Genetic Manipulation of the Rabbit Heart via Transgenesis

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Background—Transgenesis using cardiac-specific expression has been valuable in exploring cardiac structure-function relationships. To date, cardiac-selective studies have been confined to the mouse. However, the utility of the mouse is limited in certain, possibly critical, aspects with respect to cardiovascular function.

Methods and Results—To establish the potential validity of transgenic methodology for remodeling a larger mammalian heart, we explored cardiac-selective expression in transgenic rabbits. The murine α- and β-cardiac myosin heavy chain gene promoters were used to express a reporter gene, and transgene expression was quantified in cardiac, skeletal, and smooth muscles as well as in nonmuscle tissues. Although neither promoter exactly mimics endogenous patterns of myosin heavy chain expression, both are able to drive high levels of transgene expression in the cardiac compartment. Neither promoter is active in smooth muscle or nonmuscle tissues.

Conclusions—Directed organ-specific expression is feasible in a larger animal with existing reagents, and cardiac-selective transgenic manipulation is possible in the rabbit. (Circulation. 2000;101:1715-1721.)

Key Words: myosin genes muscles

The study of the cardiovascular system has benefited significantly from the use of gene-targeted and transgenic mice. Multiple aspects, including cardiac development, the conduction system, the development of coronary artery disease, the adrenergic system and sarcomeric proteins, have been explored. In vivo models have been invaluable in providing integrative data regarding physiological and pathological states in the heart, such as cardiac hypertrophy and dilation.

Because of the ease with which the mouse genome can be manipulated and the relatively low cost of maintaining large colonies, most molecular investigations of the cardiovascular system have used mice. However, small rodents do not accurately reflect crucial facets of human cardiovascular physiology. Indeed, a number of experimental models aimed at duplicating human pathological states by expressing the most abundant gene promoters were used to express a reporter gene, and transgene expression was quantified in cardiac, skeletal, and smooth muscles as well as in nonmuscle tissues. Although neither promoter exactly mimics endogenous patterns of myosin heavy chain expression, both are able to drive high levels of transgene expression in the cardiac compartment. Neither promoter is active in smooth muscle or nonmuscle tissues.

To more fully understand and appreciate the structure-function relationships of the cardiac contractile proteins, it would be beneficial to move selected models into larger mammalian transgenic animals, and the rabbit is a reasonable choice. Gestation is short (30 days), and sexual maturity occurs relatively quickly (20 to 24 weeks). The rabbit is already used to study a variety of human heart diseases, and transgenic animals can be made. At the molecular level, rabbit atria express α-MHC at all developmental stages, whereas the ventricles express both α- and β-MHC isoforms, with β-MHC the predominant adult isoform. Thus, MHC expression closely parallels that of the human heart. In addition, modalities available for clinical evaluation of human cardiac function can be readily adapted.

Initially, transgenic investigations in the mouse made use of non–tissue-specific promoters. To avoid the confounding effects of systemic expression, reagents were developed to limit transgene expression to the heart. For high levels of...
cardiac-specific expression, the α-MHC promoter has been widely used and closely mimics endogenous expression patterns. Both the mouse α-MHC and β-MHC promoters share ~85% homology with the respective rabbit promoters in the proximal 600 base pairs (J.R. et al, unpublished data). Because the proximal promoters appear to be largely responsible for cardiac specificity, we hypothesized that the mouse promoters might be useful in remodeling the protein complement of the rabbit heart. To this end, the ability of the mouse α- and β-MHC promoters to drive high levels of transgene expression in the rabbit was determined.

