Distinct Regulation of Developmental and Heart Disease–Induced Atrial Natriuretic Factor Expression by Two Separate Distal Sequences

Thomas Horsthuis, Arjan C. Houweling, Petra E.M.H. Habets, Frederik J. de Lange, Hamid el Azzouzi, Danielle E.W. Clout, Antoon F.M. Moorman, Vincent M. Christoffels

Abstract—Nppa, encoding atrial natriuretic factor, is expressed in fetal atrial and ventricular myocardium and is downregulated in the ventricles after birth. During hypertrophy and heart failure, Nppa expression is reactivated in the ventricles and serves as a highly conserved marker of heart disease. The Nppa promoter has become a frequently used model to study mechanisms of cardiac gene regulation. Nevertheless, the regulatory sequences that provide the correct developmental pattern and ventricular reactivation during cardiac disease remain to be defined. We found that proximal Nppa fragments ranging from 250 bp to 16 kbp provide robust reporter gene activity in the atria and correct repression in the atroventricular canal and the nodes of the conduction system in vivo. However, depending on fragment size and site of integration into the genome of mice, the fetal ventricular activity was either absent or present in an incorrect pattern. Furthermore, these fragments did not provide ventricular reactivation in heart disease models. These results indicate that the proximal promoter does not provide a physiologically relevant model for ventricular gene activity. In contrast, 2 modified bacterial artificial chromosome clones with partially overlapping genomic Nppa sequences provided appropriate reactivation of the green fluorescent protein reporter during pressure overload–induced hypertrophy and heart failure in vivo. However, only 1 of these bacterial artificial chromosomes provided correct fetal ventricular green fluorescent protein activity. These results show that distinct distal regulatory sequences and divergent regulatory pathways control fetal ventricular activity and reactivation of Nppa during cardiac disease, respectively. (Circ Res. 2008;102:849-859.)

Key Words: Nppa ▪ atrial natriuretic peptide ▪ heart development ▪ hypertrophy ▪ cardiac disease ▪ heart failure

A variety of mechanical, hormonal, and genetic stimuli can cause cardiac enlargement and eventually heart failure. In response to these stimuli, the ventricular myocytes increase in size and activate a hypertrophy response gene program. This gene program is characterized by induction of cardiac contractile protein genes and reactivation of a “fetal” gene program, normally only active during cardiac development (reviewed elsewhere1–2). Of the known genes that are reactivated, Nppa, encoding atrial natriuretic factor (ANF), is probably the best characterized.2,3 Nppa is expressed specifically in the myocardium of the atria and ventricles of the embryonic and fetal heart and is the first marker of their formation.4,5 After birth, Nppa expression is downregulated in the ventricles, where it is reactivated again in situations of cardiac stress.

Proximal promoter fragments (0.5 to 3.4 kbp) of Nppa of human, rat, mouse, and Xenopus have been shown to drive atrial and fetal ventricular expression both in vivo6–11 and in cell cultures12,13 and to provide a hypertrophy stress response in cardiomyocyte cell cultures and after injection as plasmid DNA in the ventricles of failing dog hearts.11,14,15 As such, the proximal Nppa promoter has become the most widely used model promoter that has importantly contributed to revealing transcriptional networks involved in cardiac gene regulation during cardiac development, health, and disease (reviewed elsewhere16–18). Nonetheless, the Nppa promoter fragments have been shown to lack hypertrophy responsiveness in vivo.8 From these findings it has been concluded that the fetal gene program is regulated by sequences within the 0.7-kbp proximal promoter fragment, whereas the reactivation of the “fetal” gene program during disease is regulated by sequences residing outside this fragment and therefore by a divergent pathway. However, a recent study indicated that the ventricular activity is absent from an even larger promoter fragment,19 hindering any conclusions regarding the divergence of fetal and hypertrophy pathways. In addition, this...
lacking function would make the proximal promoter a physiologically less relevant readout for the activity of cardiac transcriptional pathways. To address these issues, we assessed the regulatory activity of the Nppa locus in transgenic mice in detail. We found that the proximal Nppa promoter fragments, currently frequently used in studies of cardiac development and disease, lack critical regulatory functions for ventricular activity during development and in disease. Furthermore, the fetal activity of Nppa is regulated independently from its stress response and reactivation during cardiac disease, both processes requiring distal regulatory sequences. Therefore, ventricular activity before birth and reactivation during disease are regulated by divergent transcriptional pathways.

