, 2001). By contrast, other FGFs, such as FGF-1, FGF-2, FGF-8, FGF-12, FGF-13, FGF-16 are expressed in the heart during both embryonic and adult stages. These will be detailed next.

Postnatal and Adult Heart

During embryogenesis, cardiac myocytes undergo proliferative growth also known as hyperplasia. By contrast, after birth, the mitotic activity decreases and the heart reaches the required dimensions and function by growth through an increase in size (hypertrophy) of a fixed number of cardiac myocyte. In addition the cardiac myocytes undergo significant binucleation (DNA synthesis, mitosis but no cytokinesis) in the postnatal period. These events are likely influenced by a complex series of

signals such as growth factors, nutrients or events (e.g., changes in circulation, oxygen exchange and demand of the organ) that affect growth and development and correlate with the changes seen in the neonatal heart. The current belief is that the postnatal period is characterized by a very short phase of exclusively hyperplasia, followed by a phase (6-14 days in rat) and in which hyperplasia and hypertrophy of myocytes overlap (Li *et al*, 1996). After 14 days, growth is almost exclusively hypertrophic, coinciding with the total inhibition of synthesis of the cell cycle protein cyclin A (Brooks *et al*, 1998). It is important to underline that this postnatal hypertrophy is a physiological and adaptive one. While pathological hypertrophy is the subject of many studies, physiological hypertrophy that occurs naturally after birth is less studied, despite the fact that it can provide a good model for understanding the mechanism by which cardiac growth is regulated.

FGF-1 and FGF-2 are expressed in the heart during both embryonic and adult stages. Both play a very particular and important role in modulating myocytes phenotype (Schneider *et al*, 1992). In addition, FGF-2 expression during different development stages exhibits a particular pattern. The "long" form of FGF-2 is predominant in the immature myocardium suggesting that this form is associated with cell proliferation, myocardium organization and hyperplasyc phenotype, while the "short" form of FGF-2 is more abundant in the mature myocardium, being linked with hypertrophy, in both physiological and pathological (ischemic injury, inflammation, fibrosis) (Kardami *et al*, 1995). FGF-8 is also expressed in heart in many species,

playing important roles in left-right axis specification during embryogenesis (Reifers *et al,* 2000; Alsan *et al,* 2002). It is also expressed in adult heart (Schmitt *et al*, 1996). FGF-12 and FGF-13 are also expressed in adult heart. FGF-12 is expressed only in the atrial chambers and FGF-13 is expressed in both the atrium and ventricle. Their expression starts in embryogenesis and continues in adult, but their level of synthesis decreases (Hartung *et al*, 1997).

1.2.2 Tissue specific control of gene expression

Cells from different tissues within an individual differ dramatically in their RNA and protein content, in spite of having identical DNA sequences. The achievement of the complexity that characterizes an adult organism requires a harmonized coordination of genes to be expressed at the right time, place and level. The basic functionality of a cell requires the expression of "housekeeping" genes that are normally expressed in all cells of all tissues, but the specialized functioning of a particular cell type involves the expression of specific genes that are expressed only in that cell type. Tissue-specific regulatory mechanisms present orchestrate the complex process of gene expression and function. These mechanisms include (1) chromatin remodeling that allows accessibility of transcription complexes to the gene locus, (2) regulation of gene expression by the specific transcription factors for spatial and temporal control, and (3) regulation of the

mRNA itself, involving alternative splicing and/or mRNA stability. The gene products themselves can be further regulated through specific posttranslational modifications, protein export or secretion, and cleavage and activation by a particular enzyme (convertase).

The process of chromatin remodeling allows the accessibility of specific transcription factors (referring to tissue/temporal-specific transcription regulatory proteins) to the gene regulatory DNA sequence (promoter). However, tissue specific genes expression also requires the presence of binding sites for such specific factors within the promoter sequence. In this way, cells can change the expression of their genes (eg.FGF-16 gene) in response to a multitude of internal and/or external factors or events that also can act at a certain stage of development or within a certain tissue.

1.2.3 Overview of cardiac-specific transcription factors

For a long time, there has been an intense interest in understanding the molecular mechanisms that control cell specification and terminal differentiation of the developing heart. These mechanisms are not only very complex, but also highly conserved throughout evolution. The pathway of cell specification starts with external inductive signals and just like instruments involved in playing a certain symphony and in perfect harmony, each of the specific transcription factors plays its own part, at its own moment to specify the endocardium, pericardium, myocytes and conductive system. Layer after layer, process after process, the complexity of the heart is achieved. The transcription factors involved in heart organization and functionality are numerous. Here will be provided an overview of some of these, including the NK gene products, the Myocytes Enhancer Factor (MEF2) family of transcriptional regulatory factors and the GATA family of zinc finger transcription factors.

NK Homeodomain Factors

The homeobox gene *tinman* (*tin*) was first identified in *Drosophila* during development, encoding for a protein belonging to the NK-2 class. This DNA binding protein class is characterized by a 60 amino helix-turn-helix motif and is related to the HOX homeobox genes that regulate embryonic patterning. NK-2 proteins recognize and bind to a specific DNA sequence (5'-TGAAGTG-3') (Table 1, page 21). After identification in *Drosophila*, other homologous members were identified in the vertebrates as well. There are now five identified members of the Nkx-2 gene family that are expressed exclusively in the vertebrate heart. From these, Nkx 2.5 (also called Csx) is the only member expressed in cardiac progenitor cells of all vertebrates examined during embryogenesis (Harvey and Rosenthal, 1999, pp: 113-125).

The importance of this protein family in heart and vascular system development during embryogenesis is revealed by the fact that Nkx2.5 knock-out mice die by heart malfunction, while in human Nkx2.5 mutation determine severe atrial defects leading to major defects of septation and atrioventricular node function (Harvey *et al.*, 2002; Schott *et al*, 1998). Nkx2.5 also plays an important role in myocardial maturation and in the transition of myocytes from a hyperplastic to a hypertrophic phenotype (Harvey *et al.*, 2002). Nkx2.5 is a significant regulatory factor for the expression of atrial natriuretic factor (ANF), a fundamental element for cardiac hypertrophy (Komuro,

2001). It also controls the expression of other cardiac factors, such as MEF2, HAND1, MLC-2v, α-cardiac actin (α-CA) that are involved in both cardiac development and cardiac hypertrophy/cardiac phenotype (Jamali *et al*, 2001). Nkx2.5 expression is regulated by bone morphogenic protein (BMP) 4 and 2 and by transforming growth factor-β (TGF-β) (Farrell and Kirby, 2001).

Transcription factor	**DNA binding sequence**
Nkx2.5	5'-TGAAGTG-3'
MEF-1	5'-TATAAAA-3'
MEF-2	5'- CTAAAAATAA-3'
SRE	5'-CCATATTAGG-3'
EGR	5'-GCGGGGGCG-3'
GATA	5'-(A/T)GATA(A/G)-3'
NFκβ	5'-GGGGACTTTCCC-3'

Table 1: The specific DNA binding sequence for some of the best known transcription factors involved in the control of cardiac gene expression bind.

Myocytes Enhancer Factor (MEF2) family of transcriptional regulatory factors

The MEF2 family of transcription factors belongs to the MADS-box proteins family (Firulli and Thattaliyath, 2002). The MADS-box proteins family is a highly evolutionary conserved protein domain found in yeast plants and vertebrates. Its name derives from the first identified members of this family: *M*CM1, *A*gamous, *D*eficiens and *S*erum response factor (Firulli and Thattaliyath, 2002). The MADS-box proteins family represents a large group of transcription regulatory elements that starts to be expressed in the heart during embryogenesis, immediately after the cardiac cells are specified (Zheng *et al*, 2003). Together with Nkx 2.5, they represent early myocytes markers. A characteristic 29 amino acid sequence at the NH_2- terminal end forms the MEF2 domain that together with the adjacent MADS domain, is essential for DNA binding (Zheng *et al*, 2003). MEF2 factors bind either as homo- or heterodimers to a specific DNA consensus sequence: C/TTA(A/T)$_4$TAG/A in many of the regulatory regions of muscle- and heart-specific genes (Zheng *et al*, 2003) (Table 1, page 21). Vertebrates have four distinct MEF2 genes (MEF2 A to D) and all are homologous to D-MEF2 in *Drosophila* (Firulli and Thattaliyath, 2002). These genes are encoded on different chromosomes and are highly expressed in muscle during development. MEF2C, however is unique in that it is the only one that is also expressed in cardiac

myocytes. MEF2C expression in cardiomyocytes starts in early embryogenesis (day 7.5 in mouse) (Molkentin *et al*, 1996) and plays an essential role in early cardiogenesis. The MEF2C null mouse shows many vascular defects and the heart tube fails to organize itself (Lin *et al*, 1997). Subsequently, myocardium does not develop normally and many of the cardiac genes, such as ANF and α-cardiac myosin heavy chain (α-cMHC), are not expressed at all or are expressed inappropriately (Lin *et al*, 1997). The importance of MEF2C in heart chamber organization is also revealed by the fact that this factor is not expressed at the same level in atria and ventricles. MEF2C expression is more active in atria, suggesting that this factor contributes to the chamber-specific organization. This pattern of expression is also maintained in adult heart (Zheng *et al*, 2003).

