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GATA-4 Mediated Upregulation of Kv 4.2 Gene Expression in Mouse Heart

by

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Abstract

Kv 4.2 gene encodes a voltage-dependent K^+ channel that contributes to Ito. We evaluated Kv 4.2 gene expression in mouse heart by using RNase protection assay. To investigate the mechanisms that control transcription and expression of Kv 4.2 gene in cultured myocytes, we have cloned 3 KB of the 5'-flanking region of Kv 4.2 gene from genomic DNA of mouse. The transcription start site was mapped using Primer Extension and RNase protection. Two major transcription start sites are located at 288/297 base pairs upstream of the translation start sites. The 5'upstream region contains 5 GATA-4 binding sites. Since GATA-4 contributes to the regulatory cascade leading to cardiogenesis, we sought to address its contribution to regulation of Kv 4.2. Three deleted 5'-upstream regions of the Kv 4.2 gene were incorporated into the reporter Luciferase basic constructs. Reporter constructs were transfected into fetal, day 1 and day3 cardiac myocytes with the Renilla plasmid acting as control. Luciferase activity is quite weak but detectable in reporter constructs. Co-transfecting myocytes with GATA-4 produced a significant increase in the luciferase activity. In addition we co-transfected GATA-4 and reporter constructs into NIH3T3, HEK and H9C2 cells. Of these, only the H9C2 cells have a cardiac myocyte lineage. Co-expression with GATA increased luciferase activity only in the H9C2 cells. In conclusion GATA-4 regulates expression of Kv 4.2 in mouse cardiac myocytes and cells of a cardiac lineage.

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Selected Abbreviations and Acronyms

APD	**action potential duration**
4-Ap	**4-aminopyridine**
DPE	**downstream promoter element**
GTFs	**general transcription factors**
HATs	**histone acetyltransferase**
HDACs	**histone deacetylatransferase**
Ik1	**inward rectifier K^+ current**
I_{to}	**transient outward K^+ current**
PCR	**polymerase chain reaction**
PIC	**pre-initiation complex**
RPA	**RNase protection assay**
RNA P II	**RNA polymerase II**
R-T PCR	**RNA was reverse transcribed into cDNA as PCR template**
TAFs	**TATA-binding protein associated factors**
TBP	**TATA-binding protein**
TK	**thymidine kinase**

Chapter I

INTRODUCTION

I-1. PHYSIOLOGIC AND MOLECULAR PROPERTIES OF CARDIAC VOLTAGE-GATED POTASSIUM CHANNELS

1. Ion channels are important in generating the cardiac action potential

The heart is a complex organ, where muscle activity is controlled by a specialized conduction system. The repetitive contraction of the heart requires a set of proteins involved in ion transport, contraction, and metabolism. Voltage-gated ion channels are responsible for generation of electrical signals in neurons, heart, and other excitable cells[1]. The cardiac action potential is generated by the composite activity of many ion channels, pumps, and transporters (Figure 1). In most cardiac myocytes, the depolarization phase (phase 0) is mediated by a rapid influx of Na^+ through voltage-gated Na^+ channels [2]. A subsequent rapid, but incomplete, repolarization is achieved in phase I via K^+ efflux through transiently activated K^+ channels. This early outward current sets the initial plateau potential, thus influencing the behavior of subsequently activated ion channels and the duration of the actional potential. The plateau of the action potential (phase II) is characterized by minimal net ion flow due to the balance of an inactivating Ca^{2+} inward current and K^+ efflux through slowly activating voltage-gated channels. In phase III, the K^+ permeability increases with time and ultimately results in complete repolarization. Phase IV represents the time period between action potentials, and it is here that the inwardly rectifying K^+ current plays an important role in setting the resting membrane potential and thus controlling myocardial cell excitability [3].

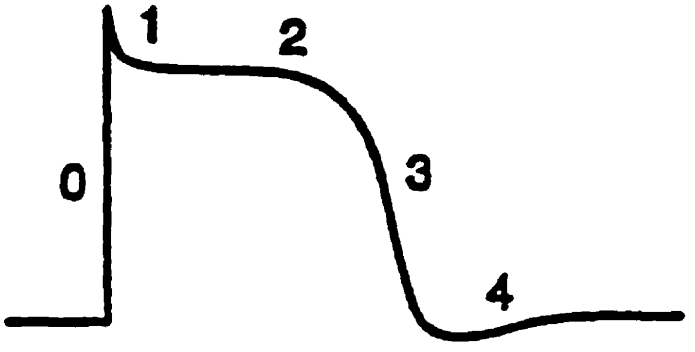


Figure 1. The phases of ventricular action potential: phase 0, rapid depolarization; phase 1, early repolarization; phase 2, plateau; phase 3, late repolarization; phase 4, return to the resting membrane potential. Several voltage-gated K^+ channels have been identified in heart. Of them, Ito and Ik play an important role in action potential repolarization. Ito is responsible for phase 1 repolarization.

2. The physiological properties of transient outward K^+ channels and their role in ventricular repolarization

Cardiac voltage-gated K^+ channels function to control the duration of the cardiac action potentials. K^+ channels in heart are more diverse than other types of ion channels. It is now clear that individual myocardial cells can express multiple types of K^+ channels. Barry and Nerbonne [4] have reported that difference in the types and /or densities of K^+ channels expressed contribute to determining the variability in action potential waveforms recorded in different regions of the heart. Moreover, it is well demonstrated that changes in the densities and/or the properties of K^+ currents are seen in a variety cardiovascular disorders.

In the mammalian myocardium, various types of depolarization-activated outward K^+ currents have been distinguished on the basis of differences in time and voltage-dependent properties and pharmacological sensitivity [5]. Depolarization-activated K^+ channels are particularly prominent in repolarization of cardiac myocytes. They can be classified into two major groups based on physiologic properties: 1). Rapidly activating and inactivating transient outward currents are referred to as Ito; 2). Delayed, slowly activating and slowly inactivating outward currents are referred to as Ik.

The present studies focus on the transient outward channel (Ito). In many mammalian species, including humans, the transient outward currents have been described in heart [5-7]. These currents are characterized by their rapid activation, inactivation, and their sensitivity to 4-amino-pyridine (4-AP) in the millimolar concentration. Transient outward (Ito) K^+ channels open (activate) within a few milliseconds after membrane depolarization but then rapidly close (inactivate). The Ito

channels play an important role in the early phase of repolarization in cardiac action potential.

3. Molecular characterization of voltage-gated K^+ channel α subunits

Channels are integral membrane proteins, which allow inorganic ions such as K^+, Na^+, Ca^{2+} and Cl^- ions to cross membranes. The voltage-dependent K^+ (Kv) channels consist of α and β subunits. α subunits are sufficient to form functional K^+ channels. The first voltage-gated K^+ channel α subunit cloned was from the ***Shaker*** locus in ***Drosophila***[8] Examination of deduced amino acid sequence revealed ***Shaker*** to be an integral membrane protein of approximately 70 kDa[9]. Later identified were three other subfamilies of voltage-gated K^+ channel subunit genes known as ***Shab***, ***Shaw***, and ***Shal*** genes. Together these genes make up the Kv channel family [10,11,12,13]. During the past decade, potassium channel protein structure and function have been investigated in detail [14]. Many studies using biological mutagenesis and patch-clamp recording have provided us with a detailed topology for K^+ channels [15, 16, 17]. They are thought to have six membrane spanning segments, termed S1 through S6, with both the amino- and carboxyl-termini located intracellularly (Figure 2). The region between the S5 and S6 segments (the pore-region) is thought to participate in forming the ion conduction pathway; while the S4 segment forms a major part of the voltage-sensor. Sequence analysis further revealed a high degree of homology between an arginine-rich region in the fourth membrane-spanning domain of ***Shaker*** and a corresponding region in voltage-gated Na^+ and Ca^{2+} channels. The ***Shaker*** protein is much smaller than the voltage-gated Na^+ and Ca^{2+} channel proteins (***Shaker*** protein is 70 kDa compared with Ca^{2+} and Na^+ channel proteins 180-240 kDa). It appears similar to one of the four homologous channels

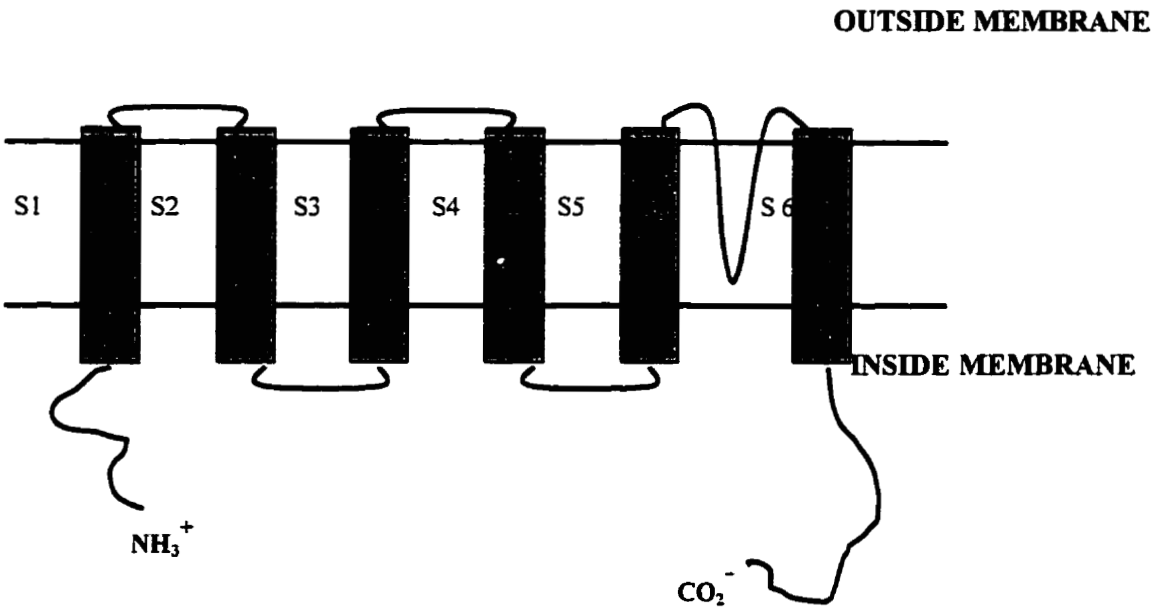


Figure 2. Simplified illustration of the proposed membrane topology of K^+ channel α subunits. Kv family members have six transmembrane domains and a hydrophobic pore (P or H5) region between S5 and S6; both the N- and C-termini are intracellular. The arginine-rich fourth membrane-spanning domain (S4) contributes to voltage-gated channel gating. Four such subunits are required to form a functional K^+ channel.

protein. Hence, K^+ channel is thought to consist of tetramers with each subunit contributing to the pore wall.

I-2. THE VOLTAGE-GATED POTASSIUM CHANNEL GENES

I-2-1. The extended Kv channel gene family

The cloning of the ***Shaker*** gene of ***Drosophlia melanogaster***, a structural gene for a voltage-dependent potassium channel, has allowed the identification of a large number of genes in mammals that encode K^+ channel proteins. Using hybridization protocols as well as polymerase chain reactions, the clones from vertebrate cDNA as well as genomic libraries of ***Shaker***-related K^+ channels have been extensively investigated. These studies were predominantly carried out with rat cDNA libraries and have recently been extended to clone and characterize human voltage-dependent K^+ channels. Using ***Shaker*** probes, Tempel [18] and Baumann and colleagues [19] isolated the first mammalian homologues of the ***Shaker*** gene. In the next decade, eighteen genes divided into four families (Kv1-Kv4) were identified to encode voltage-gated K^+ channels in mammal [20]. Most of these genes segregate into four clearly defined gene subfamilies, each of which (Kv1.1-1.7, Kv2.1-2.2, Kv3.1-3.4, and Kv4.1-4.3) is structurally and evolutionarily related to one of the four fly genes (***Shaker, Shab, Shaw and Shal***). Two additional mammalian genes have been isolated (IK8/Kv5.1 and K13/Kv6.1) for which fly homologues have not yet been identified [21].

In the Kv 4 (***Shal***-related) subfamily, the full-length ***shal***-related K^+ channel cDNAs have been reported [22]: Kv 4.1 in mouse, Kv 4.2 and Kv 4.3 in rat. The sequence of ***Shal*** proteins has been highly conserved throughout evolution. Mammalian Kv 4

proteins show >80% identity in their membrane-spanning core sequence to their fly homologue. The N-terminal domain of Kv 4 proteins is also highly conserved (63-73% identity) and the C-terminal tail contains 5-7 conserved regions interrupted with areas that are less similar.

I-2-2. Cardiac Kv channel genes and functions

Cloning of cardiac K^+ channel has been reported in different species. Kv1.2 and Kv1.4 were isolated from rat heart [23, 24] and rat brain [25, 26]. The ferret and human cardiac homologues of Kv1.4 are 98% identical to the rat protein [27, 28]. Rat Kv1.5, Kv2.1 and Kv 4.2 also were cloned from both heart and brain [29, 30, 31] The human Kv1.5 (hKv1.5) channel was cloned from ventricle, atrium and from an insulinoma cell line [32] and is only 86% identical to the rat channel. Most of nonidentity occurs in the NH2- and COOH- terminal regions, which have 64 and 75% identity, respectively. Canine and mouse Kv1.5 have been cloned from colonic smooth muscle and heart [33, 34]. These two proteins show 85% overall identity with the human homologue.

The relationship between cloned gene subunits and functional myocardial K^+ channels has been explored in heterologous expression system (the use of oocytes from South African clawed toads) [35]. These oocytes are large enough to be injected with exogenous mRNA and are capable of synthesizing the resulting foreign protein. Using this approach, it has been determined for a number of cardiac K^+ channel α subunits that contribute to the formation of various types of K^+ currents/channels. The time and voltage-dependent properties and the pharmacological sensitivities have been characterized in these K^+ channels as well. This approach has met with success, and some clear insights have been provided.

In order to define the subunits underlying cardiac Ito, Kv1.4 and Kv 4.2 had been tested in heterologous expression systems. The cardiac clone found to encode an Ito like current was Kv1.4 and Kv 4.1, 4.2 or 4.3 in rat [23,31] and human [36]. Heterologous expression of either Kv 1.4 or Kv 4.2 reveals rapidly activating and inactivating K^+ currents similar to Ito. However, recovery from steady-state inactivation of heterologously expressed Kv 1.4 or Kv 4.2 is much slower than that of Ito. Co-expression of either Kv 1.2 or Kv 1.5 with Kv 1.4 increases the rate of recovery, which suggests that functional Ito channels might be hetero-multimeric, composed of Kv1.4 with Kv 1.2 and / or Kv1.5 [37]. Subsequent studies by Dixon and McKinnon[38] revealed that Kv 4.2 message levels vary through the thickness of the ventricular wall in a manner similar to that seen for Ito, which suggests that Kv 4.2 more likely underlies Ito than Kv 1.5.

I-2-3. Study of *Shal*-related channel gene expression

The differences in the action potential waveforms of atrial and ventricular myocardium are a classic example of how a variety of K^+ currents subserve these functions in myocardial cells. Part of the variance in the action potential waveforms of atrial and ventricular myocytes is due to differences in the expression of voltage-gated K^+ channel genes [39, 40]. Some investigators have performed quantitation of *Shal* related subfamily in different species, as well as in cells isolated from different regions of the heart in the same species [38,41]. Dr McKinnon has reported that Kv 4.2 mRNA was expressed in rat at significantly higher levels in ventricle than in atria and was expressed in a steep gradient across the left ventricular wall, with 8.4 times higher in epicardium than in papillary muscle. There are species-specific differences in voltage-dependent K^+

channel expression. For example, significant levels of mRNA encoding the rapidly in activating K^+ channels Kv 1.4, Kv 4.2 and Kv 4.3 have been observed in rat ventricle [41], whereas in canine and human ventricle, there is no detectable Kv 4.2, but mRNA encoding Kv 1.4, Kv 1.5, Kv 3.4 and Kv 4.3 is present [42]. Based on the data of mRNA quantitation in *Shal* related subfamily, Kv 4.3 has emerged as the leading candidate K^+ channel gene encoding the cardiac Ito in large mammals such as canine and humans. However, Kv 4.2 has been known as the dominant K^+ channel gene encoding the cardiac Ito in small mammals such as rat and mice.

I-3. REGULATION OF GENE EXPRESSION

The primary level of gene control is the regulation of gene transcription-- the step from DNA to RNA. Eukaryotes have three distinct RNA polymerases that catalyze transcription of nuclear genes [43]. Ribosomal RNA is transcribed by RNA polymerase I; messenger RNA is transcribed by RNA polymerase II, and tRNA (and other small RNAs) by RNA polymerase III. In eukaryotic cells, the protein-encoding genes are transcribed into messenger RNA (mRNA) by a specific RNA polymerase II and transcriptional initiation is governed by DNA sequence elements.

I-3-1. The basal transcription apparatus consists of RNA polymerase II and general factors:

The initiation stage of messenger RNA synthesis is a major part for regulation of gene expression. In eukaryotes, initiation is governed by DNA sequence elements comprising several functional classes. These include 1) The core promoter element,

which contains the binding site for RNA polymerase II (RNAPII) and controls the location of the site of transcription initiation; 2) The upstream promoter elements and enhancers, which regulate the rate at which RNA PII initiates new rounds of transcription from the core promoter. Polymerase II cannot recognize its target promoter directly, instead, it relies on general transcription factors and transcriptional activators[44].

a). **Basic properties of core promoter elements**: The 5' region immediately upstream of the transcription start site is referred to as the core promoter. The key DNA element that is essential for transcription by RNA PII is the core promoter sequence. which generally encompasses the transcription start site (within about -30 relative to the +1 start site) and is sufficient to direct the accurate initiation of transcription. The most common of these elements, which can function independently or synergistically, are the TATA box (a wide range of A/T rich sequence) located near position –30 to –25, and a pyrimidine rich initiator located near the transcription start site [45].

The fundamental importance for RNA P II localizing the core promoter sequence are TATA elements. This finding is consistent with the fact that TATA-binding protein recognizes the minor groove of DNA, where protein- DNA interactions are typically influenced by A/T-content, but not by specific nucleotide sequence [46]. Breathnach and Chambon[47] have reviewed evidence describing protein-coding gene transcription related to TATA elements. They found that TATA functional required the sequence TATAAA. This consensus sequence was subsequently confirmed by functional studies of TATA mutants in the past decade [48]. For example, experiments with mutant and wild type recombinant DNA constructs show that a single-base change (e.g. G or A substituted for the second T) significantly decreases in vitro transcription of TATA-containing

promoters. More recently, however, it has become clear that numerous A/T-rich sequences can impart considerable TATA activity [49,50].

The term 'initiator' was first used in relation to RNA II transcription by Grosschedl and Brinstiel in 1980, to describe a 60 base pair region spanning the transcription start site of the TATA-containing sea urchin histone H2A gene [51]. Mutant analysis performed in the early 1980's with other TATA-contain promoters revealed highly variable phenotypes in initiator elements [52, 53]. In brief, the sequence requirements for initiator activity were poorly conserved, but a central "C" and "A" nucleotides usually were present and often surrounded by several pyrimidines, and a strong preference for a T at the +3 position was noted.

It has become increasingly clear that many promoters do not contain consensus TATA boxes, which is referred to as "TATA-less promoter". These types of promoters are typically associated with genes that are constitutively expressed in the cell. Most of these genes contain a GC-rich sequence (about 20 to 50 nucleotides) that lie within the first 200 base pair upstream of start site. A DNA binding factor called SP1 recognizes GC rich sequence, and genes lacking the TATA box may rely on these GC rich sites and the SP1 proteins bound to them to start transcription. Recent studies have proposed that SP1 is required to combine the basal transcription machinery to TATA-less promoters via interaction with the TFIID complex [54]. The study of several TATA-less *Drosophila* promoters provided the first evidence for functional critical downstream promoter element (DPE) [55]. These downstream promoter elements were suggested to have a consensus sequence A/G G A/T C G T G. Deletion of sequences 20-40 bp downstream of the transcription start site completely abolished these promoters activity. These studies

indicated that in the core promoter region, the TATA element is of fundamental importance for gene transcription. However, there are other pathways responsible for TATA-less promoter gene transcription.

b). Basic properties of general transcriptional factors: Since RNA P II cannot recognize its target promoter directly, additional factors are required for accurate transcription from even the strongest core promoters. This requirement was first demonstrated with purified RNA PII and a crude fraction from human cells in 1979 [56], and subsequent fraction studies led to the first demonstration of multiple RNA PII specific factors in1980 [57]. At present, there are six well-characterized general transcription factors (GTFs) that have been isolated from human, rat, *Drosophila* and yeast [58]. The cDNAs encoding almost all of the component polypeptides have been isolated. The designations, polypeptide compositions and general functions of these factors, along with those of RNA PII, are summarized in **Table-1** [58].