Materials and Methods

Transgenic Mice

The transgenic promoter–reporter lines −0.7rLacZ and −3/+4mCre and the αMHC-Gal4 heart failure mouse model have been described previously.9,19,20 The −0.7rLacZ construct was targeted as a single copy to the hypoxanthine phosphoribosyltransferase (Hprt) locus as described (Hprt−0.7rLacZ) (Figure 1A and 1B). To generate the −0.7mLacZ construct, we replaced the rat Nppa promoter sequence by that of the mouse Nppa promoter sequence in the −0.7rLacZ construct (Figure 1D). Subsequently, to make the −0.7/+4mLacZ construct (Figure 1D), we replaced upstream sequences in the −3/+4mCre fragment with this −0.7mLacZ construct. The −3mCre construct was generated by truncating the −3/+4mCre construct in the third exon, proximal from the neuron-restrictive silencer element (NRSE) (Figure 1D). A −380/+138 rat Nppa promoter fragment was PCR-amplified and fused to the −230/+126 cTnl-LacZ construct flanked by insulator sequences previously described to generate the 0.25rNppacTnILacZ construct (Figure 1D).

Two BAC clones of a mouse 129 SvJ BAC library (Incyte, St Louis, Mo) harboring Nppa were end-sequenced to establish the genomic sequences they contain. The BAC clone 337 ranged from −27 to +127 kbp and clone 336 from −141 to +58 kbp relative to the transcription start site of Nppa (Figure 1C and 1D). At the transcription start site of Nppa of both BACs, we replaced sequence cc.cac.gcc.agg.ATG.ggc by Egfp using the BAC modification protocol kindly provided by Gong, Heintz, and colleagues.22 Subsequently, the −11/+5mEgfp construct was generated from the modified BAC336-Egfp construct (Figure 1D).

An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org.

Results

The Activity of the Proximal Nppa Promoter Is Context-Dependent

To assess the spatiotemporal activity profile of the widely used 0.7-kbp Nppa promoter fragment, it was targeted as a single copy to the Hprt locus (Figure 1A and 1B). This locus is transcriptionally accessible throughout development and adult life, allowing promoter constructs to retain their activity and specificity. Furthermore, variations in activity resulting from variation in copy number and site of insertion in the chromatin are ruled out.21 Expression of the lacZ reporter driven by the promoter was restricted to the heart throughout development (Figure 2). Onset of transgene expression was observed at embryonic day (E)8 at the ventral side of the heart tube (not shown), comparable with the onset of Nppa expression.9 From E9.5 onward, both the reporter gene and the endogenous Nppa gene were expressed in atrial and ventricular chamber myocardium but not in the atroventricular canal and outflow tract (Figures 2, 3, and 7). Nppa expression is absent from the sinoatrial and atroventricular node.23,24 Analysis of E17.5 embryos revealed that the Hprt−0.7rLacZ construct also was not active in these components (Figure 3I through 3L). However, the transgene was ectopically active in the sinus horns and mediastinal atrial myocardium (Figure 2G through 2I and 3I). After E9.5, ventricular Nppa expression becomes restricted to the trabeculated myocardium, and after birth, it largely disappears (Figure 3). In contrast, before and after birth, the Hprt−0.7rLacZ construct remained active in a transmural pattern in the ventricles (Figures 3, 4A, and 4D). These findings indicate that although the 0.7-kbp promoter drives important aspects of the pattern of Nppa, it lacks regulatory sequences for the correct fetal ventricular pattern and postnatal downregulation and for correct repression in the mediastinal atrial myocardium and sinus horns.

