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CHAPTER V

Targeting of ANF promoter fragments to the HPRT locus reveals their regional activity in the heart

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ABSTRACT

The *in vivo* analysis of cardiac gene function relies greatly on the use of transgenesis. However, due to the integration in a random genomic locus of the DNA and the unpredictable copy number, the specificity, pattern and level of the transgene differs between distinct lines that harbour the same construct. Therefore, the conventional method is of limited use and in particular not suitable to study small regulatory DNA sequences or to investigate the consequences of single nucleotide mutations. We introduced regulatory DNA fragments of the *ANF* gene into the *hypoxanthine phosphoribosyltransferase (HPRT)* locus by homologous recombination in embryonic stem (ES) cells. This approach guarantees a single copy of the transgene integrated at a defined location in the genome. The activity and pattern of the 0.7 kb *atrial natriuretic factor (ANF)* promoter integrated in the *HPRT* locus was evaluated against that of the endogenous *ANF* gene and of randomly integrated *ANF* transgenes. The pattern of expression provided by the 0.7 kb *ANF* regulatory region is similar to the endogenous *ANF* expression pattern. Compared to random integration, *HPRT*-targeted ANF transgenic mice display a strong and selective expression in the heart. In addition, when integrated in the *HPRT* locus, transgene expression solely depends on the regulatory DNA elements of the integrated construct. Preliminary results of mutants and smaller regions of the *ANF* promoter region integrated in the *HPRT* locus suggest that this method is also suitable to examine the activity of relatively small regulatory DNA sequences as well as to study the effect of single nucleotide mutations. Our findings indicate that the *HPRT* targeting system is a powerful tool for the *in vivo* analysis of cardiac regulatory DNA sequences.
INTRODUCTION

The atrial natriuretic factor (ANF) gene encodes atrial natriuretic peptide, a cardiac-specific peptide hormone that in the mature heart is secreted by the atria and exhibits potent diuretic, natriuretic and vasorelaxant effects. In addition, the hormone plays an important role in the regulation of body fluid pressure and blood pressure (Ballermann and Brenner 1986; Needleman and Greenwald 1986), and serves as a marker for cardiac hypertrophy during which the gene is strongly up-regulated in the ventricles (Chien et al. 1991). The ANF gene shows a characteristic spatial and developmental pattern of expression. In the mature heart ANF gene expression is restricted to the atrial appendages and the peripheral ventricular conduction system. However, during development its expression is specific for the forming atrial and ventricular chambers in the heart, thereby serving as a marker for the forming chamber myocardium (Christoffels et al. 2000b). Studying the regulatory pathways that control the ANF chamber-specific expression could provide valuable insights in the process of chamber formation.

Transient transfection analysis of distinct ANF promoter fragments in ventricular, atrial and non-cardiac cells indicate that the 0.7 kb fragment of the rat ANF regulatory region is sufficient to drive cardiac expression (Argentin et al. 1994). Furthermore, it was demonstrated that the ANF promoter region is composed of distinct modules (Durocher and Nemer 1998) and interacts with several general and cardiac-specific transcription factors (Grepin et al. 1994; Lyons et al. 1995; Lin et al. 1997; Durocher and Nemer 1998; Tanaka et al. 1999; Charron et al. 1999; Morin et al. 2000; Molkentin et al. 2000; Hiroi et al. 2001; Bruneau et al. 2001; Habets et al. 2002). To date, our knowledge of the regulation of the ANF gene in vivo depends on conventional transgenesis in which a variable number of transgene copies are integrated into random genomic loci. These analyses have shown that the 0.7 kb fragment of the rat ANF promoter and the 0.5 kb region of the human ANF promoter mimics endogenous ANF gene expression (Field 1988; Seidman et al. 1988; Knowlton et al. 1995; Habets et al. 2002).
A construct that is randomly integrated into the genome is affected by the genomic environment. This results in variable levels of transgene expression that are often not correlated with copy number, in mosaic expression, in ectopic expression and in silencing by the surrounding chromatin. Therefore, transgenic lines generated by this method are only of limited use. For example, to increase the reliability of an obtained transgene expression pattern, distinct transgenic lines need to be generated that harbour the same transgene construct. Furthermore, as the severity of the problems mentioned generally increases when decreasing the size of the regulatory DNA fragment to be analysed, random transgenesis is less suitable for studying relatively small regulatory DNA fragments and not suitable to study consequences of inactivation of a single DNA binding site, unless the consequences of the mutation are very pronounced.

