Expression and regulation of the atrial natriuretic factor encoding gene \textit{Nppa} during development and disease

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Abstract

The endocrine function of the heart was shown by the identification of a potent inhibitor of renal tubular NaCl reabsorption, atrial natriuretic peptide/factor (ANP), in atrial extracts. Since then, \textit{Nppa}, the gene encoding ANP, has become an important tool for molecular and developmental biologists. Expression of \textit{Nppa} is an early and specific marker for the differentiating working myocardium of the atria and ventricles of the developing heart. Around the time of birth, ventricular \textit{Nppa} expression is down-regulated, to be reactivated in response to a variety of cardiovascular disorders. Many aspects of the regulatory pathways that control \textit{Nppa} gene expression during cardiac development or disease have been revealed. However, several fundamental issues remain to be resolved. Particularly, the regulatory mechanisms underlying the ventricular \textit{Nppa} expression in the embryonic heart and the reactivation in the failing ventricle remain unclear. Here we review current knowledge on the transcriptional regulation of the \textit{Nppa} gene.

1. Scope

Atrial natriuretic peptide was first identified as a very potent inhibitor of renal tubular NaCl reabsorption that is produced and excreted by the heart [1]. Peptide processing, receptor function, clearance, renal functions and studies of mouse models with altered natriuretic peptide levels have been extensively reviewed [2,3]. Facilitated by its high level of expression, 1% of total atrial mRNA [4], the cDNA for \textit{Nppa} was cloned already in 1987 [5] and the first studies using the \textit{Nppa} promoter in transgenic mice were conducted one year later [6], shortly after the establishment of transgene technology. These studies indicated that a small promoter fragment is sufficient to drive reporter expression in cell culture and in vivo. The developmental expression pattern of \textit{Nppa} appeared to be highly dynamic [7], and was linked to the local differentiation of working myocardium in the developing heart tube [8]. Reactivation of ventricular \textit{Nppa} expression is part of a conserved adaptive change in molecular phenotype in response to heart failure that serves both diagnostic and potential therapeutic options [2,9].

The relevance for physiology and pathophysiology, along with the small size of the gene and the early availability of its compact promoter have turned \textit{Nppa} into an extensively used model for exploring pathways that regulate cardiac gene expression during development and disease. Here we provide an overview of these studies, focusing on the transcriptional regulation of \textit{Nppa}.

2. Evolutionary origin of the mammalian natriuretic peptides

Three mammalian members of the natriuretic peptide gene family have been identified, \textit{Nppa}, \textit{Nppb} and \textit{Nppc},...
encoding ANP, BNP and CNP, respectively. Four distinct C-type natriuretic peptide genes have been identified in two teleosts, the medaka *Oryzias latipes* and the puffer fish *Takifugu rubripes* (Fig. 1) [10]. Based on these findings, a theory of the evolution of the natriuretic peptide genes has been put forward; Chromosomal duplications of an ancestral natriuretic peptide gene gave rise to four CNP genes, CNP-1-4 [10]. *Nppb* and *Nppa* are derived from CNP-3 through tandem duplications. Chicken has lost *Nppa* [11], whereas mammals have lost CNP-3. CNP-4 has been retained in humans as *Nppc* [10].

3. Expression pattern of *Nppa*

The developmental *Nppa* expression pattern has served to gain insight into the transcriptional program governing cardiac chamber development. The early embryonic heart is a simple tube that loops and rapidly elongates by recruiting mesenchyme at its venous and arterial pole. The myocardium of the heart tube has a primary (primitive, embryonic) phenotype and initially does not express *Nppa*. At very specific locations in the heart tube myocardium differentiates further to form the working myocardium, which rapidly expands by proliferation to form the chamber compartments (‘ballooning concept’ [8]). Because of the process of looping and chamber expansion, these ventricular and atrial chambers are seen to develop at the outer curvatures. The expression of *Nppa* is initiated in the developing atrial and ventricular working myocardium. More specifically, at 8.5 days of mouse development, *Nppa* expression of the future ventricular chambers is first observed at the ventral side of the middle portion of the tube. The expression in the future atrial chamber is first observed at 9.25 days of development in the dorsal caudal region. The myocardium flanking the differentiating and expanding chambers retains the primary, *Nppa* negative, phenotype and by now forms the recognizable inflow tract, atrioventricular canal, outflow tract and the interconnecting inner curvatures [8,12]. *Nppa* expression in the cardiac chambers is essentially conserved in the developing human, mouse, rat, fish and *Xenopus*, with the exception of detectable *Nppa* expression in the inflow tract of *Xenopus* (Fig. 2) [7,8,12,13,83].

