Molecular remodeling of cardiac contractile function

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James, Jeanne, and Jeffrey Robbins. Molecular remodeling of cardiac contractile function. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2105–H2118, 1997.—A number of techniques are now available that allow the contractile apparatus of the heart to be altered in a defined manner. This review focuses on those approaches that result in germ-line transmission of the remodeling event(s). Thus the desired modifications can be propagated stably throughout multiple generations and result in the creation of stable, new animal models. Necessarily, such stable changes need to be performed at the level of the genome, and two distinct but complementary approaches have been developed: transgenesis and gene targeting. Each results in the stable modification of the mammalian genome. Via gene targeting or gene ablation of sequences encoding various components of the sarcomere, the contractile apparatus of the heart can be altered dramatically. Ablating a gene may lead to a loss in function, which can help establish a function of the candidate sequence. Gene targeting can also be used to effect changes in the sequences encoding a functional domain of the contractile protein or at a single-amino acid residue, resulting in the establishment of precise structure-function relationships. With the use of transgenesis, the contractile apparatus of the heart can also be significantly remodeled. These approaches are rapidly creating a group of animals in which altered contractile protein complements will lead to a fundamental understanding of the structure-function relationships that underlie the function of the heart at the molecular, biochemical, whole organ, and whole animal levels.

contractile proteins; genetics; transgenic; gene targeting

THE PROTEINS of the contractile apparatus both underlie and reflect the different physiological demands that are placed on a particular muscle or muscle type. Although all striated muscles share a common protein pool that is highly conserved, the different fiber types are characterized by particular, and often unique, contractile protein isoform complements (reviewed in Ref. 97). The diversity of these proteins is impressive and can arise either from unique genes, which are members of large isogene families (4, 95), from alternative splicing of a primary transcript (35, 117), or from a combination of the two (57). For example, for the thin-filament protein tropomyosin (TM), although multiple (four) genes exist and are partially responsible for isoform diversity, each primary transcript also gives rise to multiple mRNAs through well-defined tissue-specific patterns of differential splicing (58, 117). The most common mode of generating isoform diversity is illustrated by the myosin heavy chains (MHCs), which, by mass, are the major proteins found in striated myocytes. These proteins are represented in the different fiber types by multiple isoforms that are encoded by unique, but closely related, genes, and the isoform content is characteristic of a particular muscle fiber type (13). As is the case for most of the sarcomeric genes, a primary level of control is exerted at the level of gene activation, and the transcriptional patterns of these genes are controlled in a tissue-specific (9, 14), muscle type-specific (67, 87), and developmental stage-specific manner (19, 32) such that the different muscle types have a unique and characteristic complement of MHC.

The presence of multiple isoforms of the proteins that make up the thin and thick filaments was first inferred from protein data (27, 42, 62, 115) and confirmed by the isolation and sequencing of the unique genes or cDNAs and is now an accepted tenet of muscle diversity. Extensive sequence data for multiple isoforms of most of the known contractile proteins have been gathered for a number of species such as human, rat, rabbit, mouse, and chicken, and the general characteristics of these gene families are well understood (reviewed in Ref. 97). Muscle type-specific proteins exist for the
MHCs, the myosin light chains (MLCs), the actins, components of the troponin (Tn) complex (TnT, TnC, and TnI), myosin binding protein C, titin, nebulin, α-actinin, and TM (reviewed in Refs. 4, 97). Extensive correlations have been made concerning the presence of a certain sarcomeric protein isoform with the functional and physiological profile of a particular muscle. In vitro approaches, in which a normal isoform is replaced by a mutated species via adenoviral infection (114) or an isoform domain substitution is performed (118; reviewed in Ref. 78), have underscored the functional consequences of isoform substitution and diversity. However, demonstrating that the different isoforms uniquely define the contractile behavior of a particular muscle type, particularly at the whole organ and whole animal levels, has remained illusive.

In the mammalian heart, the different contractile protein isoforms that can be expressed in response to a spectrum of external and internal stimuli are believed to underlie the changing physiological profiles this organ can undergo. Although differences in function are inferred, the sequences of the different isoforms are almost entirely conserved. For example, two actin isoforms, α-cardiac and α-skeletal, the products of unique genes that are located on different chromosomes, are transcribed in the heart, and their expression patterns are tightly controlled. The α-skeletal isoform is abundant in the developing heart but is downregulated in most species during cardiac maturation as α-cardiac actin is upregulated and becomes the predominant thin-filament protein (in humans, α-skeletal actin continues to be expressed in the adult heart). In the normal adult mouse heart, α-cardiac actin predominates, but in the BALB/c strain, a naturally occurring genetic lesion at the actin locus leads to increased amounts of the α-skeletal isoform relative to α-cardiac actin (31). The hearts of this strain are hypercontractile, with increased hemodynamic activity compared with other mouse strains in which the actin complement consists mostly of the α-cardiac actin isoform (41). These two proteins differ at only 4 amino acid sites out of 375 residues (107, 108); however, two of these changes are localized at the amino terminus (Asp-Asp-Glu-Glu for α-cardiac actin vs. Asp-Glu-Asp-Glu for α-skeletal actin), and this is thought to be a critical region for thin-thick filament interaction.

A striking correlation between a particular cardiac contractile protein isoform and the functional profile of the heart was first delineated for the MHC family. There are three cardiac MHC isoforms, the relative amounts of which depend on the animal, developmental stage, hormonal status, and chamber. The isoforms, termed V1, V2, and V3 on the basis of their mobilities in polyacrylamide gels, are the product of two genes termed α and β, with V1 corresponding to an α.α dimer, V2 to an α.β dimer, and V3 to the β.β species. Since the initial observations of Barany (5), who correlated adenosinetriphosphatase (ATPase) activity of myosin with the speed of muscle shortening, extensive correlations have been documented between the cardiac MHC composition and the speed of contraction (24, 44, 82, 98). Compared with the α.α homodimer, V1, V2 and V3 show diminished ATPase activities, and generally (exceptions being, e.g., the guinea pig and some avian species), as the ratio of V1 to (V2 + V3) increases, heart rate also increases. Thus the human ventricle, which is predominantly V3, has a resting heart rate of 60–80 beats/min, whereas the adult mouse heart, which contains essentially pure V1, contracts at 500–550 beats/min.