To study the regulation of the ANF gene in vivo in a controlled fashion, we have explored the use of the hypoxanthine phosphoribosyltransferase (HPRT) targeting system developed by Bronson and colleagues (Bronson and Smithies 1994). The approach is based on homologous recombination into the HPRT locus of embryonic stem (ES) cells that lack a functional X-linked HPRT gene. The lack of this gene product causes cells to die when grown on a selective medium containing hypoxanthine, aminopterin and thymidine (HAT). The targeting vector, containing the transgene construct of interest, can reconstitute this deletion upon homologous recombination. This allows the selection of ES cells with a correctly integrated transgene construct in HAT medium. Chimeric mice are generated by injection of these ES cells in blastocysts, and used for further germline transmission in order to establish a transgenic line. So far, successful HPRT targeting experiments have been performed using a variety of general and tissue-specific promoter sequences (Bronson and Smithies 1994; Hatada et al. 1999; Evans et al. 2000; Guillot et al. 2000; Cvetkovic et al. 2000). However, no detailed HPRT targeting studies that involve relatively small or heart-specific promoter sequences are available. In this study, the activity and pattern of several distinct ANF promoter constructs was established using this approach and compared to the endogenous ANF expression.
Amongst these constructs is the 0.7 kb ANF promoter region that was also used for the generation of transgenic mice by random integration.

The results presented in this report demonstrate that targeted integration of a transgene in the HPRT locus is a powerful tool for the analysis of (small) heart-specific promoter sequences in vivo. In contrast to random integrations, consistent high expression levels of reporter gene expression were observed in the heart and ectopic expression was absent. Furthermore, the ANF promoter was active in a spatial and temporal pattern similar to that of the endogenous gene.

MATERIALS & METHODS

Targeting constructs
Three constructs with different ANF promoter sequences were used to generate the targeting vector. The first plasmid, ANF-nlacZ, contains the 0.6 kb human growth hormone polyadenylation signal, the $-638/+70$ ANF promoter fragment, a chimeric intron from the pCI vector (Promega), $\beta$-galactosidase (lacZ) with a nuclear localisation signal (nlacZ) and the polyadenylation signal from the bovine growth hormone gene. The second construct, ANFmutNKE-nlacZ, is identical to the ANF-nlacZ construct, with the exception of a 4-bp substitutional mutation of the NKE located at position $-250$ of the ANF promoter as described (Habets et al. 2002). The mutation was generated using the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene). The third construct, atrial ANF-nlacZ, contains the 0.6 kb human growth hormone polyadenylation signal, the $-380/+70$ ANF promoter fragment, a chimeric intron from the pCI vector (Promega), nlacZ and the polyadenylation signal from the bovine growth hormone gene. The vector that was used to target the HPRT locus (pMP8SKB) (Bronson and Smithies 1994) was a generous gift from Sarah Bronson (Pennsylvania State University, College of Medicine).
Cell lines and culture
The HM-1 cell line (derived from a 129/Ola mouse strain) was kindly provided by David Melton. This ES cell line has a male karyotype and contains a 55 kb deletion in the X-linked HPRT gene that includes the promoter and exons 1 and 2 of the HPRT gene (Magin et al. 1992; Tsuda et al. 1997). These cells were cultured on gamma-irradiated murine embryonic fibroblasts (MEFs) in BHK-21 medium (GIBCO BRL; Life Technologies, The Netherlands) supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 1mM Sodium Pyruvate, 0.1mM β-Mercaptoethanol, non essential amino acids and $10^3$ U/ml ESGRO-LIF (Chemicon International Ltd; Harrow, United Kingdom). 1.3 x $10^7$ ES cells were transfected with twenty five to thirty micrograms of purified, linearised DNA by electroporation in 0.2 ml PBS at 0.8 kV and 1 μF (Gene Pulser Apparatus; Biorad) and plated onto two 8.5-cm petri dishes. After a 16 hrs recovery period, the selection medium containing 0.1 mM hypoxantine, 0.4 μM aminopterin, and 16 μM thymidine (1 x HAT; GIBCO BRL; Life Technologies, The Netherlands) was added. After 8-10 days of selection individual colonies were picked, propagated and individual clones were frozen and used for the isolation of DNA as described (Laird et al. 1991). The clones were screened for the transgene construct by PCR and Southern blot analysis using the 0.25 kb Rsal fragment from intron 3 of the murine HPRT gene as probe (Bronson and Smithies 1994). Correctly targeted clones were used for karyotyping and subsequently injected into blastocysts.

