Yin Yang 1 (YY1) is a transcription factor that can repress or activate transcription of the genes with which it interacts. In this report we show that YY1 is a negative regulator of the α-myosin heavy chain (αMyHC) gene, which, with βMyHC are the molecular motors of the heart. αMyHC mRNA and protein levels are down-regulated in hypertrophy and heart failure, and this is thought to be detrimental for cardiac contractility. We show that YY1 specifically interacts with the αMyHC promoter and that overexpression of YY1 in cardiac cells represses the activity of the αMyHC promoter. We also show that the 170–200-amino acid region of YY1, important for its interaction with histone acetyl transferases and histone deacetylases, is important for its repressive activity and that YY1 deleted in this region is an activator of the αMyHC promoter. Moreover, we show that YY1 levels and DNA binding activity are increased in failing human left ventricles and in a mouse model of hypertrophic cardiomyopathy, where αMyHC levels are decreased. These results suggest that YY1 is a negative regulator of αMyHC gene expression.

The cardiac myosin heavy chains (MyHCs),1 α and β, differ in the rate of ATP hydrolysis, with αMyHC having an ~2-fold greater rate than βMyHC (1). The contractile velocity of cardiac muscle correlates with MyHC isofrom composition, suggesting a causal relationship between MyHC isoform composition and cardiac contractility (1). Cardiac myosin composition varies among species, with small rodents expressing predominantly αMyHC and large mammals expressing predominantly βMyHC. Independent of species, antithetical transitions in cardiac MyHC isofrom composition have been observed in response to various pathological and physiologic stimuli. Many of these stimuli result in cardiac hypertrophy accompanied by changes in gene expression and increases in cardiac myocyte size. In rodent models, pathological stimuli result in down-regulation of αMyHC and up-regulation of βMyHC (2). In the normal human heart, ~20–30% of total MyHC mRNA consists of αMyHC mRNA, whereas in the failing heart, αMyHC expression represents less than 2% of total MyHC (3–5). At the protein level, αMyHC in normal hearts constitutes ~11% of total MyHCs, but it is undetectable in the failing heart (6). Moreover, in humans, increases in αMyHC isofrom expression are closely associated with improvements in left ventricular function and chamber remodeling, in contrast to the expression of several of the other genes known to regulate contractile function or pathological hypertrophy (7). Therefore, rodent and human models of hypertrophy and failure have directionally similar MyHC isoform shifts.

In contrast to pathological stimuli, the hypertrophy induced by exercise (physiological hypertrophy) is accompanied by an induction of αMyHC and repression of βMyHC in rats (8) and in C57/B16 mice (9). Furthermore, this hypertrophy is beneficial and is associated with cardiovascular conditioning (9, 10). Recent evidence suggests that even a small difference in MyHC isofrom composition in the heart can have important consequences for contractility. Transgenic mice expressing 12% of their cardiac myosin as βMyHC have decreased contractility and myofibrillar ATPase (11). In contrast, isolated cardiac myocytes that express only 12% of their MyHCs as α have 52% greater power output than those expressing only β (12). Thus, the decrease in αMyHC in human heart failure may play an important role in the reduction of contractility observed in the failing heart and is likely to be attributable, at least in part, to transcriptional regulation.

Through transfection, gene injection, and transgenic experiments, it has been possible to identify a number of regulatory sites in the rodent αMyHC promoter region (13, 14, reviewed in Ref. 15). Although most of these elements act positively, there is one report characterizing an element that acts as a repressor of the rat αMyHC promoter. This element, located in the first intron of the gene, is an ets binding site (16) but is not present in either the proximal or distal promoter elements of the human αMyHC promoter.2 Relatively little is known about the regulatory elements of the human αMyHC gene. However, the proximal promoter regions (~340/+20 bp) of the human and rat αMyHC genes show 80% sequence identity, including most of the potential DNA-binding sites (Fig. 1). Previous studies have shown that the ~340/+20 bp promoter region of the rat αMyHC gene is sufficient to direct cardiac-specific expression in cells (17). This fragment contains binding sites for GATA4, MEF2C, SRF, TEF, and thyroid hormone (15).