The pattern of the Hprt−0.7rLacZ construct was compared with that of 2 lines in which the −0.7r-kbp promoter lacZ construct was randomly integrated in the genome. Both lines showed similar cardiac expression during development, including ectopic expression in the sinus horns and mediastinal myocardium, albeit that the ventricular activity was relatively weak. In addition, the −0.7rLacZ promoter fragment was ectopically active outside the heart (Figure 2). At birth, line A (−0.7rLacZA) expressed lacZ homogeneously in the atria, whereas some activity was present in the left ventricle (Figure 4B). In the adult heart, the atrial expression had become patchy and the ventricular activity had disappeared (Figure 4E). In line B, expression in the atria was patchy at the day of birth, whereas expression in the ventricles was no longer detectable (Figure 4C). In the adult hearts of this line, only a few atrial cells still expressed the construct (Figure 4F). These observations indicate that the previously observed postnatal ventricular downregulation of the proximal Nppa promoter fragments may not be specific to the ventricle and may be dependent on the site of integration.

Key Regulatory Functions of Nppa Reside Within a Small “Atrial” Module, Whereas Extension of Proximal Promoter Fragments Diminishes Ventricular Activity

Previous studies indicated that the 0.7-kbp Nppa promoter fragment is organized in 3 modules, a ventricular and developmental module, an atrial module, and a basic cardiac promoter.12,25 We tested the contribution of the ventricular module in vivo by placing a 0.25-kbp fragment containing only the atrial module upstream of a cTnl promoter fragment (0.25rNppacTnI) (Figure 1D) that is normally always expressed in the atroventricular canal and only shows limited expression in the atria.9 This resulted in an expression profile comparable with that of the 0.5rNppacTnI fragment containing both the atrial and the ventricular module (Figure 7 and Figure 1 in the online data supplement),9 with repression of
the cTnI promoter fragment in the atrioventricular canal and activity driven in the atria and in the left ventricle. These findings indicate that the regulatory sequences responsible for the atrial and ventricular activity of the 0.7-kbp proximal Nppa promoter reside within the atrial module and that the ventricular module does not contribute significantly.

We previously showed that a 7-kbp mouse Nppa fragment (H11002/H11001 mCre), which includes the 0.7-kbp fragment, is
virtually inactive in the ventricles (Figure 7 and supplemental Figure IIA). By truncation experiments, we tested whether a ventricular repressor either upstream (located between −3 and −0.7 kbp) or downstream (located between −0.7 and +4 kbp) could explain the lack of ventricular activity. Both the up- and downstream truncated construct (Figure 1D) showed almost exclusive atrial expression, similar to /H11002/7rLacZ transgenes (Figure 7 and supplemental Figure IIA through IIC). These findings argue against a repressor located within the 7-kbp fragment that inhibits ventricular expression.

The 7-kbp Nppa fragment is from mouse, whereas the 0.7-kbp promoter fragment that showed activity in the ventricles is from rat. The rat proximal promoter contains marked sequence differences with those of mouse and human. To examine whether these differences could account for the lack of ventricular activity in the larger constructs, we tested the 0.7 kbp of the mouse Nppa promoter (−0.7mLacZ) (Figure 1D). Similar to the rat 0.7-kbp promoter, this construct was active in both the atria and the ventricles, was correctly inactive in the atrioventricular canal and outflow tract, and was ectopically active in the sinus horns (Figure 7 and supplemental Figure IID). Thus, the lack of ventricular expression of the larger constructs cannot be explained by a species difference.

In search for lacking sequences responsible for ventricular activity, we subsequently generated a larger construct of 16 kbp (−11/+5mGfp) (Figure 1D). However, this construct appeared completely inactive in the ventricles as well (Figure 7 and supplemental Figure III). Taken together, our data indicate that enhancers outside a −11- to +5-kbp fragment are required for ventricular activity.