The expression of MEF2C is regulated at multiple levels: transcription, alternative splicing and postranslation. There are numerous factors that control MEF2C expression in the heart and among them Nkx 2.5 seems to play an important role (Jamali *et al*, 2001). In turn, MEF2 factors and especially MEF2 C regulates the expression of many cardiac genes. Together with dHAND, another important transcription regulatory factor during cardiogenesis, MEF2 C regulates the transcription of ANF and (α-cMHC) (Zang *et al*, 2004; Adolph *et al*, 1993). Through this, MEF2 C plays an important role not only in heart development, but also in myocyte phenotype and hypertrophy.

The GATA family of Zinc finger transcription factors

The GATA family includes a group of transcription regulatory proteins which are expressed starting from early embryogenesis to adulthood and which are involved in many physiological and pathological processes. These DNA binding proteins were named by the consensus DNA sequence where they bind: (A/T)GATA(A/G) (Table1, page 21). They are characterized by the presence of two adjacent zinc finger domains (Pikkarainen *et al*, 2004; Patient and McGhee, 2002). This family includes, in vertebrates, 6 members (GATA-1 to 6) and they are expressed in different tissues. There is some overlap in both expression and function for some of the members. While GATA1-3 seem to be essential for determining erythroid and other blood cell lineages (Ohneda and Yamamoto, 2002), GATA 4-6 are very important for cardiogenesis and for heart physiology (Pikkarainen *et al*, 2004).

Like Nkx2.5, GATA-4 is an transcription regulatory element and early marker for developing murine cardiac cells. It is expressed from embryonic day 7 in precardiac mesoderm and continues to be expressed during formation of the cardiac tube, in endocardium and in myocardium (Heikinheimo *et al*, 1994). Its expression continues in the heart during throughout life and increases in some pathological conditions, such as pressure-overload induced hypertrophy (Bär *et al*, 2003).

In contrast, GATA-5 is expressed during embryogenesis in heart, but later is expressed only in the endocardium, but not in the myocardium. GATA-6 has a similar pattern of expression to GATA-4 (Firulli and Thattaliyath, 2002). These three GATA family members share a high level of similarity in amino acid sequence, especially within the zinc finger DNA binding domain, but they diverge at the c-terminal (Firulli and Thattaliyath, 2002) possibly resulting in the differences in function. GATA-6 seems to be fundamental for the development, as GATA-6 null mice die in early developmental stages due to major morphological defects, such as lack of parts of the visceral endoderm and showed abnormal development of the embryonic ectoderm (Koutsourakis *et al*, 1999). Transgenic mice with an inactive GATA-4 gene also die during development, but for a while the deficiency is compensated by an increase in GATA-6 expression, which however cannot rescue the animals. GATA-5 null mice survive till later in adulthood and present only minor urogenital tract abnormalities in females (Firulli and Thattaliyath, 2002).

In spite of partial overlap and a certain level of redundancy of GATAs in the heart, each of these three members plays a particular and important role in heart physiology. GATA-4 is a mediator of cardiomyocyte differentiation, proliferation and survival (Peterkin *et al*, 2005) and clinical studies have indicated a role in human congenital heart defects, such as cardia bifida and atrial septal defects when GATA-4 gene is mutated (Peterkin *et al*, 2005). GATA-4 and GATA-6 also seems to be involved in apoptosis and adult cardiomyocyte survival during hypertrophy (Peterkin *et al*,

2005). Studies of cardiogenesis using *Xenopus* showed that GATA-6 is required for differentiation of the cardiac lineage during embryogenesis. The requirement of GATA-6 appears to be in the maturation of the cardiac progenitors rather than in their initial induction (Peterkin *et al*, 2003).

All of GATA 4-6 are involved in the regulation of genes implicated in heart development and heart physiology. In many cases, GATAs do not act on their own, but in combination with other regulatory proteins. GATA-4, for instance, regulates the activity of several cardiac-specific promoter genes, such as α-MHC, ANF, BMP, corin (an enzyme that is essential for ANF processing in the heart), troponin I and C and even some cardiac-specific forms of muscarinic receptors (Pikkarainen *et al*, 2004). However, most of the time, GATA-4 interacts with another regulatory proteins, such as Nkx 2.5, GATA-6 or serum response factor (SRF), and cooperatively regulate myocardial gene expression (Charron *et al*, 1999; Durocher and Nemer, 1998). GATA-4 binding within the α-MHC promoter region is essential for the activation of this promoter in the heart (Farrell and Kirby, 2001). In the case of ANF promoter, Nkx 2.5 recruits GATA-4 and they must physically interact with each other in order to bind and activate this promoter (Farrell and Kirby, 2001).

Interestingly, on turn GATAs are also regulated by Nkx 2.5 (Molkentin *et al*, 2000). GATAs also regulate the expression of some FGFs, especially during embryogenesis in the heart, but also within other tissues (Ohuchi *et al*, 2005; Iwahori *et al*, 2004).

The discussed regulatory factors represent only a few of the most important in cardiac specific gene expression. Others include the helix-loop-helix proteins dHAND and eHAND transcription regulatory proteins, retinoic acid, thyroid hormones, Serum Response Factor (SRF), T-box transcription factors (Tbx), and nuclear factor κ-B (NF κ-β) (Table 1). In the context of this work the investigation of other factor expression and function in the heart will be limited to those already presented.

1.3 Rationale, Hypothesis and Objectives

FGF-16 is a relatively poorly characterized member of the FGF family. Its pattern of expression suggests that it plays a role in the growth and development of the newborn heart. The cardiac-specific and postnatal induction of FGF-16 expression is unique among FGF family members and suggests that FGF-16 expression is very precisely controlled in terms of site of production (spatial control) and stage of development (temporal control). This implies that the FGF-16 promoter interacts with cardiac-specific factors in a precise manner during the postnatal period. However, to date there is no report on the structure and control of the FGF-16 promoter in any tissue and for any species. Thus, the characterization of the FGF-16 promoter sequence and the identification of the transcription initiation site are important steps in understanding the regulation of this factor in the process of heart development and postnatal cardiac physiology.

Therefore it was ***hypothesized*** that the expression of murine FGF-16 is regulated at the transcriptional level and that the sequences that control the fine postnatal expression of this factor in the heart are contained within 6kb upstream of the translation initiation site.

The main ***objectives*** of this thesis were (1) the identification of mFGF-16 promoter region and the transcription initiation site used by this factor for its expression in the heart, and (2) the verification of the identified promoter through a functional assay.

To achieve these objectives, three specific aims were pursued:

Aim 1: To analyze the DNA sequence upstream of the murine FGF-16 start codon in order to identify putative transcription initiation sites (and potential regulatory sites), using a genomic clone containing the mouse FGF-16 gene locus.

Aim 2: To isolate messenger RNA from postnatal mouse hearts and use it to RT-PCR and RNA blotting analyses, using regions flanking putative FGF-16 transcription initiation sites as radiolabeled probes.

Aim 3: To design DNA constructs containing a hybrid luciferase gene (GFP/Luc) and different lengths of the FGF-16 promoter sequences (identified in aim #1) intended to include or exclude putative regulatory sequences. These constructs will be used to transiently transfect neonatal rat cardiac myocytes (*in vitro* assay) in order to assess cardiac expression and promoter functionality.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cloning the upstream mouse FGF-16 gene sequence

A sequence of about 6.2 kb of the 5'-flanking DNA sequence of mFGF-16 gene upstream of its ATG start codon was cloned in our laboratory (Karen A. Detillieux and Peter A. Cattini) using a mouse genomic DNA library (JCRB Gene Bank) and based on mFGF-16 cDNA provided by David Sontag (Sontag and Cattini, 2003). Three positive BAC clones were subsequently isolated and characterized by our lab (747bp KpnI/NaeI, 3.6kb XhoI/NaeI and 6kb KpnI/NaeI).

Further search and characterization of this sequence in relation to FGF-16 expression represents the subject of this thesis.

2.2 RNA Blotting

2.2.1 RNA isolation and blotting

Total RNA was isolated from mouse heart (6 weeks old) using an RNA isolation kit (fibrous tissues) from Qiagen, Canada according to the manufacturer instructions. Total RNA (60 μg) was precipitated with 3M sodium acetate (10% vol) and 95% ethanol (2X final vol.) and pelleted, washed with 70% ethanol, dried and resuspended with sterile RNAase free distilled H_2O. Once resuspended, 1μl of RNA solution was resolved on a minigel (1% agarose) to check the quality of RNA. Once the quality of RNA was confirmed, a reaction mix, composed of MOPS, formaldehyde, deionized formamide and sterile RNAase free dH_2O was added to the of RNA solution. The cocktail was then heated for 20 minutes and after RNA loading buffer was added, it was the electrophoresed on 1.5% (wt/vol) denaturating agarose gel containing formaldehyde and then blotted over night to a nitrocellulose membrane using 20x SSC (3M NaCl and 0.3 M Na citrate). The nitrocellulose membrane was then baked for 1h at 80°C for cross-linking.