Establishing a cause-and-effect relationship for a unique cardiac isoform with a particular functional profile of the heart at the organ and animal levels remains an elusive goal. Similarly, assigning structure-function relationships to different domains in the various contractile proteins themselves is also largely unresolved within the whole organ context. Although in vitro approaches have been used productively to define unique functional roles to the different domains of various contractile proteins, these studies have not, with rare exceptions, been extended to in vivo models. The importance of this deficiency is underscored by our current inability to understand the etiology of the cardiomyopathies that have been mapped to mutations in β-MHC (34), the regulatory and essential MLCs (88), myosin binding protein C (112), cardiac TnT, and α-TM (113). Thus the functional consequences of isoform shifts or of expression of mutated contractile proteins remain at the descriptive and correlative levels.

Overcoming this deficiency has become pressing, not only in terms of achieving a basic understanding of cardiac structure-function relationships but also because the above mutations in the sarcomeric proteins have been genetically mapped to disease loci. A number of approaches are currently being tested that may eventually result in our ability to modify the protein output of the cardiomyocyte. These include the catheter-mediated infiltration of a replication-deficient adenovirus (6), injecting genes directly into the heart (15), and myocyte or embryonic stem cell transfer to the heart (21, 49, 53) among others. These developing and potentially important therapeutic modalities will not be considered here because they are restricted to the somatic tissue, are not transmitted via the germ line, and cannot be used to establish stable animal models with a remodeled heart. Currently, then, our abilities to stably modify the mammalian genetic apparatus are largely restricted to either transgenesis and/or gene targeting. These techniques as applied to the study of the heart are carried out almost exclusively in the mouse because of practical considerations of both the cost in terms of the animal husbandry involved and the short generation time of the model (21-day gestation, 4–6 wk to sexual maturity). With the use of molecular genetic techniques, it is relatively straightforward to modify a cDNA or gene sequence encoding a contractile protein. If one subsequently uses the construct to generate an animal that expresses the transgene only in the heart, one can directly link a primary structural change in a domain of a cardiac protein, or isoform switch, to the resultant phenotype. Although many secondary effects and compensatory mechanisms may
be called into play to maintain cardiac output, the phenotype is, nevertheless, often informative and necessarily reflects its primary genetic etiology. In this review, we will outline the basic approaches currently being used by illustrating relevant models in which the cardiac protein complement has been modified and the functional profile of the heart has been remodelled.

**GENE TARGETING AND CONTRACTILE PROTEIN REMODELING**

Gene targeting offers the investigator the opportunity to replace the endogenous DNA sequence at a particular site with an exogenously prepared (and presumably altered) DNA. Conventional gene targeting approaches depend on homologous recombination of the electroporated DNA into totipotent embryonic stem cells. The electroporated DNA usually contains a selectable marker, and after subsequent selection procedures, those cells that have undergone homologous recombination can be identified via suitable molecular screening procedures such as Southern analysis or polymerase chain reaction (72, 76, 100, 101). There are now many variations on the basic theme, but the salient points remain more or less invariant. The DNA to be inserted consists of extensive regions of homology that flank the target site and contain at least one, and usually two, selectable markers. The targeting event is planned such that the endogenous sequences are disrupted, with DNA elements critical for the transcription and/or translation of the gene being targeted and replaced by either the selectable marker or an innocuous, noncoding sequence. This results in a null allele, that is, a gene locus that cannot produce a translatable transcript and/or a functional polypeptide. The gene ablation or targeting allows one to study the consequences of recessive alterations if the subsequent animals are bred to homozygosity in a “loss-of-function” or “gene knockout” experiment (16). This strategy is most often used to determine whether a particular gene has a critical function or to explore the potential gene dosage effect by ablating a single allele. If the targeting results in a translatable, but altered, transcript, a poison peptide can result, which interferes with the function of the endogenous protein, resulting in a “dominant negative” targeting. Additionally, homologous recombination in embryonic stem cells can be used to insert, at a defined site, a large fragment of DNA incorporating a single entire transgene (10) such that its activity and function(s) can be studied free from the confounding effects of copy number and varying chromosomal contexts (see below).

The literature now contains many examples of genes important for general cardiac development and function that have been ablated. Frequently, the targeted genes encode polypeptides that are involved pleiotropically in cardiac development or basic structure. Although the ablation can have a major impact on the contractile apparatus, the deficit and/or remodeling is not a primary consequence of the null mutation but rather reflects a more basic deficit that occurred in normal cardiac development. Examples include the hox-1.5 knockout, which results in widespread cardiovascular abnormalities reminiscent of DiGeorge syndrome (18), the retinoic acid-receptor α-knockout, resulting in numerous congenital cardiac abnormalities (103), ablation of the muscle-specific LIM protein (2), which leads to a dilated cardiomyopathy, and the endothelin-1 (56) and connexin 43 knockouts (92). Ablation of these genes, the functions of which are apparently needed for normal cardiac development and establishment of the inherent structure of the myocardium, has been tremendously informative but is not directly germane to understanding the structure-function relationships that underlie normal and abnormal cardiac contraction. Experiments targeted directly at the in vivo modification of contraction are fairly limited (Table 1), and salient examples are discussed in detail below.

The value of gene ablation and the resultant loss-of-function approach for delineating the role of a protein in regulating cardiac contraction is illustrated by the phospholamban gene knockout (65). Phospholamban is a small (52-amino acid) phosphoprotein in which the actions are linked in cardiac muscle to the activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump. Ablation of the gene and the subsequent loss of this protein resulted in hyperdynamic cardiac functional parameters with increased positive and negative first derivatives of ventricular pressure development (±dp/dt) values and enhanced ventricular filling. The affinity of the pump for Ca\(^{2+}\) was also increased, and with the use of the heterozygote nulls, which contained ~40% of the wild-type phospholamban levels, a dose-response curve for these effects was established (reviewed in Ref. 54).