Distal Regulatory DNA Regions for the Ventricular Pattern and Fetal Activity

To identify distal regulatory sequences, we used 2 Nppa containing BAC clones of 150 to 200 kbp, respectively, with 85 kbp of overlapping sequences (Figure 1C and 1D). An enhanced green fluorescent protein (EGFP) encoding reporter gene (Egfp) was inserted at the translation start site of Nppa in both BACs. Transgenic mouse lines were generated carrying the modified BAC clones. EGFP expression in 5 independent mouse lines carrying BAC337-Egfp showed similar spatiotemporal patterns but different expression levels (Figure 5C, 5D, 5G, and 5J). All lines showed ectopic expression in the neural tube (Figure 5C). In situ hybridization on sections showed robust expression of Egfp in the atria but also ectopic expression in the sinus horns and in the mediastinal myocardium (Figure 5G), in a pattern similar to that of the −0.7rLacZ constructs. In the ventricles, the spatial pattern of Egfp expression resembled the endogenous Nppa pattern, restricted to the trabeculae after E9.5 (Figure 5G and 5J). However, the observed ventricular fluorescence and the
signal in in situ hybridization were very low when compared with the atria (Figure 5D and 5G). Three weeks after birth, ventricular fluorescence was no longer detectable (Figure 6D through 6F).

Two independent mouse lines carrying \textit{BAC336-Egfp} showed a similar pattern and intensity of expression. Expression was restricted to the heart, with abundant expression in the atria and ventricles (Figure 5A, 5B, 5F, and 5I). The pre-rv lvlv

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\caption{Comparison of expression patterns of endogenous \textit{Nppa} and the \textit{Hprt-0.7LacZ} transgene. A, C, E, G, and J, Section in situ hybridization of \textit{Nppa}. B, D, F, H, I, K, and L, Section \beta\,-galactosidase staining. avn indicates atrioventricular node; c, compact myocardial layer; la, left atrium; oft, outflow tract; lv, left ventricle; mm, mediastinal myocardium; ra, right atrium; rsv, right systemic vein; rv, right ventricle; san, sinoatrial node; tr, trabeculated myocardium.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Postnatal expression of the \textit{Hprt-0.7LacZ} transgene compared with the \textit{\beta}-galactosidase lines A and B. Whole mount \beta\,-galactosidase staining. Ventricular expression in the \textit{Hprt-0.7LacZ} transgene was not downregulated after birth and remained active both in atria and ventricles (A and D). The expression in both \textit{\beta}-LacZ lines was inactivated in the atria as well as in the ventricles (B, E, C, and F). Arrowhead in B indicates the small amount of ventricular activity present at the day of birth in \textit{\beta}-LacZ line A. Arrowhead in F indicates the few atrial cells still expressing \textit{\beta}-LacZ in the adult atrium of line B. la indicates left atrium; lv, left ventricle; oft, outflow tract; ra, right atrium; rv, right ventricle.}
\end{figure}
and postnatal patterns of expression of \( \text{Nppa} \) and \( \text{BAC336-Egfp} \) were identical, including absence of expression from the mediastinal myocardium and sinus horns (Figure 5E, 5F, 5H, and 5I) and downregulation of ventricular activity after birth (Figure 6A through 6C), indicating that this BAC clone contains all regulatory sequences involved in fetal spatiotemporal \( \text{Nppa} \) gene regulation.

To assess whether the BAC sequences provide correct fetal ventricular activity and perinatal downregulation, the atrial and ventricular expression levels of \( \text{Nppa} \) and \( \text{Egfp} \) were
quantified before and after birth (Figure 5K). At E17.5, ventricular expression of both Nppa and BAC336-Egfp were \( \approx 5\% \) of atrial expression. After birth, both were downregulated in the ventricles to 1% of atrial expression. In contrast, in E17.5 embryos carrying BAC337-Egfp, ventricular Egfp expression was <1% of atrial Egfp expression and remained at this low level after birth. Subsequently, we calculated the ratios of Egfp and Nppa mRNA levels in E17.5 atria and ventricles of 2 BAC336- and 3 BAC337-Egfp transgenic lines (Figure 5L). In both BAC336-Egfp lines, the ratio of the levels of Egfp and Nppa mRNA in the atria was similar to the ratio in the ventricles, whereas in all BAC337-Egfp lines, the ratio of Egfp and Nppa mRNA in the ventricles was \( \approx 10\% \) of the ratio found in the atria. These findings indicate that BAC337-Egfp lacks a ventricular enhancer that is mainly active before birth; this enhancer is located, instead, within the unique sequences of BAC336-Egfp (Figure 7).