2.2.2 Detecting FGF-16 mRNA

One set of primers (Table 2, page 35) were designed using the "Amplify" computer program for the synthesis (PCR) of a DNA probes upstream the putative TATA1 box. (Fig. 4).The single-strand oligonucleotides were obtained from Invitrogen and Qiagen. As these primers were designed to amplify specific DNA fragments to be subcloned and then use as probes, they also carried EcoRV/Hind III restriction endonuclease sites.

PCR fragments were subcloned into the EcoRV/Hind III of Bluescript (SK) vector; for use as probes, these fragments were cut from the DNA plasmid using EcoRV/Hind III and gel band purified using DNA and Gel band purification kit (Amersham, Bioscience). The two designed DNA fragments, together with FGF-16 cDNA (624 bp) as positive control were used to detect mFGF-16 mRNA on blots. A mass of 50 ng of each DNA fragment and FGF-16 DNA were radiolabeled to an activity >1X 10^9 cpm/ μg using a random prime protocol (Promega Prime-a-Gene kit from Promega, USA). Blots were prehybridized for 24h at 42°C with about 15ml solution containing 50% deionized formamide, 0.75M NaCl, 75mM sodium citrate, 10% Denhardt's solution, 50 mM $NaPO_4$ pH=6.5, 0.1g SDS, 10 mg /100ml tRNA, 50mg/100ml salmon sperm DMA. Blots were then hybridized with a similar solution along with yeast tRNA, salmon sperm DNA and SDS, 10 g/100ml dextran sulphate and

radiolabeled probes. After 24 h of hybridization at 42°C, blots were washed three times with 0.1XSSC (15mM NaCl and 16.66 mM Na citrate) and 0.1%SDS for 15 min each at 65°C, then air dried. The blots were then assessed by autoradiography.

2.3 (Reverse Transcriptase) Polymerase Chain Reaction

Total RNA was isolated from skeletal muscle and heart of 6 week old mice as described in section 2.2.1. Primer sets (Table 2, page 35) were designed using the "Amplify" computer program for the synthesis (RT-PCR) of DNA specific probes (Fig. 4).The single-strand nucleotides were obtained from Invitrogen and Qiagen.

For reverse transcription, 1 μg of RNA was incubated for 2h at 37°C in 10 μl reaction mixture containing 2μl of 5X RT Buffer (Gibco–BRL, Canada), 1 μl of 0.1M dithiothreitol (DTT- Gibco –BRL, Canada), 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Gibco–BRL, Canada), 2μl 2.5 mM deoxynucleotides (Pharmacia, Canada), 16 units (0.5 μl-31,200 u/ml) of RNA-guard (Pharmacia, Canada), 2μl of random hexadeoxynucleotide primers (pdN6 from Pharmacia, Canada), 1 μl of 0.1% bovine serum albumin fraction V (0.01% w/v final concentration), (Sigma), and 0.5 μl (5%) DMSO (Sigma).

Amplicon	Sequence
"B" (-709/-514)	sense: 5′CCCAGAAGCTTGCAGCGGGAGGATGCGTGGGAG 3′ antisense: 5′ CTACGAATTCCAGTGAGGCATAAC 3′
"C" (-999/-765)	sense: 5′ GCTATGGAATTCGGATGTGAGCACAGGGTGC 3′ antisense: 5′ TCACACGAAGCTTGCGCTGGCCGCCTTCCC 3′
"N" (-1699/-765)	sense: 5′ TCCCGGAATTCCCAGACCCTTTAAGCG 3′
-223/+21 DNA fragment	sense: 5′CGCCAGGTACCACCCCTGACGGCCGCTCTTC 3′ antisense: 5′GGCTTAGACCTCGAGGGGTGGAGGCTAAG 3′
52 bp fragment	sense: 5' CATGGAGATCATTTTTCAAAAAGATCGCTTTC TATGTCTTAGCCTCGCTAGC 3' antisense: 5' GTACCTCAAGTAAAAAGTTTTTCTAGCGAAAG 3'
GAPDH primers	sense: 5' TGAAGGTCGGTGTCAACGGATTTGGC 3' antisense: 5' CATGTAGGCCATGAGGTCCACCAC 3'

Table 2. The sequence of the primers sets used to amplify the DNA fragments of interest.

Amplification by polymerase chain reaction (PCR) was carried out using specific sense (forward) and antisense (reverse) primer sets for each DNA sequence of interest (Table2). PCR was set up in 50µl mixture containing either 1µl RT reaction or 250ng genomic DNA or 100ng plasmid DNA, as appropriate, 1µM (final concentration) of each sense and antisense primer, and 2 units of Taq DNA polymerase (Qiagen kit) and the buffers included in the PCR amplifier kit (Qiagen, Canada) according to manufacturer's instructions. PCR reactions were carried out in a thermocycler for 30 cycles, consisting of denaturation at 95°C for 5 min, annealing at 60-65°C for 45 sec, and extension at 72°C for 1 min. For a control, samples were also processed in the same way but in the absence of either RT-treatment or in the absence of the corresponding DNA template. The reaction products (both RT-PCR and PCR) were separated on agarose gels and visualized by ethidium bromide staining.

2.4 Subcloning, Sequencing and Plasmid construction

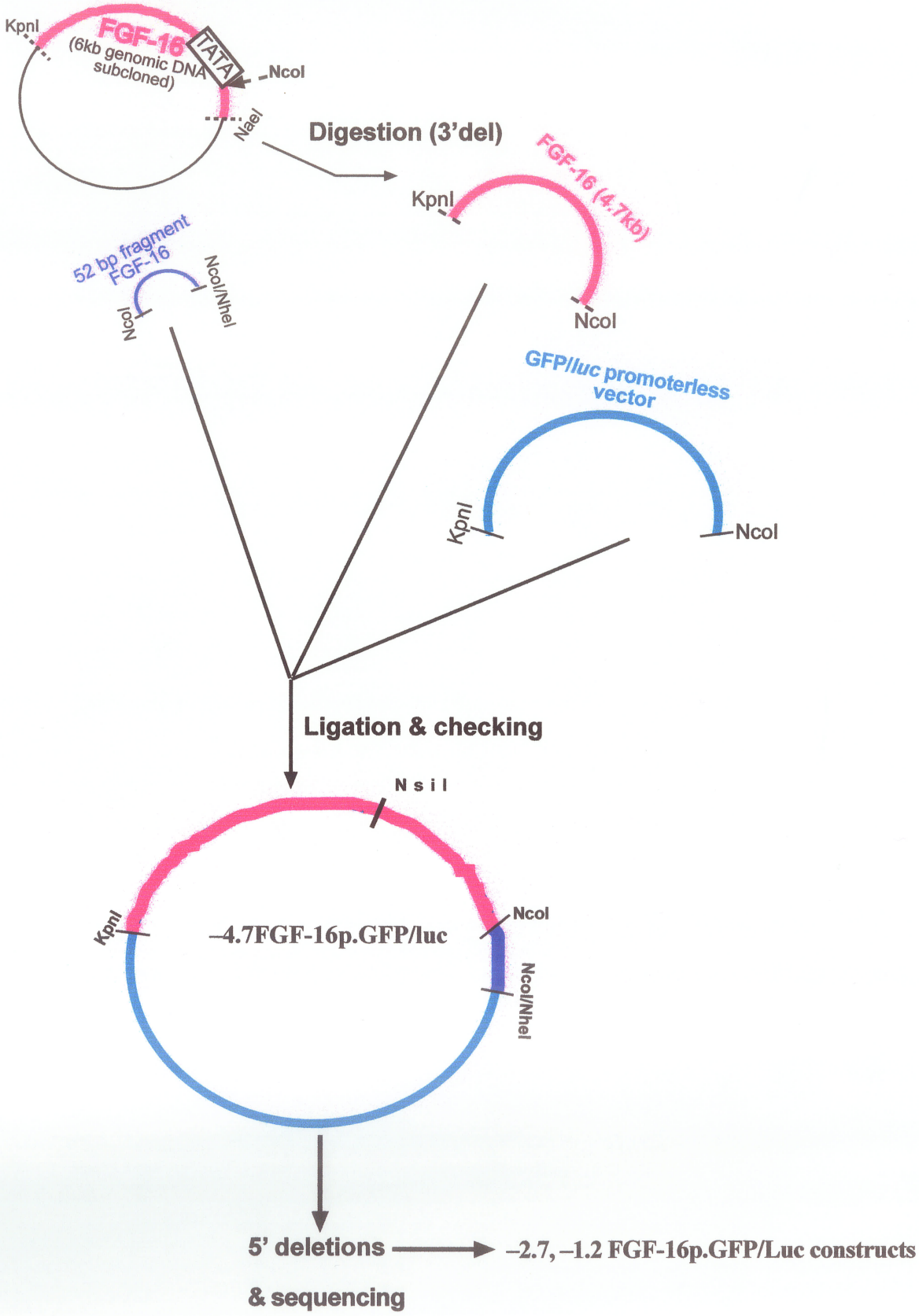
A total of five plasmid DNA constructs were generated with varying lengths of FGF-16 promoter sequences upstream of a GFP/*luc* reporter gene (FGF-16 p.GFP/luc).