Yin Yang 1 (YY1) is a transcription factor that has been shown to regulate a variety of promoters (reviewed in Ref. 18). In cardiac myocytes, it has been shown to act largely as a

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1 The abbreviations used are: MyHC, myosin heavy chain; YY1, Yin Yang 1; HDAC, histone deacetylase; HAT, histone acetyltransferase; RA, retinoic acid; NRVM, neonatal rat ventricular myocyte; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; HCM, hypertrophic cardiomyopathy.
2 C. Sucharov, unpublished observations.
YY1 Is Increased in Human Heart Failure

**MATERIALS AND METHODS**

**Antibodies**—YY1 antibody (SC-7341X) was purchased from Santa Cruz Biotechnology. The alkaline phosphatase anti-mouse (115-053-146) was purchased from Jackson Laboratories. The αMyHC antibody was a gift from Dr. Kathy Schreiber, Myogen.

**Plasmid Constructs**—The −454/+32 bp fragment of the αMyHC promoter was cloned into the pG3 basic vector (Promega). Mutations were created in the YY1 binding sites by generating oligonucleotides (see “EMSAs”) containing the mutation of interest and amplifying the fragments containing the mutation by PCR. The YY1 expression construct was a gift from Dr. Michael Atchison (University of Pennsylvania). The YY1 170-200del construct was a gift of Dr. Edward Seto (University of South Florida). The DNA constructs were purified using the Qiagen method.

**Cell Culture and Transfection**—Neonatal rat ventricular myocytes (NRVMs) were prepared according to the method described in Waspe et al. (28). Briefly, 150,000 cells/well were plated in 12-well tissue culture plates coated with gelatin. Eighteen h later, the medium was changed to minimum Eagle’s medium supplemented with Hank’s salt and t-glutamine. 20 μM Hepes pH 7.5, penicillin, vitamin B12, bovine serum albumin, insulin, and transferrin were added to the medium. Transfections were carried out by the FuGENE 6 (Roche Applied Science) method according to the manufacturer’s recommendations; 0.75 μl of FuGENE-0.25 μg of plasmid DNA was transfected in each well. In the cotransfection experiments, the total amount of DNA was kept constant by the addition of a plasmid containing the cytomegalovirus (CMV) promoter not driving the expression of any gene. H9C2 cells were maintained according to American Type Culture Collection recommendation. Transfection in H9C2 cells was done by the FuGENE 6 method; 1.8 μl of FuGENE-0.6 μg of DNA was transfected in each well on a 24-well plate. Retinoic acid (RA) differentiation of H9C2 cells was done by treating the cells with 10 nM RA every day for 5−7 days in medium with 1% fetal bovine serum.

**Preparation of Protein Extracts**—Protein extracts from human normal and failing (idiopathic cardiomyopathy) left ventricles and transgenic hypertrophic cardiomyopathy (HCM) mice and littermate controls, 4 months of age, were prepared according to Molkentin and Marklan (29) with minor modifications. 0.5 g of tissue was homogenized with 1% fetal bovine serum.

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YY1 Is Increased in Human Heart Failure

YY1 binds to the human αMyHC promoter in a specific manner. A, EMSA of the YY1 probe + NRVM nuclear extract. Lane 1, YY1-1 probe; lane 2, YY1-1 probe + NRVM nuclear extract; lane 3, addition of the YY1 antibody; lane 4, YY1-2 probe; lane 5, YY1-2 probe against NRVM nuclear extract; lane 6, addition of the YY1 antibody; lane 7, YY1-3 probe; lane 8, YY1-3 probe against NRVM nuclear extract; lane 9, addition of the YY1 antibody. B, the mutant probes do not bind to YY1. Lane 1, YY1-1; lane 2, YY1-1M; lane 3, YY1-2; lane 4, YY1-3M; lane 5, YY1-3; lane 6, YY1-3M. C, competition experiments were done to show specificity of binding. Lane 1, YY1-3 probe; lane 2, YY1-3 probe + NRVM nuclear extract; lane 3, addition of 20-fold excess of the wild-type unlabeled competitor; lane 4, addition of 50-fold excess of the wild-type unlabeled competitor; lane 5, addition of 100-fold excess of the wild-type unlabeled competitor. D, competition with the mutant, double-stranded oligonucleotide. Lane 1, YY1-3 probe; lane 2, YY1-3 probe + NRVM nuclear extract; lane 3, addition of 20-fold excess of the mutant unlabeled competitor; lane 4, addition of 50-fold excess of the mutant unlabeled competitor.