Of these general transcription factors (GTFs), TFIID plays a central role in RNA PII transcriptional regulation. TFIID is a multi-protein complex that is composed of TATA-binding protein (TBP) and a set of TATA-binding protein associated factors (TAFs). The general functions of TFIID are core promoter recognition and transcriptional activation. TFIID appears to be the only GTF that has sequence-specific DNA binding activity. In the most general case, mRNA production begins with TFIID recognizing and binding tightly to the core promoter TATA element [59]. In addition, TFIID has been reported as a target for many activators. This critical role is played by TAFs [60]. At least eight TAFs subunits have been characterized [61]. These TAFs factors are essential for stimulation of transcription by activators. Numerous interactions have been observed in

vitro between activators and TAFs[62]. Based on these findings, it was proposed that activators act in part by targeting TFII D subunits for recruitment to promoters and TAFs may make protein-protein interactions with other general transcription factors that would enhance the stability or activity of the transcription. TFIIA is also thought to play a role to stabilize binding of the TBP and TAFs to the core promoter. When the TFIID- DNA complex is formed, it can be recognized by single TFIIB [63], which interacts with both TBP and RNAPII to act as a molecular bridge. TFIIB also interacts with TFIIF, the factor that delivers RNAPII to the initiation complex. TFIIE factor directly interacts with RNAPII, and TFIIF [64]. TFIIH is thought to be involved in separation of DNA strands around the transcription start site and a protein kinase responsible for extensive phosphorylation of the carboxyl-terminal domain of RNAPII at every round of transcription initiation [65]. In the presence of nucleotide triphosphates (ATP), strand separation at the transcription start site occurs to give an open complex, the C-terminal domain of the large subunit of RNAP II is phosphorylated, and RNAP II initiates transcription and is released from the promoter.

A pre-initiation complex (PIC) assembly model is proposed to illustrate the formation of transcription [66]. This model indicates that transcription regulatory protein present as an intact structure termed RNA polymerase holoenzyme. This holoenzyme consist of RNA P II, GTFs and other intermediate proteins (activator /suppressor). In this model, a set of proteins comprised of RNA PII and the general transcription factors (GTFs) is termed the basal apparatus. This basal apparatus can recognize the core promoter and initiate transcription. The general initiation factors TFIIB, -D, -E, -F, and –H must co-assemble on the core promoter with RNAP II before transcription begins as

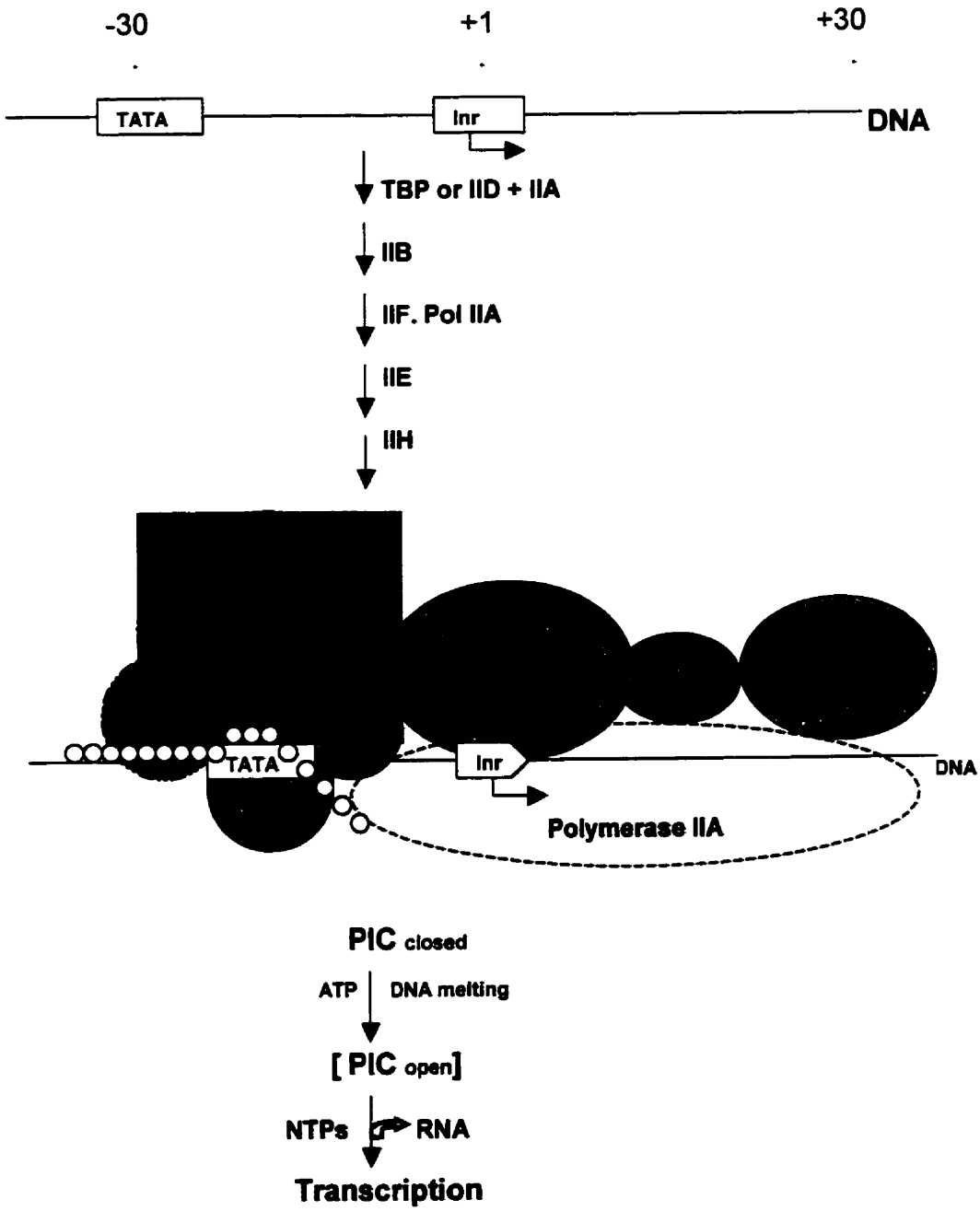
Table 1. General transcription initiation factors *

Factor		Subunits	kDa	Function
TFIID	TBP	1	38	Recognizes core promoter (TATA) and promoters TFIIB binding.
	TAFs	12	15-250	Regulatory functions can be positive or/and negative for TFII D.
TFIIA		3	12,19,35	Stabilization of TBP binding; stabilization of TAF-DNA binding.
TFIIB		1	35	Promote TFIIF-pol II binding
TFIIF		2	30, 74	Regulatory function is to target pol II to promoter.
TFIIE		2	34, 57	Stimulate TFIIH kinase and ATPase activities
TFIIH		9	35-89	Stimulate Helicase, ATPase, CTD kinase activities.
RNA Pol II		12	10-220	Catalytic function in RNA synthesis.

* The subunit compositions and polypeptides sizes are described for the human factors. The homologues for all the factors have also been identified in rat, *Drosophila* and yeast.

Figure 3. Model for pre-initiation complex (PIC) assembly and function on a TATA-containing core promoter. In this model, the transcription start-site is determinated by the TATA-binding protein (TBP) and TFIID. The PIC assembly starts with TFIID and followed by a series of B, F, E, H into PIC. The core promoter constitutes the DNA target for RNAPII, general factors enter the PIC in association with RNAPII in the form of a holoenzyme complex. The stable (closed) form of the PIC is activated in an ATP-dependent manner to form an unstable (open) complex. In the presence of NTPs, RNA PII begins to initiate transcription.

Figure 3.


-30
+1
+30
TATA
Inr
DNA
TBP or IID + IIA
IIB
IIF. Pol IIA
IIE
IIH
TATA
Inr
DNA
Polymerase IIA
PIC closed
ATP
DNA melting
[PIC open]
NTPs
RNA
Transcription

the PIC assembly, once the PIC is formed, it will start to activate the transcription. In fact, every core promoter employs the same basal apparatus and all the subunits are conserved from yeast to human [67] (Figure 3).

I-3-2. Enhancers contain bidirectional elements that assist initiation:

In addition to promoters, other DNA elements regulate the transcription as well. A number of DNA regulatory elements referred to as enhancers are located 5' or 3' of the promoter. These regulatory elements are highly varied. Molecular genetic approaches have been used to identify and characterize DNA sequences required for eukaryotic gene regulation. These sequences consist of arrays of short (10-to 12-base pair) recognition elements that interact with specific transcription factors. They contain one or more sequences for interaction with DNA binding regulatory proteins. The location and orientation of an enhancer are flexible with regard to the gene. They can be near or at a great distance upstream or downstream from the core promoter. In at least some cases. the activity of a promoter is enormously increased by the presence of an enhancer.

Transcription directed by core promoter elements in the absence of regulatory components has been defined as basal transcription. Accordingly, it has been observed that some regulatory proteins can interact with general transcriptional factors(GTFs) (Figure 4). These regulatory proteins are called activators. The level of transcription can be modified by activators binding to conserved DNA elements. The protein-DNA complex forms a regulatory network in which several factors interact to regulate gene transcription.

It seems likely that certain holoenzyme components provide specific interaction surfaces for activators and others for repressors. For example, in an SV40 enhancer study,

in vitro mutagenesis experiments revealed that SV40 enhancer consists of two functional domains designated A and B [68]. Mutations within subregions in each domain leads to a significant decrease in transcription in vivo. The A and B domains alone have weak enhancer activity, but duplication of either domain separately or in combination leads to a high level of activity [68, 69]. Investigators have shown that in the absence of activators, expression of most genes is silenced both *in vivo* and *in vitro*, or can only support a relatively low level of transcription *in vitro*. However, in the presence of gene-specific activators and gene expression can be significantly increased. Therefore, the mechanisms involved in RNA polymerase II holoenzyme exist to permit binding of a large range of regulatory capabilities. Protein- DNA interactions and protein- protein interactions make the gene regulation more complex. This capability provides the opportunity of regulating the same apparatus in different ways at different promoters.

I-3-3. Chromatin and DNA Methylation are involved in the regulation of transcription:

Recently, there are many advances in understanding of the role of chromatin structure in the regulation of transcription by RNA polymerase II. The fundamental building block of chromatin is the nucleosome, which is comprised of an octamer of histone proteins (two molecules each of histones H2A, H2B, H3, and H4) and 146bp of DNA wound around the octamer. These histone proteins are highly conserved between different species. Before activating factors can contribute to transcription regulation, they have to interact with regulatory DNA sequences of a target gene. However, most genes are packaged into nucleosomes with extensive DNA-protein interaction. In chromatin studies, Kadonaga[70] has proposed some chromatin remodeling machines that link to

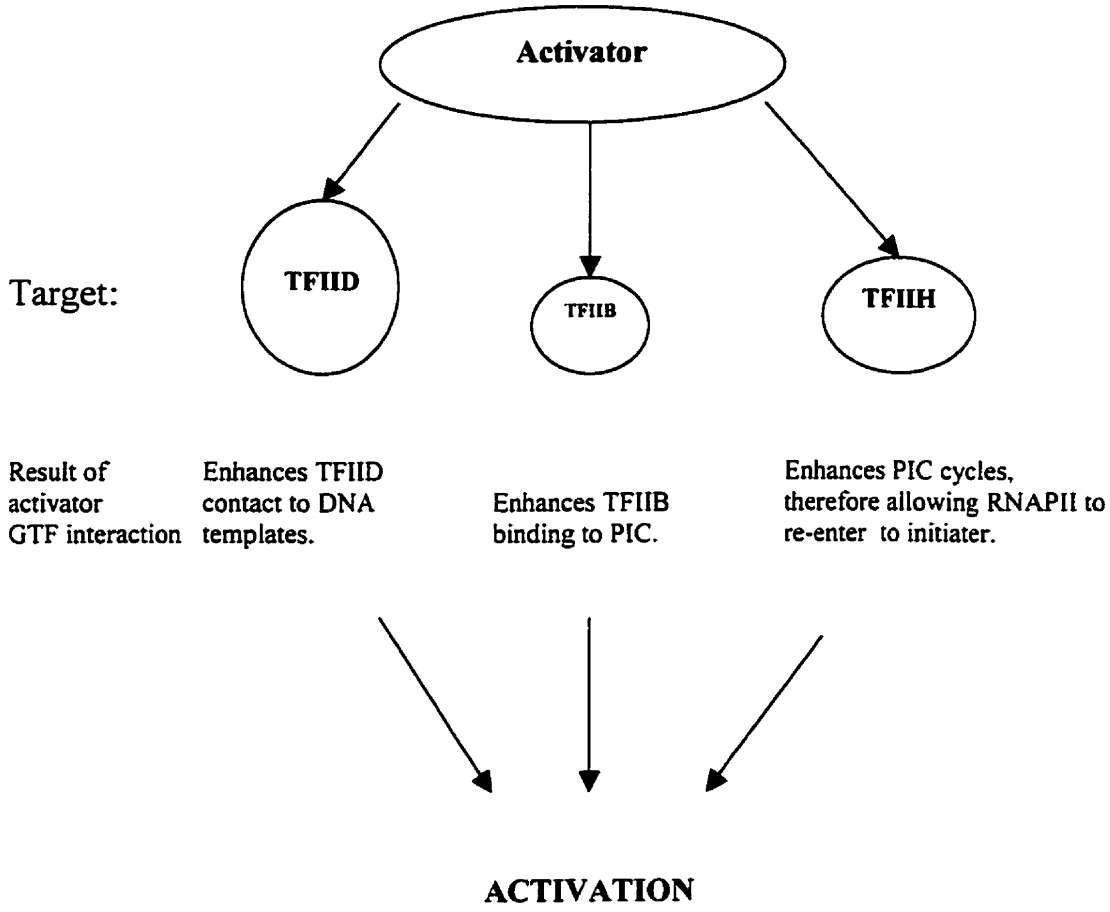


Figure 4. Many activators interact with TFIID, TFIIB, and TFIIH. Listed are the mechanisms through which activation might occur via each of these targets.

chromatin and transcription, in his review, it has been known that the packaging of genes into chromatin represses basal transcription and transcriptional activator function to counteract chromatin mediated repression. Kingston [71] reported that the regulatory proteins interact directly with nucleosomes either to stabilize or destabilize nucleosome structure, leading to either repression or activation of the transcription. Many investigators have reported that the histone acetylation plays an important role in gene activation and inactivation [72]. Together, these studies demonstrated that chromatin structure is an important component of gene expression.

For instance, the histones may stimulate transcription by presenting the DNA to activating factors. Alternatively, they inhibit transcription by their interaction with regulatory elements. Bresnick reported that the promoter of mouse mammary tumor virus gene is organized as an array of five positioned nucleosomes. In this configuration the promoter is inactive. Binding of the glucocorticoid receptor leads to nucleosome disruption and to transcription of the gene. Clearly, the nucleosome structure contributes to transcriptional regulation by organizing the DNA in a reproducible way. The mechanism is thought to be acetylation and deacetylation of histones[73]. The enzymes that acetylate histones are referred to as histone acetylatransferase, or HATs, while the enzymes that deacetylate histones are called histone deacetylatransferase, or HDACs. The histones in general possess a globular core domain, required for histone-histone interactions central to nucleosome formation, and highly charged, unstructured tail domains that protrude and form the octamer. These tails are important both for histone-DNA interactions and for interactions with other nonhistone proteins. Neutralization of

the positive charge within the histone tails by acetylation provides a reversible mechanism for regulation these interactions.

Methylation of CpG islands in promoter regions is a mechanism to modify gene activation during development and in a tissue restricted manner [74]. DNA is modified after synthesis by the enzymatic conversion of many cytosine residues to 5-methylcytosine. The majority of the conversion of cytosine residues in eukaryotic DNA is found in dinucleotide sequence CpG (5'-CG; C, cytosine; G, guanine). A crucial determinant of repression is the density of methyl-CpGs near the promoter. Methylation is a reversible sequence-specific methylation and demethylation process, by which the binding of transcription factors is effectively blocked, thereby controlling the interaction of regulatory sequences with the basal transcriptional apparatus. It was also found that methylation of promoter regions inhibits transcription by recruitment of histone deacetylatransferase and other transcriptional repressors [75].

In addition, two proteins have been identified capable of binding methylated region (MeCP1 and 2). MeCP1 was found to repress transcription of genes with methylated promoter region [76]. Li [77] reported that mutation of the DNA methyltransferase gene results in embryonic lethality. The homozygous embryos were stunted, delayed in development, and did not survive past mid-gestation 10 days. Although little is known of the molecular mechanisms that control sequence-specific methylation and demethylation, this result indicated that methylation is important during development.

I-4. THE STUDY OF THE K^+ CHANNEL GENE REGULATION

Transcriptional regulation of mammalian voltage-gated K^+ channel may play an important role in regulating the resting membrane potential and length of the action potential by determining the cell-specificity and level of expression of these proteins in cardiac and neuronal cells [78,79]. Many mammalian genes encoding voltage-gated K^+ channels have been isolated as discussed above. However, very little is known about the mechanisms that regulate the transcription of these genes.

The molecular mechanisms involved in regulating K^+ channel gene transcription and translation have been the focus of several recent studies. In Kv1.5 gene regulation studies, Mori, et al has investigated the cis-regulatory sequences that determine the cell-specific expression of Kv1.5 gene. They found that a DNA element containing a dinucleotide repetitive sequence (Kv1.5 repressor element; KRE) repressed the expression of Kv1.5-CAT reporter gene constructs in transient transcription of COS-7 and CHO cell lines, while expression of this construct in GH3 cells was not affected [80]. Deletion of the sequence fragment that contains the KRE element, resulted in a marked increase in CAT activity in COS-7 cells, to a level similar to that in GH3 cells. In CHO cells, CAT activity is also increased 3-7 fold. They also found that the promoter of Kv1.5 lacks a TATA box, has several transcription start sites, and contains a cAMP response element located within 5'-noncoding sequences that can modulate the cAMP -induced transcriptional response of Kv1.5-CAT reporter gene constructs. These observations establish that a dinucleotide repetitive sequence is capable of forming part of a cell-specific silencer element in a mammalian gene.

Moreover, several studies found that Kv1.5 gene transcription can be induced by physiological stimuli (e.g., hormones, depolarization), drugs (e.g., dexamethasone) and pathophysiologic condition (e.g., hypertension). Levitan et al [81] has examined the effects of physiological stimuli on channel gene transcription. They elevated the concentration of KCL in cultured GH3 cells, causing membrane depolarization. They used northern blots to analysis Kv 1.5 RNA expression at different times. They found that membrane depolarization inhibits Kv 1.5 gene transcription. Takimoto et al[82] also reported that dexamethasone and stress significantly increase cardiac Kv 1.5 mRNA expression. Since dexamethasone is a glucocorticoid receptor agonist, this effect is likely to be mediated by direct stimulation via a glucocorticoid-responsive element (GRE) located in the upstream region of the Kv 1.5 gene [83]. Matsubara[84] found that Kv 1.5 gene is down regulated in hypertrophied ventricular myocytes. These studies established that physiological and pharmacological conditions could alter transcriptional activity to alter K^+ channel gene expression.

Kaczmarek et al have characterized the promoter of a K^+ channel, Kv3.1 expressed in the PC12 neuronal cell line [85]. They have localized the essential promoter region to a highly GC-rich region containing four Sp-1 binding sites. Deletion analysis in NIH3T3 cells suggests that multiple silencing and enhancing elements are involved in the cell type-specific expression of this gene. The regulatory elements included a cAMP/calcium response element (CRE) and an AP1 element in the upstream region of the promoter. The promoter activity is enhanced by a cAMP analog and a calcium ionophore. Deletion of the CRE-like element at position –252 eliminated the enhancement of promoter by cAMP. These results suggest that the transcription of the

Kv3.1 channel may be regulated by neurotransmitters that elevate cAMP levels in neurons.