Two of the plasmid constructs (-4697/+21 and -2796/+21) were generated using the initial subcloned genomic 5'-flanking DNA sequences upstream of mFGF-16 translation initiation site (6kb upstream of the ATG codon), followed by digestion with convenient enzymes (NcoI, position -31). A DNA fragment of 1030bp was removed by NcoI digestion from the 3'-end of the initial FGF-16 p.GFP/luc constructs, followed by the insertion of a 52bp customer made oligonucleotide fragment (Qiagen, Canada) (Fig.3):

The 52 bp fragment corresponds to the FGF-16 promoter sequence from position -31 to +21, reported to the transcription initiation site. Each of the single-stranded oligonucleotides was obtained from Invitrogen. Double stranded oligonucleotide (Qiagen, Canada) was generated by mixing equal quantity of sense and antisense oligonucleotides and boiling them for 5 minutes, followed by overnight cooling to room temperature. The ends of the newly generated fragment were phosphorylated using a mixture containing T4 Poly nucleotidekinase/ATP (New England Biolabs) according to the manufacturer instructions.

Fig.3: The steps of reporter gene construction.

The plasmid constructs were generated using the initial subcloned genomic 5'-flanking DNA sequences upstream of mFGF-16 translation initiation site (6kb upstream of the ATG codon), followed by digestion with convenient enzymes (NcoI, position -31). A DNA fragment of 1030 bp was removed by NcoI digestion from the 3'-end of the initial FGF-16 p.GFP/luc constructs, followed by the insertion of a 52 bp customer made oligonucleotide fragment. The insertion and orientation of this 52 bp fragment was checked using specific restriction enzymes. Once generated, the initial construct was used to generate the rest of the construct.


KpnI
FGF-16
(6kb genomic DNA subcloned)
TATA
NcoI
NaeI
Digestion (3'del)
FGF-16 (4.7kb)
KpnI
NcoI
52 bp fragment FGF-16
NcoI/NheI
NcoI
GFP/luc promoterless vector
KpnI
NcoI
Ligation & checking
NsiI
KpnI
–4.7FGF-16p.GFP/luc
NcoI
NcoI/NheI
5' deletions
–2.7, –1.2 FGF-16p.GFP/Luc constructs
& sequencing

The phosphorylation reaction was stopped using 1μl of 0.5M EDTA for each 30μl phosphorylation mix, followed by phenol/IAC (isoamyl alcohol:chloroform=24:1) extraction and precipitation with 3M Na acetate (10% volume) and 95% ethanol (2X volume). The fragment was pelleted, washed with 70% ethanol, dried and resuspended with sterile TE solution, and then the ligation was set up using T4 ligase (New England Biolabs) according to the manufacturer instructions.

The insertion and orientation of this 52bp fragment was checked using specific restriction enzymes (Fig.3). Once generated, -2796/+21 FGF-16 p.GFP/luc construct was used to generate the third construct (-1224/+21 FGF-16 p.GFP/luc) by digesting with NsiI (Fig. 3) followed by removing -2796/-1224 DNA fragment from the 5'-end, gel band purification using DNA and Gel band purification kit (Amersham, Bioscience) and relegation. The smallest construct (-203/+21FGF-16 p.GFP/luc) was generated by PCR using the appropriate primers (Table 2, page 35), followed by cloning in the same –p.GFP/luc vector. Each of the inserts and the quality of the plasmids were checked before proceeding to transient transfection using site-specific restriction enzymes digestions

2.5 Myocyte cell culture

Ventricular myocytes were isolated from one day old rat hearts as previously described (Doble *et al*, 1996; Iwaki *et al* 1990), using enzymatic digestion (0.01% w/v) with trypsin (Worthington Biochemicals), collagenase (Worthington Biochemicals) and DNase I (Worthington Biochemicals) using a spinner flask, followed by fractionation on a Percoll gradient

Briefly, pups were killed by decapitation, the hearts were excises and placed in a Petri dish with PBS-glucose (for 500ml solution: 4g NaCl, 0.1g KCl, 0.3g Na_2HPO_4, 0.1g KH_2PO_4, 10g glucose). The atria were removed carefully and the ventricles were cut in small pieces and washed clean of the blood. The pieces were transferred to a spinner flask and digested eight rounds of 10 minutes each using 10ml PBS/glucose/enzymes. After each round of digestion, the supernatant from the flask was transferred in a clean autoclaved bottle containing 10ml FBS (Gibco-BRL) to stop the digestive process. When all pieces were completely digested, the resulting mixture of cells in PBS-glucose and FBS were passed through a nytex filter and centrifuged for 2 minutes at 1500 rpm (HNS centrifuge) at room temperature. The supernatant was removed and the pellet was resuspended with 8ml 1X ADS (6.8 g/l NaCl, 4.7 g/l HEPES, 0.14 g/l NaH_2PO_4, 1g/l glucose, 0.4g/l KCl, 0.2g/l MgSO4). The suspension was centrifuged (2000g for 30 minutes at room temperature, JS5.3 centrifuge) through a

Percoll gradient. After fractionation, the top layer containing non-myocytes was removed and discarded; the bottom layer containing myocytes was resuspended in Ham's F10 Nutrient Mixture (Sigma-Aldrich), then centrifuge again for 2 minute at 1500 RPM (HNS centrifuge). The supernatant was removed and the pellet is resuspended in media (10% Horse serum/Gibco-BRL, 10% FBS, 1% Penicillin & Streptomycin /Gibco-BRL, 79% Ham's F10). Cells were counted using a hemacytometer and then plated on 60mm collagen coated plates with a density of 1.4 million cells/plate (Fig.8A).

2.6 Transient transfection

Cells were transfected 24h after plating. The medium was changed to one containing high glucose DMEM (Gibco-BRL, Canada) with 15% FBS and 1% Penicillin/Streptomycin. Calcium phosphate/DNA precipitation method was use for gene transfer. To each plate 0.35 ml of DNA mix buffer (10µg of the test DNA plasmid, 21µl of 2M $CaCl_2$, dH2O and 0.175 ml Hepes buffer) was added, according to the protocol (Doble *et al*, 2000) modified and detailed by the laboratory of Dr. E. Kardami. Cells were also cotransfected with 20ng TKp.RLuc. The use of renilla luciferase in combination with firefly luciferase corrects for variation in transfection efficiency After 24h, the cells were washed twice with 1X ADS and the medium was replaced with a growth medium containing: 10µl ascorbic acid (1mg/ml, Sigma-Aldrich), 1% Insulin-Transferin-Selenium-A (Gibco-BRL), 0.66% Albumin (Sigma-Aldrich), 0.5% FBS (Gibco-BRL), and 96% DMEM-F12 (Gibco-BRL), as well as 1% antibiotic. The cells were maintained in this medium for another 24h before harvesting.

2.7 Reporter gene assay

Cells were collected using a cell scraper and lysed using lysis buffer (100mM Tris, 0.1% Triton-X-100). For the luciferase assay, a Luciferase Assay System kit (Promega, Madison, WI, USA) was used, according to the specification of the manufacturer. The specific luciferase activity was read by luminometer using a dual luciferase program (firefly luciferase gene and renilla luciferase).

2.8 Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA). Complete reporter gene assays were repeated tree times for the whole set of luciferase hybrid constructs (Fig. 8) and the appropriate controls (Fig. 8). For each of the constructs, three plates were transfected and harvested at the same time and under the same conditions. Complete data sets from each of the experiments were statistically analyzed the values was considered statistically significant if $p < 0.05$. For each group of tests standard error (SE), standard deviation (SD) and mean were also analyzed using "INSTAT" program.

CHAPTER 3: RESULTS

3.1 Identification of the mouse FGF-16 promoter region

Two transcription factors databases were searched:

- Transfac[http://www.generegulation.com/pub/databases.html#transfac], and
- Genomatix [www.genomatix.de])

in order to identify within the 6.2 kb DNA of previously cloned murine FGF-16 5' flanking sequence upstream of the ATG start codon of the murine FGF-16 gene those sequences that are important in many genes for forming the transcriptional complex containing RNA Polymerase and the general transcription factors to initiate the process of transcription.