The sinus node and atrioventricular node are derived from the primary myocardial inflow tract and atrioventricular canal, respectively, and indeed never express *Nppa* [14,15]. In contrast, the peripheral ventricular conduction system and bundle branches, which are derived from the ventricular chambers (trabecular component), express *Nppa* from the earliest stages of ventricular differentiation onward [15]. During fetal development of mouse, rat and human hearts ventricular *Nppa* expression becomes restricted to the trabecular component, and subsequently is down-regulated, first in the right ventricle and later in the left ventricle. Around the time of birth ventricular *Nppa* expression is further downregulated to very low levels, while atrial expression remains high [4,16]. Although the embryonic expression pattern of *Nppa* is highly conserved between different species, the ventricular down regulation in mice was reported to be complete at 15.5 days of development, while in rat the ventricular down regulation was found occurs between day 1 and day 10 after birth [16]. However, recent findings indicate that in both mouse and rat ventricles, downregulation of ventricular *Nppa* occurs only after birth [11].

4. Regulation of heart and chamber-specific expression

4.1. Regulatory DNA sequences of *Nppa* in cultured cells

Transfection experiments with rat *Nppa* promoter–reporter gene constructs in cultures of cardiomyocytes of different developmental stages have led to the identification of functional regulatory domains (Fig. 3) [17]. Activity of upstream rat *Nppa* promoter fragments of 3.7 or 3.4 kbp was observed in both transfected embryonic atrial and ventricular cells, while in cells isolated from neonatal hearts, these constructs showed activity in atrial but not in ventricular cells [17,18]. No activity was observed in non-cardiac cells. These activity patterns are similar to the pattern of endogenous *Nppa* gene activity in these cultured cells. Transfection of 2.5 or 0.4 kbp human *Nppa* promoter–reporter constructs resulted in similar activity, encoding ANP, BNP and CNP, respectively. Four distinct C-type natriuretic peptide genes have been identified in two teleosts, the medaka *Oryzias latipes* and the puffer fish *Takifugu rubripes* (Fig. 1) [10]. Based on these findings, a theory of the evolution of the natriuretic peptide genes has been put forward; Chromosomal duplications of an ancestral natriuretic peptide gene gave rise to four CNP genes, CNP-1-4 [10]. *Nppb* and *Nppa* are derived from CNP-3 through tandem duplications. Chicken has lost *Nppa* [11], whereas mammals have lost CNP-3. CNP-4 has been retained in humans as *Nppc* [10].

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Fig. 2. Expression of cTnI, Mlc2a and Nppa in serial sections of Xenopus and mouse embryos detected by non-radioactive in situ hybridisation. cTnI and Mlc2a are shown as myocardial markers (A – C). In the linear heart tube, no Nppa is detected (D), and during subsequent stages remains confined to atrial and ventricular chamber myocardium (E, F). Arrows in E and F indicate developing atrial appendages. Nppa is not expressed in the atrioventricular canal or outflow tract, and in rat and mouse heart no Nppa expression is observed in the inflow tract. A 638 bp promoter fragment drives chamber specific expression of the LacZ reporter. (G,H). ht: heart tube; ift: inflow tract; oft: outflow tract; la: left atrium; ra: right atrium; lv: left ventricle; rv: right ventricle.