Few other studies have defined the K^+ channels promoter. Schoots examined the human K^+ channel Kir 3.1[86]. This is the first promoter that has been isolated and characterized for an inwardly rectifying K^+ channel. They established the transcription initiation site and found that the promoter region lacks a TATA box, but contains GC-rich elements as well as various putative transcription factor-binding sites. Wymore.et al, [87] have characterized the transcription unit of mouse Kv1.4, a voltage-gated K^+ channel gene expression in brain and heart. They found that decreased expression of the transcript is due to the presence of five ATTTA repeats in the 3' noncoding region, which inhibits translation. Thus transcriptional and post-transcriptional regulation of Kv1.4 may modulate the levels of functional Kv1.4 channels.

Study of K^+ channel gene regulation in cardiac cells is a new field. Transcriptional activation of K^+ channel genes during development or in response to extracellular signals also involves the regulated assembly of multi-protein complexes on enhancers and promoters. A key issue in understanding this process is how a relatively small number of different transcription factors can achieve the high level of specificity required to regulate the complex patterns of gene expression in K^+ channel genes.

I-5. TRANSCRIPTION FACTOR GATA-4 PLAYS AN IMPORTANT ROLE IN REGULATING CARDIAC GENE EXPRESSION

GATA transcription factors are important regulators of both hematopoiesis (GATA-1/2/3) and cardiogenesis (GATA-4). The consensus DNA sequence (A/T)

GATA(A/G), referred to as the GATA element, was first identified in studies of erythrioid-specific gene transcription [88, 89]. An erythroid transcription factor (GATA-1) that binds specifically to GATA cis-elements was identified and shown to contain a novel DNA-binding domain consisting of two similar zinc fingers [90, 91]. Subsequently, molecular cloning of the cDNAs encoding two additional factors (GATA2/3) had been carried out by cross-hybridization to GATA-1 cDNA[91]. The cDNA for each subfamily of GATA factors (GATA4/5/6) was discovered by cross-hybridization of cDNA clones to sequences within the highly conserved DNA-binding domain [92, 93]. Recently, a novel member of this family, GATA-4 was found to be expressed in the heart and in endodermally derived tissues [94] whereas the mRNA for GATA-4 is absent in skeletal muscle cells. Therefore, on the basis of its expression pattern, it was suggested that the GATA-4 protein might interact with GATA regulatory cis-elements of cardiac-cell specific gene. The gene encoding GATA-4 factor is transcriptionally regulated in a tissue-restricted manner [94].

GATA-4 is present in the Xenopus presumptive cardiac ventral mesoderm prior to formation of the cardiac tube [95] and is present in the mouse embryo at a similar stage. The early expression of GATA-4 in the heart suggests that this gene plays an important role for cardiac development in cardiogenesis. However, the understanding of the target genes regulated by GATA factors is still unclear.

Several promoters from myocardial-specific genes are now characterized and shown to be important for directing cardiac-specific and GATA-dependent transcription (**Table 2**). These include gene encoding the rat A- and B- type natriuretic genes [96,97]; the rat and human α-myosin heavy-chain genes [98]; the murine cardiac-specific troponin-C

gene [99]; the quail slow myosin heavy-chain gene [100]; the homebox gene Nkx2-5 [101] and the Na^{+}-Ca^{2+} exchanger gene [102].

In α- myosin heavy-chain study, Dr Molkentin et al established that GATA-4 interacts with the α-MHC GATA sites to stimulate cardiac muscle –specific expression. Mutation of the GATA-4 binding sites either individually or together decreased activity by 50 and 80% in adult myocardium, respectively. Furthermore, overexpression of GATA-4 lead to the expression of co-transfected α-MHC promoter /enhancer construct in skeletal muscle cells, but not in fibroblasts. This finding suggests that absence of α-MHC expression in skeletal muscle in vivo may be due to the absence of GATA-4 in these cells.

GATA-4 has been implicated in the transcriptional regulation of the cardiac troponin C promoter. In the presence of GATA-4 factor, troponin C promoter activity increased 75-to-90 fold. Again, mutation in the relevant GATA binding site results in a 70% reduction in activity. These data demonstrated that GATA-4 is an important regulating factor for cardiac-specific gene regulation.

Olson et al has reported that the homeobox gene Nkx2-5 has a GATA-dependent enhancer. This gene has multiple functions and plays an important role in cell migration and embryonic development. Nkx2-5 gene is expressed predominantly in the mouse cardiac progenitor cells early in development. In the adult mouse, the mRNA is present in heart [101]. The homeobox protein expression in the heart is believed to be a key factor during the process of cardiogenesis. In Olson's study, they found that transcription of

Table 2. Defined GATA-4 dependent transcription in cardiac muscle genes.

Target gene	Regulatory elements	Ref
natriuretic protein	-100 GTC**TGATAA**ATC**AGATAA**CCC -76	(97)
α-myosin heavy chain	-254 CCC**TGATA**CATA**GATAT**GGGG –273	(98)
Troponin C	-119 CTG**AGATTA**CAG –108	(99)
Homeobox protein Nkx2-5	-9366 GG**AGATAA**GA-ACT**TTATC**TTC –9261	(101)
Na^{+}-Ca^{2+} exchanger gene	-54 CAC**AGATAA**GCAG -40	(102)

The GATA binding sites (bold) are critical for promoter activity and interact with GATA-4 in vitro.

Nkx2-5 gene in heart is related to the distal enhancer GATA-4 elements. There are two GATA-4 binding sites located in –9261 to-9366. Deletion of these two binding sites caused a lost of all cardiac Nkx2-5 gene expression. This result reveals a novel GATA-dependent mechanism for activation of Nkx2-5 transcription in the developing heart.

In addition, Menick et al[102] recently reported that GATA is one of the novel elements in the regulation of cardiac expression of the Na^{+}-Ca^{2+} exchanger gene (NCX1). The Na^{+}- Ca^{2+} exchanger is an integral membrane protein and plays a major role in calcium efflux in heart. Under physiological conditions, the exchanger functions as an antiporter, moving sodium and calcium in opposite directions across the cell membrane. NCX1 mRNA levels are up-regulated in both cardiac hypertrophy and failure [103, 104]. To understand the mechanisms of NCX1 gene in transcription level, the feline NCX1 gene promoter has been characterized [105]. Analysis of the DNA sequences in the 5' upstream region of NCX1 promoter revealed a number of elements that may be involved in regulation, such as E-box, GATA, CarG, MEF-2, M-Cat and AP2 elements. There are two GATA elements located at –80 and –50 in the 5' upstream region. To identify whether these GATA elements control cardiac NCX1 gene expression, point mutations were made within both –80 and –50 GATA elements. Mutated reporter constructs were transfected into rat neonatal myocytes. The luciferase activity was reduced >90% compare to wild-type constructs [102]. This result indicated that GATA-4 plays a critical role for NCX1 gene expression in rat neonatal cardiomyocytes.

However, the transcriptional activities of the GATA proteins are modulated by interactions both with transcription factors and with transcriptional coactivators and repressors. For example, the C-terminal zinc finger of GATA-1 interacts specifically with

SP1 and other transcription factors to synergistically activate erythroid specific gene expression [105]. Recently, three related GATA proteins, Friend of GATA-1(FOG1); U-shaped (USH); and friend of GATA –2(FOG2) have been shown to interact specifically with the N-terminal of Drosophila GATA-1, and the N-terminal of GATA-4, respectively [106]. This interaction appears to modulate specifically the transcriptional activity of GATA in either a positive or negative way. Therefore, GATA-4 as an important regulatory element may regulation in three ways: transcriptional regulation of the genes themselves, specific interaction with target elements, and interaction with other transcription factors.

Summary

Electrophysiological studies have clearly demonstrated the presence of diverse types of K^+ channels which are prominent in repolarization of cardiac myocytes. Molecular studies have revealed the detail of topology of Kv K^+ channel structures. Eighteen voltage-gated K^+ channel genes have been cloned. The relationship between cloned genes and functional myocardial K^+ channels has been explored in heterologous expression system. During the past decade, major advances have been made in understanding the mechanisms that switch genes on and off. In spite of the exact transcriptional mechanisms are not entirely understood. Nevertheless, recent advances have been provided insight into transcription by polymerase II and regulatory activators in eukaryotic cells, chromatin and DNA methlation are also play the important roles in the regulation of gene expression. The study of K^+ channel gene expression in cardiac cells is a new field. The mechanisms involved in regulating K^+ channel gene transcription

have been examined in several recent studies. K^+ Channel Kv1.5, Kv 3.1, and Kv1.4 have been investigated. It seems reasonable that defining the molecular complement of the functional K^+ channels in myocardial cells will require a combination of physiological, molecular, genetic, immunohistochemical, and biochemical approaches. With a combination of these approaches, it seems certain that during the next decade, cardiac physiologists will succeed in defining the molecular structures of various types of K^+ channels expressed in myocardial cells.

Chapter II

OBJECTIVES

A great deal of work has been done in molecular clones of voltage-gated K^+ channels, and a variety of experimental strategies have defined functional domains within the channel proteins. However, little effort has been made to define the mechanisms that control transcription and functional expression of these important molecules.

In cardiac ion channel gene expression, an important question is how a cell type-specific gene expression can be induced. The development of methods for cloning and characterizing individual genes has provided the opportunity to study these mechanisms at the molecular level. The current challenge is to understand how specific protein-DNA interactions regulate gene expression in cardiac myocytes. In particular, to define the roles of specific activator involved in cardiac channel gene regulation.

II-1. STATEMENT OF PROBLEMS

It is generally accepted that the Kv 4.2 gene is important for contributing to cardiac Ito. Changes in Ito contribute to action potential change in heart disease [107], and Ito is a target for many antiarrhythmic drugs [108, 109]. Because of its important role in cardiac electrical activity, major efforts have been made to understand the biophysical properties and the molecular basis of Ito. It has been reported that the experimental models of hypertrophy in cat [110,111,112], guinea pig [113], rabbit [114], and rat [115] have shown the changes in cellular electrical activity associated with experimentally induced hypertrophy. The most consistent and pronounced change described is a prolongation of action potential duration caused by reduction of Ito density. Several recent studies have reported that the Ito current density was significantly reduced in cells from the infarcted heart and from the congestive heart failure model [116,117]. They found that the heart failure in human and in animal models is associated with prolongation of action potential duration (APD). Whether decreased transcription is linked to the decreased density of the Ito is not clear. The level of Kv 4.3 mRNA decreased by 30% in failing hearts compared with nonfailing controls. In contrast, the levels of Kv1.5 and Kv1.4 mRNA were increased in the arrhythmogenic myocytes with time after coronary artery occlusion [118].

It is obvious from the examples discussed above that disease conditions can and do alter ion channel expression.

II-2. PREVIOUS STUDY

The gene encoding a K^+ channel subunit that underlies the Ito has been described in rat . Kv 4.2 cDNA and Kv 4.3 have been cloned from rat heart. On the basis of analysis of mRNA transcribes in mammalian ventricles, Kv 4.2 is the important cardiac K^+ channel gene encoding Ito in rat. Kv 4.2 gene expression has been reported in the human, canine, and rat heart, however, the mouse heart Kv 4.2 gene has not been evaluated.

We hypothesize that the presence of regulatory elements within the 5'-flanking region of Kv 4.2 gene contribute to the regulation of Kv 4.2 in mouse heart.

II-3. OBJECTIVES

To investigate the above hypothesis, several strategies have been undertaken in the present study.

Quantitative analysis of Kv4.2 mRNA expression: Before cloning Kv 4.2 5'-flanking region, I tested whether the Kv 4.2 gene was expressed in mouse heart by using an RNase protection assay.

Cloning and characterization of the Kv 4.2 promoter: To understand the mechanism of regulation of Kv 4.2 gene expression, I cloned Kv 4.2 gene's 5'-flanking region from a mouse genomic library. I investigated the location of the Kv 4.2 transcription initiation sites and found putative regulatory elements in this gene.

Cell cultures, DNA transfected to mouse neonatal myocytes: I used Kv 4.2 5'-flanking region reporter constructs to investigate cardiac ion channel gene transcription. These

reporter constructs were transfected into fetal, 1 day, and 3 days myocytes. Relative promoter activity was detected by measuring the ratio of luciferase/renilla activity.

Investigation of Kv 4.2 gene tissue-restricted expression: To investigate whether Kv 4.2 gene expression has tissue restricted expression, I used Kv 4.2 5'-flanking region reporter constructs transfected into H9C2 cell line, NIH 3T3 cell line, and HEK cell lines.

Chapter III

QUANTITATION OF KV 4.2 mRNA EXPRESSION

III-1. INTRODUCTION

Before cloning and studying the mouse Kv 4.2 gene regulation, we raised the question of whether the Kv 4.2 gene is expressed in mouse heart? In spite of the great deal of work done in quantitation of *Shal* related subfamily in different species, previous work had focused on the human, canine, and rat heart, the mouse heart has not been evaluated. The present study is to determine whether the Kv 4.2 gene is expressed in mouse heart.

III-2. MATERALS AND METHODS

Preparation of RNA from mouse atrium and ventricle: All RNA samples were prepared from fetal (18 day embryonic), neonatal (1, 3,14 day) and adult CD-1 mice. Each RNA sample was produced from pooled dissections from six animals. For preparation of RNA from ventricle, only the lower quarter of left and right ventricular muscle, from the apex of the ventricle was used. The ventricular dissection consisted of

epicardial muscle. Tissue samples were first carefully moved to the PBS solution to remove excess blood and then homogenized in 4M guanidinium thiocyanate [119].

The method that was used to separate RNA from the guanidinium homogenate is described as follows: 1) 3.6 ml of the homogenate was layered onto a 1.4-ml pad of 5.7M CsCl in a Beckman SW 50.1 centrifuge tube (Beckman Instruments, Inc). 2) Centrifuge the SW 50.1 rotor at 42000 rpm for 18 hrs at 18°. The supernatant solution was then carefully removed. The pellet was dissolved by vortexing extensively in SET buffer(10 mM Tris-Hcl, pH7.4, 5 mM EDTA, 0.1% SDS). 3) The RNA was precipitated by adding 0.1 volume of 2M sodium acetate (pH5.4) and 2.5 volumes of 100 % ethanol. Store at – 20° for at least 2 hr. 4) The RNA was centrifuged at 15000 rpm for 15 min. Dissolve the pellet in DEPC-treated water and store at -70°C. Estimate the yield by measuring the absorbency at 260 nm in a spectrophotometer.

RNase Protection Assay: RNase protection assays were performed essentially as described by Krieg and Melton[120]. The aim was to determine whether Kv 4.2 mRNA was expressed in mouse heart and to compare the level of expression at five different stages. RNA samples were arranged in a developmental manner (Fetal, 1,3,14 days and adult). For each sample, 10ug of total RNA was used in the assay. The probe was made by using DNA template which was prepared by subcloning 300 bp of a Kv 4.2 mouse cDNA fragment into pBluescript II SK (Stratagene). This fragment is contained part of the Kv 4.2 coding region. In this experiment, we used a piece of cytoplasmid ß actin cDNA as a probe (~200bp) to test actin RNA expression as quantitative control in mouse ventricle.

III-3. RESULTS

Kv 4.2 gene is expressed in mouse heart: In current studies, the voltage-gated K^+ channel Kv 4.2 gene expression in mouse heart was observed by RNase protection assay. We have found that Kv 4.2 mRNA is expressed in mouse heart and is increased during development (Figure 5).

III-4. DISCUSSION

Several investigators have reported the Kv K^+ channel gene expression. RNase protection assays confirmed the presence of Kv1.2, Kv1.4, Kv1.5, and Kv 4.2 in adult rat heart. Our study indicates that Kv 4.2 gene does exist in mouse heart and the developmental changes in mRNAs encoding voltage-gated K^+ channels occur in the mouse heart. These data thus contribute to our progress in identifying Kv K^+ channel gene expression in mouse heart.

Chapter IV

CLONING AND CHARACTERIZATION OF THE MOUSE KV4.2 PROMOTER

IV-1. INTRODUCTION

Libraries of DNA fragments from genomes of numerous organisms have been constructed in genomic cloning systems as diverse as yeast artificial chromosomes (YACs) [121], cosmids [122], bacteriophage P1 [123], P1 artificial chromosomes (PACs) [124], and bacterial artificial chromosomes (BACs) [125]. Detailed descriptions of these cloning systems are beyond the scope of this thesis. In brief, the genomic clones are copies of DNA from chromosomes. The most common procedure for selecting specific clones from

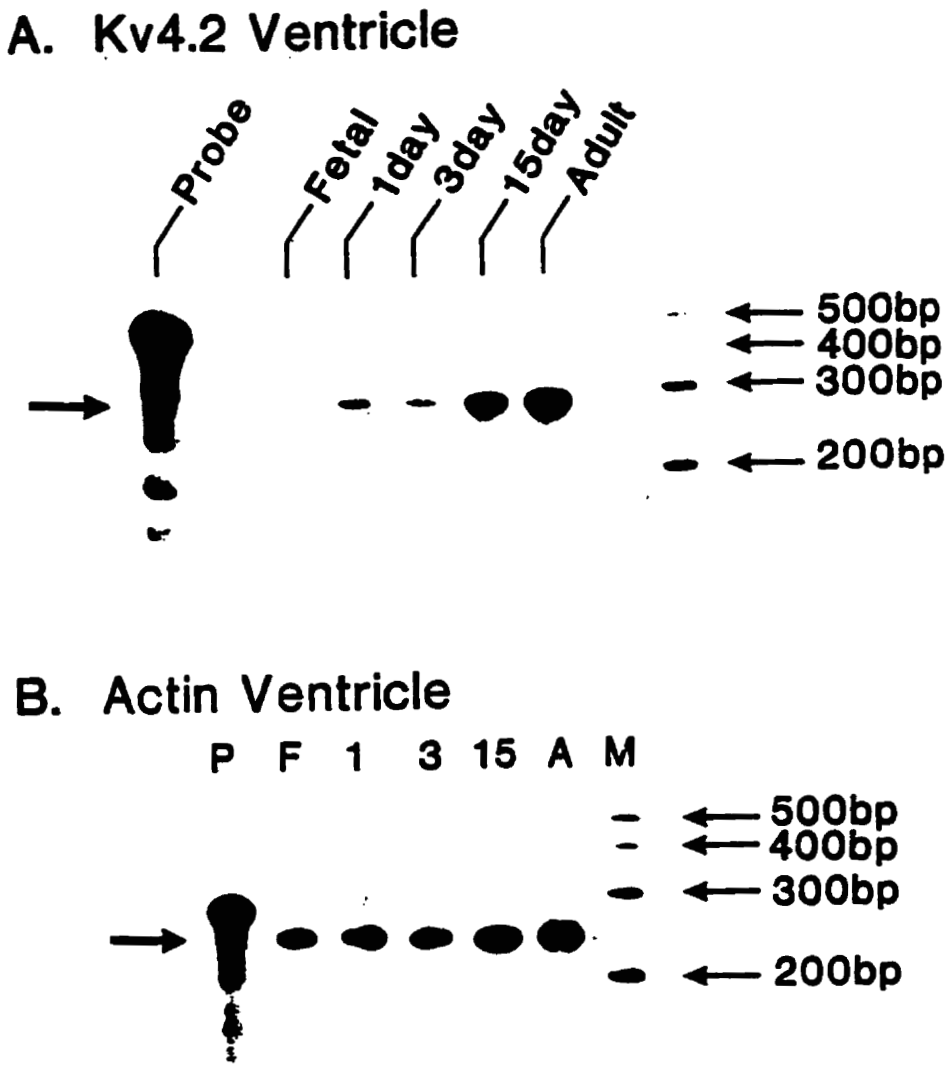


Figure 5. Kv 4.2 K^+ channel mRNA expression in mouse ventricular muscle determined by RNase protection analysis. Panel A shows developmental expression of Kv 4.2 in mouse ventricle. Panel B shows actin RPA product as quantitative control in mouse ventricle. In panel A to B lane 1 represents probe; lanes 2 to 6 represent fetal, 1-day, 3-day, 14-day and adult respectively; and lane 7 represents RNA molecular weight markers. Arrows indicate the RPA products.

genomic DNA is to use bacteriophage λ. The DNA of the phage is about 50 kb long and only non-essential region is replaced with foreign DNA without impairing the ability of the phage to infect and reproduce in most *E. coli* cells. A genomic library is a collection of recombinant molecules, maintained either in phage particles or in plasmids growing in bacteria, that includes all DNA sequences of a given species. The bacteriophage P1 cloning system can accept inserts in the 70-100 Kb and has the convenience of being commercially available [126]. This capability significantly increases the useful tools that are available for cloning and analyzing complex eukaryotic genomes.