A TATA box sequence was discovered 306 bp upstream of the FGF-16 start codon in the mouse genomic sequence. A CAAT box sequence was also identified 337 bp of the ATG codon, upstream of the TATA box sequence. In addition, a GC box (443 bp upstream of the ATG codon), as well as some potential binding sites for another

cardiac and muscle specific transcription factors were also identified. As this TATA box was the first candidate considered, it was designated as TATA1 (Fig. 4).

In order to determine if TATA1 represents the putative transcription initiation site of mFGF-16, total RNA was purified from adult mouse heart and used for RNA blotting.

One set of primers was designed (Table 2, page 35) for the PCR synthesis of a DNA probe downstream (-277 to -134, reported to the ATG start codon) of the putative TATA1 box (Fig. 4). In order to have confidence in this probe, the PCR fragment was tested using DNA blotting by a slot blot that was hybridized with the plasmid containing the cloned mouse genomic DNA upstream of the mFGF-16 coding sequence.

Once this DNA fragment was verified as being from the FGF-16 gene, the, it was used along with the mFGF-16 cDNA to detect mFGF-16 mRNA by RNA blotting. The -709/-514 (reported to the ATG codon) DNA fragment (also called amplicon B), upstream of the putative TATA1 box gave a clear positive signal and of the expected size (Sontag, 2005) when hybridized to the mouse heart RNA (Fig. 4; Fig 5; Fig.9).

This result indicated that the transcription initiation site is in fact upstream of this TATA1 box (position -306) and that the sequence containing TATA1 is actually part of the coding region for FGF-16 and downstream of the transcription initiation site.

Fig.4: Schematic representation of cloned genomic sequences upstream of the ATG start codon of the murine FGF-16 gene. Sequence analysis using available transcription factor databases revealed the presence of two putative promoter regions identified by the presence of TATA and CCAAT sequences, at positions -306 (TATA1) and -1125 (TATA2) relative to the ATG start codon. B, C, M and N represent DNA regions used in identifying the location of transcription initiation.

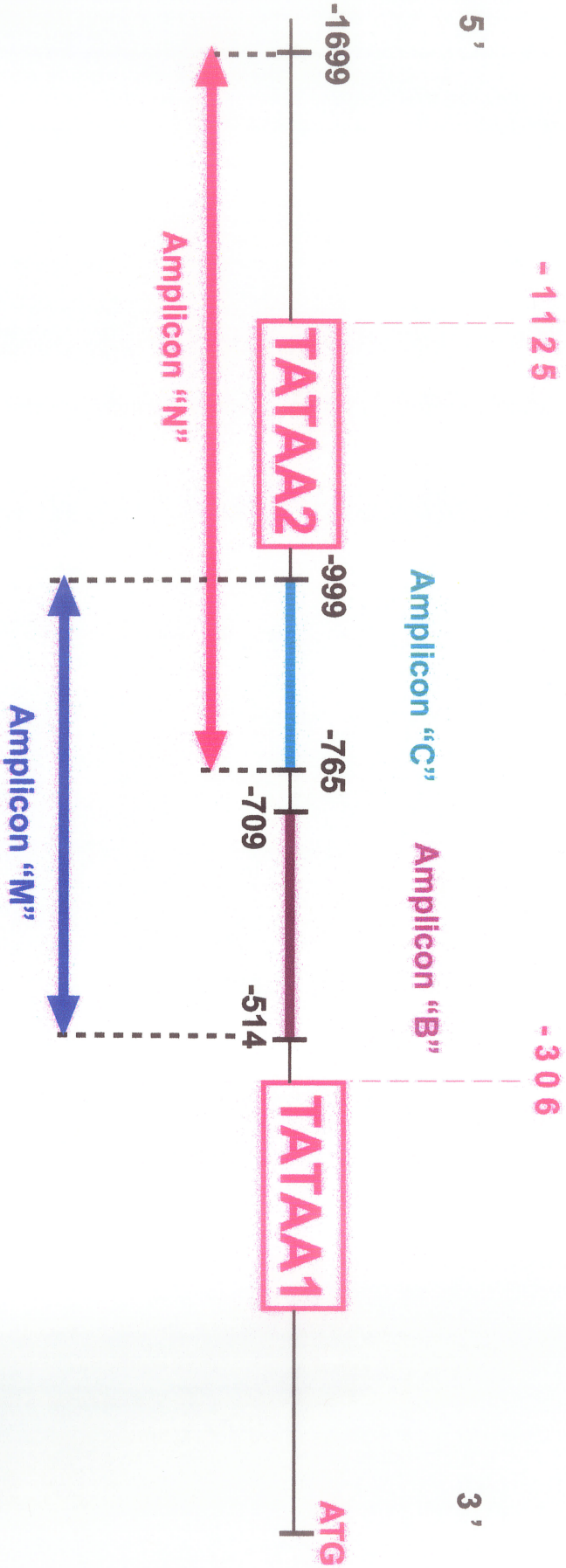
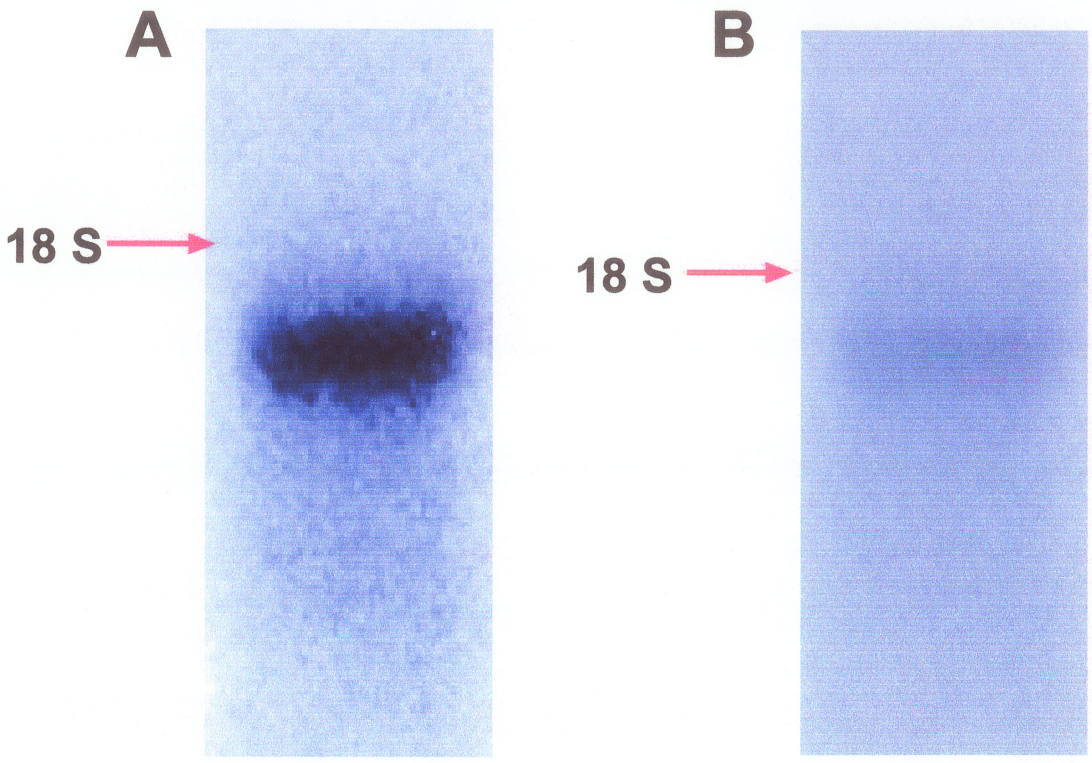

5'
-1699
-1125
TATAA2
Amplicon "N"
-999
Amplicon "C"
-765
-709
Amplicon "M"
Amplicon "B"
-514
-306
TATAA1
ATG
3'

Fig.5: From adult mouse heart, RNA blotting and hybridization was carried out using (A) the FGF-16 cDNA and (B) the "B" region from Figure 3 as radiolabeled probes. Hybridization of heart RNA to the amplicon B probe indicates that this probe represents part of the transcribed region and that that transcription initiation occurs upstream of the TATA box sequence located at position -306 (relative to the ATG start codon). The point of migration for 18S RNA (1869 nt) is indicated as a size reference.


A
18 S
B
18 S

3.2 Localization of the mouse FGF-16 promoter

Taking into account that the reported size of the FGF-16 mRNA is about 1.8 kb lengths (Sontag and Cattini, 2003) the research was extended to a larger upstream fragment of DNA (1.7kb).

A second putative TATA box candidate was discovered 1125 upstream of the FGF-16 start codon (ATG) in the mouse genomic sequence. As this was the second TATA box investigated, it was called TATA2. Once more a CAAT box sequence placed upstream of the TATA2 box (position-1165 reported to ATG start codon) was o identified. Binding sites for other transcription initiation factors were found nearby. Potential binding sites for some cardiac- and muscle-specific transcription factors, namely MEF2 (position -1034, reported to ATG start codon), as well as MyoD (position -1340, reported to ATG start codon) and GATA factor (position -1260, reported to ATG start codon) were also identified (Fig. 7).

