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Hairy-related Transcription Factors Inhibit GATA-dependent Cardiac Gene Expression through a Signal-responsive Mechanism*

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Combinatorial actions of transcription factors in multiprotein complexes dictate gene expression profiles in cardiac development and disease. The Hairy-related transcription factor (HRT) family of basic helix-loop-helix proteins is composed of transcriptional repressors highly expressed in the cardiovascular system. However, it has remained unclear whether HRT proteins modulate gene expression driven by cardiac transcriptional activators. Here, we have shown that HRT proteins inhibit cardiac gene transcription by interfering with GATA transcription factors that are implicated in cardiac development and hypertrophy. HRT proteins inhibited GATA-dependent transcriptional activation of cardiac gene promoters such as the atrial natriuretic factor (*ANF*) promoter. Adenovirus-mediated expression of Hrt2 suppressed mRNA expression of *ANF* and other cardiac-specific genes in cultured cardiomyocytes. Among various signaling molecules implicated in cardiomyocyte growth, constitutively active Akt1/protein kinase Bα relieved Hrt2-mediated inhibition of GATA-dependent transcription. HRT proteins physically interacted with GATA proteins, and the basic domain of HRT was critical for physical association as well as transcriptional inhibition. These results suggest that HRT proteins may regulate specific sets of cardiac genes by modulating the function of GATA proteins and other cardiac transcriptional activators in a signal-dependent manner.

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Cardiac transcription factors play essential roles in regulating tissue-specific gene expression during proper development and function of the heart (1–3). The expression profiles of distinct sets of cardiac genes are altered in cardiac disease, indicating the importance of transcriptional regulation in response to disease stimuli (1–3). Transcription factors form multiprotein complexes, and combinatorial actions of transcription factors in such complexes dictate the specificity of downstream gene expression. For example, the physical and functional interaction among various cardiac transcription factors including MEF2, NKX2.5, TBX5, and GATA4 can be regulated by upstream cellular signaling and is likely to be impaired in patients with congenital heart disease (4–7).

Members of the Hairy-related transcription factor (HRT)[1] family of repressors, also known as Hesr, Hey, CHF, gridlock, and HERP (8–13), are highly expressed in the heart and vasculature. HRT proteins have a basic helix-loop-helix (bHLH) motif, an orange domain, and a conserved C-terminal tetrapeptide motif and show the highest structural similarity to Hairy and Enhancer of split in flies and the mammalian HES family proteins (8–13). Expression of the *HRT* genes is activated by Notch signaling, suggesting a role for HRT proteins as transcriptional mediators of Notch signaling in the cardiovascular system (14–17). Mutations of the *HRT2* ortholog in zebrafish, *gridlock*, result in defects of aortic development, and misexpression of *gridlock* favors the development of arteries over veins (12, 17). In mice, targeted disruption of *Hrt2*/*Hey2*/*CHF1* causes ventricular septal defects and other congenital cardiac anomalies (18–20), indicating that Hrt2 plays an essential role in cardiovascular development.

HRT proteins preferentially bind to an E-box DNA element and negatively regulate transcription (14, 15). In addition, HRT proteins associate with other transcription factors and specifically inhibit their transactivation capacity independent of DNA binding (11, 14, 21). Although HRT proteins are highly expressed in the developing and adult heart, it remains unknown whether HRT proteins modulate gene expression driven by cardiac transcriptional activators such as GATA proteins (22–25), which are implicated in cardiac development and growth (1–3).

Here, we show that HRT proteins physically associate with cardiac-enriched GATA transcription factors and inhibit GATA-mediated cardiac gene transcription. The present study suggests that signal-dependent modulation of the interaction

[1] The abbreviations used are: HRT, Hairy-related transcription factor; ANF, atrial natriuretic factor; bHLH, basic helix-loop-helix; GSK3β, glycogen synthase kinase 3β; HES, Hairy and Enhancer of split; MEF2, myocyte enhancer factor 2; MyHC, myosin heavy chain; SRF, serum response factor.