For a number of reasons, data dealing directly with remodeling the heart via ablation or modification of the genes that encode elements of the contractile apparatus are limited. Importantly, one might expect that ablation of a critical component of the contractile apparatus would be lethal. A functional heart is critical to fetal development by embryonic days 11–12, and if the heart is seriously compromised, the fetus rarely survives past embryonic day 12.5. Thus homozygous animals carrying the null mutation at both alleles would not come to term but rather would die during embryogenesis and be resorbed. Before one goes to the trouble of making a targeted animal, it is also prudent to have the analytic infrastructure in place for the necessary analyses, and these have been largely lacking for an assessment of cardiac function in utero. Our abilities to discern cardiac contractile function in the intact, midgestation embryo, as reflected by both stroke volume and heart rate, have been quite limited but are developing rapidly (23, 48). To date, however, in the absence of being able to rigorously analyze a resultant phenotype, these genes have not been the ones of choice for ablation experiments. Gene ablation of the sarcomeric sequences can, however, be productively applied to answer a limited subset of such questions as, Do gene dosage effects occur when only one functional copy of the gene is present, and can other closely related isoforms that are coexpressed during development compensate for the particular isogene deficit in either the...
humans, rat, and rabbit, with MHC cardica thick and thin filaments, MHC and actin, appear to be antithetically regulated (39, 60, 67). The intervening region, 4-500 base pairs, contains the transcriptional regulatory apparatus for a gene is downregulated in the ventricles and b gene is upregulated (66, 81). However, b-MHC expression can be reactivated in the heart during a hypertrophic response (22) or by the induction of a hypothyroid state (37), with a concomitant downregulation of a-MHC. It was reasoned, therefore, that ablation of a-MHC with a commensurate decrease in the transcriptional activation of the promoter might be compensated for by maintenance of b-MHC expression in both cardiac compartments, resulting in a major remodeling of the protein complement of the heart. However, no homozygote nulls survived to term, and a study (45) of the embryos revealed that death occurred at 12-13.5 days of gestation. Ultrastructural analyses of the developing fetal homozygote null atria revealed major fiber disarray and a lack of a well-defined sarcomeric structure. No significant, compensatory up-regulation of b-MHC could be detected in this compartment. The ventricles, in which b-MHC is normally expressed during this developmental stage, were overtly normal, although their ultrastructural architecture was subtly modified (J. Robbins and H. Osinska, unpublished data).

Table 1. Remodeling cardiac contractile apparatus

<table>
<thead>
<tr>
<th>Gene targeting</th>
<th>Phenotypic Consequences</th>
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<tbody>
<tr>
<td>Phospholamban ablation (54, 65)</td>
<td>+/- are viable and have hypercontractile hearts; +/- are hypercontractile but less so than +/-, thus establishing a gene dosage effect for the protein in regulating cardiac contractility</td>
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<tr>
<td>a-MHC ablation (45)</td>
<td>+/- are embryonic lethal; ln +/- a-MHC mRNA decreased 50%, protein decreased 16-40%. Variably penetrant cardiac phenotype; decreased contractile function in working hearts and fibrosis. Mild hypertrophic response on molecular level in ~50% of +/-</td>
</tr>
<tr>
<td>a-Cardiac actin ablation (55)</td>
<td>-60% +/- embryonic lethal; others die within 2 wk. Decreased a-actin mRNA and protein in +/-. Increased vascular smooth muscle and skeletal actins in both +/- and +/-, but +/- heart function is normal</td>
</tr>
<tr>
<td>a-MHCArg403-Gln tag and exchange (33)</td>
<td>+/- die soon after birth; +/- are affected, confirming FHC mutation is dominant. Asymmetric LV hypertrophy with significant LA hypertrophy. Myocyte disarray and progressive fibrosis. Gender differences noted (male &gt; female). Decreased contractile function in working hearts (decreased LV function)</td>
</tr>
<tr>
<td>Tnc linked to rat a-MHC promoter (650 bp) (73)</td>
<td>Partial replacement of cardiac Tnc, with stnc in some cardiomyocytes. Decrease in adenosine sensitivity of tension-pCa relationship</td>
</tr>
<tr>
<td>MLC-2v linked to mouse a-MHC promoter (12, 83, 84)</td>
<td>High levels of transgene transcript (4- to 10-fold increase in ventricle compared with normal) but no increase in MLC-2v protein levels. Complete atrial MLC-2a to MLC-2v shift. Atrial myocytes show increase in unloaded shortening velocity. No overt pathology or hypertrophy; slight decreased LV function</td>
</tr>
<tr>
<td>MLC-2f linked to mouse a-MHC promoter (38)</td>
<td>High levels of transgene transcript (4- to 10-fold increase in both compartments compared with endogenous isoforms). No increase in overall MLC-2 protein levels. Complete MLC-2a to MLC-2f shift; partial MLC-2v to MLC-2f shift. No overt pathology or hypertrophy; significant decreased LV function</td>
</tr>
<tr>
<td>ELC-1a linked to mouse a-MHC promoter (J. Robbins, unpublished data)</td>
<td>High levels of transgene transcript (4- to 15-fold increase in both compartments compared with endogenous isoforms). No increase in overall ELC-1 protein levels. Complete ELC-1a to ELC-1a shift. No overt pathology or hypertrophy; significant increased LV function</td>
</tr>
<tr>
<td>TM linked to mouse a-MHC promoter (80, 85)</td>
<td>High levels of transgene transcript (150-fold increase in both compartments compared with endogenous isoform). 34-Fold increase in b-TM protein to 55% of total TM in cells, resulting in partial a- to b shift. Working heart preparation shows significantly decreased LV relaxation times. Myofilament Ca2+ activation is affected</td>
</tr>
<tr>
<td>Troponulin linked to mouse a-MHC promoter (M. A. Sussman, unpublished data)</td>
<td>High protein levels (7- to 20-fold increase in both compartments compared with endogenous isoform). Dilated cardiomyopathy</td>
</tr>
<tr>
<td>a-MHCArg403-Gln + 468-527 linked to rat a-MHC promoter (3,300 bp) (109)</td>
<td>High protein levels (7- to 20-fold increase in both compartments compared with endogenous isoform). Dilated cardiomyopathy</td>
</tr>
<tr>
<td>g-Enteric actin linked to mouse a-MHC promoter (55)</td>
<td>Modest levels (26-50% of endogenous) of transgene transcript. Low levels (2-12%) of protein. Biventricular hypertrophy, with 1 male showing dilated cardiomyopathy</td>
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Nos. in parentheses, ref. no. MHC, myosin heavy chain; FHC, familial hypertrophic cardiomyopathy; LV, left ventricular; LA, left atrial; Tnc, troponin C; stnc, skeletal Tnc; MLC, myosin light chain; TM, tropomysin; ELC, essential light chain; bp, base pair.
significant increase (5- to 10-fold) in β-MHC transcripts could be detected in the ventricles of some adults, a level of transcription sufficient to compensate for the deficit of the α-transcript did not occur. Close examination of the hearts at the molecular, cellular, and whole organ levels revealed that significant modifications in the heart were present in a subset of the animals. In all of the heterozygotes, reduction in the α-MHC transcripts was reflected at the protein level, with deficits of 15–40%. In approximately one-half of the heterozygotes, a significant hypertrophic response could be detected at the molecular level, with transcripts for β-MHC, atrial nature factor, and α-skeletal actin significantly elevated. Examination of the gross pathology of the sections revealed significant fibrotic lesions in the adult hearts, and at the ultrastructural level, clear focal points of fiber dysgenesis and sarcomer shortening were quite common. Alterations in overall sarcemer structure were also reflected by alterations in titin transcript levels. These molecular and cytological pathologies had functional consequences at the whole organ level. Left-sided heart function was measured with the isolated working heart preparation (37), and clear deficits in contractility and relaxation (±dp/dt) were detected. The affected hearts were also unable to respond to increased preload and did not exhibit a Starling response, indicating that the classic length-tension relationship that underlies cardiac muscle function was significantly compromised. However, these deficits did not present in all of the heterozygote null animals, and ~40% showed only minor functional deficits. The underlying mechanisms responsible for this variable penetrance are unknown but are usually ascribed to modifier genes present in the heterogeneous genetic backgrounds of the different animals. However, this critical point remains unresolved. In summary, the MHC knockout established that a deficit in α-MHC transcript accumulation did not result in activation of the β-MHC locus to the level necessary to sustain normal cardiac function. Haploinsufficiency of the major adult cardiac MHC led directly to significant cardiac remodeling, resulting in detectable pathology and compromised cardiac function.