Identification of expressed sequences within genomic DNA is a hurdle in the characterization of complex genomes. How do we define regulatory sequences that govern gene transcription? The study of genes and other regulatory sequences in clones from these systems has invariably been conducted using subcloning strategies. The mapping of genes has also relied upon PCR and hybridization analysis of insert DNA subcloned into small vecters.

IV-2. MATERIALS AND METHODS

IV-2-1 Library Screening

Kv4.2 mouse genomic library: In our Lab, Dr Jim Lees-Miller had cloned Kv 4.2 mouse cDNA coding region. A 180 base pairs RT-PCR fragment was used to probe the mouse genomic library. The CGAT Genome Resource Facility in Toronto completed this part of the work. They used the bacteriophage P1 cloning system to isolate the genomic DNA and isolated six PACs clones with Kv 4.2 sequences (These are 444D13, 473H1, 514D12, 613M24, 643P3, and 658C13).

IV-2-2 Genome subcloning and sequencing

Southern blots analysis: To clone the specific Kv 4.2 promoter region, we harvested 658C13 PAC cloned DNA, and digested with restriction enzyme Nco I (New England Biolabs Inc), RcA I (New England Biolabs Inc), Scal I (Gibcol BRL), BamH I (Gibcol BRL) and EcoR I (Gibcol BRL). 7ug of DNA in each sample are digested with different restriction enzyme and run in a 1% of agarose gel with lambda/ Hind III DNA marker. The gel stained with ethidum bromide and photographed with a ruler laid alongside the gel, so that the band positions can be identified on the membrane. The gel treated with 0.25 M HCL solution, then the DNA transfer into the H^+ nylon membrane for over night. 0.4M NaOH solution was used as the transfer solution.

The 300 base pair fragment in pBS derived from mouse cDNA was labeled by the [32P] d-CTP as the probe. After DNA transferred, the nylon membrane was hybridized with probe at 55^0 C for 6 hrs. Hybridization solution consist of 1x Naphosphate:EDTA ; 1% BSA; 5% SDS; 0.1mg/ml salmon sperm DNA; 30% formamide and 5% dextrisan sulfate. Membrane washed with 0.1% SSC and 0.1 % SDS at 60^0C for 30 min twice and dried for autoradiography.

Cloning the Kv 4.2 upstream region: Several bands were detected by hybridization on southern blots (Figure 6). We located the 3kb of NcoI fragment in the gel and isolated these DNA, then cloned into pGL3 basic vector NcoI site(Promega). Restriction mapping of Kv 4.2 5'flanking region in this 3kb fragment is performed by southern blots analysis.

Sequencing the Kv 4.2 upstream region: To sequence the 3kb of the Kv 4.2 upstream fragment, several subclones were constructed by using convenient restriction

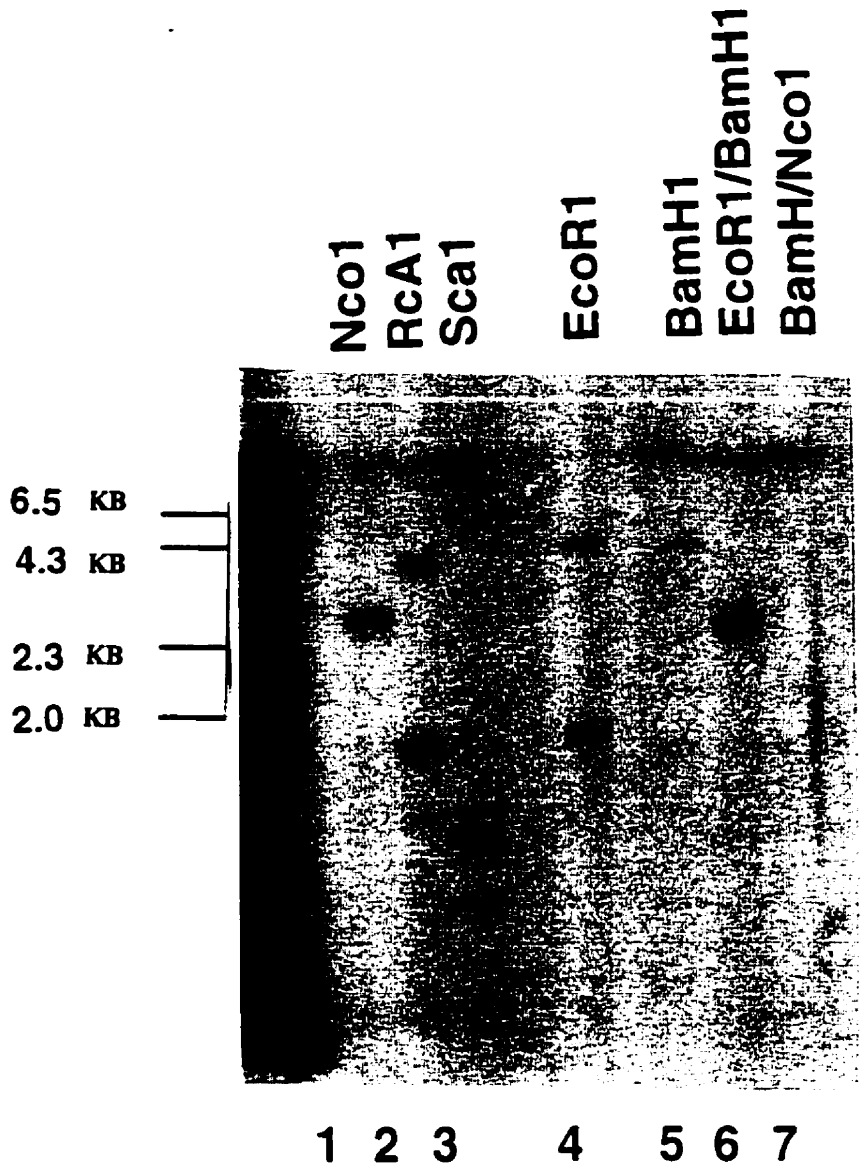


Figure 6. Shows the genomic clone 658c13 DNA digested with restriction endonucleases and southern blot analysis with Kv 4.2 specific probe shows the different size of bands from digestion. Lanes 1 to 7 represent DNA digested by Nco I, RcA I, Scal I, EcoR I, BamH I, EcoR I/BamHI, BamHI/ NcoI respectively. DNA molecular markers are indicated in the left.

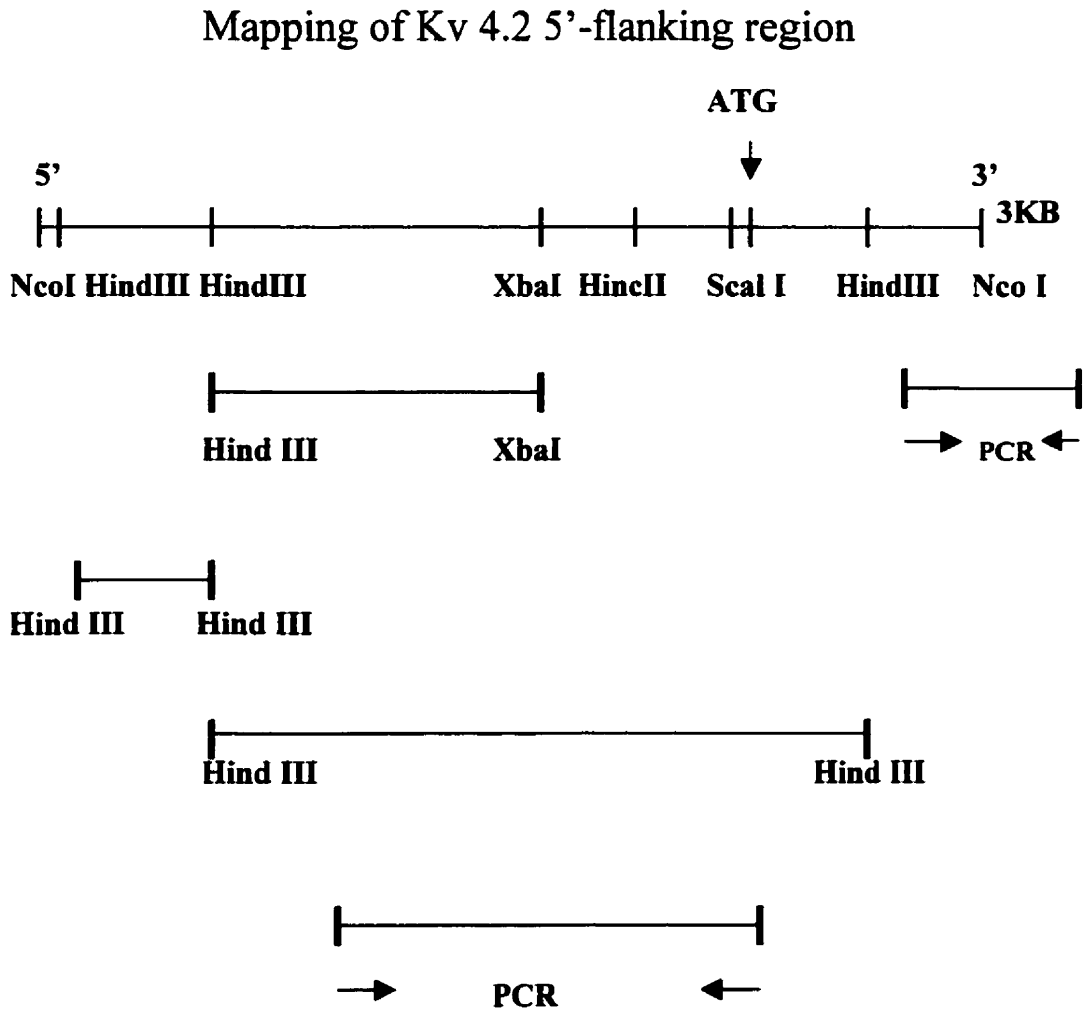


Figure 7. Construction of Kv 4.2 5'- flanking region. The schematic representation of the restriction mapping of the Kv 4.2 gene. Arrows in the first row represent the location of translation initiation codon ATG. Several clones indicate the subclones in Kv 4.2 5'-flanking region for sequencing. Arrows in subclones represent the PCR primers.

enzymes (Figure 7). We cut the whole fragment with restriction enzyme Hind III, and subcloned the Hind III fragments into pBluescript. The AT cloning system (Invitrogen) was used to clone the PCR products. Several primers have been used in this study, (these are T7/T3, pGL3basic, Kv42G3', Kv42G5', Kv423.5, Kv42Pro.5, Kv42P5-2, and MKv42.P3.). Minipreps were prepared from the clones and were sequenced either by the dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemical Corp.) or by cycle sequencing using the Ampli-Cycle sequencing kit (Perkin-Elmer) as specified by the manufacturer using an annealing temperature of 55 ° C. The orientation and junction sequences were verified by sequencing the plasmids.

IV-2-3 Promoter reporter Construct

Four luciferase-containing constructs have been used in this study (Figure 8). A) the pGL3basic vector has no promoter or enhancer. It includes only the luciferase gene with upstream multiple cloning sites for introducing the putative promoter fragment. B) the enhancer vector contains (from 5' to 3') the SV40 enhancer, a multiple cloning site for introducing the putative promoter-containing element, and the luciferase reporter gene. C) the pTK Luc vector was constructed by inserting the thymidine kinase promoter into pGL3basic vector. It contains pTK promoter and rest of pGL3basic vector elements. D) the control vector contains the SV40 promoter and enhancer, a multiple cloning site for introducing the putative promoter-containing element, and the luciferase reporter gene. Several 5'-flanking fragments from the Kv 4.2 gene have been cloned into the pGL3-enhancer or heterologous promoter constructs using appropriate restriction enzymes (Figure 9).

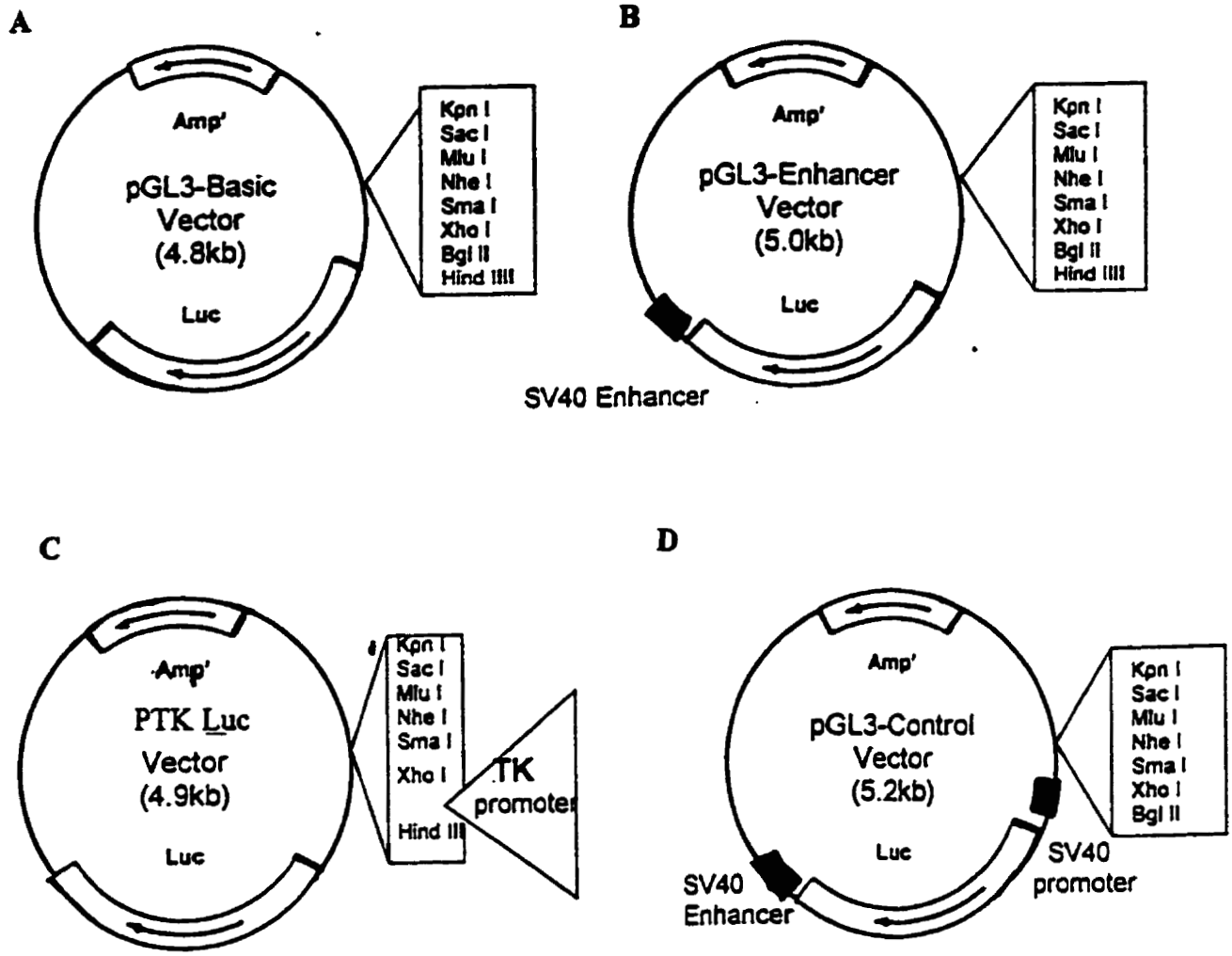


Figure 8. This figure show the four pGL3-vectors containing luciferase construct maps. Additional description: *luc* cDNA encoding the modified firefly luciferase; Amp, gene conferring ampicillin resistance in *E. coli*. Arrows within *luc* and the Amp gene indicate the direction of transcription. A) represents the negative control construct. B) represents pGL3 enhancer construct. C) represents PTK Luc construct. D) represents positive control construct.

Kv 4.2 promoter reporter constructs. We have subcloned 3 fragments from the 5'-flanking region into the pGL3basic vector. The fragment NcoI/NcoI (+277/-1993) contains the Kv 4.2 upstream region to the protein translation start site. This fragment NcoI site was created at the start codon by PCR-based mutagenesis. It contains about 2000 bp of the whole core promoter and upstream region shown as Figure 9-b-1. The fragment NcoI/Hinc II (+277/-127) is Hinc II to the protein translational start site; this construct only contains Kv 4.2 core promoter region without the enhancer containing region shown as Figure 9-b-2. The fragment NcoI/Hinc II(-1993/-127) contains Kv 4.2 5'- upstream enhancer containing region without core promoter region show as figure 9-b-3.

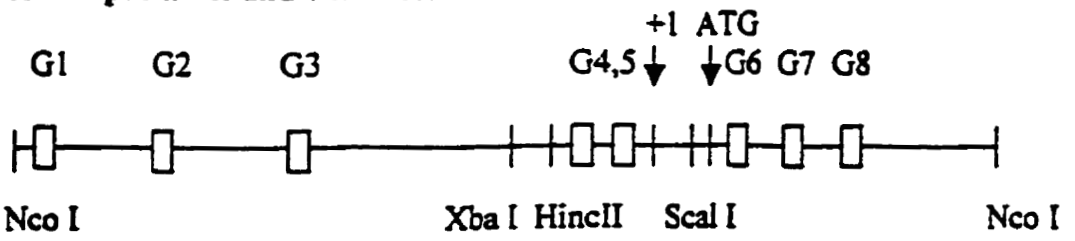
Heterlogous promoter reporter constructs. Two of the Kv 4.2 gene upstream regions were cloned into heterologous TK (thymidine kinase) promoters. (PTK LUC construct was kindly obtained from Dr.B.E. Markham). The TK promoter is approximately 150 bp and is cloned into pGL3 basic plasmid at the Xho I/Hind III sites resulting in the construct pTKLUC. The fragment HincII/NcoI (-127/-1993) which contain the Kv 4.2 enhancer containing region was cloned into TK promoter shown as Figure 9-c-1. The fragment ScaII/NcoI (+134/-1993) which contains the Kv 4.2 core promoter and enhancer containing region was cloned into TK promoter shown as Figure 9-c-2.

The pGL3 enhancer vector contains SV40 enhancer. One of the Kv 4.2 gene upstream region (NcoI/Nco1 +277/-1993) was cloned into pGL3 enhancer construct shown as Figure 9-d.

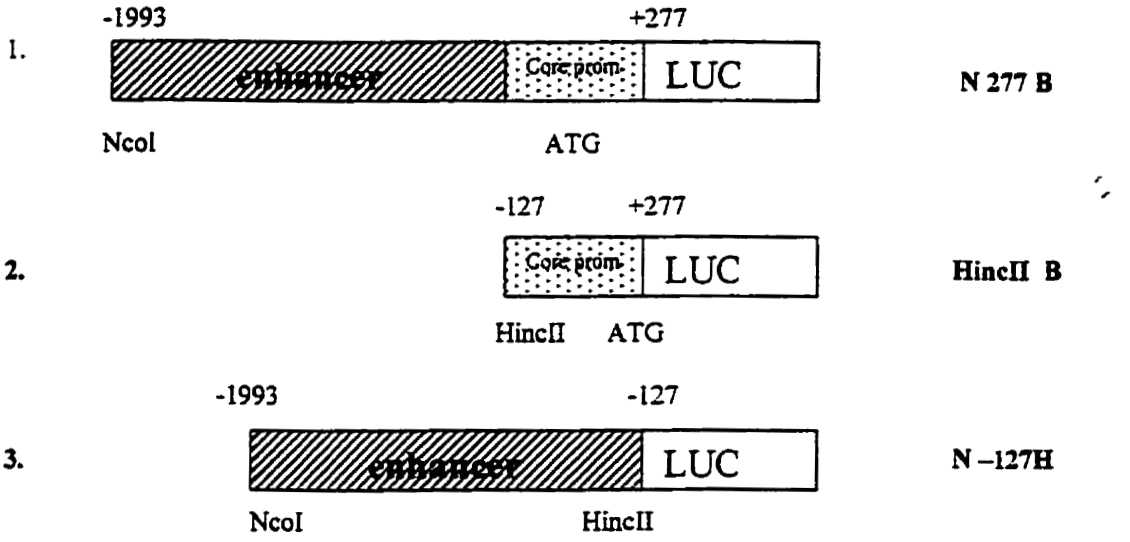
Figure 9. Mouse Kv 4.2 genomic DNA containing the 5'-flanking region. A shows the schematic representation of the Kv 4.2 gene containing the translation initiation codon ATG site , transcription start site (+1), and eight putative GATA-4 sites. B to D shows the Kv 4.2 promoter-reporter constructs used in transfection experiments.