Fig. 3. Schematic representation of the proximal Nppa promoter and genomic locus containing Nppa and Nppb and the flanking genes Clcn6 and MGC33867. Interactions between transcription factors involved regulating Nppa activity and the corresponding cis-elements are shown. Most elements shown have been first identified in the rat Nppa promoter and are conserved in mouse and human.
indicating functional conservation of the \textit{Nppa} promoter between these species \cite{19}.

Sequences located between 700 and 136 bp upstream of the transcription start site were found to be crucial for activity in cardiac cells, and a 136 bp upstream promoter fragment was highly active in non-cardiac cells \cite{17,20}. Deletion from $-700$ to $-380$ did not affect promoter activity in embryonic and 1 day postnatal ventricular cells. However, in 4 day postnatal ventricular myocytes this deletion caused a decrease similar to that seen when the entire region between 700 and 136 was deleted. These data were integrated into a model in which three regulatory domains are recognised, a proximal basal promoter that extends to $-137$ bp, an atrial and embryonic enhancer located between $-300$ and $-380$, and a ventricular enhancer that is located between $-380$ and $-600$ \cite{20}. The \textit{cis}-elements identified in these regulatory domains will be discussed in detail.

4.2. Activity of \textit{Nppa} promoter fragments in transgenic mice

To identify regulatory elements of the \textit{Nppa} promoter mediating cardiac specific expression in vivo, transgenic mice have been generated with promoter–reporter gene constructs integrated into the genome (Fig. 4). The proximal human \textit{Nppa} promoter ($-500/+77$ bp) was fused to the SV40 large T antigen \cite{6}. In adult mice the antigen was not detected outside the heart. Expression was always observed in the atria and in addition in ventricles of two of the six reported lines, indicating that this short promoter fragment is sufficient to confer cardiac restricted expression and, in addition, to drive expression in the atria and to a lesser extent in the ventricles.

Transgenic mice carrying a 2.4 kbp rat \textit{Nppa} promoter–reporter construct showed reporter activity in both the atria and ventricles \cite{16}. In addition, low levels of expression were observed in the hypothalamus. Ventricular activity decreased from 2–5% of atrial activity at day of birth to 0.025% of atrial activity at postnatal day 11, while atrial levels remained constant. Since ventricular down regulation of \textit{Nppa} in mice has been reported to be complete before birth \cite{16}, it was concluded that the rat promoter in transgenic mice is regulated like the endogenous rat \textit{Nppa} gene, and that \textit{cis}-elements in the \textit{Nppa} promoter region of rat and mouse have evolved differently. However, recent studies indicate that \textit{Nppa} expression in the mouse ventricles is downregulated after birth, like that in rat \cite{11}. Furthermore, when compared to atrial levels, ANF levels in the adult rat ventricle are much higher (approximately 1% of atrial levels) than the levels of ventricular transgene expression, suggesting that not all sequences required for ventricular activity were included. Mice carrying 638 or 3003 bp of the rat \textit{Nppa} promoter showed reporter activity in postnatal day 1 ventricles and, in addition, showed detectable expression in the brain. The ventricular levels were 8–24 fold less in adult animals \cite{21}. Based on these quantitative findings it was concluded that both the 638 and the 3003 bp promoter fragments control correct neonatal ventricular expression and subsequent inactivation. However, it has become clear that transgenic constructs are inactivated with ageing \cite{22}. Since the atrial expression levels of the reporter gene in transgenic mice were not compared between day 1 and adult animals, conclusions regarding the ventricular down-regulation should be drawn with care.

The observations that 638 bp of the rat promoter is active in both atria and ventricles before birth and in atria of adult animals led to the conclusion that this fragment contains all regulatory sequences required for regulating the spatiotemporal expression pattern of \textit{Nppa}. However, the expression pattern of a 638 bp promoter reporter construct showed significant differences with the endogenous pattern. In contrast to the endogenous gene, the reporter gene was found to be expressed in the sinus horns of the inflow tract. Furthermore, expression of the reporter gene remained detectable in the compact ventricular myocardium at later stages of development.
stages of embryonic development [23]. In addition, ventricular downregulation of the fragment during fetal stages was not observed. These differences with the endogenous pattern indicate that additional regulatory sequences involved in the spatiotemporal expression pattern must be located outside this fragment. When 7 kbp of Nppa regulatory sequences, from −3.2 kbp to +3.7 kbp relative to the transcription start site, were used to drive expression of the Cre gene, expression was observed in the atria and sinus horns of 6 independent transgenic lines from ED10.5 onward [24]. Surprisingly, no significant activity was observed in the ventricles of any of these lines. Preliminary data indicate that this fragment does not include a ventricular repressor, but that the enhancer for ventricular activity is located outside this fragment (unpublished observations of the authors laboratory).