The gene encoding the major thin-filament cardiac protein, α-cardiac actin, has also been disrupted by homologous recombination in embryonic stem cells (55). In the mouse heart, both the α-cardiac and α-skeletal actins are coexpressed, with transient expression of the vascular smooth muscle isoform also occurring during fetal cardiac development (71). Although a majority of the homozygous null animals died during development, ~40–45% survived and lived for 1–14 days postbirth. The survival of these animals implied that compensatory isogene(s) transcriptional activation had occurred that partially compensated for the loss of the α-cardiac actin isoform. RNA and protein analyses confirmed that both the α-skeletal and vascular smooth muscle isoforms were upregulated in these hearts relative to both the wild-type and heterozygous animals (the latter animals showed intermediate levels of these two isoforms relative to the normal and homozygous nulls). Preliminary analyses have shown that, in the heterozygotes, there is a deficit in both the α-cardiac actin transcript and protein and a commensurate upregulation of the α-skeletal and vascular smooth muscle isoforms. The heterozygote null animal was asymptomatic, with morbidity and mortality indistinguishable from the wild-type animals. No overt phenotype presented in these animals, with the overall architecture, histology, and function of the hearts indistinguishable from the wild-type animals, indicating that probably at least a partial replacement of the normal cardiac isoform was well tolerated. However, the actual degree of cardiac isoform replacement was not determined (55).

Homologous recombination in embryonic stem cells can also be used to produce more subtle mutations such that individual domains, or even single-amino acid residues, can be effectively targeted and modified. This methodology, which was developed in parallel by a number of groups, has been termed, in its different reiterations, "hit and run" (40), "in-out" (106), "plug and socket" (59), or "tag and exchange" (3). After the locus is initially targeted, another recombinatorial event is mediated at the same site. Although technically more difficult and time consuming than conventional gene targeting, the approach is extremely powerful. It can be used to determine structure-function relationships because one is able to effect an exact change at a single, defined locus and subsequently determine the effects of the mutation within the context of the whole animal over its lifetime. A single amino acid can be modified or whole functional domains can be changed. Thus it is possible to create chimeric genes in which regions postulated to be critical for the different functionalities of the isoforms under study are changed and/or exchanged. Alternatively, mutations at the particular sites that have been characterized as causing a human cardiovascular pathology, such as familial hypertrophic cardiomyopathy (FHC), can be created, yielding a well-defined animal model of a human cardiovascular pathology.

With this approach, a model of FHC was created (33). FHC is an autosomal dominant disease that causes, with widely varying severity, ventricular hypertrophy and an enlargement of the interventricular septum. Examination of the myocytes reveals a general cellular disarray and myofibril dysgenesis. It is a common cause of sudden cardiac death in otherwise asymptomatic young individuals. As noted above, the disease is caused by multiple mutations in multiple contractile protein genes. For example, over 40 separate mutations have been located in the β-MHC gene (reviewed in Ref. 110). The characterization of these mutations and of additional mutations in other sarcomeric proteins prompted the hypothesis that FHC is a "disease of the sarcomere" (112). A current working hypothesis is that the hypertrophy is caused by compromised force development in the cardiac sarcomere (114).

Geisterfer-Lowrance et al. (33) used a two-step targeting approach to create a modified amino acid residue that corresponds to a missense mutation that has a severe effect on morbidity and mortality when present in the human β-MHC locus (Arg103 → Gin). This
mutation, like a large number of FHC mutations in the MHC locus, is located within the globular head region of the heavy chain, and crystallographic data indicate that it is close to the actin-myosin interface (90). In vitro analyses of the mutant heavy chain revealed normal ATPase activity in the absence of actin, but the maximal velocity of the actin-activated ATPase was reduced approximately fivefold (105). The regions flanking this residue are 100% conserved between the α- and β-MHC, providing a strong rationale for targeting the residue in the murine system (91, 110). The targeted allele carrying the FHC mutation in α-MHC was inserted at the locus, and the mice were made. Although the homozygous FHC mice were born and had an overtly normal heart, they died within 1 wk. As in the case for the human condition, the mutant allele was dominant, with the heterozygotes developing a slight asymmetric hypertrophy in the left ventricle (LV) and enlarged left atrium. Myocardial histology was also abnormal, with apparent myocyte disarray and progressive fibrosis. Significant gender effects were apparent, with the males being affected more severely, although both sexes showed increasing cardiac dysfunction and histopathologies as they aged. The isolated working heart preparations also showed abnormal LV function. Although the model obviously does not exactly recapitulate all aspects of the FHC phenotype, the experiments have provided a stable animal disease model and definitive proof for the genetic basis of FHC. The animals should prove extremely useful in analyzing the hemodynamics, cellular pathologies, response to stress, exercise, and electrophysiological abnormalities that may result from this mutation. They may also prove useful, if the modifier genes can be identified, in explaining the variable penetrance of the disease in both animal models and the patient population.