**Distal Regulatory Sequences Control the Stress Response of Nppa**

In the Hprt locus, ventricular activity is maintained even in the adult heart. To test whether the 0.7-kbp proximal Nppa
promoter in this transcriptionally favorable context is inducible in the failing ventricle in vivo, we subjected these mice to cardiomyopathy using a transgenic model in which Gal4 is driven by the α/MHC promoter (αMHC-Gal4). All male Gal4-positive offspring develop aspecific dilated cardiomyopathy 3 weeks after birth, which is associated with strong induction of Nppa. Although endogenous Nppa was strongly induced in double transgenic mice, the Hprt−0.7rLacZ construct was not (Figure 6G). Subsequently we tested the 7-kbp fragment, which lacks the capacity for embryonic ventricular expression but contains an NRSE implicated in induction of Nppa promoter fragments in heart failure. This −3/+4mCre construct was not reactivated in the ventricles either (Figure 6G).

In contrast, mice carrying either BAC336-Egfp or BAC337-Egfp showed strong reactivation of Egfp in the failing

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Figure 7. Overview of the activity of regulatory Nppa sequences. Black lines depict the Nppa locus, gray lines depict DNA fragments described in literature, and dark green lines fragments analyzed in this study. All fragments were studied in transgenic mice, except for the first 3 (7), which were analyzed in cultured cardiomyocytes. A indicates atrium, act., ventricular activity; AVC, atrioventricular canal; patt., ventricular pattern; HT, hypertrophy; Ref, reference; nd, not determined; na, not applicable; V, ventricle; −, no expression; +, expression; +/-, expression in some lines; ++, high activity (comparable with endogenous Nppa).
ventricles (Figure 6A, 6D, and 6G). In ventricles of BAC336-
Egfp mice, Egfp was upregulated 12-fold relative to its level
in healthy littersmates, which is similar to the upregulation
of endogenous Nppa. In mice carrying BAC337-Egfp, an excep-
tional 50-fold induction of Egfp over basic ventricular ex-
pression level was observed, the height of the induction
being, at least in part, attributable to the low basic expression
level of BAC337-Egfp in the healthy ventricle. Also, trans-
verse aortic constriction of mice carrying these BACs in-
duced the expression of both Nppa and Egfp (Figure 6B, 6C,
6E, and 6F and supplemental Figure III), demonstrating that
the regulatory sequences in both BACs mediate the response
of Nppa to pressure overload hypertrophy. These results show
that both BAC clones contain the regulatory sequences
required for the cardiac stress response of Nppa (Figure 7).

Discussion

Proximal Nppa Promoter Fragments Lack
Regulatory Functions for Ventricular Activity

In the developing heart, Nppa serves as a marker for differenti-
ettating atrial and ventricular working myocardium, whereas
its expression is excluded from the sinus horns, atrioventric-
ular canal, outflow tract, and nodes of the conduction sys-
tem.4,5,25 After birth, Nppa is expressed at very high levels in
the atria (≈1% of total mRNA), whereas its expression in the
ventricles is downregulated.27 Reactivation of Nppa expres-
sion is part of a highly conserved adaptive change in gene
expression in response to hypertrophy and heart failure,
serving both diagnostic and potentially therapeutic options.
Because of these properties, Nppa has become a widely used
model gene for studying gene regulation and monitoring phenotypic changes during cardiac disease (reviewed else-
where18,28,29). Proximal promoter fragments are frequently
used as readout tools for the activity of a wide range of tran-
scriptional pathways that control patterning of the devel-
opment heart and gene regulation in healthy, hypertrophic,
or failing ventricular myocardium (reviewed elsewhere16–18). It
is therefore important to know which regulatory functions the
proximal promoter contains. Promoter fragments ranging from −3.4 to −0.5 kbp were reported to efficiently drive
expression in the atria and, in several cases, also in the ventricles of transgenic mice and Xenopus.9–10 Furthermore,
analysis of promoter activity in transfected atrial and ventric-
ular myocytes and noncardiac cells has indicated that the
Nppa promoter is organized in 3 modules: a basic cardiac
promoter, a developmental- and atrial-specific module, and a ventricular enhancer located just upstream of the
atrial module.12,30,31 These studies have led to the present
view that the proximal Nppa promoter correctly drives
atrial and fetal ventricular expression and postnatal ven-
tricular downregulation.