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to assess if the TATA2 box represents the putative transcription initiation site of mFGF-16. The decision switch to this method of investigation was based mainly on the fact that it is a more sensitive technique compared to RNA blotting.

Total RNA from adult mouse heart, as well as total RNA from mouse skeletal muscle was isolated. A glyceraldehyde-3 phosphate dehydrogenase (GAPDH) primer set was used in order to check the quality of the isolated RNA. As the product of GAPDH genes are involved in glucose metabolism, the GAPDH gene are present in multiple copies and expressed at high and constant level in most of the tissues and therefore their sequence represent useful controls for (RT) PCR and many blotting analyses.

Primers (Table 2, page 35) for amplifying new fragment downstream (-999 to -765, amplicon "C") of the new putative TATA 2 box were designed (Fig.4). The potential of these primers to generate a PCR product was checked using mouse genomic DNA isolated from liver as a template.

The presence of amplicon "B", as well as the presence of amplicon "C" was verified using RT-PCR method, (they both represent DNA fragments downstream of TATA2 (Fig.4; Fig.6; Fig.9). In order to ensure that these amplicons are those expected, the RT-PCR products were gel purified and digested with diagnostic restriction enzyme. The appropriate digestion products confirmed that these fragments were those expected.

A second RT-PCR experiment was set up, using this time the forward primer on position -1699 (reported to the ATG codon) together with a reverse primer at position -765, reported to the ATG codon (fragment "N", which encompasses TATA2 as in Fig.

4), as well as the forward primer at position -999 (reported to the ATG codon) and the reverse primer at position -514, reported to the ATG codon (fragment "M", Fig. 4). For all primer sets, a control PCR was performed using genomic DNA as a template in order to confirm the expected size of the amplicon. An RT-PCR product was identified only for amplicon "M", but not for amplicon "N" (Fig.6; Fig. 9).

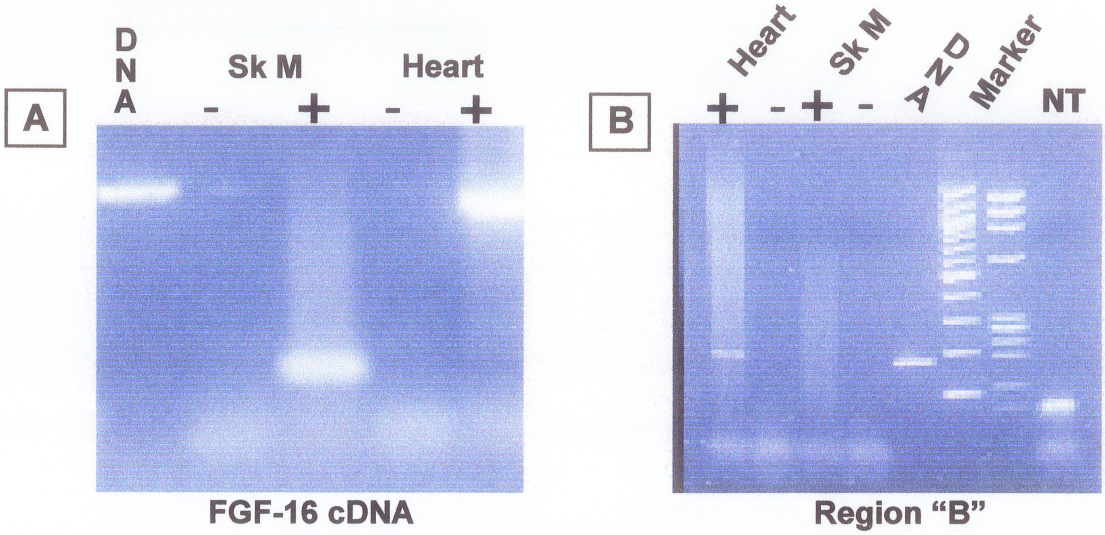
A lower molecular weight band was identified in some cases in non template (NT) or in skeletal muscle (SkM) lanes, probably representing contamination that never appeared in the heart samples. It is also important to note that when skeletal muscle RNA was used as a template for RT-PCR no products of the expected size were detected for any of the primer sets used (Fig.6). These results are consistent with the present literature about FGF-16 high specificity of expression.

Using primer extension analysis, the presence of a transcription initiation site 1073 bp upstream of the ATG start codon was confirmed (data not shown). This was designated as position +1 for the purposes of hybrid construct design (Fig.7; Fig.9)

Fig. 6: Reverse transcriptase polymerase chain reaction (RT-PCR) using RNA from adult mouse heart ("Heart") or skeletal muscle ("SkM") was used to identify the region of FGF-16 transcription initiation. PCR reactions from template material with (+) and without (-) reverse transcriptase enzyme were carried out using primers specific for (A) FGF-16 cDNA, (B) Region "B" (-709/-514, reported to the ATG codon), (C) Region "M" (-999/-514, reported to the ATG codon), and (D) Regions "C" (-999/-765, reported to the ATG codon) and "N" (-1699/-765, reported to the ATG codon), which spans the putative TATA box. In each case, PCR using DNA template ("DNA", plasmid or genomic) was set up as a positive control for amplicon size, and PCR using no template (NT) served as a negative control. The absence of an amplified signal from Region "N" indicates that transcription initiation is occurring downstream of the putative TATA2 box (Fig. 4). Using primer extension analysis, the presence of a transcription initiation site 1073 bp upstream of the ATG start codon was confirmed (data not shown). This was designated as position +1 for the purposes of hybrid construct design.

A lower molecular weight band was identified in some cases in non template (NT) or in skeletal muscle (SkM) lanes, probably representing contamination that never appeared in the heart samples, but, when skeletal muscle RNA was used as a template for RT-PCR, no products of the expected size were detected for any of the primer sets used.

Note that letter designations refer to those given in Fig.4.


A
DNA
Sk M
Heart
- + - +
FGF-16 cDNA
B
Heart
Sk M
ΔZD
Marker
NT
+ - + -
Region "B"
C
Heart
Sk M
ΔZD
NT
Markers
+ - + -
Region "M"
D
Heart
Sk M
ΔZD
Markers
NT
+ - + - Δ + - + - Δ + - + - Δ
FGF-16 cDNA
Region "C"
Region "N"

3.3 Functional assessment of the mouse FGF-16 promoter

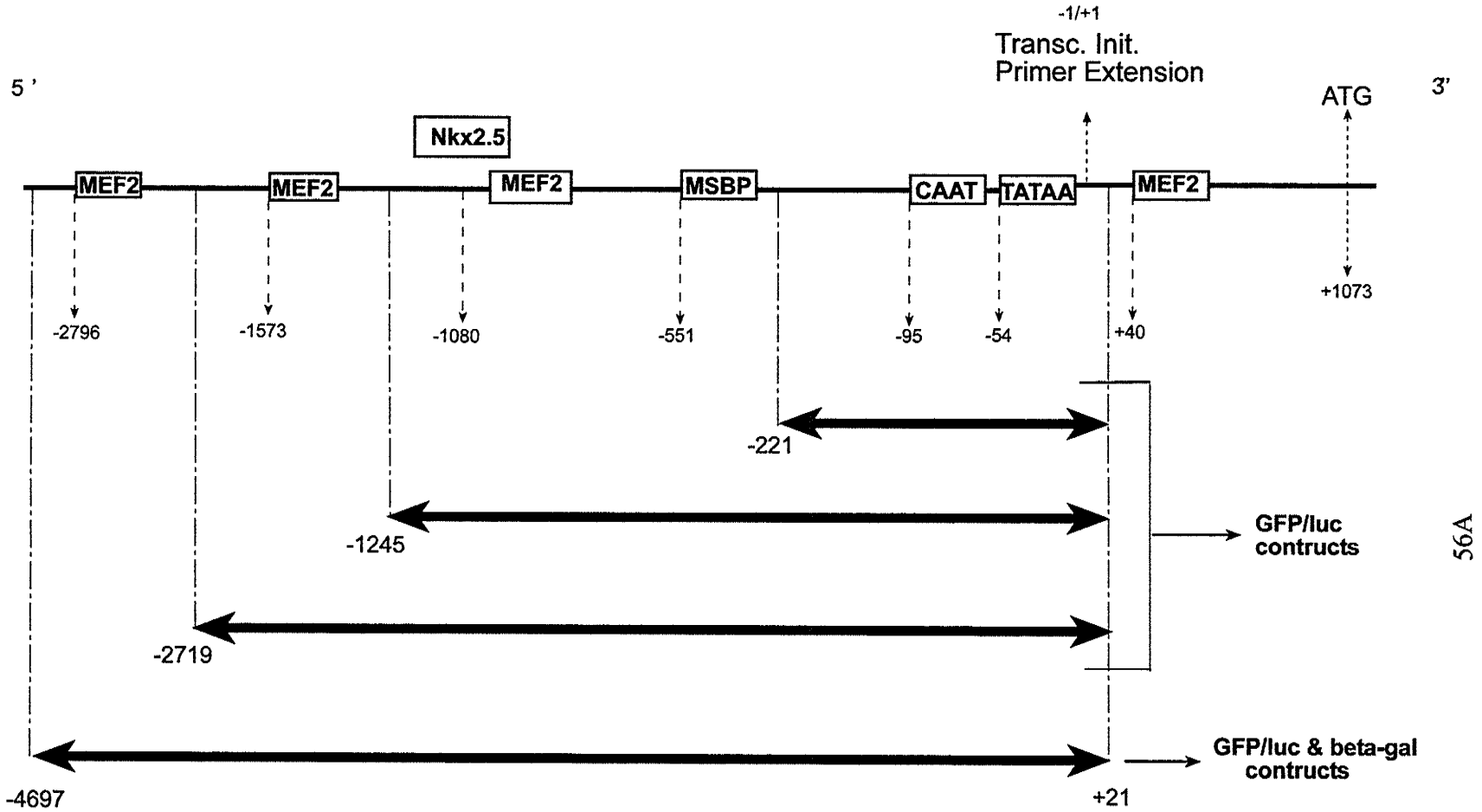
The experiments presented previously were designed to localize the promoter region and the transcription initiation site for FGF-16 expression in mouse heart. Once these were identified, the functionality of the newly identified promoter had to be verified using a cell culture system. As FGF-16 mRNA expression has been already reported in the heart (Miyake *et al*, 1998; Sontag and Cattini 2003, Sontag 2005), neonatal rat myocytes primary culture was considered a good model for testing the activity of this promoter.