Methods

Generation of Transgenic Rabbits

The constructs have been described. All experiments were performed with New Zealand White rabbits under a protocol approved by the Animal Care Committee. A doe was superovulated on day 1 of the protocol with 150 U pregnant mare serum gonadotropin delivered subcutaneously under the skin of the neck. On day 3, the donor doe was mated with a nontransgenic buck. In addition, both the donor and recipient does received 150 U of human choriongonadotropin administered into an ear vein. On day 4, the eggs were harvested from the donor doe (a terminal procedure), and the pronuclei of viable eggs were injected with purified DNA. The injected eggs were transferred into the fallopian tube of the pseudopregnant recipient, who was moved to a nesting cage 2 to 3 days before the expected delivery date. Transgenic offspring were identified by polymerase chain reaction and genomic Southern analyses with 32P-labeled CAT cDNA as probe. The founder rabbits were up to 5 months (females) or 6 months (males) old before a breeding program was begun. F1 and/or F2 offspring were used for all subsequent analyses. Diploid copy number was determined with DNA dot blots with a 32P-labeled CAT cDNA probe.

RNA Profiles

Rabbits were sedated with intramuscular ketamine, then euthanized with intravenous pentobarbital. The heart was quickly isolated, and atrial and venricular tissues were dissected, frozen directly in liquid nitrogen, and stored at −80°C until use. RNA isolation and transcript quantification have been described. Transcript-specific oligonucleotides for rabbit α-MHC (5'-CAGGCACCTCGTGTTTATTGCG-GGGTTAAACAGAGCCGGGTTTC-3'), β-MHC (5'-GCCGATC-AAACGGTCACCCAGCTATTCTCATTTCAAGCT-3'), and GAPDH (5'-CTGGAGGCCCTCGTCCTCTCTCTGCTGCTC-GCTG-3') were labeled with [32P]ATP and hybridized as described.

CAT ELISA

Dissected tissues were frozen in liquid nitrogen. For each time point, samples from a nontransgenic rabbit were also analyzed for nonspecific cross-reactivity in the CAT ELISA. Proteins were isolated by homogenization of the tissues in a small volume (200 to 400 μL) of 0.25 mol/L Tris (pH 7.8) with a Tekmar homogenizer (Tekmar Co). The homogenate was incubated at 65°C for 10 minutes, then centrifuged for 10 minutes at 12 000 rpm in a tabletop microfuge. The supernatant was transferred to a new tube, and the protein concentration was determined by the Bradford method. The ELISAs were performed with a microtiter kit according to the manufacturer’s instructions (Boehringer-Mannheim). For each time point, values were averaged and the SEMs determined.

CAT In Situ Hybridization

CAT in situ hybridization was performed on samples of papillary muscle. The staining protocol was modified such that the anti-CAT-digoxigenin antibody was preabsorbed to rabbit heart powder (obtained from acetone precipitation) instead of mouse embryo powder.

Transgenic Rabbit Generation

| Eggs recovered | 1403 |
| Viable eggs injected | 1000 |
| Potential founders (F0) | 120 |
| Stillborn | 33 |
| Liveborn | 87 |
| Confirmed F0 animals | 11 |
| Number of lines with transgenic offspring | 8 |
| Number of lines with detectable transgenic expression | 4 |

Results

Generation of Transgenic Rabbits

Of 1000 reimplanted embryos, 8.7% resulted in live births. Of the 87 kits born alive, 11 were transgenic, for an overall efficiency of ~1% (Table). Our success rate for generating transgenic mice is ~25%. Thus, our current success rate with rabbits is substantially less than with mice but similar to what has been reported by others. Compounding the difficulty of the basic animal husbandry was germ-line mosaicism among the founders. This necessitated generating multiple litters before a stable F1 breeder could be obtained. This significantly extends the time line for experiments; because only low numbers of F1 offspring were available, it was necessary to wait until the F2 generation to establish a useful cohort of age-matched animals for analysis. Seven α/CAT founders were generated, of which 6 transmitted the transgene to the F1 generation and 3 had detectable CAT expression. Four β/CAT founders were generated, but 2 failed to produce transgenic offspring. The remaining 2 founders passed the transgene to offspring, but only 1 line had detectable CAT expression. For the analyses reported below, the 3 α/CAT lines and 1 β/CAT line that both transmitted and expressed the transgene at detectable levels were used to derive experimental cohorts.