Generation of transgenic mice, genotyping and phenotyping
For all constructs chimeras were generated by microinjection of correctly targeted ES cells into C57Bl/6 (Harlan, The Netherlands) blastocysts as described (Hogan et al. 1986). For the ANF-nlacZ construct male chimeras were crossed with FVB females (Harlan, The Netherlands) for germ line transmission. The transgenic female pups, 129/FVB hybrids that have received the 129 ES cell genome, were subsequently bred to FVB mice. The offspring was genotyped by Southern blot analysis and PCR on DNA prepared from either the yolk sac or the toes.
For Southern blot analysis we used the 3 kb NeoI/XbaI fragment of the lacZ reporter gene as a probe (Sambrook et al. 1989). For PCR analysis, primers specific to lacZ sequences were used (lacZ+, GCA TCG AGC TGG GTA ATA AGC GTT GGC AAT and lacZ-, ACT GCA ACA ACG CTG CTT CGG CCT GGT AAT) according to standard procedures (Christoffels et al. 2000a). Embryos were stained for β-galactosidase activity as described (Franco et al. 2001). Whole-mount in situ hybridisation and in situ hybridisation on sections was performed as described (Moorman et al. 2001) using the cDNA probe for ANF (Zeller et al. 1987).

RESULTS

Generation of HPRT targeted ANF transgenic mice

To target the 0.7 kb fragment of the rat ANF gene as a single copy into the HPRT locus, we used the pMP8SKB vector (Figure 1) (Bronson and Smithies 1994).

![GFP reporter gene ANF](image)

**FIGURE 1: Schematic representation of gene targeting to the HPRT locus.**

(A) The ANF promoter construct integrated into the targeting vector pMP8SKB. Boxes represent exons and arrows indicate the location of transcription start sites for the ANF gene and the HPRT gene.

(B) Homologous recombination event between the HM-1 HPRT locus and the targeting vector results in integration of a single copy of the transgene construct at the HPRT locus as shown in panel (C). Location of the probe used for Southern blot analysis is indicated.
This targeting vector contains two regions of homology upstream and downstream of the HPRT transcription start site to facilitate recombination, the promoter and the first and second exon of the HPRT gene, and a multiple cloning site for insertion of the ANF construct (Figure 1A). The targeting vector was transfected into HM-1 cells, which have a deletion in the HPRT locus including the promoter and exons 1 and 2 of the HPRT gene (Magin et al. 1992; Tsuda et al. 1997). Successful homologous recombination corrects HPRT deficiency, restoring the ability of HM-1 cells to grow in HAT medium, and simultaneously inserts the ANF sequence upstream of the HPRT promoter (Figure 1B and C). After 10-days of selection in HAT medium a hundred resistant colonies were picked and propagated for 6-8 days and individual clones were frozen for DNA isolation. Fifty clones were screened by PCR for the presence of the transgene using specific primers and forty-nine appeared to be transgene positive (data not shown). Three individual clones were used for Southern blotting to verify a single integration event at the HPRT locus (data not shown). After karyotyping, two out of three correctly targeted HM-1 clones were expanded and used to generate chimeric mice by blastocyst injection. Nine male chimeric mice were obtained with varying degrees of HM-1 cell contribution assessed by coat colour. The chimeric mice with moderate degree of HM-1 cell contribution were most efficient in transmitting the HM-1 genome when mated with female FVB mice. Because HM-1 cells are male cells and the HPRT locus is located on the X chromosome, only female offspring from the initial cross can receive the transgene. These mice were used to further establish a transgenic line.