Transgenic Xenopus embryos carrying 0.6 or 3.4 kbp Xenopus Nppa promoter–reporter gene constructs showed expression restricted to the developing heart, both in atria and ventricles. Expression was first observed at stage 34/35 and became restricted to the atria at about stage 49. However, reporter activity was also observed in the outflow tract, in contrast to endogenous expression, indicating that also in Xenopus regulatory sequences outside of the 3.4 kbp upstream promoter are involved in regulating the endogenous Nppa expression pattern [25].

A 5.1 kbp rat Nppa construct conferred only a fraction of the activity of the 3.6 kbp fragment when transfected into rat ventricles in vivo. This was due to the silencing effect of two AP-2 sites located 4 kbp upstream [26]. These findings confirm the existence of regulatory elements outside the proximal promoter region.

5. Transcription factors regulating the proximal Nppa promoter

The Nppa promoter has been used extensively as a model to identify and study transcriptional regulatory pathways involved in cardiac gene regulation. Transient transfection assays and transgenesis have been deployed to study the regulatory role of transcription factors and their corresponding cis-elements. Here we provide an overview of the cis-elements and transcription factors involved in regulating the Nppa gene (Fig. 3).

5.1. Serum response factor (Srf)

Two serum response binding elements (SRE) are located within the 700 bp Nppa promoter. Srf is a MADS box transcription factor expressed in cardiac, skeletal, and smooth muscle cells, and is capable of activating different sets of target promoters by association with several cofactors. Interactions between Srf and Nkx2-5, Gata4, −5, and −6 and Myocardin have been reported [17,25,27–29]. Srf binding to the SRE is inhibited by Hop, a homeodomain (only) protein. In Hop mutant mice a number of Srf target genes including Nppa, Nppb and α-skeletal actin (SkA) are upregulated [30,31].

5.2. Nkx2-5

Several NK-homeobox binding elements (NKE) have been identified within the Nppa promoter. Nkx2-5 is a member of the NK homeobox factor gene family that plays a critical role in cardiac development. Mutations in human NKX2-5/CSX are associated with atrial septal defects and atrioventricular conduction disorders, reviewed in Ref. [32]. Nkx2-5 cooperates with a number of other factors (see below). Nkx2-5 is expressed in the precardiac mesoderm, the embryonic and the adult heart [33]. The NKEs were found to be required for cardiac specific expression of Nppa reporter fragments in transfection experiments [34–36]. However, in transgenic mice carrying Nppa-cTnI reporter constructs, mutation of an important NKE at −250 did not result in loss of activity but in ectopic expression in the atrioventricular canal [8]. In addition, in transgenic Xenopus embryos carrying 0.6 kbp Xenopus Nppa promoter, mutation of the NKE resulted in only a minor reduction of cardiac expression while the reporter gene became ectopically expressed in non-cardiac tissues. Mice lacking Nkx2-5 show arrest of cardiac development after looping and die after ED10.5. Both Nppa and Nppb expression in the developing ventricles was abolished while atrial expression remained detectable [33,37], indicating that Nkx2-5 is required for ventricular but to a lesser extent for atrial Nppa expression.