Our present data reveal that the proximal promoter frag-
ments lack important regulatory functions required for fetal
ventricular activity. All promoter fragments analyzed were
active in the atria and correctly inactive in the atrioventricular
canal and outflow tract of the developing heart and in the
nodes of the mature heart (Figure 7). The “atrial” module in
the context of the cTnI promoter was found to be largely

sufficient to provide these characteristics (Figure 7 and
supplemental Figure I). These results provide in vivo support
for previous studies showing that the activity of the promoter
is mediated by T-box factors and Nkx2–5 that act on sites
within this atrial module.9,32,33 In contrast, the ventricular regulatory characteristics appeared to largely lack from prox-
imal promoter fragments. Firstly, the fetal transmural pattern
of expression of the Nppa gene was not recapitulated in
transgenic mice. Secondly, whereas ventricular activity was
observed in the favorable genomic context of the Hprt locus,
in randomly integrated constructs, it was weak and absent in
the context of larger Nppa fragments (up to −11/+5 kbp).
Thirdly, the activity of the 0.7-kbp promoter was not cor-
rectly downregulated in the ventricles after birth. In the
context of the Hprt locus, the Nppa promoter fragment
remained active, and in the randomly integrated constructs,
the inactivation was nonspecific, as also the atria lost expres-
sion. Finally, analysis of the BAC transgenic mice revealed
that fetal ventricular expression requires 2 distinct distal
sequences. The ventricular transmural pattern requires addi-
tional regulatory sequences located between −27 to −11 kbp
and/or +5 to +58 kbp relative to the Nppa gene, whereas
fetal ventricular activity is provided by a distinct strong fetal
ventricular enhancer that we located more upstream between
−141 to −27 kbp (Figure 7). Therefore, the atrial module
within the proximal promoter may provide only residual
ventricular activity, which is unmasked in a favorable genomic
text. Taken together, we conclude that the activity pattern of the Nppa proximal promoter region is very
useful to study atrial gene activity and repression in the nodes
of the conduction system but does not represent a physi-
ologically relevant readout for ventricular gene regulation in
the developing and adult heart.

Distinct Regulatory Sequences and Divergent
Pathways Drive Fetal Activity and Stress Response

The proximal promoter of Nppa is responsive to hypertrophic
stimuli in some experimental settings11,14,15 but does not
respond to ventricular hypertrophy in transgenic mice in
vivo.5 Our analysis confirms and extends these observations
in a different heart disease model. Firstly, we tested a larger
fragment that contains an NRSE that has been implicated in
the hypertrophy response of Nppa. Although this NRSE is
sufficient to mediate a response to hypertrophic stimuli in
vitro through neuron-restrictive silencer factor,26 our results
indicate this is not the case in vivo. Secondly, in previous
studies, the postnatal expression and the inducibility of Nppa
promoter fragments has been tested in mice carrying ran-
domly integrated promoter constructs, which, according to
our findings, may become downregulated by postnatal silenc-
ing that is not specific to the ventricles. We circumvented this
potential problem by testing the inducibility of the 0.7-kbp
promoter in the context of the Hprt locus that stays transcrip-
tionally accessible throughout life. Again, the transgene was
found to be nonresponsive in the cardiac disease model.
Taken together, all data consistently indicate that the prox-
imal promoter region lacks critical sequences that mediate the
induction of Nppa, rendering the fragment completely non-
responsive to cardiac disease in vivo.
Based on the assumption that the proximal promoter fragments drive fetal ventricular activity, previous studies indicated that reactivation of the fetal gene program during cardiac disease is regulated by pathways distinct from those that regulate prenatal ventricular activity. As discussed above, our analysis shows that also the fetal ventricular activity functions are lacking from the proximal promoter fragments. Therefore, the previous conclusion regarding the divergent pathways was premature.