In an effort to characterize the possible negative and positive regulatory regions within the mFGF-16 5'-flanking DNA, the newly identified promoter sequence was searched again using two databases:

- Transfac (http://www.generegulation.com/pub/databases.html#transfac) and
- Genomatix (www.genomatix.de)

Based on the position of the cardiac-specific and muscle regulatory elements identified by database search, a series of hybrid luciferase reporter genes were generated with varying lengths of the FGF-16 5'-flanking DNA. (Fig.7)

Fig.7: Hybrid FGF-16 p.GFP/luc plasmid constructs. A series of hybrid constructs was generated based on the relative location of the transcription initiation site and the putative sites of the transcription factors that might be involved in FGF-16 transcription control. Each of the FGF-16 genomic fragments shown was placed upstream of a green fluorescent protein and firefly luciferase (GFP/luc) coding sequence in a promoterless vector (-p.GFP/luc). Each of the constructs was verified using diagnostic restriction enzyme digestion and then used to transiently transfect neonatal rat cardiac myocytes.


-1/+1
Transc. Init.
Primer Extension
5 '
3'
ATG
Nkx2.5
MEF2
MEF2
MEF2
MSBP
CAAT
TATAA
MEF2
-2796
-1573
-1080
-551
-95
-54
+40
+1073
-221
-1245
-2719
GFP/luc
contructs
GFP/luc & beta-gal
contructs
-4697
+21

56A

Each of the FGF-16 inserts was placed upstream of a green fluorescent protein/ firefly luciferase (GFP/Luc) coding sequence in a promoterless vector (-p.GFP/luc). Each of the resulting constructs was checked by digestions with site specific restriction diagnostic enzymes. In addition, the quality of the plasmid, including the absence or low level of open circular plasmid forms, or denaturated supercoils, no contamination with bacterial genomic DNA during isolation, appropriate concentration was also checked before transient transfection since poor quality plasmids can compromise the gene expression. Possible deletions were also verified using specific restriction enzymes analysis.

The plasmids (–4.7, –2.7, –1.2 and –0.2FGF-16p.GFP/Luc; position are reported to the transcription initiation site) were used to transiently transfect neonatal rat cardiac myocytes. Hybrid -4.7, –2.7 and –1.2FGF-16p.GFP/Luc genes demonstrated significant promoter activity in transfected myocytes compared to a promoterless gene (-pGFP/Luc), representing random transcription initiation. In contrast, no significant activity of -0.2FGF-16p.GFP/Luc was observed (Fig.8). The results indicate that the region between positions -1224 and -203 (reported to the transcription initiation site) contains sequences necessary for FGF-16 expression in the cardiac myocytes.

Fig 8: FGF-16 promoter activity in cultured rat neonatal cardiac myocytes.

A. Neonatal rat cardiac myocytes culture at the right density for transient transfection;

B. FGF-16 promoter activity in cultured rat neonatal cardiac myocytes. Cells were transfected with TKp/luc and CMVpGFP/luc for comparison. Basal activity (random transcription initiation) was determined using the promoterless vector (-p.GFP/luc). To normalize for transfection efficiency, cells were cotransfected with a Renilla luciferase expression vector (TKp.RLuc). For each plasmid, the mean and standard deviation are shown (number of plates = 3). Values were compared to –p.GFP/luc and considered statistically significant if $p<0.05$ (*). Note that in the case of CMVpGFP/luc, the actual relative values of luciferase expression are 100X higher than shown. The results indicate that the region between positions -1224 and -203 (reported to the transcription initiation site) contains sequences necessary for FGF-16 expression in the heart.

CHAPTER 4: DISCUSSION

There is no report on the structure and function of the FGF-16 promoter in any tissue and for any species. FGF-16 is expressed in brown adipose tissue during embryonic development but the expression of FGF-16 mRNA after birth occurs specifically in the heart (Miyake *et al*, 1998). This pattern of expression correlates with postnatal cardiac developmental changes and suggests that FGF-16 plays an important role in the growth, development and physiology of the heart and that the transcriptional regulation of FGF-16 gene might play an important role in the expression of this factor in the postnatal heart. Thus, the characterization of the FGF-16 promoter sequence and the identification of the putative transcription initiation site represent important steps in understanding the place of this factor in the general picture regarding heart development and heart functioning.

4.1 Identification of mFGF-16 transcription initiation site

Based on the information achieved by database searching and some preliminary data it was hypothesized that the FGF-16 promoter region is contained within the 6kb of the genomic DNA sequence upstream translation initiation site (ATG codon) that was previously cloned. Within that sequence two potential TATA box sequences were identified: one in position -306 (relative to ATG codon position, called "TATA1") and one in position -1125 ("TATA2") (Fig.4; Fig.9).In the effort to identify the actual transcription initiation site, total RNA from mouse heart was isolated and radiolabeled probe (-709/-514relative to ATG codon, fragment "B", Fig.4; Fig.9). This probe was hybridized with RNA blots. If this TATA1 box sequence is the one used by transcriptinal complex to initiate FGF-16 expression in mouse heart, fragment "B" upstream of TATA1 would not have been detected. However, fragment "B" gave clearly positive signals when hybridized to the mouse heart RNA. This indicated that the transcription initiation site is in fact upstream of this TATA1 box (position -306, reported to ATG codon) and that this sequence is actually part of the transcribed region for FGF-16 and downstream of the transcription initiation site.

Attention was then focused on the second TATA box candidate (TATA2). RT-PCR was performed using heart RNA and appropriate primers for synthesis of fragment "B" (Fig.6), which showed a positive result using RNA blotting (Fig.5), and primers for

PCR synthesis of the DNA fragments that flanked TATA2 candidate. Again, the presence of "B" fragment was detected, this time as an RT-PCR product. These data suggested that this DNA fragment is part of the coding region of FGF-16, placing it downstream of the transcription initiation site. The presence of fragment "C", as well as the presence of fragment "M" and absence of fragment "N", that span the TATA2 box, as RT-PCR products, clearly indicated that the FGF-16 transcription initiation site and promoter region is contained within the -1452/-710 DNA region, reported to the mFGF-16 start codon. (Fig.6; Fig.9)

Fig.9: Schematic representation of the cloned genomic sequences upstream of the ATG start codon of the murine FGF-16 gene. B, C, M and N represent DNA regions used for identification of transcription initiation site. The data achieved by RNA blotting and RT-PCR are indicated accordingly.

The data achieved by RNA blot and RT-PCR were validated by primer extension analysis of total RNA from heart and this revealed a single transcription initiation site located 1073 bp upstream of the start codon. (Fig.7; Fig.9) The identification of the FGF-16 transcription initiation site in this region is consistent with the size of the identified FGF-16 transcript (1.8 kb) in mouse heart (Sontag, 2003). Interestingly, by calculation, this also suggests that the 3'untranslated region and polyA tail of the mRNA might be small in size: the average poly A tail is 250 bp (Dreyfus and Regnier, 2002) and there is about 1 kb of untranslated sequence in the heart FGF-16 mRNA. The coding region is 624 bp (Sontag and Cattini, 2003), leaving less than 150 bp for 3' untranslated sequence.