5.3. Gata

The proximal Nppa promoter contains two binding sites for Gata zinc finger transcription factors, GATAp (proximal) and GATAd (distal) [20]. Both sites are capable of binding Gata4 and Gata6, which synergistically induce Nppa reporter constructs. Furthermore, Gata4 activates the Nppa promoter in synergy with myocyte enhancer factor −2c (MEF2c), Tbx5, SRF, and Nkx2-5 [38]. Jumonji (JMJ), a transcription factor that physically associates with Nkx2-5 and Gata4 inhibits the synergistic activation by these factors [39]. Friend of Gata-2 (FOG-2), a zinc finger transcription factor, that is expressed in ventricles and to a lesser extent in the atria, was found to repress trans-activation of the Nppa promoter by Gata4 [40]. Gata4 deficient embryonic stem (ES) cells showed reduced potential to differentiate into cardiac myocytes while Gata4 over-expressing P19 embryonic carcinoma cells showed increased differentiation of beating cardiomyocytes [41]. Ventral folding is disrupted in Gata4 deficient mice and as a consequence formation of the heart tube and foregut fails. Two independent heart tubes were formed that express Nppa indicating that although heart tube formation is disrupted, differentiation of chamber myocardium is initiated in these mice [38].
5.4. Mef2c

Mef2c is a member of the myocyte enhancer factor –2 family of MADS box transcription factors and binds to A/T-rich sequences of several muscle specific genes. Mice lacking Mef2c did not express Nppa and hearts did not undergo looping [42]. Mef2c contributes to Nppa activity directly through binding to the low affinity A/T-rich sequence [43,44]. In addition, Mef2c is recruited by and physically interacts with Gata4 and in synergy activates the Nppa promoter. Mutation of the A/T rich sequence or GATAd resulted in a significant decrease of synergistic activation, whereas mutation GATAp completely abolished synergy [44].

5.5. T-box factors

During the last few years the importance of the T-box transcription factor family members Tbx5, Tbx2, Tbx3 and Tbx20 in regulation of Nppa has become apparent. Three T-box binding elements (TBE) have been identified in the proximal Nppa promoter [45,46]. Tbx5, which is mutated in Holt–Oram syndrome, was found to interact with these sites, and to associate with Nkx2-5 to form a heterodimer which synergistically activates the Nppa promoter [45,46]. The TBE-NKE module at −250 appeared to be dominantly required for the synergistic activation. Mice haploinsufficient for Tbx5 showed a marked reduction in the expression of Nppa and the chamber-specific Csx40 gene, while mice completely lacking Tbx5 did not express these genes anymore [45]. In addition, Tbx5 is expressed in a pattern completely overlapping that of Nppa. Taken together, these findings show that Tbx5 is an important activator of Nppa.

At the early stages of heart development Tbx20 expression overlaps with Tbx5 expression, however at later stages of cardiac development, Tbx20 expression becomes largely complementary to that of Nppa [47]. Tbx20 function in regulation of Nppa remains unclear. In one study Tbx20 was reported to interact with Gata4/Gata5 and Nkx2-5 to activate the 700 bp Nppa promoter fragment [48]. Binding of Tbx20 to the TBE sites occurred with much lower affinity than observed for Tbx5. In contrast, when co-transfected with a 3003 bp Nppa promoter fragment, Tbx20 was found to have no effect on Nppa activity when co-transfected with Gata4 or Nkx2-5 and was found to moderately inhibit Nppa activation by Tbx5 in a dose dependant manner [47].

Mutation of either the TBE or NKE located at −259 and −250, respectively, of a Nppa-CtnI reporter construct resulted in ectopic expression in the atrioventricular canal in vivo, indicating that 1) Nppa is normally actively repressed in the atrioventricular canal, as opposed to not activated, and 2) that the repression is mediated by the same TBE-NKE required for activation by Tbx5/Nkx2-5 [23]. Tbx2 and Tbx3, which is mutated in ulnar mammary syndrome, act as repressors of the Nppa promoter by interacting with the TBEs in the Nppa promoter. These factors form a ternary complex with Nkx2-5 and the TBE-NKE site. Furthermore, they were found to efficiently inhibit the synergistic activation by Tbx5, Nkx2-5 and Gata4. Tbx2 and Tbx3 are expressed in the primary myocardium that refrains from chamber differentiation, strictly complementary to the pattern of Nppa expression [14,23,49]. Transgenic analysis indicated that Tbx2 is responsible for repression of Nppa in the primary myocardium, and for repression of chamber differentiation. Mice ectopically expressing Tbx2 in the primary heart tube, completely failed to form chambers and to express Nppa, Cx40 and Csl whereas mice lacking Tbx2 showed ectopic expression of Nppa, Csl, and Cx40 in the atroioventricular canal [49,50]. Taken together, Tbx2, −3 and −5 pattern and function accounts for restriction of Nppa expression to the chamber myocardium through activating and repressing interactions with the proximal Nppa promoter.