RESULTS

The YY1 Transcription Factor Binds to Several Sites in the Human αMyHC Promoter Proximal Region—YY1 has a loose consensus binding site, with the 5′CCAT3′ core sequence being essential for binding (33). The −452-bp fragment of the αMyHC promoter was scanned for potential YY1 binding sites using the Vector NTI Suite v. 6.0 (InforMax). Three regions containing the 5′CCAT3′ core were found (−425/−413 (YY1-1), −370/−360 (YY1-2), and −325/−310 (YY1-3)). To test whether these regions were capable of interacting with the YY1 transcription factor, 30-bp probes containing the putative YY1 binding sites were created and used in EMSAs with nuclear extracts prepared from NRVMs (see “Materials and Methods” for probe description). Fig. 2A shows the result of EMSA experiments using the three potential YY1 binding regions in NRVMs. A prominent complex was observed for all three probes (Fig. 2A, lanes 2, 5, and 8). To confirm that this complex contains YY1, an anti-YY1 antibody added to the reaction prevented the formation of the complex (Fig. 2A, lanes 3, 6, and 9). The YY1 antibody was used against GATA4 and Sp1 binding complexes, and no alteration to their binding ability was observed, consistent with the specificity of the antibody (data not shown). To characterize further the interaction of YY1 and the αMyHC promoter, mutations in the YY1-1, YY1-2, and YY1-3 binding sites that should disrupt YY1 binding were designed (see “Materials and Methods”). The mutant oligonucleotides were labeled and used as probes against NRVM nuclear extracts. The mutants failed to interact with any proteins in the extract (Fig. 2B, lanes 2, 4, and 6). Specificity of this binding was tested using the wild-type and the mutant double-stranded oligonucleotides as cold competitors. As seen in Fig. 2C (lanes 3, 4, and 5), increasing amounts of the YY1-3 wild-type site as a cold competitor decreased YY1 binding in a concentration-dependent manner. Addition of the mutant cold competitor did not affect YY1 binding, suggesting that the binding is specific (Fig. 2D, lanes 3 and 4).

The YY1 Transcription Factor Is a Repressor of αMyHC Promoter Activity—YY1 has been shown to act as a positive or negative regulator of transcription (18). To test the effect of YY1 on the activity of the αMyHC promoter, cotransfection experiments were performed in NRVMs. As shown in Fig. 3A, −452 bp of the αMyHC promoter driving the expression of the luciferase gene was cotransfected with increasing amounts of a YY1 expression construct. Dose-dependent repression of promoter activity was observed with 0.04 to 0.2 μg of CMV-YY1 DNA transfected into the cells (Fig. 3A). Because YY1 can act as a positive or negative regulator of transcription depending on the promoter and/or cell context, we tested whether its repressive effect on αMyHC promoter activity was specific for NRVMs. H9C2 is a rat atrial embryonic cell line that has characteristics of both skeletal and cardiac muscle (34). Differentiation of H9C2 cells with RA has been shown to activate a more cardiac phenotype (34). Undifferentiated H9C2 cells were cotransfected with the αMyHC promoter and CMV-YY1. As shown in Fig. 3B, YY1 significantly increases the activity of the αMyHC promoter in H9C2 cells. Interestingly, upon differentiation of H9C2 cells by RA, YY1 becomes a repressor of the αMyHC promoter. Cotransfection of a YY1 expression construct, and the αMyHC promoter-luciferase construct into...
HeLa cells also resulted in an increase in the activity of the promoter (data not shown). These results suggest that repression of αMyHC promoter activity by YY1 occurs in a cell type-dependent manner.

EMSA experiments showed that the αMyHC promoter has three sites that can interact with YY1. To test whether abolishing the interaction of the YY1 transcription factor with the αMyHC promoter would result in an up-regulation of this promoter in cells, point mutations in all three YY1 binding sites and all possible combinations were generated in the context of the αMyHC promoter. The resulting constructs were transfected into NRVMs. As shown in Fig. 4A, each of the mutant constructs showed 2–4-fold higher activity when compared with the wild-type promoter construct. Combinations of mutations in the different sites did not result in an increase in the activity of the promoter when compared with mutations in single sites, suggesting that a single YY1 site is sufficient for complete repression by YY1 (see “Discussion”).