Figure 9.

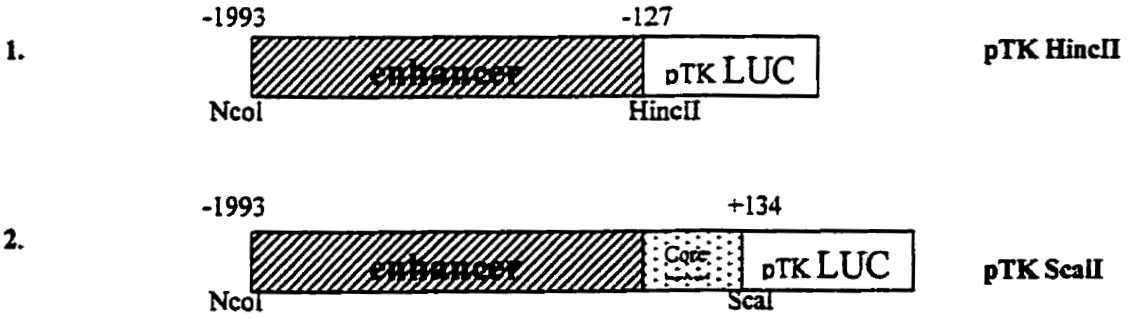
A. KV4.2 promoter and enhancer location



B. Construction of KV4.2 5' flanking region in luciferase reporter vector



C. Clone KV4.2 5' flanking region in pTK-promoter vector



D. Clone KV4.2 5' flanking region in pGL3 enhancer vector

IV-2-4 Location of the KV4.2 gene transcriptional start sites

Primer Extension In primer extension analysis, the total RNA was isolated by the methods described above. mRNA was made by Oligotex™ (Qiagen). An oligonucleotide (5' GTGTCTTGCCATGTCTGGA A3'), which was antisense to the region from +450 to +470 of mouse Kv 4.2 genomic DNA was radiolabeled by T4 polynucleotide kinase (Gibco BRL) with gamma-(^{32}P) ATP (600Ci/mmol). mRNA (5ug) isolated from mouse heart was denatured at 65° C. The primer was annealed with mRNA and extended using Supertrascription II reverse transcriptase (Gibco-BRL) at 37°C for 1h. The synthesized cDNA was denatured at 65°C and analyzed by electrophoresis on 6% acrylamide gels. The same primer was used for the DNA sequence ladder run on the same gels. The size of the cDNA reflects the distance from the primer to the 5'-end of the RNA.

RNase Protection Assay In the RPA, to determine transcription start sites, a (^{32}P) UTP-labeled antisense RNA probe was synthesized using T3 polymerase from pBS KS linearized with Xba I. The resulting probe is complementary to the 686 bp of the 5'-upstream region in Kv4.2 genomic clone. Subsequent procedure was similar to that described above.

Location of the regulatory elements of the KV4.2 promoter

I have used a MatInspector V2.2. program to search for potential binding sites in the Kv4.2 gene sequence.

IV-3. RESULTS

Genomic construct of mouse Kv 4.2 5'flanking region

To determine the structure of the 5'-upstream region of the K v4.2 gene. NcoI fragments of the genomic clones were subcloned into pGL3basic vectors. These subclones were then restricted and subjected to Southern analysis. The data are shown in **Figure 7**. Using restriction enzyme mapping and Southern blotting, we determined that 3kb of the Kv 4.2 upstream region fragment contains approximately 2.2kb of the 5'-noncoding region and 0.8 kb of coding region. The structure and mapping of the 5'-upstream of Kv 4.2 gene is shown in **Figure 7**. Cloning various restrictions enzyme cut fragments from Kv 4.2 gene generated a series of deletion constructs as show in **Figure 9**.

Characterization of the Kv 4.2 upstream region:

To characterize the promoter region of Kv 4.2, we have identified the transcription start sites using primer extension analysis and RNase protection assays.

For primer extension analysis, we had designed an antisense oligonucleotide (Experimental Procedures), corresponding to the sequence of Kv4.2 coding region 5'-end. Extension of this primer yielded two major fragments, which indicated the major transcription start sites in the upstream region. Two transcription start sites were identified in the mouse heart corresponding to 297 and 287 base pairs upstream of the AUG codon (**Figure 10**).

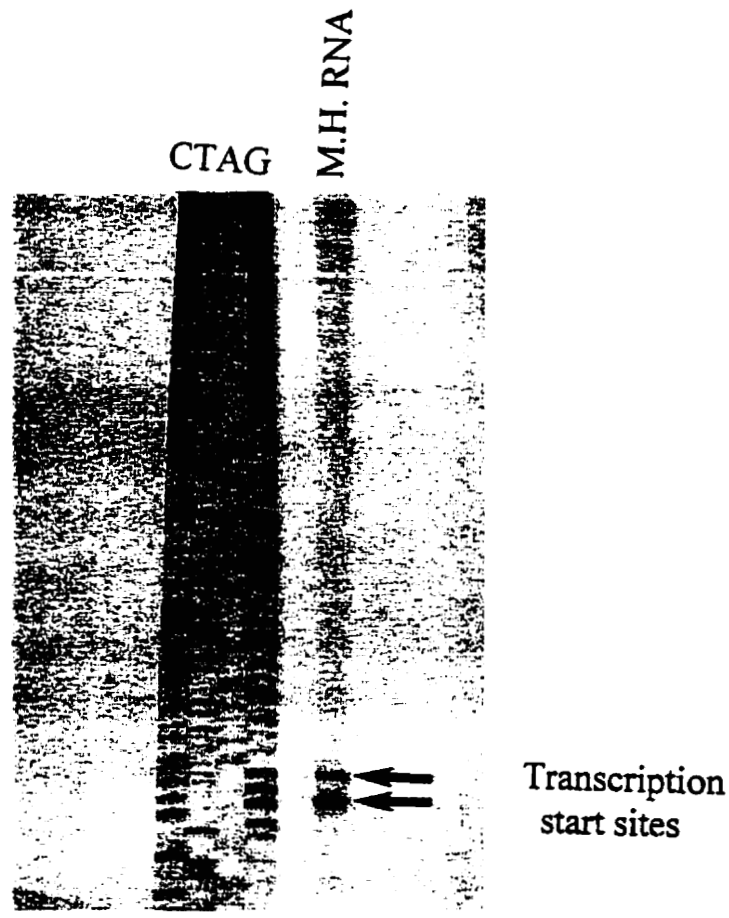


Figure 10. **Identification of the transcription start sites of the Kv 4.2 gene.** This figure shows the primer extension assay. A labeled oligonucleotide was hybridized with 5ug of the poly A RNA isolated from mouse heart. The transcription start sites localizes in the region 287-297 base pairs upstream of the translation start site. The nucleotide corresponding to the extended fragment is indicated by two arrows on the right, and flanking sequence is shown on the left.

For RNase protection assays, the length of the protected bands in the RNase protection assay should indicate the distance from the start of the mRNA to the 3'-end of the probe. We used Hind III fragment (as described above) as the probe. We found that the size of the band (~600) is in agreement with the results detected by primer extension (**Figure 11**). There were also some other minor sites lying in the upstream region of the two major sites. We found it difficult to determine the exact position of these minor sites, since they did not appear to agree with those detected by RAP analysis. It is therefore not clear whether these minor sites represent real, alternate transcription start sites.

The sequence surrounding the Kv 4.2 transcriptional start sites contain no apparent TATAA sequence. However, in the 5'-flanking region at position –92 a TATAA- like motif was observed (**Figure12**). Eight consensus sites for GATA were found in this genomic sequence. The 5'-upstream region contains 5 putative GATA-4 binding sites beginning at position -1836, -1627, -1106, -83, and –68, which have previously been demonstrated to be important for activity of other cardiac-specific gene promoters [123,124]. Further inspection reveals that this region contains a number of putative binding sites for other transcriptional factors. One consensus Car-G binding site at position +657, two SP-1 binding sites at –1211, -826, and several CANNTG (E-box) motifs were identified (**Figure 12**). These elements may be involved in the transcriptional regulation of the Kv 4.2 gene as well.

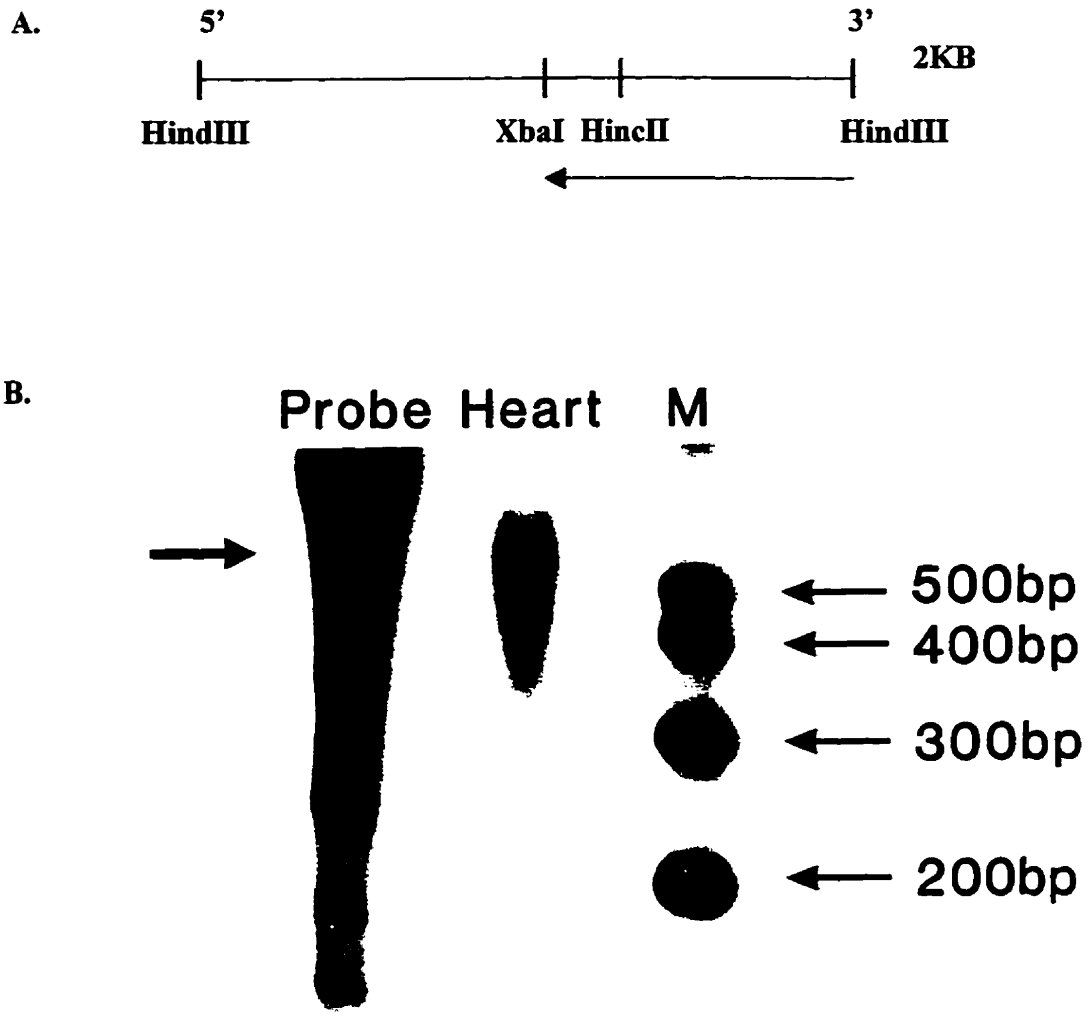


Figure 11. **RPA assays shows the transcription initiation sites of mouse Kv 4.2 gene.**

A shows the genomic map of Kv 4.2 5'-flanking region. The Hind III site to XbaI site is 1kb that span over the transcription start sites. **B** shows RNase protection. Lane1, undigested probe alone; lane 2, heart RNA, protect a ~600bp fragment of probe from digestion; Lane 3, RNA marker. Arrow in left indicates the RPA product.

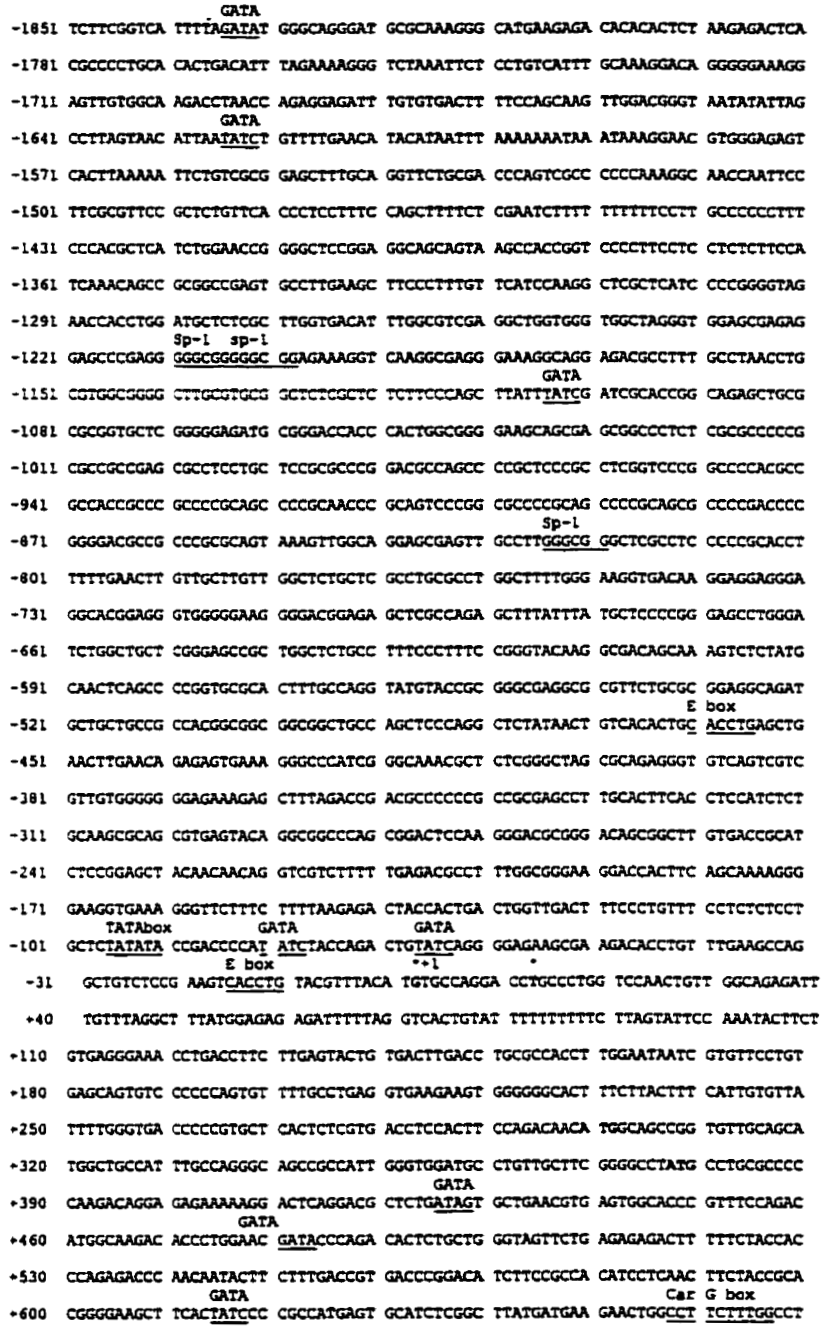


Figure 12. 5'Flanking sequence of mouse heart Kv4.2 gene. Numbers represent nucleotide position relative to the first major transcription start site (+1). The other major transcription start site is also labeled with an asterisk. One TATA box, one CarG- like, three SP1 elements, three E-box, and eight GATA binding sites are underlined. The translation start sites in bold letters.

IV-4. DISCUSSION

To understand the mechanisms underlying transcriptional control of the Kv 4.2 gene, we have cloned the promoter region of the Kv 4.2 K^+ channel gene from mouse genomic DNA. We report the location of mouse Kv 4.2 gene transcription start sites from both primer extension and RNase protection assay. Together, these results demonstrate the existence of two principal transcription initiation sites in the Kv 4.2 gene. This is the similar to what has been reported for other cardiac genes. Kv 3.1 and Na^+-Ca^{2+} exchanger gene have been reported to have two major and several minor transcription start sites[86, 103].

By analysis the putative regulatory elements in the 5'-flanking region of the Kv 4.2 gene, we noted that the Kv 4.2 core promoter region sequence has no TATAA box. However, two putative SP1 binding sites had been found in the upstream region. This result is in agreement with what has been reported from other cardiac K^+ channel genes. All of the Kv1.5, Kv3.1 and Kv1.4 genes have no TATA box. All of them contain putative SP1 binding sites. These data suggest that SP1 factor is important for regulation of the TATA-less promoters in cardiac genes.

Several cardiac-specific gene studies have demonstrated that many regulatory elements are required for cardiac gene expression. Markham [127, 128] has reported a number of α-myosin heavy chain (α- MHC) gene upstream regulatory elements. These upstream regulation elements play an important role in the regulation of the α-MHC gene that encodes a cardiac muscle- specific protein in heart and skeletal muscle, such as GATA binding site; M-CAT site [129]; AT-rich site; and CarG box site [130]. These elements interact with GATA-4; M-CAT binding factor; AT-rich factor and serum

response factor, respectively, and their functions are positive regulatory factors. An E-box site that binds myocyte-specific enhancer binding factor-2 (MEF2) function as a negative regulatory factor.

A number of transcriptional regulatory elements have been found in Kv 4.2 sequence. There are eight putative GATA binding sites; one CarG binding site; three SP1 sites; and five E-box binding sites in this gene, which may play positive or negative roles in gene transcription. Since GATA-4 contributes to the regulatory cascade leading to cardiogenesis, and all cardiac gene expression has been reported to be upregulated by GATA-4, we sought to address its contribution to the regulation of Kv 4.2 gene.

To compare the mouse with human Kv 4.2 genomic sequence, we found that the ***Shal*** protein has been highly conserved. There is 81% identity between human and mouse Kv 42. Of the eight GATA-4 regulatory binding sites, only number 7 is identical between human and mouse. However one CarG box and two SP1 binding sites are identical between human and mouse sequences (**Figure 13**).