Transgenic Expression in α-MHC Transgenic Rabbits

To analyze mouse promoter activity in transgenic rabbits, CAT was quantified with an ELISA. This method was chosen as a standardized and reproducible method to quantify the amount of CAT protein so that the promoter activity obtained in the rabbit could be compared directly with previous data from the mouse. In our experience, promoters that result in CAT levels of 300 to 500 pg protein/μg total protein are capable of driving transgene expression at rates that are sufficient to replace the most abundant proteins in the cardiomyocyte, including the myosins and other components of the contractile apparatus. The transcriptional patterns of α-MHC in the rabbit heart have been defined previously. Normally, α-MHC is the only isoform expressed in the atria throughout development. Endogenous α-MHC gene expression is initially high in the ventricle but is gradually replaced by the β-MHC isoform as the animal matures. There is substantial animal-to-animal variation, however, and the ratios of the 2 isoforms can differ.
dramatically, depending on the individual as well as the region of the ventricle from which the sample is derived. We confirmed these expression patterns in the New Zealand strain chosen for transgenic analyses and found excellent agreement with previously published data (data not shown).

Atrial CAT expression in the 3 α-CAT lines was determined at different developmental times (Figure 1A). Line 286 (2 transgene copies) had very low levels of CAT in the atria at all time points tested. Line 222 (8 copies) showed a progressive increase in the amount of CAT present in the atria to 271 ± 16 pg CAT/μg protein at 16 weeks, the oldest age assayed. Line 290 (14 copies) initially had high levels of CAT in the atria (806 ± 91 pg CAT/μg protein), with attenuation of expression over time to very low levels at 16 weeks. Thus, none of the 3 α-CAT lines mimicked endogenous atrial α-MHC expression. Although the murine promoter shows copy number–dependent and position-independent expression in the mouse, this does not appear to be the case in the rabbit atria. At the oldest time point examined (16 weeks), line 290 shows lower levels of atrial CAT expression than line 222 (8 copies). No significant differences presented between the left and right atria. Ventricular expression was also determined (Figure 1B). Line 286 had modest expression levels. CAT expression was undetectable in line 22 ventricles at 3 to 5 days but was upregulated, albeit to a very low level, at 4 to 6 weeks and >16 weeks. Line 290 demonstrated robust CAT expression in the ventricles 3 to 5 days after birth and, like the endogenous gene, was progressively downregulated during postnatal development.

To determine homogeneity of CAT expression in the ventricle, in situ immunohistochemistry was performed on papillary muscle from 8-week-old F2s derived from the high-expressing line 290 (Figure 2). On a gross level, CAT was evenly distributed throughout cross sections of the muscle. Although we cannot rule out patchy expression on a myocyte-to-myocyte basis (which could reflect endogenous expression patterns), transgene expression clearly was not restricted to isolated regions of the left or right ventricle.

**CAT Expression in Nonmuscle, Nonstriated Muscle, and Skeletal Muscle Tissues**

For the promoter to be useful in remodeling the heart in a selective manner, its activity in nonmuscle tissues and in nonstriated muscle types should be minimal. To assess this, CAT levels were determined in protein extracts from 6
normal rabbits have significant expression of CAT expression. For example, at 16 weeks, CAT in the masseter ranged from 204 to 1386 pg CAT/μg protein and in the diaphragm from 858 to 1462 pg CAT/μg protein. Promoter activity in the masseter and diaphragm was not surprising, because these muscles normally express the α-MHC isoform. However, CAT expression in the soleus greatly exceeded that of the masseter and diaphragm, with values ranging from 2338 pg CAT/μg protein at >16 weeks to 6746 pg CAT/μg protein at 4 to 6 weeks. This was unexpected, because soleus is often considered the “purest” slow muscle, expressing β-MHC almost exclusively. Indeed, we were able to detect only trace amounts of endogenous α-MHC in this muscle tissue. The data show that, although the promoter is capable of driving high levels of transgene expression in striated muscle, fiber type specificity is not maintained.