Tissue-specificity of the ANF-HPRT transgenic line

We next analysed the tissue-specific expression of the 0.7 kb ANF-HPRT transgenic line and compared it with the expression of the endogenous ANF gene and three previously established multicopy 0.7 kb ANF transgenic mouse lines generated by random integration (Figure 2) (Habets et al. 2002). Only two of the lines with the randomly integrated construct did express the transgene. Whole mount X-gal staining of ED10.5 ANF-HPRT embryos showed
abundant transgene expression in the heart (Figure 2B and F) in a pattern similar to the pattern of ANF mRNA (Figure 2A and E).

**FIGURE 2:** HPRT-targeted mice with the 0.7 kb ANF regulatory region mimics endogenous ANF gene expression.

(A) Whole mount in situ hybridisation of an ED10.5 embryo showing endogenous ANF mRNA expression.

(B) Transgene expression of the 0.7 kb ANF regulatory region targeted to the HPRT locus in an ED10.5 mouse embryo.

(C,D) Transgene expression of the 0.7 kb ANF regulatory region generated by conventional oocyte injection at ED10.5. Note the extensive ectopic expression in various embryonic structures.

(E) A dorsal view of endogenous ANF expression at ED 11.5.

(F-H) A dorsal view of transgene expression at ED10.5 in an ANF-HPRT embryo (F), and in hearts of mice generated by random integration (G,H).

Compared with the conventional ANF transgenic lines, the ANF-HPRT line displayed a stronger reporter gene activity (compare Figure 2B and F with 2C, D, G and H). In the two random ANF transgenic lines ectopic expression is present in mesodermal tissues (somites and the tail region) and in neural tissues (Figure 2C and D), whereas ectopic expression is absent from the ANF-HPRT line (Figure 2B).
Within the ANF-HPRT line we observed that the males with one targeted allele in absence of a wild-type allele (hemizygous males; Figure 3A and C) show transgene expression in all cells. In females with both a targeted HPRT allele and a wild-type allele (heterozygous females; Figure 3B and D) the number of transgene-expressing cells is reduced due to the X-chromosome inactivation.

**FIGURE 3**: Random X-inactivation of HPRT-targeted ANF-nlacZ transgene expression.

(A,C) In hemizygous males no mosaic expression is observed.
(B,D) Transgene expression is subjected to random X-inactivation and, as a consequence, heterozygous females display mosaic β-gal expression in whole mount preparations (panel B) and serial sections (panel D).

*Spatial-temporal expression pattern of the ANF-HPRT-nlacZ transgenic line*

Next, we assessed whether the 0.7 kb ANF-HPRT transgenic line recapitulates the temporal and spatial expression pattern of the endogenous ANF gene.
Heart-specific reporter gene expression and endogenous ANF expression were analysed by X-gal staining of embryos and sections and by nonradioactive in situ hybridisation of embryos and sections (Figure 4 and 5).

**FIGURE 4:** The *HPRT*-targeted 0.7 kb ANF regulatory region mimics the spatial and temporal pattern of endogenous ANF gene expression.

(A) A ventral view of ANF transgene expression at ED8.0.
(B, D and F) Endogenous ANF expression at ED8.0 (B), ED9.5 (D), ED11.5 (F).
(C) A lateral view of ANF transgene expression at ED9.5.
(E) A dorsal view of ANF transgene expression at ED11.5.