Figure 13. Comparing the homology between human and mouse genomic nucleotide sequence of promoter region of the Kv 4.2 gene. The nucleotide sequence of mouse Kv 4.2 is derived from mouse genomic clone. The nucleotide sequence of human Kv 4.2 is obtained from GenBank. In the sequence comparisons, dashed lines indicate residues identical to human sequence, and dots indicate deletions. The sequence reveals the interspecies conservation in both upstream region and coding region.

```
Human          GCT TGTATGTGTT ACGTTTGGTT TTAAGGATGA TTCCTAATGA
Mouse          C-A -TGG---TAA -A-C------ --------A-- CA-T-T-ATG

Human     CTCGGGGGCG GGGTACGCGA AAAACAAAA. AGGTTGGTAG CGACAATTTT
Mouse     ACT------- ------A--- -G-C-----T C-------CCA -T--------

Human     CCTGTGTCTC TCTACCTCAG TAACCCCTCA TACGAACACC TTTAACTCTT
Mouse     -----C---- CTC-A----- -C----T--- CC-GG----- ---TCT-TC-

Human     TTAACTATTT TAAATATGCG CAGTGATGCG CAGAGAGGAT GAAGGGGACG
Mouse     -CGGTC---- --G-----G- ---G------ --A--G-C-- ---.-A---A

Human     CAAACTTTCA GAGACGCACG CCCCTTGACG ATGACATTTA GAAAAGGG.C
Mouse     --C---C-A- -----T---- -----GC--A C--------- --------T-

Human     TCCACATCCC TGTTATTCGT AAAGGAC..G GGAAAAGGAG TTGTGTCAAG
Mouse     -AA-TTCT-- ---C---TGC -------AG- --G------- ----------

Human     ACCTCGATCG AGGAGAGT.. CTCTGATTTT TTGGCAAGTT GGACAGATGG
Mouse     ----AACCA- ------T-TG TGTGAC---- CCA------- ----G-G-AA

Human     CATATCAATC CCTGCAACGT T......... ....AACAAT CTGGTTTTGA
Mouse     T----T-GC- TTAGT---A- -AATATCTGT TTTG------ -ATAA---A-

Human     AAATAAATAA AAACAGAAAG AGGAAATGCA CTTAAAAATT CCGCGTCGGA
Mouse     ---A-T-A-T ---GGA-CGT G---G-GT-- ---------- -T-TCG----

Human     GCTTTGCGGG TCTTGCCTTC CAGTCGCCCC CAGAAGACAA CCAATTCCTG
Mouse     -------A-- -TC---GAC- ---------- -CA---G--- ---------T

Human     CGCATTCCGC TCCGCTCACC CTCCTTCCCA GCTTCTCTCG .........C
Mouse     ---G------ --T-T----- ------T--- ----T----- AATCTTTTTT

Human     TCTCTTTCCC CCCATTTTCC CACGCTCGCC TTGAACCGGG ACTCGAGAGG
Mouse     -T-TCC-TG- ---CC----- -------AT- -G-------- G---CG----

Human     CAGAAGCAAG GCACGGGTCC CCTTTCTCCT CTCCTCCACC AAACCACCGC
Mouse     ---C-T---- C---C----- ----C----- ---T---AT- ----AG----

Human     AGCCCAG.CA CGTGAAGCTT CCCTTTGTTC ATCCGAGGCT CGCTTTTCTC
Mouse     G------TGC -T-------- ---------- ----A----- ----CA-C-

Human     CCCAGCAGAT CCACCTGGAT GCTCTCCCTC GGTGACATTT TGCGCCGGGG
Mouse     -GGG-T---A ---------- ------G--T ---------- G---T--A--

Human     TTGGTGGGTG GCTGGGGTGG AGCGAGAGGA GCCCGAGGGG GCGGGGGCGG
Mouse     C--------- ---A------ ---------- ---------- ----------

Human     AGAAAGGTCA AGCCGAGGGA AAGGCAGGAG ACGCCTTT.C CTAACCTGCG
Mouse     ---------- --G------- ---------- --------G- ----------

Human     TGGCGGGGCG TGCGCGCGGT TATTTATTTA TTTTCTCCTT ATTTATTGAT
Mouse     ---------T ----T----C -C-CGC-C-C --CC-AG--- ------C---

Human     CGCACTAGCA GAGCAGCGCG GGGAGC.CCG GGGAGATGCA GGACCACCCA
Mouse     -----CG--- ----T----- C--T--T-G- ---------G ----------

Human     CTGGCGGGGA AGCAGCTAGC AGCCCTCCCG CGCCCCCGCG CTGCCGAGCG
Mouse     ---------- ------G--- G------T-- ---------- -C--------

Human     CCTTCTGCCT CCGCGCTCGG ACGAGAGCCC GTGCCGGCCC CGGCCCCGGC
Mouse     ---C----TC -GCGC-CG-A CGCCAGC--- -CT--C---T ---T------

Human     CCCACCGCGC CAACGCCGCC CGCCCGGCCG CCCCGCAGCC CCGCCGCCCC
mouse     -----GC--- --C----CG- -C-G-A---. -G--AA-C-G -A-T---GG-

Human     GCAGCCCCGC ACCGCGCTGG CCAGGCTCCC GCGACAGTGG CCCCGCAGT.
Mouse     --CC-G-A-- C----AGC-C --C-A-C--G -G---GCC-C -G-------A
```

```
Human     AAGTTGGCAG GAGCGAGTCC CCT...CCGT TCTCGCCTCC CCCGCACC.T
Mouse     ---------- --------TG ---TGGG--G G--------- --------T

Human     TTTGAACTTG TTGCTGCT.. GCTCTGCTCG CCTGCGCCTG GCTTTT.GGA
Mouse     ---------- -----TG-TG ---------- ---------- ------G---

Human     AGGTGAAAAG GAGGAGGGAG GCACGGAGGG ATGGGGGAAG GGAAAGAAGA
Mouse     ------C--- ---------- ---------- .--------- --G-C-----

Human     GCTCGCTTGA GCTTTATTTA TGCTCTCTCG GCGCATCGGA TTCGGCTGCT
Mouse     ------CA-- ---------- -----C-CG- -A--C-G--- -CT-------

Human     CGCGAGCTGC TTTCTCTCCT CTTCCCTTTC CGGGTGCACG GCGAGGAGAA
Mouse     --G----C-- -GG----G-C T--------- -----A--A- ----CAGC--

Human     AGTCTCTATG CAACTAAGCC CCGGCGCGCA CTTGGCCAGG TATGTACCGC
Mouse     ---------- -----C---- ----T----- ---T------ ----------
Human     GGGAGCGGCG CGTTCTGCGC GGAAGCAGAT GCTGCTGCCG CCACGGCGGC
Mouse     ---C------ ---------- ---G------ ---------- ----------

Human     GGCGGCTGCC AGCTCCTGAG CTCTGTAACT GTCACACTGC ACCTGAGCTG
Mouse     ---------- ------CAG- ----A----- ---------- ----------
Human     AACTTGAAAA GAGAGTG.AA GGGGCGATTG GGCGAACGCT .TTTGGCAGA
Mouse     --------C- -------A-- ---C----C- ---A------ C-CG---TAG

Human     CACAGAGGGT GTTTGTAGAC GTGGGGGAGG AGA..... ATCTCTATTA
Mouse     -G-------- --CA--C-T- --T-T--G-  G--GAAAGAG C-T-AG-CCG

Human     ACGCCCCCCA CCGTAACCAC TGCACATCAC CTCCATCTCT GCAAATACAG
Mouse     ---------G ---CG---CT -----T---- ---------- ----GCG---

Human     CCCGAGGAGT AGAGGCAGCA GCAGCTGGAC CCCCAAAGAG AGACGTGGGG
Mouse     -GT---T-CA G-C---CCAG CGGA--CC-. ........-- G----C---A

Human     CAGCGGC.TG TGACCGCATC TCCTGAGCTA CAACAACAGG TCGCCTTTTT
Mouse     -------T-- ---------- ---G------ ---------- ---T------

Human     GAGACTCCTT TGGCGGGAAG GGCTACTTGG AAAGGAAGGT TTGAAAGAGT
Mouse     -----G---- ---------- -A-C----.. .......... ......C--C

Human     GAGAAGGGTA GGTGTAAGGG TTCCCTAATT CGTCGAAAGA ATTCTATTGG
Mouse     A-A-G---A- ----A----- ---TT-CT-- TAAGAG-CT- CCA-GACT-

Human     GTGACTCTCG TTCGTCTTCT CTATCCTACA CTCCACATAC TGACCCTATA
Mouse     --TGACT-TC CCTG-T-C-- --C----..G ---T-T---- C-----C---

Human     TTATCCAGAC TGTGCCGGGG AGAAATCAAA AACACCTGTT TGAAGAAA..
Mouse     -CTA------ ---AT-A--- GAG--GCG-- G--------- -----CC-GG

Human     .......CGG CTGCACCTGT GTGCTTATTT GTGCCAGAGG GTGGCCTAGC
Mouse           --A AGT------- ACGT---CA- -------GAC C--C---G-T

Human     CCACCTGCAG GAAGAGATTT GGCTGGGTTC TGTTGAGGGT GATTGTTAGG
Mouse     ---A---TT- -C-------- --TT-A-C-T -A-G---A-A ----T-----

Human     ACGTTGTATT TTGTTGCCAT TA..TTCCAA ATACCTGTCT TGGAGGGAAA
Mouse     T-AC------ --T--TTTC- --GTA-T-C- -ATA--TCTG --AG--A--C

Human     GTTGCCCTTC TGAGAACTGT GACTTTACCA GGAGCCCTAT CTTGGAATAA
Mouse     C-GA--T-CT ----T----- -----G---. ..T--G-C-C ----------

Human     GAGTTACACC TCTGGACCAC GTTTCTCACT AGTACTTTGC TTGACTGGAG
Mouse     TC--...GTT C---TGAGCA -G-C-C-C-C ---GT----- C---GGT--A

Human     GAAGTGGGTG ACTTTTGGCT GCTTCGGTGA CCCATTGT.. ....AGACGC
Mouse     --------G- G-AC--TCT- A---TCA-TG TGTTA-T-TG GGTG-CC-C-
```

```
Human   CTCGTTACCC TTCTTCCTTC CGCTTCAAGT AATCATGGCG GCGGGGGTGG
Mouse   G-GC-C--T- -CG-GA-CT- -A----C--A C-A------A ---------T-

Human   CAGCGTGGCT GCCTTTTGCA AGGGCAGCGG CTATCGGGTG GATGCCTGTG
Mouse   ----A----- ---A-----C ---------- -C-T------ ---------T

Human   GCCTCGGGGC CTATGCCGGC TCCCCCGAGG CAGGAGAGGA AAAGGACCCA
Mouse   --T------- -------T-- G---------A ---------- -------T--

Human   AGATGCTCTC ATTGTGCTGA ATGTGAGTGG CACCCGCTTC CAGACGTGGC
Mouse   G--C-----G -A-------- -C-------- ------T--- -----A----

Human   AGGACACCCT GGAACGTTAC CCAGACACTC TACTGGGCAG TTCTGAGAGG
Mouse   -A-------- ------A--- ---------- -G-----T-- ---------A

Human   GACTTTTTCT ACCACCCAGA AACTCAGCAG TATTTCTTTG ACCGTGACCC
Mouse   ---------- ---------- G--C--A--A -C-------- ----------

Human   AGACATCTTC CGCCACATCC TGAATTTCTA CCGCACTGGG AAGCTCCACT
Mouse   G--------- ---------- -C-C------ ------G--- -----T----

Human   ATCCTCGCCA CGAGTGCATC TCTGCTTACG ATGAAGAACT GGCCTTCTTT
Mouse   ----C----- T--------- --G-----T- ---------- ----------

Human   GGCCTCATCC CGGAAATCAT CGGCGACTGC TGTTATGAGG AGTACAAGGA
Mouse   ---------- -A-----T-- T--------- ---------- ----------

Human   TCGCAGGCGA GAGAACGCCG AGCGCCTGCA GGACGACGCG GATACCGACA
Mouse   C--------G -----T---- -------C-- ---T--T--A --C--T----

Human   CCGCTGGGGA GAGCGCCTTG CCCACCATGA CTGCAAGGCA GAGGGTCTGG
Mouse   ATA-A--A-A ---T--GC-- ---------- ----T----- ----------

Human   AGGGCCTTCG AGAACCCCCA CA.CCAGCAC GATGG
Mouse   C-------T- ----T----- --C-------- C----
```

Chapter V

CELL CULTURE, TRANSFECTION OF PROMOTER REPORTER CONSTRUCTS, AND LUCIFERASE ASSAYS.

V-1. INTRODUCTION

Isolated cardiac myocytes maintained in primary culture have been used as a model of the myocardium for 20 years. Burrows (1912) is the first person who placed pieces of explanted embryonic chick hearts in culture and observed that single individual cells migrated away from the explant [131]. After another 43 years, Cavanough (1955) isolated and maintained chick embryo myocytes [132]. It was not until 1960 that Harary and Farley became the first to culture mammalian myocytes [133]. In gene regulation studies, we want to know "how are genes and their encoded proteins regulated?" Specifically, the mechanism of channel gene regulation in cardiac myocytes remains largely unknown. With the recent advances and current interest in using molecular biological techniques to investigate cardiac physiology and expression of channel proteins, culturing cardiac myocytes are becoming an increasingly important technique.

To define regulatory sequences that govern gene transcription, promoters and enhancers have been assigned specific roles by means of in vitro assays. In these test systems, cloned DNA sequences are reintroduced into cell culture on specific plasmids carrying a reporter gene and are examined for their ability to activate expression of reporter gene. These simple and reproducible assays allow us to analyze regulatory elements in detail by noting the effects of deleting, adding, or changing DNA sequences

within the regulatory elements. When a specific type of cell is studied in isolation, it is possible to determine whether or not a given set of nucleotides can regulate transcription in a tissue-specific pattern.

In the Dual- Luciferase™ reporter assay system (Promega), two individual reporter enzymes within a single system can be measured. Typically, the "experimental" reporter reflects the effect of the specific promoter conditions on the level of gene expression, while the activity of the co-transfected "control" reporter provides an internal control by which each value within the experimental set can be normalized. Normalizing the activity of the promoter to the internal control effectively eliminates inherent variability of cell number and transfection efficiency. Furthermore, internal controls can negate assay-to-assay variability arising from inconsistencies in the transfer volume of samples being assayed.

V-2. MATERIALS AND METHODS

Cell culture for primary myocytes:

The primary myocyte cell cultures were assessed at three stages: fetal (18 day embryonic), neonatal (1 and 3day). Ventricular myocytes from fetal, 1 day and 3 day mice were isolated using the procedure for isolating neonatal rat ventricular myocyte described by Chiamvimonvat et al [134].

Cell isolation procedure: Cardiac myocytes were isolated from 0, 1 and 3 days neonatal CD-1 mouse. Animals were killed by decapitation and hearts excised under sterile conditions into a filter-sterilized (0.2um pore size) normally Ca^{2+} free minimum essential medium (Gibco, New York) plus (mM): $NaHCO_3$, 23.8; $MgSO_4$, 1.2; and DL-

carnitine, 1.0; pH 7.4 at room temperature. Atria and great-vessel tissues were carefully removed. The ventricles were washed and minced in the Ca^{2+} free medium containing 0.16 mg/ ml collagenase (Yakult, Tokyo, Japan), 10mg/ml bovine serum albumin A2153; Sigma, USA) and 20mM taurine. The tissues were then incubated with 2ml of the enzyme solution in a dry bath heater (Fisher Scientific, Ontario, Canada) and stirred continuously at 37°C. The supernatant was removed and enzyme solution replaced at 5-min intervals. The supernatant obtained after the first 15-min was discarded. Thereafter, the supernatants were placed in 199 culture medium (SIGMA) plus Na HCO_3, 26mM; 15% fetal bovine serum (HYCLONE laboratories Inc UT, USA); 0.05mg/ml Insulin; 10^5 IU/ml Penicillin; and 100ug/ml of Gentamycin; adjusted to pH 7.4. The cells were centrifuged at 2000 r.p.m. for 5 min and resuspended in the culture medium. The cells were plated in a tissue culture dishes and maintained in an incubator at 37 °C in humidified air with 5% CO_2 for 20 min, and then re-plated at density of 1.0 to $2.0x10^5$ per milliliter in tissue culture dishes in order to remove endothelial cells which attached to the original dishes during this 20 min interval. Finally, cells were maintained at 37 °C in humidified air with 5% CO_2 until needed.

Transfections and assays:

After overnight culture, cardiomyocytes were transfected. A few hours prior to transfection fresh medium was added. Cells were washed with PBS twice and were transfected with 2.0ug DNA (isolated using the QIAGEN maxi-prep) for 3h by the **Superfect** method (QIAGEN), and placed into fresh medium, each plate was cotransfected with 0.2ug of Renilla Luciferase report gene DNA as an internal control.

Twenty-four hours post-transfection, the cells were washed with 1x PBS, harvested, and lysed for 20 min in 100ul of lysis reagent (PROMEGA), the cell lysates were centrifuged, and the supernatants were assayed using the Dual-Luciferase Assay Kit (PROMEGA) in a 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Cell culture for cell lines:

NIH-3T3 mouse fibroblasts cell line, Human embryonic kidney (HEK) cell line, and H9C2 rat ventricular cell line (ATCC CRL1446, cardiac myoblastes from rat, passage numbers 16-21) were grown in a density of about $2x10^5$ cells/cm^2 and cultured as monolayers in Dulbecco's modified Eagle's medium (GIBCO BRL). The culture medium was supplemented with10% fetal calf serum (HYCLONE), 2mM glutamine, 100IU penicillin, and 100ug/ml streptomycin under an atmosphere of 5% CO_2 in air saturated with water vapor at 37°C. The medium was replaced every 2 days.

Transfections and assays in cell lines:

All cell lines was cultured for 14-20 days until they reached a density of about $2x10^5$ cells/cm^2. A few hours prior to transfection fresh medium was added. Before transfection, cells were washed using PBS for 2 times. Cells in each plate were transfected with 2.0ug DNA (isolated using the QIAGEN maxi-prep) for 3h by the Fu GENE™ reagent from BOEHRINGER MANNHEIM, and placed into fresh medium. Each plate was cotransfected with 0.2ug of Renilla Luciferase report gene DNA as well for internal control. Twenty-four hours post-transfection, the cells were washed with 1x PBS, harvested, and lysed for 20 min in 100ul of lysis reagent (PROMEGA), the cell lysates were centrifuged, and the supernatants were assayed using the Dual-Luciferase Assay Kit (PROMEGA) in the same way as myocytes.

Data analysis:

In transfection studies, all promoter reporter constructs were co transfected with Renilla luciferase reporter construct to correct for differences in transfection efficiency and the variability of the cells in each plate. Luciferase and renilla luciferase activities were assayed using the Dual-Luciferase Assay Kit (PROMEGA) in a luminometer. Each promoter reporter construct was been transfected in four independent times. In each time, promoter reporter construct was transfected into three individual plates of cells. All the data are normalized using luciferase activity/ renilla luciferase activity. The finally result is the average luciferase activity/renilla luciferase activity in four groups.

V-3. RESULTS

Analysis of the Transcription activity of the 5'-upstream Kv4.2 gene promoter region- *In vitro* studies designed to functionally define the Kv 4.2 promoter domain sequences necessary for initiating transcriptional activity were carried out by using Luciferase reporter expression analyses. These studies were undertaken by using the SuperFect method to transfect the different promoter -reporter constructs into fetal and neonatal cardiac myocytes. In this experiment, pGL3-control vector which contains SV40 promoter and SV40 enhancer construct was used as a positive control; pGL3 basic vector which has no promoter or enhancer construct was used as a negative control (**Figure 14**). Our data indicate that the fetal and neonatal cardiac myocytes can be transient transfected. The positive and negative control promoter activity is in good manner.

To determine whether the 2.0 KB of the 5'-flanking region of Kv 4.2 gene is necessary for the promoter activity, we have made three deleted reporter constructs +277/-1993 pGL3basic (**N277B**), +277/-127 pGL3basic (**HincII B**) and –1993/-127 pGL3basic (**N-127H**). We used these constructs transfected into fetal, 1 day, and 3 days myocytes and found that Kv 4.2 gene promoter activity is quite weak but detectable compared with positive and negative control (**Figure 15**).

Deletion of the Kv 4.2 core promoter region N-127H and deletion of the Kv 4.2 enhancer containing region HincII B constructs both observed low luciferase activities. However, N277B contains both the Kv 4.2 core promoter region and enhancer regions resulting in a modest but significant increase in the ratio of luciferase/renilla. (**Figure 15**). This may indicate that both the core promoter plus enhancer region are essential to turn on the promoter activity region.

GATA-4 induces expression of Kv4.2 gene in myocytes:

In the current studies, GATA-4 plasmid was most kindly obtained from Dr Markham. This plasmid was generated by an EcoR I fragment containing GATA-4 sequence from approximately 485 to 2203, It encodes the full length GATA-4 cDNA in a pMT2 vector. Co-transfection of myocytes with GATA-4 and the three Kv 4.2 promoter reporter constructs (described above) produced an increase in the Luciferase activity in all three reporter constructs compared with no GATA-4 factors. Specifically, in the +277/-1993 pGL3basic reporter construct, there is about 3-7.5 fold increase in the Luciferase activity (**Figure 16**). These data indicate that the GATA-4 factor regulate the transcription of Kv 4.2 gene in cultured primary neonatal myocytes. Statistical analysis shows that this increase represents a significant difference. (P=0.024, N=6).