The analysis of 2 BAC transgenic mice revealed that ventricular Nppa regulation requires a multipartite ventricular regulatory module. This module consists of the proximal 0.25-kbp atrial module driving residual ventricular expression and at least 2 additional distal sequences (Figure 7). Whereas BAC337-Egfp provided the correct fetal transmural ventricular pattern, and was appropriately reactivated during disease, its ventricular activity before birth was similarly low as in the adult heart, indicating that BAC337 lacks a ventricular enhancer that induces activity before birth. In contrast, BAC336-Egfp activity mimicked all aspects of ventricular Nppa regulation, including fetal transmural pattern, abundant fetal activity, perinatal downregulation, and reactivation in hypertrophy and in heart failure. These results indicate that shared sequences, residing within both BAC clones, may regulate the typical fetal transmural (trabecular) ventricular pattern and reactivation in cardiac disease (Figure 7). Importantly, this analysis also revealed that the sequences required for activity in the fetal ventricle are located in a different genomic region, more than ~27 kbp upstream (Figure 7). Therefore, the reactivation in the failing ventricle and the fetal ventricular activity are regulated by distinct distally located sequences and, consequently, divergent pathways converging on these sequences. Further mapping will be required to locate the discrete cis elements and factors that mediate these respective activities.

Acknowledgments
We thank Dr Leon de Windt and Hans van der Linden for their contributions. We thank the facility for genetically modified mice (Marian van Roon) for generating the transgenic mice.

Sources of Funding
This work was supported by Netherlands Heart Foundation grant 96.002 (to V.M.C. and A.F.M.M.), Netherlands Organization for Scientific Research Vidi grant 864.05.006 (to V.M.C.), and European Union FP6 contract “Heart Repair” LSHM-CT-2005-018630 (to V.M.C. and A.F.M.M.)

Disclosures
None.

References


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Circ Res. 2008;102:849-859; originally published online February 14, 2008; doi: 10.1161/CIRCRESAHA.107.170571

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTARY DATA

Materials and methods

Transgenic mice, supplementary data

Hprt-0.7rLacZ

Of the targeted Hprt-0.7rLacZ two independent transgenic lines were established in which we verified a single integration event at the Hprt locus by Southern blot. Both lines showed identical expression patterns.

-3mCre

To generate the -3mCre construct we truncated the -3/+4mCre construct upstream of the NRSE, by cloning the human growth hormone polyadenylation signal into the PvuI site located in the third exon of Nppa.

Bac336-Egfp and BAC337-Egfp

The two-step BAC modification protocol previously described by Shiaoching Gong and Nathaniel Heinz,1 consists of two homologous recombination steps. After both the co-integration and the resolution step correct recombination was verified by Southern blot using a hybridization probe against Egfp. The BAC-Egfp constructs were purified using a CsCl gradient following a protocol also kindly provided by Shiaoching Gong and Nathaniel Heinz.

-11/+5mEgfp

To generate the -11/+5mEgfp construct, we started with the modified BAC336-Egfp construct. Using conventional restriction enzymes we cloned a fragment reaching from the first natural SalI site at -11 kbp upstream of Nppa, to the first natural XhoI site at +5 kbp downstream of Nppa.
From all short randomly integrated constructs vector sequences were removed and constructs were injected into pronuclei of zygotes of FVB mice and these were re-implanted into pseudo-pregnant foster mothers by use of standard techniques. Undigested circular BAC constructs were injected into pronuclei to generate transgenic mice.