5.6. Irx4

Irx4 is a member of the Iroquois homeobox family of transcription factors and is expressed in the embryonic ventricle [51]. Neonatal mice lacking Irx4 did not show down-regulation of SkA and Nppa after birth. However, Irx4 expression overlaps with Nppa throughout development and the Nppa expression pattern before birth in Irx4 null mice was not affected. Therefore, before birth, Irx4 might lack essential cofactors necessary for repressing ventricular Nppa activity after birth [52,53].

5.7. Baf60c

Smarc3 encoding Baf60c, a subunit of the BAF complexes, is expressed in the embryonic heart and is required for heart formation. Baf60c is involved in recruitment of the BAF chromatin remodelling complexes and in transfection experiments activates the Nppa promoter in synergy with Tbx5, Nkx2-5 and Gata4. Mice with impaired Baf60c function show several cardiac malformations with reduced levels of Nppa and Nkx2-5 expression [54].

5.8. Pitx2

Mutation or ectopic expression of the homeobox factor Pitx2, which is mutated in Rieger syndrome, results in disrupted right–left axis determination, cardiac looping disorders and isomerism. Pitx2 works synergistically with Nkx2-5 to activate Nppa promoter–reporter constructs in cell culture [55]. However, Pitx2 is expressed in only a sub domain of Nppa expressing regions in the developing mouse heart [56], suggesting that although Pitx2 is required for cardiac development, its role in Nppa regulation is only minor.
5.9. Hand2

Hand2, a basic helix-loop-helix transcription factor, was reported to activate Nppa in synergy with Nkx2-5 in vitro [57]. However, in looped hearts expression of Hand2 is predominantly found in the right ventricle and outflow tract, almost complementary to the Nppa pattern [58], and mice lacking Hand2 showed normal expression of Nppa [58]. These data indicate that the contribution of Hand2 to Nppa gene activity in vivo is limited.

6. Nppa regulation in cardiac disease

6.1. Nppa regulation and induction in cell cultures

Induction of Nppa is a conserved feature of ventricular hypertrophy [9]. In vitro models used to gain insight into pathways involved in the hypertrophy response will be considered here. Hormonal, genetic and mechanical stimuli result in a hypertrophic response, characterized by activation of immediate early response genes such as c-fos, c-jun, and c-myc, followed by reactivation of an ‘embryonic’ gene program, including the genes Nppa, SkA, tropomyosin and β-myosin heavy chain [9]. A c-fos/c-jun heterodimer was found to directly bind the AP-1 site at −496 to −489 of the Nppa promoter, suggesting a direct interaction between the early-immediate genes and the Nppa promoter [29]. In addition, several factors implicated in regulation of developmental Nppa expression have been found to mediate inducible expression of Nppa in the hypertrophic ventricle, reviewed in Ref. [59], (see Fig. 3A).

6.1.1. Glucocorticoid response

Two glucocorticoid responsive elements (GRE) mediating glucocorticoid induction in transfected cells, have been identified within the 1 kb upstream promoter region of rat Nppa [60]. ANP production showed a similar increase in rat hearts in response to glucocorticoid (dexamethasone) treatment when compared to left ventricular hypertrophy [61].