Figure 14. Functional analysis of control constructs in fetal, 1day, and 3 day myocytes. Group 1 from left to right is no transfection background; Group 2 is pGL3basic as the negative control; Group 3 is pGL3 control plasmid which contain SV40 promoter and SV40 enhancer construct as a positive control; In each group, three bars represent the Fetal, 1day, and 3 day myocytes. All constructs were cotransfected with renilla luciferase reporter construct to correct for differences in transfection efficiency. The luciferase activity is normalized using renilla luciferase activity.

Figure 14.

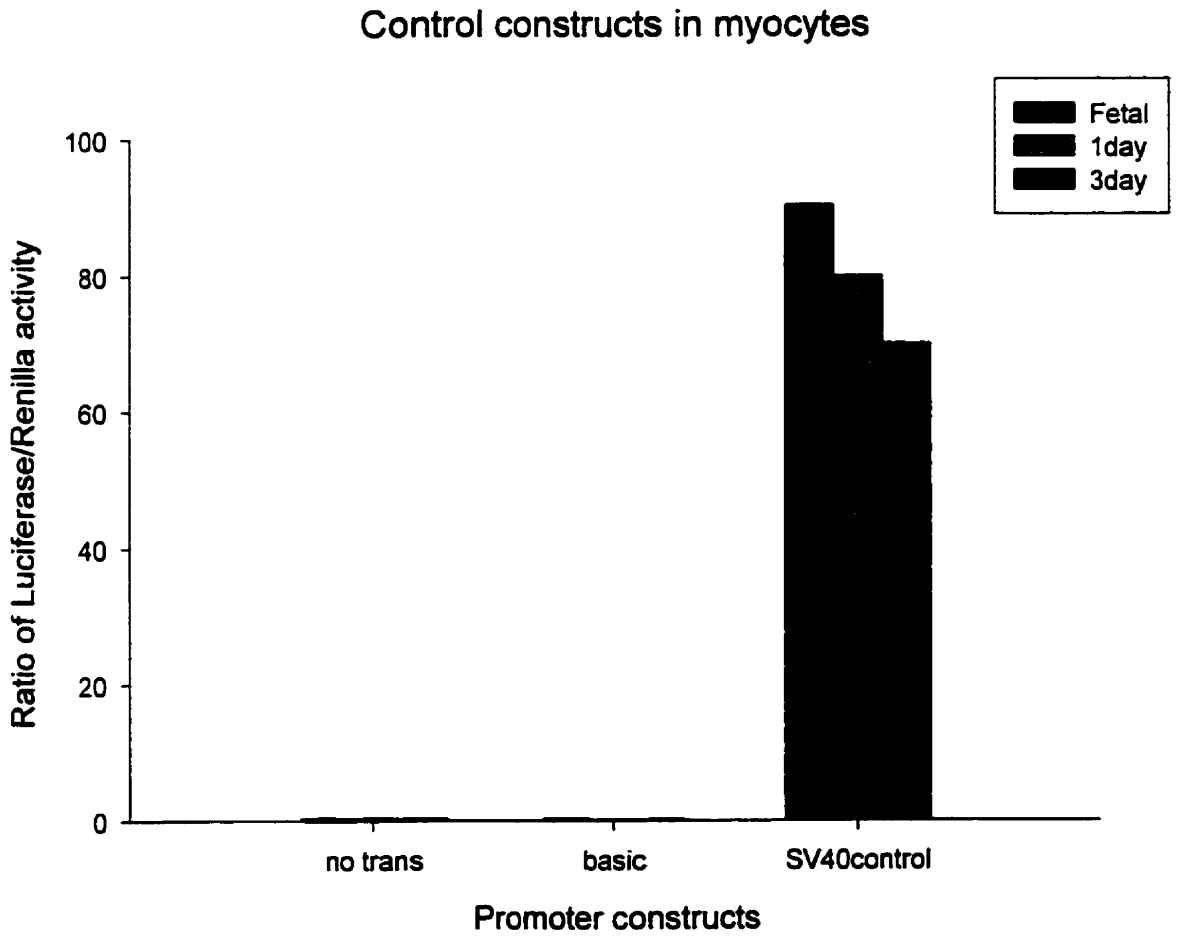

Control constructs in myocytes
Fetal
1day
3day
100
80
60
40
20
0
Ratio of Luciferase/Renilla activity
no trans
basic
SV40control
Promoter constructs

Figure 15. **Deletion analysis of the mouse Kv 4.2 Promoter.**

To determine whether the 2.0 kb of the 5'-flanking region of Kv4.2 gene is necessary for the promoter activity. Three Luciferase reporter constructs were transfected into fetal, 1day, and 3 day myocytes by SuperFect method.

Promoter-reporter constructs are arranged as follow:

Basic represents the pGL3basic which has no promoter and enhancer region as the negative control. **HincII B** represents the pGL3basic plus Kv4.2 core promoter region. **N-127H** represents the pGL3basic plus Kv4.2 enhancer containing region but no core promoter region. **N277B** represents the pGL3basic plus Kv4.2 core promoter and enhancer containing region.

Kv 4.2 gene promoter activity is quite weak but detectable in all of them. However, the **N277B** pGL3basic construct was generated the highest luciferase activity which may indicate that this partial sequence is essential to turn on the promoter activity region. Error bars indicated standard deviations. (N= 4).

Figure 15

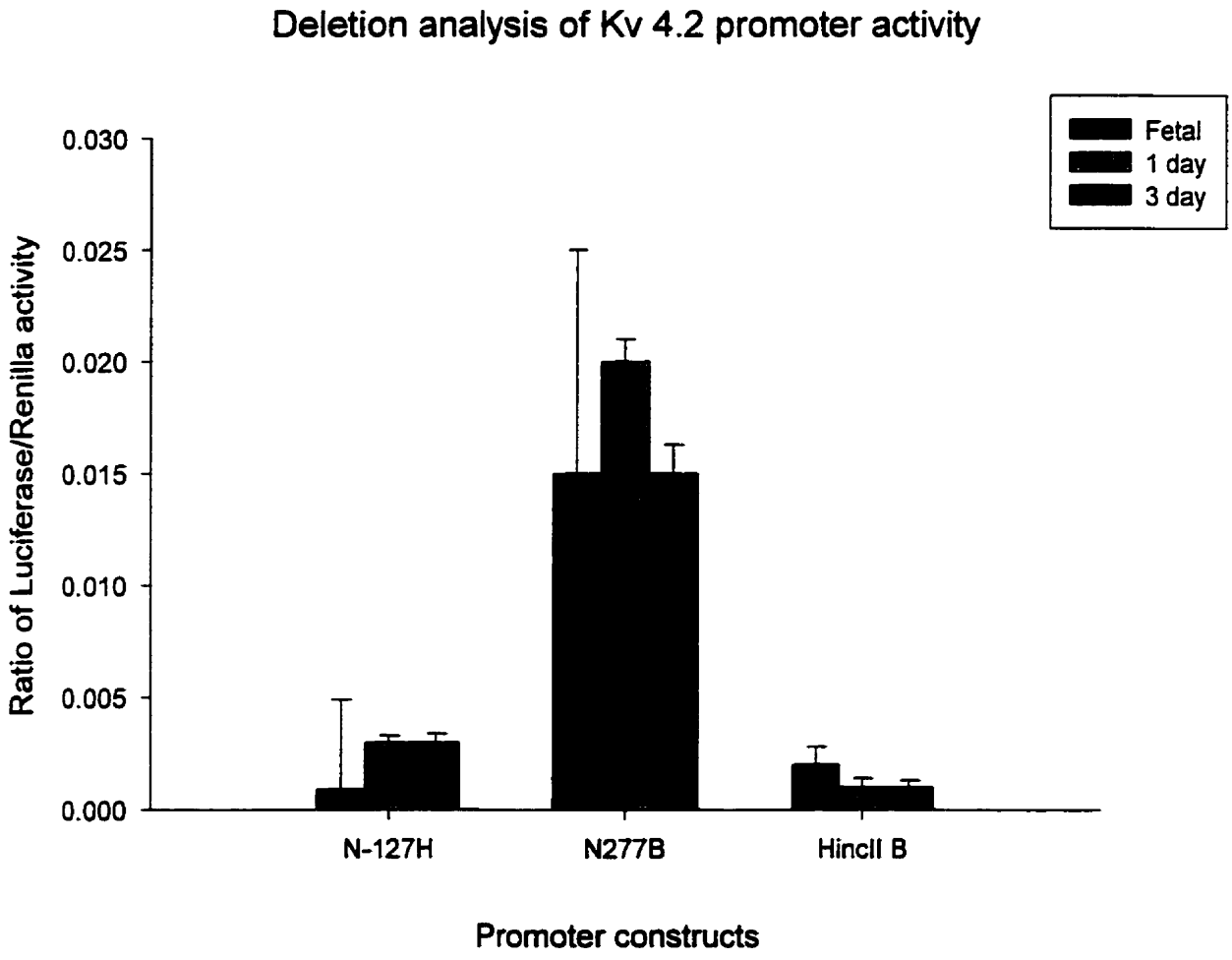
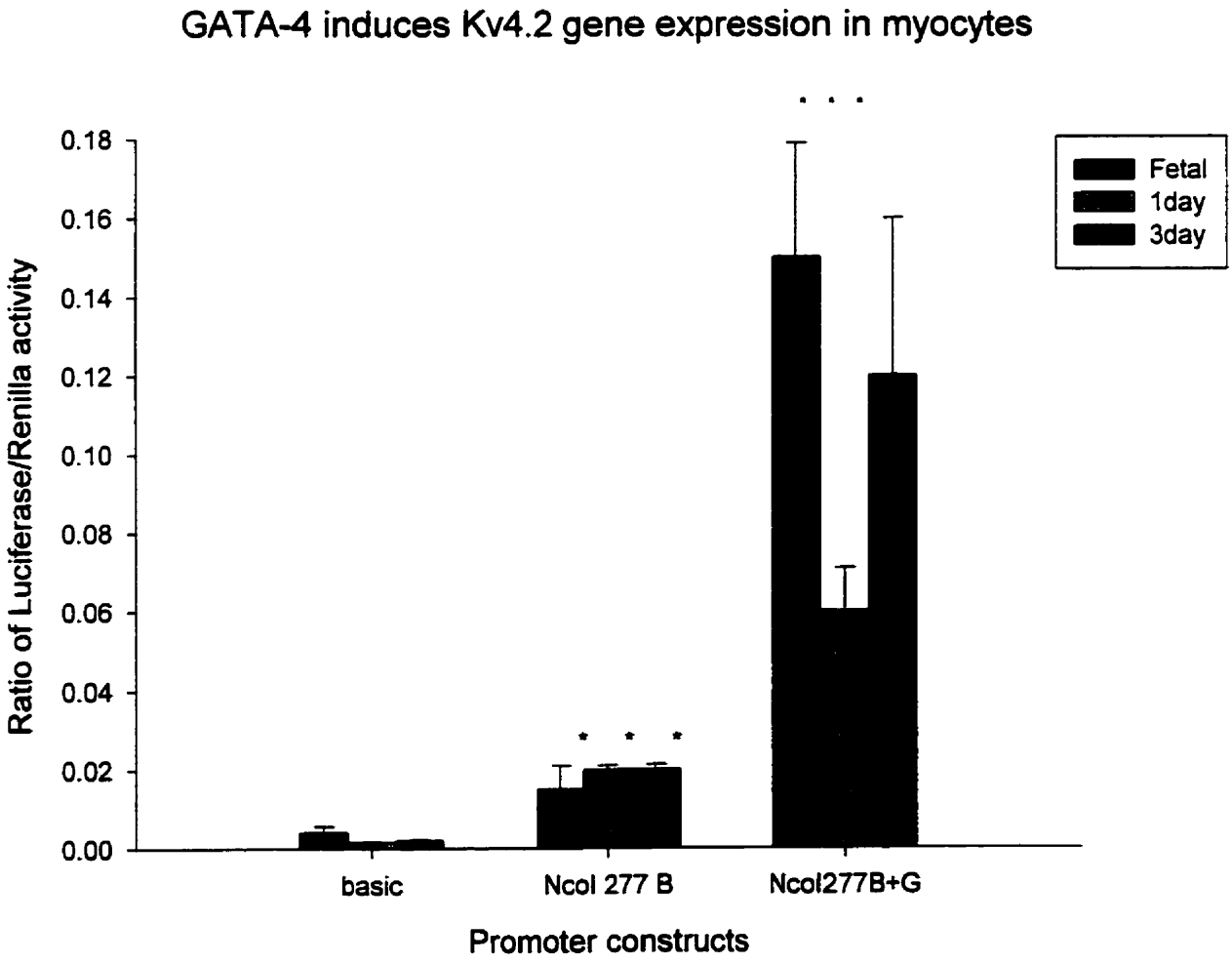

Deletion analysis of Kv 4.2 promoter activity
Fetal
1 day
3 day
Ratio of Luciferase/Renilla activity
0.030
0.025
0.020
0.015
0.010
0.005
0.000
N-127H
N277B
HincII B
Promoter constructs

Figure 16. **GATA-4 induces expression of Kv 4.2 gene in myocytes:** NcoI 277 basic reporter construct was transfected into fetal, 1day, and 3 day myocytes. Co-transfection with GATA-4 significantly increases luciferase activity.

Figure16.



* designates a significant difference (T test) comparing negtive control(basic) and Kv 4.2 promoter construct(NcoI B).

. designates a significant difference (T test) comparing with/without GATA-4 in NcoI 277B construct transfection.

GATA-4 induces expression of Kv4.2 gene has cardiac cell specific manner:

To identify whether GATA-4 induces expression of Kv 4.2 gene in a cardiac-specific manner. N277B reporter construct has been alone and co-transfected GATA-4 into NIH3T3, HEK and H9C2 cells. The luciferase activity can be observed in all three cell lines (**Figure 17A, B**). Of these, only the H9C2 cells have a cardiac myocyte lineage. Co-expression with GATA-4 increased luciferase expression in all three cell lines, but produced the largest increase (~15x) in H9C2 cells. This observation indicated that GATA-4 factor produces its strongest regulatory effect of the Kv 4.2 promoter in cultured mouse cardiac myocytes and cells of a cardiac lineage.

However, these experiments have some limitations. 1). We did not know whether endogenous Kv 4.2 and GATA-4 exist in H9C2 cells or not. 2). We did not know whether endogenous Kv 4.2 and GATA-4 exist in NIH 3T3 and HEK cells or not as well. We will discuss these results late.

Heterologous promoter constructs in transcription regulation events:

In the current study, there are five GATA binding sites in the upstream 5'untranslated region of the Kv 4.2 gene. To understand which sequence contains essential GATA binding sites for inducing expression of Kv 4.2 gene, we use the pTK LUC plasmid to create heterologous promoter constructs.

Thymidine Kinase (TK) promoter has high activity in both cardiac muscle and skeletal muscle. pTK LUC was kindly obtained from Dr Markham. This plasmid was generated by cloning approximately 150 bp of the TK promoter fragment into the pGL3 basic plasmid at the XhoI / Hind III sites [135].

Two heterologous pTKLUC promoter constructs were generated as follows. A 2.1KB fragment Scal I /NcoI from +134/-1993 in Kv 4.2 5'- flanking region was cloned into the Sma I /Kpn I sites of pTKLUC plasmid which contains all five GATA binding sites. Another 1.9 KB Hinc II / Nco I fragment from –127/-1993 in Kv4.2 5'-flanking region was cloned into Sma I /Kpn I site of pTKLUC plasmid which contains only 3 GATA binding sites in 5'- upstream region in Figure 9.

In these studies, we found 1) Comparing the Kv 4.2 promoter reporter constructs alone, transfection of pTK promoter reporter constructs into fetal, 1 day, and 3 days myocytes increased luciferase activity. However, addition of the Kv 4.2 core promoter and enhancer containing region to pTK promoter, both pTKScal and pTK Hinc II constructs did not significantly increase luciferase activities compare to pTK promoter only construct (Figure 18). These results suggest that the repressor element present in Kv 4.2 promoter region. 2) In two of them, cotransfected with GATA-4 have significantly increased luciferase activity. The pTK Scal report construct contains 5 putative GATA-4 binding sites. The luciferase activity is increased about two to five fold mediated by GATA-4 (Figure 18). Whereas pTK Hinc II reporter construct contains 3 putative GATA-4 binding sites. The luciferase activity is increased about three to four fold mediated by GATA-4 (Figure 18). From these data, we assume that the GATA regulatory element present in the Kv 4.2 promoter 5'-upstream region.

Since heterologous enhancer element (SV40 enhancer) do not contribute to Kv 4.2 promoter activity, we do not show data here. However, the lusiferase activity produced by the Kv4.2 plus heterologous enhancer reporter construct was increased by co-expression with GATA-4.

Figure 17A. GATA-4 induces expression of Kv 4.2 gene in a tissue specific manner.

Luciferase reporter constructs were transfected into NIH 3T3 and H9C2 cell lines by the FuGENE method.

Figure 17A.

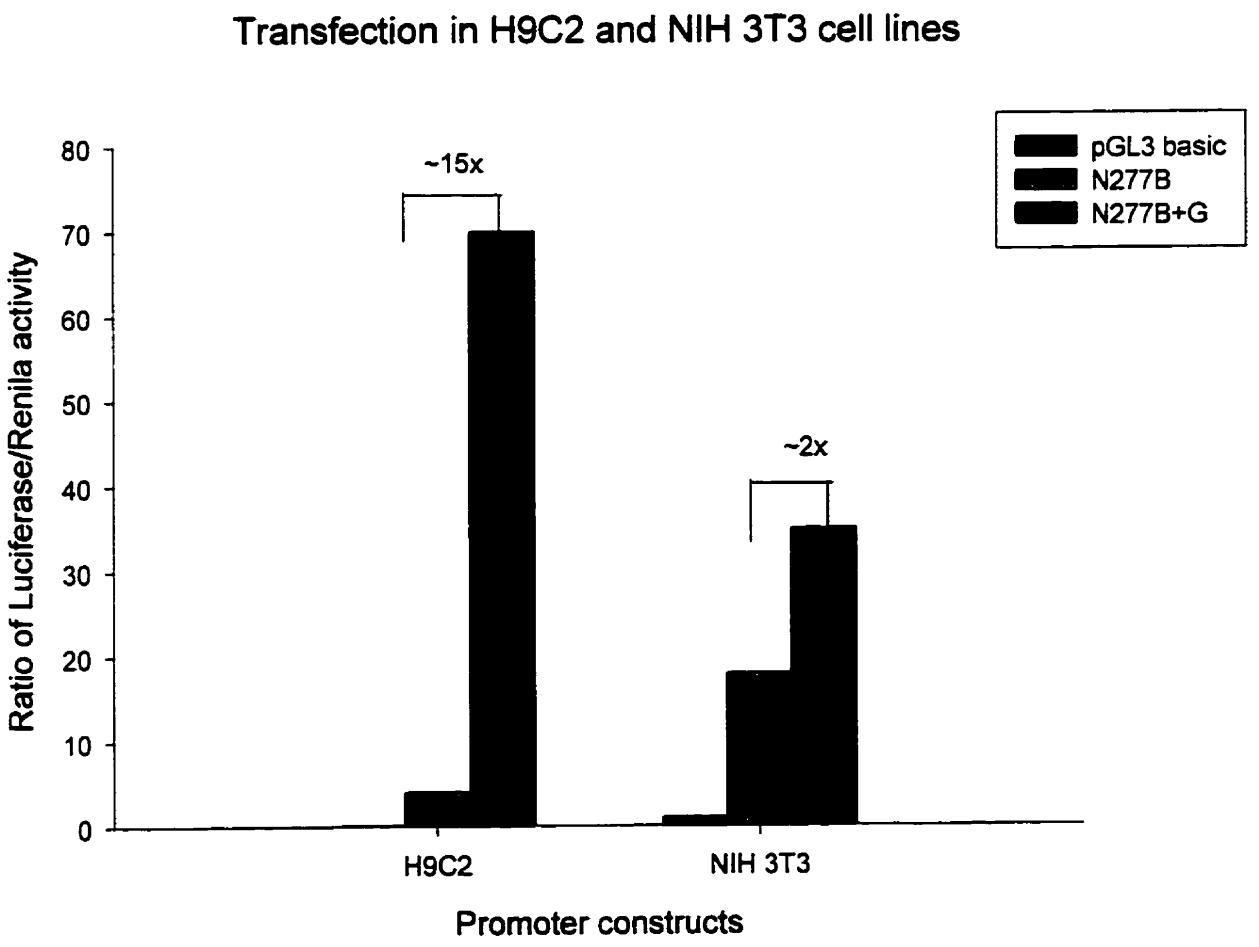

Transfection in H9C2 and NIH 3T3 cell lines
pGL3 basic
N277B
N277B+G
~15x
~2x
Ratio of Luciferase/Renila activity
80
70
60
50
40
30
20
10
0
H9C2
NIH 3T3
Promoter constructs

Figure 17B. GATA-4 induces expression of Kv 4.2 gene in a tissue specific manner. Luciferase reporter constructs were transfected into HEK cell line by the FuGENE method.