The difficulty, expense, and time commitment needed to carry out targeting of a locus involved in contractile protein function is underscored by the paucity of examples cited in the above section. An additional concern with gene targeting is the lack of target organ specificity; that is, the targeting event is propagated in all somatic cells and can, if the target gene is critical to the function of other organ or muscle systems, complicate or even confound one’s ability to dissect out the direct effects on cardiac function. To overcome this difficulty, attempts have been made to develop organ-specific gene modification using the DNA recombinase enzyme family, and significant progress with the Cre recombinase has been reported (reviewed in Refs. 70, 89). The approach is disarmingly straightforward. The DNA recombinase Cre can excise DNA in the mammalian genome with high efficiency by binding to its target sequence, a 34-base pair DNA substrate termed loxP. By flanking a target sequence with loxP sites on each side and driving expression of Cre specifically in the heart with cardiac-specific promoters (see TRANSGENESIS AND CONTRACTILE PROTEIN REMODELING), it should be possible to restrict a targeting event only to the heart by breeding a mouse carrying the loxP-flanked DNA to another line that expresses Cre in the cardiac compartment (Fig. 1). A number of laboratories have made substantial progress in the creation of the requisite mouse lines, and positive results of a cardiac-specific gene targeting have recently been reported (1). An additional refinement for conditional knockouts would be the use of a local and temporally defined delivery of Cre with, for example, an adenovirus-based delivery system, and proof of principle for this technique has been established (111).

**TRANSGENESIS AND CONTRACTILE PROTEIN REMODELING**

Transgenesis offers an alternative route for stably altering the mammalian genome such that the modifications can be transmitted through the germ line. Although the procedures are technically possible in larger animals (79), because of cost, time, and animal husbandry considerations, basic research has been largely limited to the mouse. A number of transgenes affecting the heart have been expressed by using gen-
eral or cardiac-specific promoters (reviewed in Refs. 17, 25), and models for general remodeling events such as hypertrophy [e.g., via expression of p21^{ras} or the α_{1B}-adrenergic receptor (18, 74)] have been generated. However, for the purposes of this review, the discussion will be restricted to those experiments in which the contractile apparatus is a specific target.

Currently, transgenesis is most often (but not exclusively) carried out by injecting an exogenous DNA fragment into the pronucleus of a fertilized egg. Unlike the situation for gene targeting, insertion of the exogenous DNA is random, and, generally, multiple copies are placed into the genome, usually at one site in a head-to-tail or head-to-head arrangement. Unless homologous recombination in stem cells is used (10), the insertion point cannot be controlled nor can the investigator predetermine the number of copies placed into the recipient genome. Thus, unlike gene targeting, in which the homologous recombination event can be selected, transgenesis is a random process, and the DNA is placed at sites other than where the homologous sequences are located. This means that expression of the transgene will be superimposed on that of the endogenous gene. Thus, for a phenotype to present, the transgene must yield a dominant effect. The ultimate transcriptional activity of the transgenic DNA can be copy number dependent or independent and is often influenced by the particular chromosomal location into which it is placed ("position effects"), rendering it more or less transcriptionally active. Additionally, placement of the DNA is not a benign process and can lead to significant disruption, rearrangement, or deletion of the flanking DNA, resulting in insertional mutagenic effects (99). Fortunately, these effects are most often recessive, and because analyses of the transgenic animals are usually restricted to the heterozygotes, this does not present a problem. The position-dependent and copy number effects oftentimes necessitate the analysis of multiple lines, an expensive and labor-intensive process. Nevertheless, multiple mutations and chimeric constructs can be generated, and numerous lines of mice can be made relatively rapidly (compared with gene targeting). These points recommend careful consideration of the transgenic approach for cardiac remodeling studies.

Systemic expression of a transgene can seriously complicate the resultant phenotype. The development of promoters in which expression is largely restricted to the cardiac compartment has enhanced the utility of the transgenic approach for specifically remodeling the cardiac contractile apparatus in the absence of other confounding effects on other organ or muscle systems. Not surprisingly, the transcriptional sequences of the cardiac contractile genes themselves have been used effectively to drive cardiac transgenic expression. These include the actin (11), MLC-2v (28, 43), and β- and α-MHC promoters (50, 94) among others. When choosing a promoter in which the primary purpose is to eventually effect a significant change in the contractile apparatus, a number of points need to be considered. First, the underlying transcriptional pattern of the promoter must be taken into account. For example, if the α-MHC promoter is used, transgene expression will occur in the primitive heart tube and in the developing atria throughout the latter two-thirds of gestation. It will only be activated in the ventricle just before birth (102). Thus developmental abnormalities in the heart may occur as a consequence of fetal expression and should be considered. Second, the robustness of the promoter elements must also be considered. The contractile proteins are very abundant, and this is reflected in both the steady-state transcript levels and the transcriptional activity of the genes. To effectively compete with the activity of the endogenous gene (which is normally unaffected by insertion of the transgenic DNA), the transgene must be expressed at very high levels. Although most of the above-named promoters have been characterized for their transcriptional activity in the heart and show good cardiac specificity, their utility may be limited to the expression of various signaling molecules in which the effects do not depend on high transcript accumulation. For example, a truncated version of the MLC-2v promoter drives expression specifically in the ventricle, has been used to express p21^{ras}, and has generated a valuable model of cardiac hypertrophy (43), but the levels of expression achieved with this promoter would not be high enough to replace the structural proteins in the contractile apparatus (7).