6.1.2. Adrenergic signaling

Treatment of cultured ventricular cells in vitro with phenylephrine or norepinephrine leads to a 15 fold increase of Nppa mRNA. Both −3003 and −638 bp promoter fragments were induced by α-adrenergic stimulation, whereas further truncations resulted in a decrease of inducibility [62]. Several putative cis-elements within the proximal promoter were identified, including an AP-1 site, an SP-1 site and two SREs. It was found that mutation of both SREs, or mutation of either SRE in combination with the SP-1 site completely abolished α-adrenergic inducibility in vitro [63,64]. Also the A/T-rich element has been implicated in the α-adrenergic signaling [43]. Stimulation of cultured cardiac myocytes with isoproterenol, a β1 and β2 agonist, resulted in an increase in transcription of both −3003 and −638 bp promoter fragments. β-Adrenergic signaling in the hypertrophic rat heart was found to be mediated primarily through the β1-subtype, since isoproterenol induced stimulation of ANF was inhibited by betaxolol, a specific β1 inhibitor but not by a specific β2 antagonist [65]. In accordance with these findings, transgenic mice expressing a constitutively active α-adrenergic receptor displayed elevated ventricular Nppa levels while transgenic mice overexpressing the β2 receptor showed no increased Nppa activity [66].

6.1.3. Hypoxia

In response to restricted oxygen availability, hypoxia-induced genes are activated by binding of hypoxia induced factor, HIF-1, to hypoxia-response elements (HRE). In the rat heart, Nppa is induced in the early phase of ischaemia, preceded by an increase in HIF-1α. HIF-1 was found to bind to an HRE in the 2307 bp rat Nppa promoter. Furthermore, over-expression of HIF-1α induced this Nppa promoter fragment whereas a dominant-negative isoform completely prevented hypoxia induced activation of the Nppa promoter fragment in vitro [67].

6.1.4. Endothelin-1 (ET-1)

ET-1 induces myocardial cell hypertrophy in vitro in both atrial and ventricular cells with induction of Nppa [68]. In the failing heart production of ET-1 is increased. Inhibition of ET-1 improved survival of rats with chronic heart failure and prevented cardiac hypertrophy [69]. ET-1 response in vitro requires both an intact SRE and an intact GATAp, which are juxtaposed in a 30 bp module in the proximal Nppa promoter [28]. GATA4 and GATA6 are both capable of interacting with SRF and forming a ternary complex over this 30 bp cis-element, which activated the −638 bp Nppa promoter fragment [28].

6.1.5. NRSF

Neuron restrictive silencer factor (NRSF), a zinc finger transcription factor, binds to neuron restrictive silencer elements (NRSE) located in the 3′ untranslated region of Nppa, and in the upstream regulatory sequences of Nppb and SkA. In transfection experiments, inclusion of the NRSE sequence silenced activity of Nppa and Nppb promoter constructs in transfected ventricular myocytes [70,71]. Fibronectin, an extracellular matrix protein that accumulates in the hypertrophic ventricle, inhibited NRSF binding to the NRSE upstream of Nppb [71]. Furthermore, ET-1 prevented NRSE mediated Nppa repression. Mice expressing a dominant negative isoform of NRSF driven by the heart specific α-MHC promoter developed dilated cardiomyopathy and had elevated expression levels of Nppa, Nppb and SkA. However, mice which express SRF or molecules believed to be biologically neutral, such as GFP and Gal4, under control of the α-MHC promoter also develop cardiomyopathy with elevated levels of Nppa expression [72–74]. These findings indicate additional experiments are required to elucidate...
whether elevated Nppa levels are caused directly by NRSE, or indirectly by the developing cardiomyopathy.

6.2. Hypertrophic response of Nppa reporter fragments in vivo

The ventricular reactivation of several Nppa promoter–reporter gene fragments in animal models of hypertrophy was addressed in attempts to identify promoter regions mediating the hypertrophy response of Nppa in vivo.

Transgenic mice carrying the −500/+77 Nppa promoter construct were subjected to a 35–45 mmHg pressure gradient across the thoracic aorta. In response, the endogenous Nppa mRNA levels increased 20-fold, while reporter levels remained constant, indicating segregation in regulon gradient across the thoracic aorta. In response, the endogenous Nppa mRNA levels increased 20-fold, while reporter levels remained constant, indicating segregation in regulon.