The 5' untranslated region of FGF-16 mRNA might play a role in FGF-16 expression since some putative cardiac-specific factor binding sites were also identified within this sequence (Fig.7). The expression of FGFs is regulated at multiple levels; positive and negative regulatory sequences that control FGF genes expression were identified in the promoter region of FGFs genes (Djenabi S *et al*, 1999; Murakami A *et al*, 2002), as well as in the domains located in the untranslated regions (.FGF2 and FGF4) (Bryans M *et al*, 1995; Shibata F *et al*, 1991).

Further analysis (including transfection experiments using hybrid constructs containing 5' untranslated sequences) is required in order to identify those putative positive and/or negative regulatory elements within this untranslated DNA that might

play roles in the regulation of FGF-16 expression in the heart.

The identified translation initiation site of FGF-16 in the heart was designated as corresponding to position +1, located 1043 upstream of the ATG codon. Unlike many FGFs, such as rFGF-2 hFHF-2 (Pasumarthi *et al*, 1997), mFGF-1 (Madiai *et al*, 1999, 2002) that are expressed in a multitude of tissues and whose promoters region lacks all conventional elements, such as TATA box, CAAT box, but contain GC rich regions, mFGF-16 promoter region contains within all the conventional elements of a basal promoter: a TATA box at position -54 and a CAAT box at position -95 (the specified positions are reported to the transcription initiation site) (Fig. 7).

A recent genome-wide analysis of promoters, in the context of gene expression pattern revealed that genes that have within their promoter a TATA box and no CpG islands are usually expressed in a tissue-specific manner, while those genes whose promoter lacks the presence of TATA box within their promoter are usually common genes expressed in most if not all of the tissues (Schung *et al*, 2005). Sp1 seems also to be an indicator for less-specific expression (Schung *et al*, 2005). In this perspective and further considering the pattern of expression of FGF-16, it should be expected that FGF-16 gene belongs to a relatively highly tissue-specific category of proteins. However, further data are necessary to confirm these speculations.

Finally, the existence of mRNA isoforms as result of alternative transcription initiation site usage is frequently associated with the lack of a TATA box consensus

sequence within the promoter of a gene. From this point of view, the identification of the TATA box as part of the mFGF-16 promoter is consistent with the fact that FGF-16 transcript in mouse heart is unique and no isoforms were identified. However, the presence of TATA1 box closed to the FGF-16 start codon still rises questions regarding its possible function as transcriptional initiation site used by transcription complex for FGF-16 expression in other tissues than heart or in other developmental stage than that which was investigated (i.e. postnatal and adult). Since an RT-PCR product of the expected size was detected only in heart and not in skeletal muscle RNA, there are indications to support tissue specific expression of FGF-16 from this promoter.

4.2 Confirming the functionality of the newly identified mFGF-16 promoter region

To confirm the identity of a functional FGF-16 promoter in cardiac cells, a series of hybrid luciferase reporter genes were generated with varying lengths of FGF-16 upstream sequences (–4.7, –2.7, –1.2 and –0.2FGF-16p.GFP/Luc). The different hybrid constructs were designed based on the position of cardiac-specific and muscle regulatory elements identified by database search within the newly identified promoter region (Fig.7) As FGF-16 mRNA expression has been already reported in the heart (Miyake *et al.*, 1998; Sontag and Cattini, 2003, Sontag 2005, Lavine 2005), neonatal rat myocytes primary culture is a good model for testing the activity of this promoter. The choice of rat and not mouse cells was determined by the simple availability of the culture system.

Reporter gene analysis revealed that -4.7, –2.7 and –1.2FGF-16p.GFP/Luc genes are expressed significantly above the promoterless gene (-pGFP/Luc) level in transfected cardiac myocytes; no activity of -0.2FGF-16p.GFP/Luc was observed. These data substantiated on one hand the fact that the subcloned region containing the newly identified promoter sequence has indeed a promoter activity above the promoterless gene, but also it reveals that this activity is a highly regulated one in neonatal cardiac myocytes. (Fig.8)

These data also suggest that the subcloned genomic sequences of approximately 4.7 kb upstream of the transcription initiation site (+1) may contain enough information in order to trigger the expression of the reporter gene, and by extrapolation the expression of FGF-16 *in vitro* and *in vivo*. Furthermore, the functional analysis supports the data achieved by RNA techniques (blotting, RT-PCR and primer extension) that mFGF-16 promoter is located 1073 bp upstream of coding sequences and that this promoter is functional in primary neonatal cardiac myocytes culture. The reporter gene assay also suggested that the sequences located between -1.2 and -0.2 kb upstream of this site are required for the cardiac expression of FGF-16. The pattern of expression in the functional analysis of sequential 5'-deletions of FGF-16 promoter sequence within the hybrid DNA constructs also suggests that the minimal promoter regulatory region required for basal promoter activity in cardiac myocytes is located upstream of position -203 (reported to the transcription initiation site)since the –0.2FGF-16p.GFP/Luc hybrid construct does not drive a luciferase expression above that one of the promoterless gene (-p.GFP/luc). This is consistent with the fact that within the DNA sequence located between position -221 and -1245 (reported to the transcription initiation site) a series of specific binding sites for cardiac-and muscle-related regulatory elements that might be able to facilitate FGF-16 expression in cardiac myocytes have been identified by databases search (Fig.7). However, no significant differences between the transcriptional activities of different hybrid constructs including varying lengths of the identified FGF-16 promoter sequences were observed.

Therefore, further analysis and different approaches are required in order to identify the importance of those putative positive and/or negative regulatory elements within the DNA sequence of mFGF-16 promoter region that are responsible for the regulation of FGF-16 expression in the heart, and well as to the trans-acting factors that bind to those sequences and are necessary for the transcription of FGF-16 in the heart.

4.3 Conclusions and future directions

Currently there is no report on the structure and function of the FGF-16 promoter in any tissue and for any species. Nevertheless, using techniques that use RNA, the transcription initiation site used for FGF-16 transcription in mouse heart was identified 1073 bp upstream of the ATG codon. This position was subsequently designated +1. The data obtained by transfecting hybrid luciferase reporter genes with varying lengths of the identified FGF-16 promoter sequences into the neonatal rat cardiac myocytes suggest that the subcloned genomic sequence of approximately 4.7 kb upstream of the transcription initiation site (+1) may contain enough information in order to trigger the expression of the reporter gene, and by extrapolation the expression of FGF-16 *in vitro*. The reporter gene assay also suggested that the sequences located between -1.2 and -0.2 kb upstream of the transcription initiation site seem to be required to target cardiac expression of FGF-16. In conclusion, the promoter region of mFGF-16 in cardiac tissue was successfully identified and all the achieved data supports our initial hypothesis. This is actually the first description of FGF-16 promoter sequence for any species.

FGF-16 is very intriguing, both because its pattern of expression and because very little is known about its regulation or function. An important step that should be done in this direction is to demonstrate the functionality of the newly identified mFGF-16 promoter by *in vivo* studies. The use of a transgenic mouse model will allow the

exploration of FGF-16 expression in all developmental stages, beyond that which is possible with cell line and primary culture. The hypothesis to be addressed is that the 6kb sequence upstream of the FGF-16 translation start site (including about 1kb of the 5' untranslated region) will be sufficient to mirror endogenous gene expression in vivo. In addition, the study of endogenous FGF-16 expression can be expanded, through the use of *in situ* hybridization or related techniques to determine exactly what cell types within an organ (i.e. the heart) and at what developmental stage are producing FGF-16.

Further analysis and different approaches are also required in order to identify those putative positive and/or negative regulatory elements within the DNA sequence of mFGF-16 promoter region that are responsible for the regulation of FGF-16 expression in the heart, and well as to the trans-acting factors that bind to those sequences and are necessary for the transcription of FGF-16 in the heart. Electrophoresis mobility shift assay (EMSA) and DNAase I protection assay (footprinting) can be used for further characterization of these sequences. Chromatin immunoprecipitation (ChIP) and DNAase I hypersensitivity (HS) can also be used to complement the *in vitro* studies (EMSA and footprinting) in terms of isolating putative regulatory regions *in situ* and correlating them with development- and tissue- specific chromatin-related events such as hyperacetylation. Longer term studies may examine the correlation between the temporal and spatial expression of the specific proteins that will be discovered to be involved in FGF-16 regulation and the pattern of FGF-16 expression.

FGF-16 also raises numerous questions related to its spatial pattern of expression. In this direction, studies about tissue-specificity can answer many questions. This can be assessed by both *in vitro* studies, such as reporter gene assay using non-myocytes cell lines, as well as using *in vivo* studies, such as those already discussed for the transgenic model.

Finally, but no less important, similar studies should be extend to another species, especially human, taking into account the clinical implication that might rise from the possibility of using in medical practice the physiological potential of this factor. In addition, the proteins and sequences involved in regulating FGF-16 gene expression might be important for increasing our understanding about the spatial and temporal control of gene expression in neonatal heart. From here rise the necessity of establish the function of FGF-16 in heart during development as well as postnatal and in adult.

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