Figure 17B

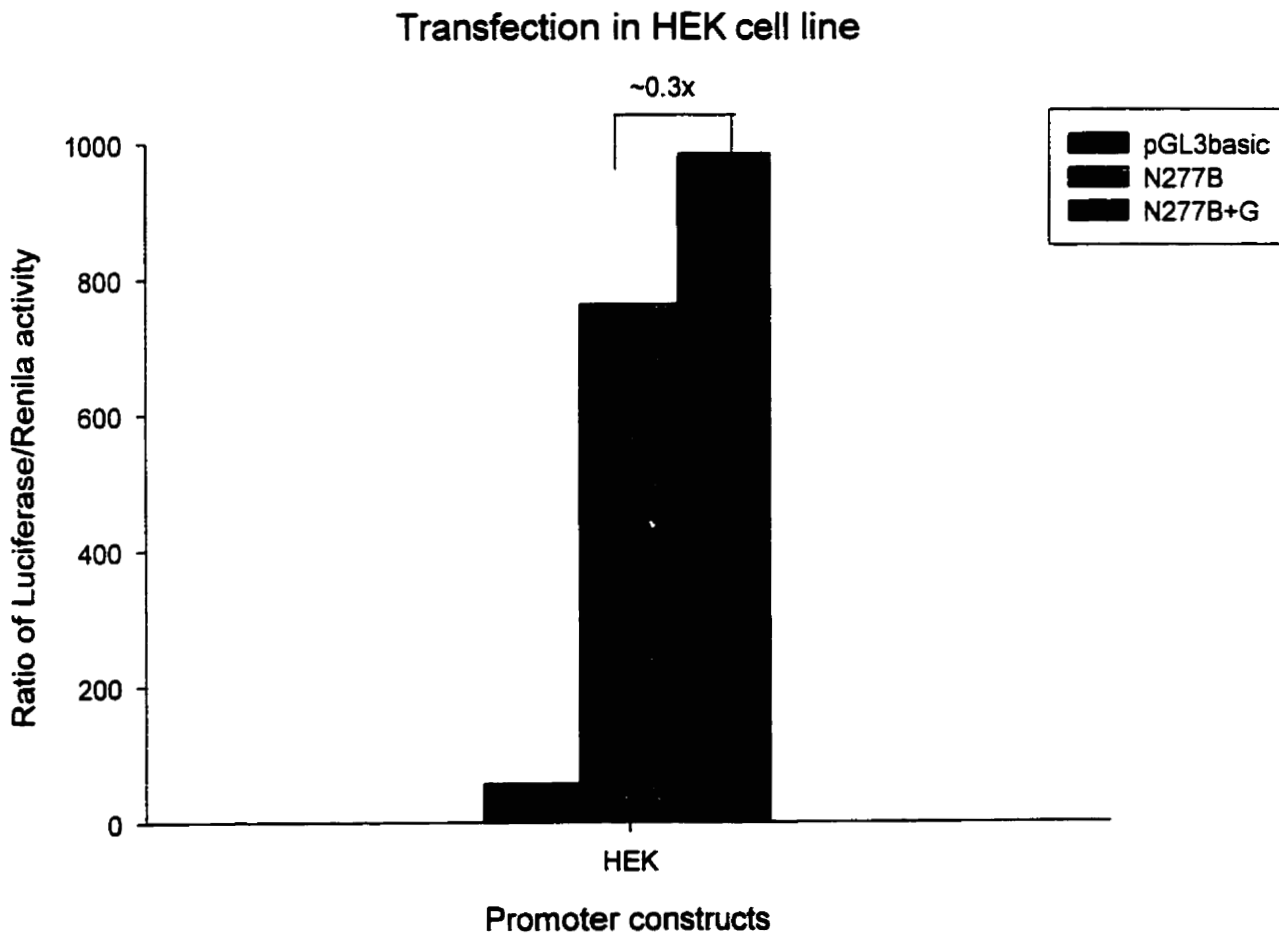
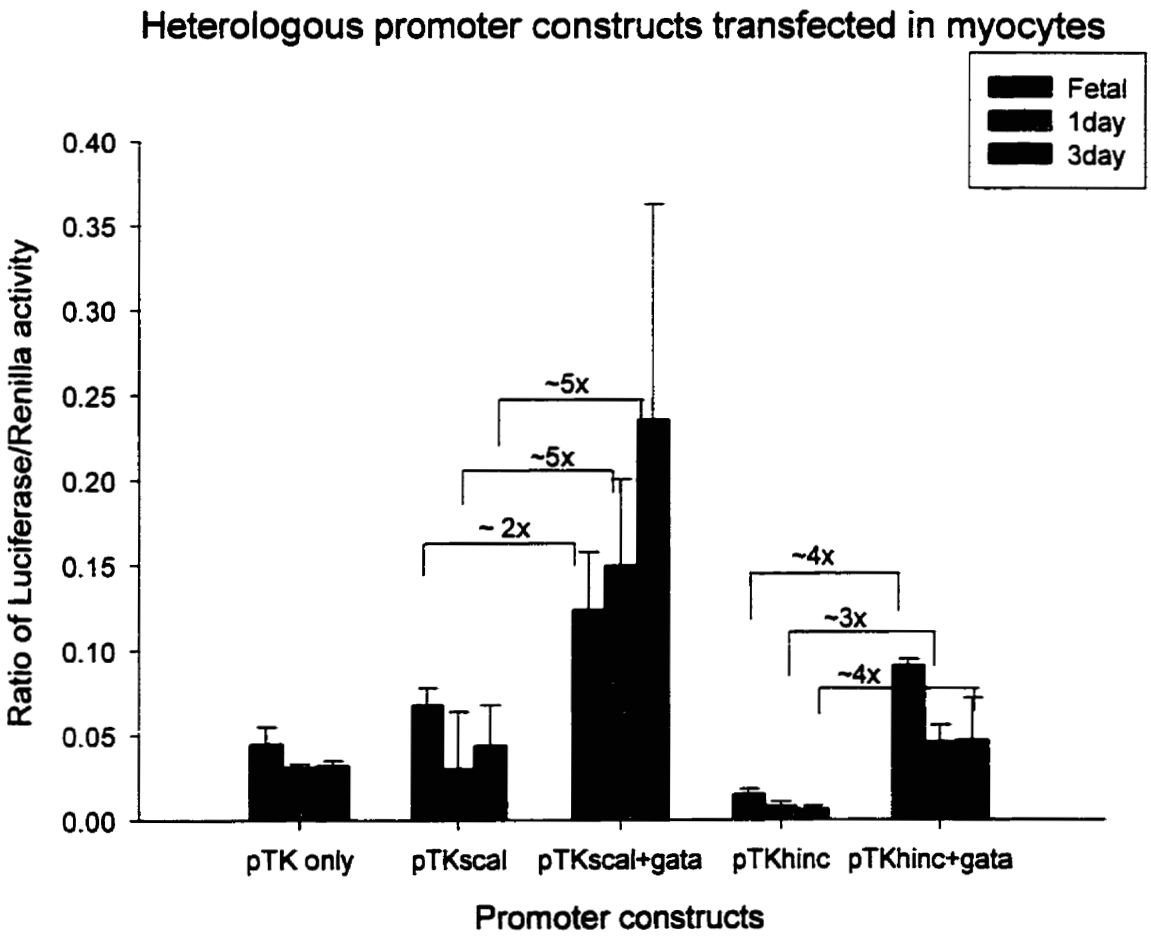

Transfection in HEK cell line
~0.3x
pGL3basic
N277B
N277B+G
Ratio of Luciferase/Renila activity
1000
800
600
400
200
0
HEK
Promoter constructs

Figure 18. **Heterologous promoter constructs transfected in myocytes.** pTK promoter reporter constructs were transfected into fetal, 1day, and 3 day myocytes by SuperFect method. 1). pTK promoter plus Kv4.2 promoter reporter constructs can increase luciferase activity comparing to Kv 4.2 promoter alone. 2) In two of them, co-transfected with GATA-4 have significantly increased luciferase activity. pTK ScaI has significantly increased luciferase activity (about two to five fold) mediated by GATA-4. Whereas pTK Hinc II has significantly increased luciferase activity (about three to four fold) mediated by GATA-4. These results suggest that GATA binding sites presented in 5'upstream region must be important for GATA-4 regulated Kv 4.2 gene expression. Error bars indicated standard deviations (N= 4).

Figure 18.


Heterologous promoter constructs transfected in myocytes
Fetal
1day
3day
Ratio of Luciferase/Renilla activity
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
~5x
~5x
~ 2x
~4x
~3x
~4x
pTK only
pTKscal
pTKscal+gata
pTKhinc
pTKhinc+gata
Promoter constructs

V-4. DISCUSSION

Thus far, studies of the cardiac gene expression have been hampered by the absence of stable cell lines that can be used for *in vitro* studies and transfection assays. We report here that we have established the mouse primary neonatal myocyte cell culture as a source for studying gene transient transfection assays *in vitro.* The use of primary neonatal myocyte cell culture provided a good opportunity to study the cardiac gene expression and regulation in mouse heart muscle cells. Transfection of mammalian cells with DNA has provided the different results that have varied with different cells. In our current studies, Superfect reagent has been successfully used to improve the transfection efficiency in mouse myocytes.

1. **Kv 4.2gene is expressed in cultured primary myocytes.** In this study, we have used promoter-reporter gene constructs to identify the elements regulating cardiac expression of the Kv 4.2 gene. Three 5'flanking region deletions were made to identify the region responsible for essential promoter activity. Deletion of the Kv4.2 core promoter region **N-127H** and Deletion of the Kv4.2 enhancer containing region **HincII B** both resulted in low luciferase activities. However, **N277B** pGL3basic construct containing Kv 4.2 both core promoter and enhancer containing regions was observed to reproducibly increase luciferase activity (Figure 15). These results indicate that Kv 4.2gene is expressed in cultured primary myocytes and 2.2 KB of upstream region sequence is essential to turn on the promoter activity. It appears that the essential promoter activity of Kv 4.2 gene may reside in this fragment.

2. **GATA-4 regulates transcription of Kv 4.2 gene.** GATA-4 elements have an

important role in the transcriptional regulation of several cardiac specific gene. In the present study, five GATA binding sites have been found in the up stream region of Kv 4.2 gene. Co-transfected GATA-4 factors with Kv 4.2 promoter reporter construct can significantly increase luciferase activity in cardiac myocytes (Figure 16). Interestingly, co-transfected GATA-4 factors with pTK promoter plus Kv 4.2 promoter reporter construct can also significantly increase luciferase activity (Figure18). In addition, co-transfected GATA-4 factors with SV40 enhancer plus Kv 4.2 promoter reporter construct can significantly increase luciferase activity as well. Together, these results indicate that the Kv 4.2 gene may be a target for transcription factor GATA-4. The five putative GATA binding sites in Kv 4.2 gene 5' upstream enhancer region may contribute to the regulation of Kv 4.2 gene expression in cardiac myocytes. Heterologous promoter constructs study has found that both pTK ScaI and pTK HincII reporter constructs are significantly increased luciferase activity mediated by GATA-4, these results indicated that the GATA binding sites presenting in 5'upstream region are necessary to act the promoter to express the Kv 4.2 gene. The mechanism responsible for GATA-4 regulation Kv 4.2 gene expression has not been determined. Future studies will provide answers to determine which GATA binding site is related to Kv 4.2 gene regulation.

3. GATA-4 plays a role in cardiac tissue-specific expression of the Kv4.2 gene.

GATA-4 was first described as a transcription factor present in the heart and in endodermally derived tissue. Because of the tissue-restricted distribution of this protein and its ability to regulate expression of several cardiac specific genes, we thought that GATA-4 might be involved in the cardiac tissue-specific expression of Kv 4.2 gene. We tested this hypothesis by performing transfection of a Kv 4.2 reporter construct in three

cell lines. The **N277 B** reporter construct alone have been transfected into NIH3T3, HEK and H9C2 cells. Of these, only the H9C2 cells have a cardiac myocyte lineage. We found that the luciferase activity can be observed in all three cell lines. We also found that H9C2 cells have very low transfection efficiency compared with HEK and NIH 3T3 cell lines (**Figure 17A, B**). However, co-expression with GATA significantly increased luciferase activity only in the H9C2 cells. This suggested that GATA-4 regulates expression of the Kv 4.2 in cultured mouse cardiac myocytes and cells of a cardiac lineage. Why is the Kv 4.2 gene not highly expressed in H9C2 cells is not clear. The explanation for the reasons may be as follows: 1). The currently studied Kv 4.2 promoter region is not complete. 2). H9C2 cell should fully support Kv 4.2 gene expression without adding GATA-4, our results indicated that H9C2 cell may lack endogenous GATA-4. 3). It is possible that there is a transcriptional suppressor factor that silences expression of this K^+ channel gene in cardiac myocytes unless GATA is present. The reason for Kv 4.2 gene highly expressed in HEK cells is not clear either. Perhaps HEK cells contain GATA-4 that fully supports Kv 4.2 gene expression. Perhaps there are some other unidentified factor up-regulated the Kv 4.2 gene that is in HEK cells but not in H9C2 cells.

Chapter VI

CONCLUSION AND FUTURE STUDIES

VI-1. CONCLUSIONS

It is exciting that, during the past decade, major advances have been made in uncovering the mechanisms that switch genes on and off. Study of the molecular events involved in the normal and pathological heart has been greatly advanced with the rapid development of molecular biology techniques. However, how a cell type-specific gene expression pattern can be induced is an unknown question in cardiovascular biology today. Detailed underlying mechanisms of Ion channel protein gene expression and regulation in cardiac muscle are still a new opening area. There are a number of reasons why these investigations have been limited. Since K^+ channel protein is in small amounts in cardiac muscle; K^+ channel promoters may not be strong promoter; and cultured cardiomyocytes are not stable. On top of this, the absence of stable cell lines is the major problem for the cardiac gene *in vitro* studies. An important improvement and advantage of our present study is that we use the cultured mouse neonatal myocytes as a new source to study the cardiac gene expression. It provides an alternate way to study early cardiogenesis *in vitro*.

In the present study, distribution of mRNAs level for Kv4.2 has been tested by using RNase protection assay in mouse. We have found that the Kv 4.2 gene is present in mouse heart and in developmental manners. To investigate the transcriptional mechanisms of the mouse Kv 4.2 gene, The 5'-flanking region of the mouse Kv 4.2 gene

was cloned, and primer extension and RPA determined the transcription initiation sites. We also characterized putative regulatory elements for Kv 4.2 gene expression. We found that GATA-4 is an important activator for Kv 4.2 gene expression in cardiac myoctyes.

VI-2. FUTURE STUDIES

Different molecular mechanisms contribute to the cardiac muscle phenotype. Although we learned a lot in the past decade, we are still far from understanding the cardiac gene programme. Further studies will likely clarify molecular mechanisms underlying the control of K^+ channel gene expression under physiological and pathological conditions.

Site-directed mutagenesis: A simple method for site-directed mutagenesis using the polymerase chain reaction has been described in detail in 1989[136]. In brief, this method uses primers, which contain one or several bases which differ from the wide-type sequence. After PCR, these mutated sequences are incorporated into the PCR product. This altered sequence is then cleaved with restriction enzymes and inserted in place of the wild-type sequence.

In order to test how and where the GATA binding sites are important to regulate Kv 4.2 gene expression, we will use site-directed mutagenesis to construct the absence of GATA binding sites in the Kv 4.2 gene, and to transfect these modified regions into cultured myocytes, and ask whether or not GATA binding sites are necessary to generate high level of Kv 4.2 gene expression.

Gel mobility shift assay: To investigate further the role of the regulatory element, future experiments will be performed to characterize GATA, E-box, and CarG regulatory

elements. The DNA binding assay using nondenaturing polyacrylaminde gel electrophoresis (PAGE) provides a simple, rapid, and extremely sensitive method for detecting sequence-specific DNA-binding proteins. In this experiment, the DNA-binding assay is based on the observation that DNA-protein complexes migrate through polyacrylamide gel more slowly than unbound DNA fragments. An oligonucleotide corresponding to the region surrounding the GATA element will be radiolabeled. If this element recognizes and binds to GATA protein in nuclear DNA extracted from myocytes, we should observe a retarded band compared with oligonucleotides lacking GATA protein binding.

A similar strategy can be used to test E-box, and CarG regulate elements as well.

***In vitro* and *in vivo* gene transfection using virus as transfectional vector:**

Transfection of cells with DNA has become an important tool for studying the expression and function of genes, particularly in mammalian genes. The most widely used transfection methods included calcium phosphate coprecipitation; electroporation; and lipsomal reagents. Success with these methods varies greatly with cell type. In our current studies, K^+ channel gene expression in cardiac myocytes is particularly difficult to establish because the cultured cardiac primary myocytes are very sensitive to general transfection reagents.

A retrovirus vector is an infectious virus used to introduce a nonviral gene into mitotic cells *in vivo* or *in vitro*. Retroviral vectors are useful in achieving stable and efficient transduction of the genes into cells that are not easily transfected, such as primary cells.

Recently, adenoviral vectors have drawn the attention of many of those involved in the field of gene transfection and gene therapy because of their practical advantages and application potential. Therefore, in the future, if we can establish adenoviral-mediated K^+ channel gene transfection *In vitro* and *in vivo*, we can expect to get high transfection efficiency by adenoviral vectors encoding reporter genes. In addition, these viral vectors provide useful tools for studying cardiac ion channel genes during development.

Expression of the mouse Kv 4.2 mRNA in pathological heart:

In our previous study, we have shown the existence of Kv 4.2 mRNA expression in normal mouse heart. The level of Kv 4.2 in pathological mouse heart still remains unknown. Several studies have identified modified cardiac K^+ channel mRNA expression with animal models of hypertension and myocardiac infarction in rat [137,138]. Recently, Dr Tomaselli et al have reported that changes in the level of Kv 4.3 mRNA in human tissues decreased by 30% in failing heart compared with nonfailing controls [139]. Moreover, Dr Olson has reported that GATA-4 is a novel transcriptional regulatory factor in mediating the changes in gene expression associated with cardiac hypertrophy [140]. Despite advances in medical therapy, heart failure, hypertension, and myocardiac infarction remain a major cause of death in the world. It is necessary to evaluate and determine expression of the mouse Kv 4.2 mRNA in pathological heart including different animal models. To understand the mechanism for the reduction of Ito in heart disease models, we have to investigate Kv 4.2 gene transcriptional regulation under pathological condition.

Cloning large region of Kv 4.2 genome:

The current study focuses on a 2.2KB upstream region of the Kv 4.2 gene. With regard to detailed restriction map in Kv 4.2 gene sequence and genomic cloning, we should clone more upstream and downstream region of Kv 4.2. In this way, we can find more related regulatory elements in Kv 4.2 sequence, and also we can understand the mechanism of Kv 4.2 promoter regulation in detail. For example, if we have cloned more of the upstream region, we could locate the promoter initiation site in a larger region. If we have more of the downstream region cloned, we could find whether an intron exists in this region. Meanwhile, we can search for the best DNA fragment required to turn on the Kv 4.2 channel promoter activity.

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