These results led to the conclusion that developmental ventricular regulation of Nppa and reactivation during disease are regulated by distinct pathways requiring distinct regulatory regions. However, in light of the possible absence of critical ventricular enhancers from the Nppa promoter fragments in question (see Section 4.2), these experiments should be repeated once these enhancers have been identified.

Rat hearts transfected in vivo with 5.1 kbp of the rat Nppa promoter exposed to acute wall stress did not show an increase in reporter activity [26]. This indicates that the hypertrophy response elements are located outside this promoter region, even though reactivation of several shorter fragments has been observed in transfected cardiomyocytes subjected to hypertrophy mimicking signals. In contrast, a 6–12 fold increase was observed after injection of a 3.4 kbp rat Nppa reporter construct into dog ventricles after aortic banding [76]. Deletion of the AP-1 site at −496 to −489 completely abolished induction, whereas fusion of the AP-1 sequence to a β-MHC promoter construct was sufficient to confer significant induction in response to aortic banding [76]. Deletion of the AP-1 site at −496 to −489 completely abolished induction, whereas fusion of the AP-1 sequence to a β-MHC promoter construct was sufficient to confer significant induction in response to aortic banding. The discrepancies in Nppa promoter response may be explained by sequences located between −5.1 and −4 kbp, by species differences between dog and rat, or by epigenetic mechanisms which do not influence injected and transfected plasmids, but do affect transgenes integrated into the genome.

7. Nppa as a marker for stem cell differentiation

Pluripotent embryonic stem (ES) cells or embryonal carcinoma derived (P19Cl6) cells have the ability to differentiate into contracting cardiomyocytes that express cardiac genes including Nppa, GATA4 and Nkx2-5 [77].

Differentiating P19Cl6 and ES cells stably expressing transgenic Tbx5 initiated beating earlier and expressed Nppa and Cx40 at significant higher levels than differentiating control cells, indicating Tbx5 enhances working myocardium differentiation of cardiomyocytes and directly or indirectly controls Nppa expression [46,78]. Based on electrophysiological properties and gene expression profiles, several types of ES cell derived cardiomyocytes may be distinguished including nodal-like, atrial-like and ventricle-like cells [79,80]. The nodal-like cells were identified and characterized using the chicken GATA6 promoter/enhancer. This construct is expressed in the Nppa negative sinoatrial- and atrioventricular nodes and in the atrioventricular bundle in transgenic mice [81]. Cardiac myocytes derived from transgenic ES cells expressing this construct were selected and found to be similar to nodal cardiac cells in vivo with regard to morphology and action potential characteristics. Gene expression characterization of these selected cells using RT-PCR showed that these cells displayed high levels of expression of Msx2, Tbx2 and Tbx3, while only very low levels of Nppa were found. These findings are in line with the expression profile of nodal cells in the developing mouse and chicken heart [14,15,23,49]. In contrast, in an ES cell line stably expressing a human Nppa-EGFP construct a sublineage of Nppa-EGFP positive cells displayed a nodal electrophysiological phenotype [82]. This result is difficult to reconcile with the fact that endogenous Nppa is never expressed in nodal cells.

8. Concluding remarks

Analysis of Nppa gene regulation has provided important insights into mechanisms governing cardiac gene regulation, chamber formation and differentiation. Nppa appears to be a selective and useful marker for the differentiating working myocardium in the embryo. Many aspects of the molecular mechanisms regulating cardiac regionalized expression, including the important role of T-box transcription factors, have been revealed. Several pathways involved in induction of gene expression in the diseased heart have been identified. However, the regulation and regulatory sequences of Nppa proved to be more complex and extensive than initially thought. In particular the regulation of ventricular expression, the perinatal down regulation, and the subsequent reactivation in the diseased heart in vivo have not been resolved satisfactorily. Therefore, current research may focus on the identification and functional analysis of the regulatory sequences of Nppa involved in these processes in vivo.

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References