Heart failure mouse model
Mice carrying the Hprt-0.7rLacZ or -3/+4mCre construct, or one of the modified BAC-Egfp clones, were crossed with the αMHC-Gal4 heart failure mouse previously described. Gal4 positive and double positive male offspring developed heart failure three weeks after birth. When dyspnoea was diagnosed, mice were terminated. On postmortal examination all Gal4 positive male mice showed an enlarged heart and a swollen, yellowish liver, signs of heart failure with congestion. Atria and ventricles of double positive hearts and of Cre, LacZ or Egfp single positive hearts of littermates were separated and RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) following the protocol of the manufacturer. First strand cDNA was synthesized using an optimized reverse transcription protocol.

Aortic banding
Transverse aortic constriction (TAC) was performed in 3- to 4-month-old mice, carrying the -3/+4mCre construct, or one of the modified BAC-Egfp clones. The aorta was subjected to a defined 25-gauge constriction between the first and second truncus of the aortic arch as described. Aged-matched unbanded animals were used as controls. When discomfort (dyspnoe, decreasing mobility) was diagnosed between the 2nd and 3rd week after TAC, mice were terminated and heart tissue was collected. On postmortal examination all mice included
in the qRT-PCR experiment had an enlarged heart and/or a stiffened, more solid appearance. RNA isolation and cDNA synthesis was performed as described above.

Animal care was in accordance with national and international guidelines.

**Quantitative real time PCR**

Quantitative real time PCR was performed using a LightCycler Real-Time PCR system (Roche Diagnostics, Almere, The Netherlands). The relative start concentration \([N(0)]\) was calculated using the following equation: \([N(0)] = 10^{\log(\text{threshold})-\text{Ct(mean Eff)}}\). Values were normalized to \(\text{Gapdh}\) expression levels.

**Non-radioactive in situ hybridisation and \(\beta\)-galactosidase activity detection**

Whole mount \(\text{in situ}\) hybridization, \(\text{in situ}\) hybridization on sections and whole mount and cryosection \(\beta\)-galactosidase activity staining were performed as described previously.\(^5\,6\)
References


Supplementary Fig. 1. A 0.25 kbp ‘atrial’ regulatory module correctly represses *Nppa* in the AVC and drives atrial and residual ventricular expression. (A) A 356 bp *cTnl* promoter fragment is always expressed in the atrioventricular canal and only shows limited expression in the atria and ventricles. (B) As previously shown,7 a fragment of -638/-138 bp of the rat *Nppa* promoter is able to impose inhibition of expression upon the *cTnl* fragment in the atrioventricular canal, while increase of expression is observed in the atria and to a lesser extent in the ventricles. (C) All elements required to inhibit expression in the atrioventricular canal and to drive the atrial and ventricular expression reside within a 0.25 kbp module of the *Nppa* promoter. ra, right atrium; la, left atrium; rv, right ventricle; lv, left ventricle; avc, atrioventricular canal; oft, outflow tract.
Supplementary Fig. 2. Correct ventricular expression requires distal regulatory sequences that reside outside a -11 to +5 kbp promoter fragment. (A) As previously shown, extension of the promoter fragment to 7 kbp (-3/+4mCre) diminishes ventricular activity. This lack of activity is not caused by a specific ventricular repressor located either up- or downstream of the 0.7 kb proximal promoter fragment as indicated by the lack of ventricular activity in the -3mCre (B) and the +4mLacZ transgenes (C). (D) A mouse -0.7mLacZ promoter fragment behaves similarly as the rat -0.7rLacZ promoter fragment, with activity in both the atria and the ventricles and ectopic expression in the sinus horns. (E) A promoter fragment of 16 kbp (-11/+5mEgfp) does not drive any activity in the ventricles either. Arrowheads point at the sinus horns. See legend to supplementary Fig. 1 for other abbreviations.
Supplementary Fig. 3. Induction of *Nppa*, *BAC336-Egfp* and *BAC337-Egfp*, but not the -3/+4mCre construct, in mice after induction of hypertrophy by transverse aortic constriction (TAC). In all mice carrying the *BAC336-Egfp* (n=3) or the *BAC337-Egfp* (n=3) construct, green fluorescent protein was visibly upregulated upon pressure overload. This finding was confirmed by quantitative RT-PCR. All groups >/=3. Error bars indicate SEM. RG, reporter gene.