IFT: inflow tract; V: ventricle; RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle; SH: sinus horns.
The onset of transgene expression was observed at ED8.5 comparable with the onset of endogenous ANF mRNA expression. However, at ED8.5 reporter gene expression is observed in the linear heart tube in a slightly broader pattern as compared to the endogenous ANF mRNA (Figure 4A and B). At stage ED9.5 the reporter gene is expressed in the atrial and ventricular chamber myocardium, whereas expression was absent from the embryonic myocardium of the AVC and OFT (Figure 4C). At ED11.5 nlacZ is expressed in both atria and ventricles (Figure 4E and Figure 5B and C). Moreover, transgene expression is restricted to the myocardial layer (Figure 5D).

At both stages (ED9.5 and E11.5), the ANF gene shows a similar pattern of expression (Figure 4D and F and Figure 5A). However, in contrast to the endogenous ANF expression,
the ANF-HPRT transgene shows expression in both sinus horns (compare Figure 4E and 4F). Furthermore, at ED11.5, ED15.5 and ED17.5, ANF-HPRT embryos show comparable levels of expression in both ventricles while endogenous ANF expression is higher in the LV than in the RV (Figure 5A, E and G). From ED11.5 onwards, ANF-HPRT transgene expression persisted in the compact outer layer of the ventricles while endogenous ANF expression becomes confined to the trabecular myocardium of the ventricles (see Figure 5B, C, F and G). Newborn ANF-HPRT mice still show extensive reporter gene expression in the atria while the expression in the ventricles is scattered (data not shown). Based upon whole mount X-gal staining, the pattern of expression in the sinus horns of ANF-HPRT mice is similar to the pattern of expression observed in mice with the randomly integrated ANF construct. Serial sections of these ANF mice still need to be analysed.

Analysis of chimeric mice
We generated a second ANF construct that contains the 0.7 kb ANF regulatory region (-638/+70) in which the -250 bp located high affinity NK-2 homeobox factor binding element (NKE) is inactivated by mutation (ANFmutNKE-nlacZ). In the heart, this NKE is recognised by the transcription factor Nkx2.5, which plays an important role in the regulation of ANF gene expression (Lyons et al. 1995; Durocher et al. 1997; Lee et al. 1998; Tanaka et al. 1999; Shiojima et al. 1999; Hiroi et al. 2001; Habets et al. 2002). The ANFmutNKE construct was targeted to the HPRT-locus, and male chimeric mice were used for germ line transmission to generate a transgenic line. The hearts of the female chimeric mice were used for whole mount X-gal staining. This provided a first indication of the activity and pattern of the ANFmutNKE-nlacZ construct. As is shown in Figure 6C, the HPRT-targeted ANFmutNKE-nlacZ construct was expressed in both atria and ventricles, comparable to the hearts of female chimeric mice in which the wild-type ANF construct was targeted (ANF-nlacZ; figure 6A and B). It should be mentioned that the patterns of expression of the distinct hearts are difficult to compare as the female chimeric mice all differ in degree of chimerism and thus harbour a
variable mix of wild-type and transgenic cells. Once the targeted transgenic line is established, transgene expression patterns can be compared with those of the ANF-HPRT mice to reveal the role of the NKE in the spatial and temporal pattern of transgene expression.