Promoters derived from the murine α- and β-MHC genes have been extensively characterized concerning their ability to drive expression in the transgenic context (38, 47, 50, 52, 73, 74, 80, 83, 93, 94). Both promoters have been sequenced completely and direct high levels of transgene expression in a manner that reflects their transcriptional patterns in vivo. An important caveat to consider in experimental design or interpretation is that both of these genes are expressed at other sites. Although it is widely appreciated that the β-MHC isogene encodes the myosin that is found in slow-type striated muscle fibers, α-MHC is also expressed at other sites including the pulmonary myocardium (46) and a restricted set of extracardiac muscles (116).

The α-MHC promoter has been used to remodel the contractile apparatus in the adult mouse heart to explore questions of isoform functionality. Metzger et al. (73) used a rat α-MHC promoter to express high levels of transgenically encoded skeletal TnC in the heart. Depression of cardiac muscle is more sensitive to acidosis than is skeletal muscle. Evidence from several experimental avenues suggested that the pH sensitivity displayed by cardiac muscle had at least a partial basis in the desensitization of the cardiac myofilament to Ca^{2+} activation. The authors hypothesized that the structural basis for the differential sensitivity lay in the unique skeletal versus cardiac TnC isoforms. In the transgenic hearts, Northern analyses and Western blots demonstrated physiologically significant accumulations of skeletal TnC transcript and protein. Expression of the skeletal isoform resulted in a shift in the midpoint of the tension-pCa relationship, a result consistent with the hypothesis that the cardiac TnC isoform plays an important role in determining the pH sensitivity of the cardiac myocyte and is at least
partially responsible for the contractile function depression that is observed after an acute myocardial ischemic episode and the resultant acidosis.

A detailed examination of the transgenic paradigm has been carried out with MLC. As is the case for many of the other contractile proteins, MLC expression is controlled in a muscle type- and developmental stage-specific manner in the heart (20). Atrial- and ventricular-specific genes and isoforms exist, and numerous in vitro and biochemical studies indicate that the unique proteins may have different functions (63, 64, 77). In addition to identifying mutations in the essential and regulatory light chains (LCs) as being associated with FHC (88), other studies have correlated MLC isoform shifts with the development of dilated cardiomyopathy (68), hypertrophy (96), and altered myosin cycling kinetics in different congenital heart diseases (75).

To explore the parameters underlying transgenic replacement, as well as questions concerning LC functionality, a number of different LC replacements were carried out in transgenic mice using the α-MHC promoter to drive expression. Questions of contractile protein stoichiometry were first addressed by expressing the ventricular isoform of the regulatory LC, MLC-2v, in both the atria and ventricle. It is well documented in genetically amenable systems such as Drosophila that gene dosage effects can alter contractile protein levels, resulting in detrimental effects on sarcomere structure and function (8, 30). Although transgenesis can drastically alter the effective gene dosage, this turned out not to be a problem for LC remodeling in the mouse heart. First, expression of the MLC-2v transgene had no effect on the steady-state mRNA levels encoding related contractile proteins, including the endogenous MLC-2v transcripts in the ventricle (84). Thus no downregulation of endogenous gene expression could be detected. Second, although the overall MLC-2v mRNA in the ventricle increased 4- to 10-fold, no commensurate increase in the protein could be detected in either the total protein or myofilament pools. Thus mRNA and protein levels were discordant (83). Further study of the system showed that the transgenic transcripts were translated as efficiently as the endogenous transcripts and that MLC-2v RNA was present in normally sized polysomes (J. Robbins, unpublished data). A working hypothesis is that the protein turnover rates of MLC-2v have been altered to maintain the overall contractile protein stoichiometry.

Although no increase in MLC-2v protein could be detected in the ventricles, complete replacement of the atrial isoform with MLC-2v occurred in the transgenic atria. Presumably due to the high levels of the transgenic MLC-2v RNA in the atria relative to MLC-2a RNA, the rate of MLC-2v protein production is high enough such that dilution effects lead to essentially complete replacement. The phenotypic effects of the replacement were not striking, consistent with the data that overall contractile protein stoichiometries had been unaffected in the mice. No overt pathology presented, nor was whole organ function, as measured in the isolated working heart, significantly altered. The histology and overall architecture of the cardiomyocytes at the cytological level and of the sarcomeres at the ultrastructural level were also unremarkable (83). However, isoform functionality was demonstrated by examination of contractile function at the atrial cardiomyocyte level. The speeds of shortening and relengthening at maximum Ca²⁺ stimulation were both increased, a result that is consistent with the higher contractile demands that are placed on the ventricle relative to the atrium (12).

Expression of the skeletal (fast) LC-2 isoform (MLC-2f) extended the efficacy of the transgenic paradigm to the remodeling of both cardiac compartments. A number of lines carrying varying copies of the transgene were made: as the number of copies increased, the steady levels of the transgenic mRNA also increased (38). Copy number dependence of transgene expression levels is promoter dependent (51) and has important implications for the physiological assessment of a cardiac contractile protein modification or isoform replacement. By creating multiple lines with varying copy numbers, the transgene will be expressed at different levels, and a complete dose-response curve for the transgenic replacement can theoretically be generated.

Importantly, transgenic expression with the full-length α-MHC promoter was homogenous at the cardiomyocyte level. However, despite approximately equal levels of MLC-2f RNA in both cardiac compartments, examination at the protein level revealed significant differences in the degree of replacement exhibited between the atria and ventricles. Although the atria showed essentially complete replacement at low or moderate copy number (3–10), the ventricles were more refractory. Even under conditions in which copy numbers were high (20–34) and the transgenic transcript was present in ~10-fold excess relative to MLC-2v, replacement was <40%. By breeding the different lines with one another such that additive mRNA accumulations resulted, an increased degree of replacement could be achieved. A working hypothesis consistent with these data and supported by in vitro replacement strategies is that the different isoforms have radically differing affinities for both their own and the ectopic contractile apparatuses. Thus MLC-2v may have a very high (relative to MLC-2f) affinity for its endogenous contractile machinery, rendering it extremely effective in competing out the transgenic MLC-2f. Presumably, the unbound protein is then rapidly degraded. By increasing the steady-state levels of MLC-2f relative to MLC-2v, one can drive the replacement more completely. Again underscoring the different functions of the unique isoforms, cardiac contractility and relaxation were both affected as measured by the whole working heart preparation, with severe deficits in both indexes presenting.