To show further that the effect of YY1 on the αMyHC promoter is attributable to a direct interaction of YY1 with the promoter, cotransfection experiments using the YY1 cDNA and the wild-type αMyHC promoter, as well as promoter constructs containing each of the sites mutated individually, or a combination of the three sites mutated, were done in NRVMs. As shown in Fig. 4B, the wild-type and singly mutant constructs were repressed by YY1, but the construct containing all three sites mutated did not respond to YY1. These results suggest that YY1 repression of the αMyHC promoter is not the result of a non-specific squelching effect attributable to the presence of an exogenous DNA into the transfection experiments. To the contrary, an αMyHC promoter construct that cannot bind YY1 has increased activity in NRVMs.

YY1 Repression of the αMyHC Promoter Is Suppressed upon Deletion of the 170–200-Amino Acid Region—The region of YY1 encompassing 170–200 amino acids has been shown to be important for its interactions with HDACs and HATs (27). A YY1 construct deleted for this region was used in cotransfection experiments with the αMyHC promoter. As shown in Fig. 5, this construct not only fails to repress the activity of the αMyHC promoter but increases the activity of the promoter 2–3-fold. These results suggest that the 170–200-amino acid region of the YY1 transcription factor is of fundamental importance to its function as a repressor of αMyHC promoter activity in NRVMs. Because this is the region that has been shown to be important in the interaction with HDACs and HATs in HeLa cells, it is possible that the function of YY1 may be regulated by these proteins in NRVMs.

YY1 Levels Are Increased in Failing Human Left Ventricle and in Mouse HCM—Considering that αMyHC expression (mRNA and protein) is decreased significantly in myocardial pathological hypertrophy and failure, we next tested the hypothesis that the amount of YY1 would be increased in these settings. Western blots were performed using protein extracts from non-failing and failing human left ventricle as well as from cardiac tissue from wild-type mice and a transgenic mouse model of HCM (35). YY1 levels are increased 2-fold in failing human left ventricle (Fig. 6A) and in the hearts from the HCM transgenic mouse model (Fig. 7A). Six different non-failing and failing left ventricle samples were used, and the experiment was repeated twice. The failing left ventricle samples came from patients 39, 66, 54, 49, 55, and 54 years of age, and the non-failing left ventricle samples came from patients 50, 19, 20, 75, 56, and 55 years of age. Four different heart samples were used for the wild-type and HCM samples. Because hypertrophy increases the general transcription/translation activity of cardiac myocytes, the loading control for the Western blots was done by measuring total protein in each lane through Coomassie staining of the polyvinylidene difluoride membranes (see “Materials and Methods”). To test whether YY1 binding activity correlated with the increase in protein levels observed in the failing hearts, the YY1-2 probe from the αMyHC gene was used as a probe in EMSAs against normal and failing extracts. As shown in Fig. 6B, binding of YY1 was increased 3-fold in the failing heart samples. There is, therefore, an anesthetic relationship between the amount and binding activity of the YY1 transcription factor and αMyHC mRNA and protein. These results are consistent with the hypothesis that YY1 up-regulation in failing hearts could be contributing to the down-regulation of human αMyHC gene expression in hypertrophy and heart failure.

YY1 Is Decreased in Exercise-induced Hypertrophy—As described earlier, exercise induces hypertrophy and has been shown to be associated with an increase in αMyHC gene expression (8, 34). Exercise has also been shown to counteract the deleterious effects of hypertension (36). Considering that YY1 decreases the activity of the αMyHC promoter in NRVMs and its levels are increased in human failing hearts, we hypothesized that the elevated YY1 levels in human heart failure would be
In this study, we attempted to gain an understanding of the molecular mechanisms whereby αMyHC is decreased in pathological hypertrophy and heart failure. The transcription factors that have been shown to be important for the regulation of the rat αMyHC promoter, i.e. GATA4, MEF2C, NFAT3, and SRF among others, are positive regulators of transcription (15). There is only one report of an ets binding region in the rat αMyHC promoter that has been shown to act as a repressor (16), but this region is not present in the human αMyHC proximal promoter region (data not shown). Here we show that YY1 binds to the αMyHC promoter in a sequence-specific manner and represses its transcription in cardiac cells. We also show that YY1 is increased in the failing human heart and in a transgenic mouse model of HCM but decreased in exercised-induced hypertrophy.