FIGURE 6: Representative X-gal stained hearts of ANF-HPRT adult female chimeric mice.
(A,B) X-gal stained adult hearts targeted with the 0.7 kb ANF-nlacZ construct. The degree of chimerism of the mouse is higher in panel A as compared to the mouse in panel B.
(C) Adult heart targeted with the 0.7 kb ANFmutNKE-nlacZ construct. (D) Adult heart targeted with the atrial ANF-nlacZ construct.
Transient transfection analysis of distinct ANF promoter regions in ventricle, atrial and non-cardiac cells suggest that the 0.7 kb ANF promoter region is composed of at least three modules, a “basal” cardiac promoter region (-138/+70 bp), an atrial region (-300/-380 bp) that is required for activity in the atrial cells and an upstream region (-638/-380 bp) required for activity in isolated ventricular cells (Durocher and Nemer 1998). To test whether these ANF promoter modules exist in vivo, we generated a construct that contains the atrial region plus the basal promoter region (-380/+70 bp). This construct was targeted to the HPRT locus and male chimeric mice were used for breeding to establish the atrial ANF-HPRT transgenic line. As shown in Figure 6D, when targeted to the HPRT locus the atrial ANF-nlacZ construct does show nlacZ expression in the heart of a female chimeric mouse. Predominant expression can be observed in the atria while expression in the ventricles is far less. Once a transgenic line is established, transgene expression patterns can be compared with the data from the ANF-HPRT mice to reveal whether the in vitro characterised atrial module is responsible for selective transgene expression in the atrial chamber myocardium during development.

**DISCUSSION**

Major problems experienced in transgene technology are transgene inactivation, mosaic expression and ectopic expression of the transgene, and the variability in level, pattern and specificity of expression between distinct lines generated with the same construct. These phenomena find their origin in the random integration of the construct in the host genome and the (in)ability of the transgenic promoter to activate expression when integrated in the chromatin. Several studies described the use of chicken β-globin insulator sequences as an appropriate strategy to protect the transgene construct from the genomic environment (Chung et al. 1993; Chung et al. 1997; Wang et al. 1997; Bell et al. 1999). These insulators are, when integrated into the genome, characterised by their ability to block the interaction with nearby regulatory
elements without stimulatory or inhibitory effects and thereby reduce inactivation by the surrounding chromatin and ectopic expression. To test the use of chicken β-globin insulator sequences we used a 356 bp fragment of the cardiac troponin I (cTnI) gene. This promoter fragment was flanked with a 1.2 kb tandem repeat of chicken β-globin insulator sequences. We observed that the number of lines expressing the transgene dramatically increased. However, ectopic expression was still observed and the level of expression was rather weak. Therefore, we focused on the generation of transgenic mice in which the transgene was targeted to the HPRT locus.

The 0.7 kb ANF regulatory region largely recapitulates the pattern of expression of the endogenous ANF gene

The HPRT gene is ubiquitously expressed and the locus is transcriptionally active and accessible in all tissues at all developmental stages of development (Bronson and Smithies 1994). Hence, transgenic constructs are integrated in a favourable genomic environment, and each independently targeted construct will experience a similar influence of higher order gene regulation. So far, inhibition of a transgene when targeted to the HPRT locus has not been observed. Using both ANF-HPRT transgenic mice and mice with a randomly integrated construct, the 0.7 kb ANF regulatory region largely mimics the endogenous ANF expression pattern and shows a selective expression in the chamber myocardium of both atria and ventricles. Transgene expression was not detectable in AVC and OFT. However, the pattern of transgene expression at ED8.0, the transgene expression in the sinus horns and the level and pattern of transgene expression in the ventricles differs from the endogenous ANF expression pattern. In both mice with the randomly integrated construct and ANF-HPRT mice reporter gene expression was clearly present at all stages in the sinus horns. Thus, most likely, the 0.7 kb ANF regulatory region lacks the sequences necessary for a correct (endogenous) expression pattern in the IFT region of the heart. Although serial sections showing the reporter gene expression in mice with the randomly integrated ANF construct still need to be analysed,
the results of the ANF-HPRT mice indicate that the 0.7 kb ANF regulatory region also does not harbour the regulatory sequences necessary for the correct ventricular ANF expression. Recently, we analysed a 7 kb fragment of the ANF regulatory region containing the 3 kb upstream and 4 kb downstream DNA sequences (de Lange et al, in preparation). However, no ventricular transgene expression could be observed in E14.5 mouse hearts. These data emphasise the complexity of the regulation of ventricular ANF expression and even suggest the location of a ventricular repressor element outside the 0.7 kb promoter fragment.