Mouse β-MHC Promoter
As noted above, β-MHC is the predominant isoform expressed in the adult rabbit ventricle. To determine whether the mouse β-MHC promoter was capable of driving significant levels of transgene expression in the rabbit, the β-MHC–cat construct was tested in transgenic rabbits. Of the 4 founders, 2 produced transgenic offspring, but only 1 line showed detectable levels of CAT. CAT ELISAs showed modest levels of expression (200 pg CAT/μg protein) in the atria (Figure 4A). Consistent with endogenous patterns of β-MHC expression, transgene expression in the ventricle was robust even as early as 3 to 5 days after birth and increased to very high levels (1739±303 pg CAT/μg protein at 4 to 6 weeks and 1643±166 pg CAT/μg protein at >16 weeks) as the heart matured. Observing the caveat that only 1 line was available for analyses, the data are consistent with the hypothesis that this promoter can drive expression at levels sufficient for efficient transgenic replacement of an endogenous contractile protein. As was the case for the mouse α-MHC promoter, the β-MHC promoter showed significant activity in selected skeletal muscles (Figure 4B). The relatively low levels of CAT found in the masseter, diaphragm, and soleus muscles at 3 to 5 days increased significantly during later developmental stages.

Endogenous Expression of α-MHC and β-MHC in Transgenic Hearts
It is formally possible that endogenous MHC expression might be suppressed by high levels of transgene expression, presumably from competition for rate-limiting factors needed for gene expression. Thus, we examined α-MHC expression in hearts derived from line 290, the highest copy number line showing the highest levels of transgene expression (Figure 5). No “squelching” occurred despite the very high levels of transgene expression (2648±902 pg CAT/μg protein). These data are consistent with results obtained in the mouse.22 In the rabbit heart, it appears that alterations in endogenous myosin gene transcription due to competition with rate-limiting amounts of transcription factors will not be a serious problem.
In addition, no abnormal pathology or histology in any of the lines was ever detected (data not shown).

Comparison of Rabbit and Mouse \(\alpha\)-MHC and \(\beta\)-MHC Promoters

Although the mouse promoters drive striated muscle–specific expression in the rabbit, they are unable to exactly mimic endogenous rabbit myosin transcriptional patterns. To explore the structural bases for these differences, the full-length rabbit \(\alpha\)-MHC and \(\beta\)-MHC promoters were cloned and sequenced. Very limited data for these sequences exist.\(^{27-29}\) Mouse MHC probes were used to screen a New Zealand White rabbit genomic library. The \(\alpha\)-MHC and \(\beta\)-MHC genes in the rabbit lay in tandem arrangement with the \(\beta\)-MHC gene immediately upstream of the \(\alpha\)-MHC regulatory region. The entire intergenic region between the \(\beta\)- and \(\alpha\)-MHC genes was sequenced (Genbank AF192305): in the mouse, this encompasses the \(\alpha\)-MHC promoter.\(^{30}\) For the mouse \(\beta\)-MHC gene, \(\approx\)5000 bases upstream of the transcriptional start site is sufficient to direct cardiac- and slow fiber type–specific expression in transgenic mice.\(^{16,19}\) On this basis, 7000 bases upstream of the \(\beta\)-MHC transcriptional start site were also sequenced (Genbank AF192306). The proximal promoters are highly conserved, with important regulatory sequences, as defined by both in vivo and in vitro assays in the mouse, being present in both species (Figure 6). A DNase I hypersensitive site previously identified in the hamster \(\beta\)-MHC promoter as playing an important regulatory role\(^{31}\) was also present in both the rabbit and mouse \(\beta\)-MHC sequences.

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**Figure 5.** Endogenous expression of \(\alpha\)-MHC (MyHC) and \(\beta\)-MHC in transgenic rabbits. RNA dot blot comparing levels of gene expression between age-matched transgenic and nontransgenic rabbits from line 290. RA indicates right atrium; LA, left atrium; V, ventricle; TG, transgenic; and NTG, nontransgenic.