Thus, in these remodeling experiments, the degree of replacement is a function of both the steady-state levels of the transcript that can be obtained and the affinity of the transgenic protein relative to the endogenous species that is being replaced for the contractile apparatus. It should be emphasized that, for contractile protein
remodeling, complete replacement will almost certainly not be necessary to effect a severe phenotype: as noted above, FHC is a dominant disease trait, and in hearts in which only partial contractile protein isoform shifts present, significant changes in contractile function occur (75).

The α-MHC promoter has also been used to modulate the α- to β-isofrom ratio of the actin-binding protein TM, which, in conjunction with the Tn complex, regulates Ca2+-sensitive interactions of the thick and thin filaments. The α- and β-striated TMs show 87% identity at the amino acid level, have a preferential ability to form α,β heterodimers (compared with the α,α or β,β species), and show varying ratios in the striated muscle fiber types. In the mouse heart, α-to-β ratios increase from 5:1 to 50:1 during maturation and the α,α homodimer is the predominant species. With the use of transgenesis to overexpress β-TM, a 150-fold increase in the message level was obtained in the heart, leading to a 34-fold increase in β-TM protein (the cardiomyocytes now consisting of 45% α and 55% β) and a resultant shift in TM content from α,α to α,β. Unlike the case observed for the thick filament-associated LCs, the massive increases in β-TM transcript and/or protein led to a decrease in the α-TM transcripts (and protein), indicating the existence of “cross talk” at the RNA or protein level (80).

Overexpression of the protein did not cause any overt cardiac pathology or changes in the expression of other contractile protein genes. Cardiomyocyte structure and ultrastructure were both unremarkable, although a slight deficit in diastolic function was noted as measured in the isolated working heart. However, force measurements on isolated cardiac muscle fiber bundles revealed that the TM isoform switch increased the ability of strong cross-bridge binding to activate the thin filament. Ca2+-sensitivity of myofilament activation was also increased, and the rightward shift of the Ca2+-force relationship, which is effected by the adenosine 3′,5′-cyclic monophosphate-dependent phosphorylation of the inhibitory unit of Tn (TnI), was significantly blunted (85). These data indicate that modulation of the α- to β-TM ratios can play a role in myofilament desensitization by phosphorylation of TnI and that thin-filament sensitivity to activation by Ca2+ is mediated, at least in part, by the relative ratios of the TM isoforms.

In addition to determining isoform function, transgenic expression can also be used to illustrate the in vivo functionality of newly defined sarcomeric proteins. Tropomodulin is an actin-capping protein that appears to be involved in the control of actin filament length in striated muscle. In vitro, it can inhibit elongation of the actin filament at the slow-growing (pointed) end (36, 104). The α-MHC promoter was used to drive varying levels of tropomodulin in the transgenic mouse heart (M. A. Sussman, personal communication). Significantly, severity of the phenotype was directly linked to the steady-state levels of the protein. Extensive myofibril dysgenesis was detected, and abnormal sarcomeric patterns, as assayed by immunocytochemistry on intact cardiomyocytes, were also obvious. At the whole organ level, a striking dilated cardiomyopathy presented, illustrating the importance of regulating tropomodulin levels and the critical role that control of actin filament length can play in the maintenance of normal myofilament structure and heart function.

Unlike the multistep homologous recombination-based strategies, creation of site-specific mutations in cDNA or a gene can be rapidly carried out in vitro, and the resultant DNA can be used in pronuclear injections to generate multiple transgenic lines. In an initial experiment, transgenesis was used in an attempt to create an animal model of FHC (109). With the use of a rat α-MHC promoter linked to a rat α-MHC cDNA that contained the Arg403 → Gln mutation, as well as an internal deletion covering amino acids 468–527, five transgenic mouse lines were generated. Although high levels of the transgenic MHC transcript could be detected in some of the lines, the amount of rat polypeptide, as detected by a slight size difference on polyacrylamide gels, was quite minor, confirming that a discordance between transgenic transcript and protein levels can present when one attempts to remodel the sarcomere. All lines showed a degree of small-vessel coronary disease, a characteristic observed in some human patients with the disease. The amount of mutant protein present in the myofilaments was surprisingly small. Estimates ranged from 2 to 12% of the total myosin for the two lines that were analyzed. Nevertheless, a phenotype presented, consistent with the observation that the FHC mutations are dominant. Gender differences were also noted; however, in contrast to the gene-targeted FHC model (33), the females exhibited the more pronounced hypertrophic response, with LV mass increased by 12% and right ventricular mass by 14% relative to nontransgenic littermates. A dilated cardiomyopathy of the LV also developed in the males of one line as the animals aged.

Although aspects of this model are difficult to interpret due to the multiple mutations present in the protein, the low level of protein produced, and the cross-species nature of the construct, the animals do demonstrate some of the phenotypic characteristics of the human condition, perhaps more so than the genetically accurate model produced by gene targeting. The relative efficacy of each of these models for elucidating the mechanisms of the developing cardiomyopathy remains to be determined in future studies.

The effectiveness of transgenically mediated isoform shifts can be enhanced significantly by breeding these animals into a null background (produced by a gene knockout). Undoubtedly, this will become a more common approach as the repertoire of relevant gene-targeted and transgenic strains grows. The effectiveness of combining transgenesis and gene targeting has been demonstrated with the actin nulls. Transgenic mice that overexpress γ-enteric actin in the heart were made and subsequently bred against the α-cardiac actin gene knockout mice such that transgenic expression occurred on the homozygous null background. Substitution of the enteric isoform rescued the
null phenotype, and the animals lived for extended periods (55). However, the unique functional profiles of the different actin isoforms were confirmed at the whole organ level. Significant differences in cardiac function, as assayed with both the isolated working heart and a closed-chest model (61), resulted from the replacement. The hearts displayed a significant hypertrophic response, were dramatically enlarged, and showed striking hypoxic contractile function. These data demonstrate that actin isoform substitutions can result in altered muscle function.