Cotransfection experiments in NRVMs suggest that YY1 is a strong repressor of human αMyHC promoter activity. Squeezing of transcription in the presence of an excess of a transcription factor is a phenomenon that has been extensively described (38). In our studies, we have three experiments that show that the repression mediated by YY1 is not attributable to a non-specific squelching effect. First, point mutations in the YY1 binding sites of the human αMyHC promoter prevented the binding of YY1 to this promoter. Mutation of these sites also resulted in its up-regulation, suggesting again that the YY1 is a transgenic mouse model of HCM but decreased in exercised-induced hypertrophy.

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Transfection experiments using the constructs containing single sites mutated as well as combinations of these sites mutated suggest that the repressive effect of YY1 on the αMyHC promoter is not additive but equivalent for all three sites. Combinations of mutations in the three different sites did not result in a further increase in promoter activity when compared with constructs containing single mutations. At this point, we do not know the reason for this finding, but it could be
attributable to the fact that repression by YY1 requires all three sites and that disruption of a single site abolishes YY1 repression activity.

Why YY1 represses transcription of the αMyHC promoter in cell types in which this promoter is normally active is not yet known. One hypothesis is that YY1 is a general repressor of transcription in cardiac cells. However, YY1 can activate transcription of brain natriuretic peptide in cardiac cells (21). Another hypothesis is that there is an equilibrium between YY1 (as a repressor) and activators of the αMyHC promoter. In a normal physiological state, YY1 repression of the promoter is counteracted by positive regulators, but in a pathological state, when αMyHC activity is repressed, YY1 is capable of overcoming the activators and effectively represses the promoter.
The mechanism by which YY1 represses or activates transcription is not clear. Various groups have attempted to characterize functional domains of YY1 that are responsible for its opposing effects. The reports in the literature are controversial, and some regions that are characterized as activators by one group are characterized as repressors by other groups (18). Recently, YY1 has been shown to be itself acetylated and deacetylated by HATs and HDACs, respectively. These studies were done in HeLa cells and two HATs, pCAF and p300, were found to acetylate the 170-200-amino acid region of YY1, whereas the C-terminal zinc finger was acetylated only by pCAF. Acetylation of this central region increases the repressive activity of YY1 and results in a more efficient interaction with HDACs (27). Acetylation of the C-terminal DNA-binding region most likely decreases the repression activity of YY1 by decreasing its affinity for DNA (27). Our results suggest that the 170–200-amino acid region plays a fundamental role in the repression of αMyHC promoter activity mediated by YY1 in NRVMs. However, preliminary studies from our laboratory suggest that in NRVMs neither pCAF nor p300 is capable of reversing the repressor activity of YY1 (data not shown). YY1 has been shown to be deacetylated by class I HDACs (1, 2, and 3) in HeLa cells. In addition to class I HDACs, cardiac cells also express the class II HDACs (4, 5, and 6). It is unknown whether the class II HDACs play a role in the repression of YY1 in cardiac cells. In these cells, the best characterized example of HDAC/HAT regulation is the MEF2C-HDAC-calciumpcalmodulin-dependent kinase pathway (39), and it is possible that YY1 regulation might follow a similar pathway in this context. It is also possible that the regulation of YY1 in cardiac cells is not mediated by acetylation/deacetylation.

The increase in YY1 protein levels in human failing heart extracts and in the HCM mouse model, where αMyHC levels are very low, is consistent with the hypothesis that it is a repressor of αMyHC gene expression in vivo. Our data are in agreement with a previous report that YY1 levels are increased in failing human heart and in animal models submitted to left coronary occlusion (40). In both cases, a limitation of the technique is that YY1 levels are not measured in individual myocytes. The decrease in YY1 levels in exercised HCM mice is of particular interest. In physiological hypertrophy in wild-type mice, there is a 10% increase in heart weight, no change in αMyHC expression, and YY1 levels do not change. However, in HCM, where there is pathological hypertrophy and a decrease in αMyHC, exercise reverses the induction of YY1 levels and increases αMyHC levels. Together, these results suggest that αMyHC levels are tightly linked to YY1.

In conclusion, our results show that YY1 and αMyHC are regulated in an antithetical manner and suggest that transcriptional activity of the αMyHC promoter is dependent on low levels of YY1 and vice versa. Our results also show for the first time a transcription factor that represses the activity of the human αMyHC promoter. Although further investigation is required, YY1 could be part of a pathway that functions to repress the αMyHC gene expression in cardiac hypertrophy and failure, contributing to the pathology of human heart failure.

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