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**Figure 6.** Comparison of mouse and rabbit \(\alpha\)-MHC and \(\beta\)-MHC promoters. A, Comparison of \(\alpha\)-MHC promoters. Homologies for proximal regions are shown. MEF-1 site is present in mouse promoter but absent in rabbit. B, Comparison of \(\beta\)-MHC promoters. Transcriptional regulatory cassettes shared between promoters are shown. *Two E-boxes present in mouse \(\beta\)-MHC but absent in rabbit sequences are indicated.
Discussion

Heterologous promoters have been used successfully to create transgenic animals, including transgenic rabbits.30 The mouse MHC promoters are capable of driving high levels of transgene expression in the rabbit but do not faithfully recapitulate the endogenous genes’ cardiac compartment– and developmental time–specific patterns. Fidelity of mouse α-MHC promoter activity with endogenous α-MHC rabbit expression would require constant high expression in the atria and, in the ventricles, robust expression in the neonatal stage that gradually diminishes as the animals age. Line 286, with 2 transgene copies, showed minimal expression in both cardiac compartments at all developmental stages. One α/CAT line, line 222, showed preferential atrial expression with increasing promoter activity as the rabbits matured but, at all developmental time points tested, had only minimal ventricular expression. Line 290 initially had high atrial expression that diminished significantly by 16 weeks of age. In this line, promoter activity in the ventricle mimicked endogenous cardiac expression patterns to some extent, although ventricular expression remained robust into adulthood. On the basis of data obtained in the mouse, we noted previously that levels of CAT expression in the 300 to 500 pg/μg protein range indicated promoter activity that was sufficient to effect replacement of abundant sarcomeric proteins.16–19,22 Assuming a similar relationship in the rabbit, the levels of expression observed in line 290 in the developing atria and mature ventricles should be more than sufficient to remodel the contractile apparatus. Although as the animal matured, atrial expression would decrease and transgenic replacement would be restricted to the ventricular compartment, this might actually be advantageous for a subset of transgenic experiments in which ventricle–specific expression is desired in the adult animal.

Cardiac-compartment specificity was not conserved across species lines for the β-MHC promoter, although the promoter did show a strong selectivity for the ventricle. Both promoters retained the ability to drive striated muscle–specific expression, and no smooth muscle expression could be detected. However, the precise striated muscle fiber–type specificity exhibited by these promoters in the mouse was not repeated in the rabbit. This may reflect differences in transcription factor pools present in the different fiber types, the different promoter sequences upstream of the proximal promoters (Figure 6), a negation of controlling factors due to position–dependent effects, or a combination of the 3.

The extension of the transgenic paradigm to the rabbit heart provides additional opportunities for studying structure–function relationships and modeling disease in the cardiovascular system. The cost of generating and maintaining a transgenic rabbit colony substantially exceeds that of a mouse colony, and mosaicism in the founder population, as well as a 6-month period before a new generation can be bred, increases the difficulty of establishing stable experimental cohorts. However, the transgenic rabbit has significant advantages over murine transgenics, with size being only the most obvious. The contractile isoform profile closely reflects that of the human heart, and the length of the contractile cycle is significantly longer, approaching that of the human neonate. Transgenic rabbits will be useful in assessing whether experimental findings in the mouse can be accurately extended to larger mammalian hearts. In addition, specific models of cardiovascular disease, as they are moved into the rabbit, may more closely mimic human pathology. For example, recent experiments in which troponin mutations associated with human familial hypertrophic cardiomyopathy were expressed in the mouse heart show that the animal is able to tolerate only minor amounts of the mutant protein before dosage becomes lethal.33,34 The increased sensitivity of the mouse, relative to the human, could reflect the differences in heart rates and cardiac cycles between the 2 species. The extremely rapid heart rate of the mouse may render the animal exquisitely sensitive to alterations in the Ca2+–handling proteins. In such an instance, modeling the disease in an animal with a slower heart rate could be more appropriate.

Acknowledgments

This work was supported by National Institutes of Health grants HL-56370, HL-41496, HL-56620, HL-52318, HL-60546, and HL-56620 (to Dr Robbins) and HL-03769 (to Dr James). We thank Katherine Tolzmann for excellent technical assistance.

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Circulation. 2000;101:1715-1721
doi: 10.1161/01.CIR.101.14.1715

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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