*Targeting the HPRT locus reveals efficient, strong and heart-specific ANF transgene expression*

Previously, the HPRT approach has been successfully used to target a variety of general and tissue-specific promoter sequences and all report a strong and specific transgene expression pattern (Bronson and Smithies 1994; Evans et al. 2000; Guillot et al. 2000; Cvetkovic et al. 2000). Furthermore, using variable tissue-specific promoters, it was demonstrated that transgene expression of constructs targeted in the HPRT locus solely depends on the regulatory DNA sequences of the integrated transgene construct (Cvetkovic et al. 2000; Guillot et al. 2000; Evans et al. 2000). Using the heart-specific ANF promoter fragment we obtained analogous results. By comparing transgene expression patterns of mice obtained by conventional versus targeted transgenic techniques we demonstrated that ANF transgene expression is stronger in ANF-HPRT animals. Integration of the ANF transgene into the HPRT locus resulted in specific expression in the heart with no ectopic expression.

Integrating a transgene construct into the HPRT locus is a very efficient technique. Cvetko et al reported an efficiency of 96% of single homologous recombination events when targeting the HPRT locus (Cvetkovic et al. 2000). Although we only tested three colonies by Southern blot, all appeared to contain a correctly integrated transgene into the genome (100%). Based upon this high efficiency number, one can scale down the ES cell
transfection and selection procedure, which allows for a fast and cost-efficient generation of distinct transgenic lines.

As the HPRT gene is located at the X chromosome it is subject to random X-inactivation (Lock et al. 1987; Gartler et al. 1992; Willard et al. 1993). Hemizygous males and homozygous females express the transgene in all cells while heterozygous females express the transgene in a reduced number of cells. These differences in number of expressing cells may permit more sophisticated analyses of transgene results, which may be beneficial when studying for example non-cell autonomous processes. To avoid the complication of X-inactivation, transgenes could be targeted to autosomal chromosomes by using a drop-in approach similar to that used for the HPRT locus (Soriano 1999; Mao et al. 1999).

Conflicting data have been reported on whether the transgene needs to be orientated in the same or reverse transcriptional orientation as the endogenous HPRT gene. Shaw-White and colleagues observed a transcriptional interference perpetrated by the HPRT gene promoter when the transgene was orientated in the same orientation as HPRT transcription and recommend a transgene orientation opposite to the HPRT gene transcription (Shaw-White et al. 1993). Others observed a consistent transgene expression in the expected tissue regardless of the orientation (Bronson and Smithies 1994; Guillot et al. 2000). In all three ANF constructs the transgene was inserted in the reverse transcriptional orientation relative to the endogenous HPRT gene and preceded by a 0.6 kb human growth hormone polyadenylation signal. However, a distinct transgenic line that comprised the 5.5 kb \textit{\alpha-myosin heavy chain (\textit{\alpha-MHC})} promoter fragment has been successfully targeted to the HPRT locus in our lab as well (Chapter 6). Although in this line the transgene orientation of the \textit{\alpha-MHC} promoter is the same as that of the HPRT gene, no adverse effects of transgene expression were observed.

\textit{Conclusions}

In this study we have shown that the HPRT targeting system provides an efficient and accurate method to investigate regulation of gene expression in
the heart. The 0.7 kb ANF promoter displayed a largely correct spatial-temporal transgene expression pattern largely similar to the pattern of the endogenous ANF gene. Furthermore, when targeted to the HPRT locus, in all developmental stages the tissue-specificity is retained and ectopic expression is absent. Preliminary results using chimeric analyses suggest that regulatory DNA fragments as small as 400 bp are active in the HPRT locus. Whether the HPRT targeting system is suitable for studying the effect of small mutations still awaits further analysis, although first observations in chimeric mice are promising. Moreover, different transgenes that are inserted into the favourable chromatin configuration of the HPRT locus will experience the same influence of higher order gene regulation. This allows for the generation of one single line per construct and may reduce the number of experimental animals and, at the same time, accelerate the characterisation of regulatory DNA sequences.

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