Isoform substitutions such as those described above can provide detailed information about isoform specificity and function. Although the presence of a transgenic message against the endogenous background can be easily detected, documentation of transgenic protein, if its expression is not ectopic to the particular cardiac compartment, can be problematic. For example, if a subtle mutation is made to study the function of a particular domain, it may not be possible to generate specific antibodies for the transgenically encoded polypeptide, especially if the substituted protein differs from the native species by only a few amino acids. These experimental problems have made use of epitope tags such as c-myc (EQLISEEDL), FLAG (DYKD- DDDDK), or vesicular stomatitis virus glycoprotein (YTDIEMNRLGK) attractive because highly sensitive monoclonal antibodies against the epitopes are commercially available. However, in analyzing any phenotypic change(s), one must always consider the possibility that the tag itself, and not the substituted isoform, caused the perturbation. Normally, the tags are placed arbitrarily at the amino or carboxy terminus to try to minimize any impact on the function of the protein. But the rationales behind these placements are usually not particularly rigorous due to a lack of high-resolution molecular modeling, and subtle changes in the function of the protein due to the tag are difficult to detect. This can be especially problematic in the cardiac sarcomere in which the proteins assume an almost crystalline arrangement. The addition of an epitope at a functionally unfortunate locus could result in a major, easily detectable effect, such as nonincorporation of the tagged protein, or could subtly interfere with sarcomere function and complicate the phenotypic analyses of the mutation to a considerable degree.

CONDITIONAL TRANSGENESIS AND CONTRACTILE PROTEIN REMODELING

As applied to cardiac contractile protein remodeling, being able to turn transgene expression on and off at will during the lifetime of an animal (conditional transgenesis) is an unrealized goal. A report of conditional transgenesis in mice using a tetracycline-transactivated system (29) prompted a number of labo-
ratories to apply this technology to the cardiovascular system. The paradigm as described is a binary system requiring two transgenes: 1) a cardiac-specific promoter driving the tetracycline transactivator (tTA) sequence coupled to the transcription activator virion protein 16 (VP16) and 2) a cytomegalovirus (CMV) minimal promoter coupled to multimers (5–7 copies) of the tetracycline operon (tetO) (Fig. 2). This chimeric promoter is then ligated to the target transgene that is to be conditionally controlled. In the presence of tetracycline, the tTA binds to the drug. Transcription of the reporter gene, dependent on the very low basal activity of the minimal promoter is "off." If tetracycline is not present in the system, tTA binds to the tetO, allowing the VP16 transactivator to increase expression driven by the CMV minimal promoter. Expression of the reporter gene can thus be turned on and off by removing or supplying tetracycline. Conditional transgenesis would be a particularly informative tool for remodeling the cardiovascular system because a significant portion of human cardiac pathology is related to compensatory mechanisms inherent in the plastic nature of the heart. For example, the pathological manifestations of FHC, massive cardiac hypertrophy that impairs diastolic relaxation and causes attendant decreased coronary filling and myocardial ischemia, are likely compensatory mechanisms, the roots of which lie in a decreased force generated by a mutated sarcomeric protein (114). If expression of the mutated protein could be cycled on and off in a controlled manner, the steps leading from initial decreased force generation by individual sarcomeres to the final clinical end point of devastating cardiac hypertrophy could be determined and better therapeutic modalities could be developed.

To date, the binary tTA system has been used successfully in tissue culture assays, but extension to the mouse heart has been more problematic. A 2.9-kilobase rat α-MHC promoter was used to drive expression of tTA-VP16 and either the firefly luciferase or β-galactosidase gene as the reporter (119). Expression of the reporter genes was limited to the heart and was appropriately extinguished with tetracycline administration. Firefly luciferase activity was induced ∼300-fold, but β-galactosidase activity was not homogeneous in the cardiomyocytes. In another study, an α-MHC promoter was used to drive cardiac-specific expression of the tTA-VP16 construct, with the CMV minimal promoter driving expression of Id1, a helix-loop-helix gene that is a negative regulator of differentiation in several different cell lineages (86). In these experiments, expression of both tTA and Id1 was shown to be limited to the heart. Id1 was expressed while the mice were off tetracycline but was not detected when their drinking water was supplemented with the antibiotic, indicating appropriate interaction of tTA and tetO. No data were presented regarding protein levels of Id1. Thus proof of principle for the tTA-VP16 system has been demonstrated in the heart. However, several important considerations need to be addressed. First, it is clear that reporter gene expression is not uniform, even between littermates, raising questions about the overall stability and reproducibility of the system. Additionally, the amount of target (conditionally expressed) protein accumulation that can be achieved is unclear. The nonhomogeneous β-galactosidase activity suggests that the tTA-VP16 system may not be able to drive sarcomeric protein gene expression at high-enough levels to remodel the cardiac sarcomere and effect a phenotype. Conditional expression of cardiac genes that encode "amplifiers" such as receptor or signaling peptides may be more amenable to this system, in which small changes in expression levels of the desired gene could result in a magnified cardiac response.

**FUTURE DIRECTIONS**

The general utility of these approaches for studying basic questions of normal and abnormal cardiac physiology should increase exponentially in the next decade as the number of animal models grows and interbreeding programs are begun. Our abilities to more precisely control both gene targeting and transgenesis will also dramatically increase. Spatial control of transgenesis is already a reality, and organ-specific knockouts are on the horizon. Temporal control of both processes will also be possible; "conditional transgenesis," in which the transgene can be cycled on and off in the heart by pharmacological intervention, has already been demonstrated (26, 86, 119). This will enable the cardiac physiologist to not only determine the functional consequences of the transgenic reagent but also study whether the heart can, with transgenic expression abolished, cycle back toward normalcy. Variations on these themes, not yet foreseen, should allow us to modulate heart function by reversible interventions at the gene and protein levels such that the molecular bases for normal and abnormal cardiac function can be determined and used to explore the mechanistic underpinnings of cardiac physiology. In all of these studies, as major and minor effects on cardiac physiology are noted, it is important to keep in mind that a mouse is not a human. Extension of some of the most valuable models to large-animal transgenics will provide both a measure of the relevance of the conclusions drawn from the murine models and suitable models for testing potential clinically important treatment modalities.

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