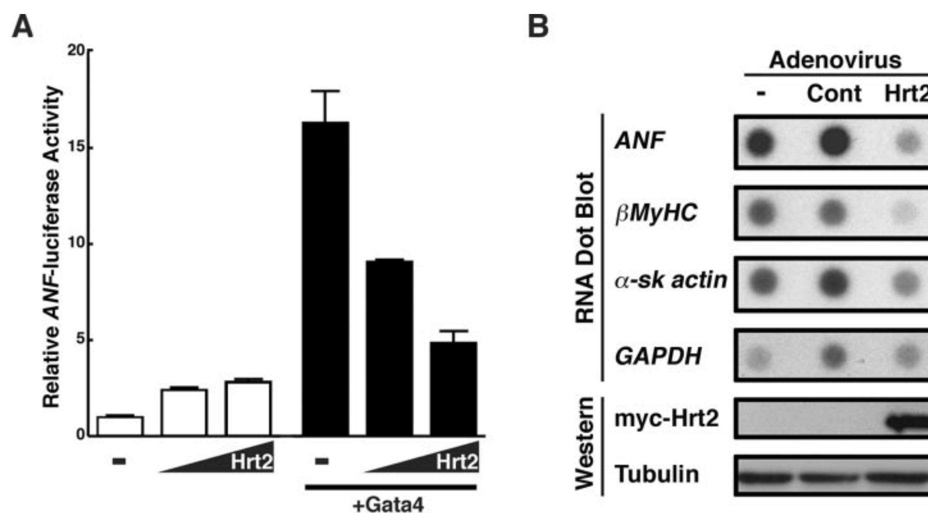

FIG. 1. **Hrt2 inhibits Gata4-dependent *ANF* gene expression.** *A*, Hrt2 suppressed Gata4-dependent *ANF*-luciferase activity. HeLa cells were transfected with an *ANF*-luciferase reporter plasmid and expression vectors encoding Gata4 or Hrt2. Gata4, 300 ng; Hrt2, 300 or 600 ng. Basal luciferase activity without Gata4 and Hrt2 expression was given a value of 1. *B*, adenovirus-mediated expression of Hrt2 inhibited mRNA expression of *ANF*, *βMyHC* and α-skeletal (*α-sk*) actin in cardiomyocytes. mRNA expression levels were examined using RNA dot blot analysis. Expression of Myc-Hrt2 was confirmed by Western blot analysis using anti-Myc antibody. Western blot analysis of α-tubulin was performed as a loading control. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. Adenovirus: −, no infection; *Cont*, control adenovirus without insert; *Hrt2*, Myc-Hrt2 adenovirus.

between HRT and GATA factors may regulate cardiac gene expression during development and disease.

MATERIALS AND METHODS

Plasmid Construction and Adenovirus Preparation—Plasmids encoding rat atrial natriuretic factor (*ANF*)-luciferase (26), rat α-myosin heavy chain (*MyHC*)-luciferase (27), mouse *Nkx2.5*-luciferase (28), mouse *Hrt2*-luciferase (14), Myc-human HRT1/-2/-3, Myc-mouse Hrt2 and its deletion mutants (14), and the constitutively active forms of calcineurin A (29), ribosomal S6 kinase 2 (30), protein kinase A (31), myr-Akt (32), and Notch1 intracellular domain (14) were described previously. An expression construct of the dominant negative form of glycogen synthase kinase 3β (GSK3β) (33) was provided by Dr. G. Crabtree (Stanford University). A luciferase reporter harboring a single GATA-binding element ((GATA)-MIS-luciferase) was provided by Dr. R. Viger (Universite Lavel, Quebec, Canada) (34). Other expression constructs were generated by PCR as cassettes flanked by unique restriction sites and were cloned into pcDNA3.1-N-Myc or -N-FLAG vectors as described previously (14).

The recombinant adenovirus encoding Myc-Hrt2 or its viral packaging control was prepared by Cre/loxP-mediated recombination of a sub360 adenoviral cosmid in 911 cells as described previously (35).

Luciferase Assays—In 6-well plates, HeLa cells were transfected using FuGENE 6 (Roche Applied Science) with 300 ng of plasmid for luciferase reporters, Myc- or FLAG-Gata4 plasmid, and Myc- or FLAG-Hrt2 plasmid. Luciferase activity was measured 40 h after transient transfection. All experiments were accompanied by Western blot analysis to verify relevant protein expression. Plasmids encoding Hrt2 mutations were titrated (600–1200 ng) such that protein expression of the mutations was comparable with protein levels of wild type Hrt2. 100 ng of plasmids encoding various enzymes was used for the initial screen, and subsequent titration revealed that 5 ng of myr-Akt plasmid was sufficient for the maximal effect.

Cardiomyocyte Preparation and Adenoviral Infection—Neonatal rat cardiomyocytes were cultured as described previously (36, 37). Eighteen hours after plating, cells were infected with adenovirus for Myc-Hrt2 and its control at a multiplicity of infection of 30/cell for 2 h and cultured in serum-free Dulbecco's modified Eagle's medium for 36 h. Total RNA was harvested using TRIzol (Invitrogen), and dot blot analysis was performed as described previously (38). Expression of Myc-Hrt2 was confirmed by Western blot analysis. Expression of α-tubulin was used as a loading control.

Co-immunoprecipitation and in Vitro Binding Assays—COS-1 cells were transiently transfected with expression constructs for Myc- and FLAG-tagged proteins using FuGENE 6. Whole cell lysate was incubated with polyclonal anti-Myc antibody (Santa Cruz Biotechnology) and protein A-Sepharose beads (Amersham Biosciences) in lysis buffer (1× phosphate-buffered saline, 1 mM EDTA, 0.5% Triton X-100). Immunoprecipitates were detected by Western blot analysis using anti-FLAG M2 antibody (Sigma) or by staining with monoclonal anti-Myc antibody (Santa Cruz Biotechnology).

For *in vitro* binding assays of Hrt2 deletion mutations, ^{35}S-labeled Hrt2 fragments were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega), incubated with the lysate of COS-1 cells transfected with Myc-Gata4 plasmid, immunoprecipitated with polyclonal anti-Myc antibody, and visualized by autoradiography. The largest ^{35}S-labeled fragments corresponded to the predicted sizes of wild type Hrt2 and its mutants, and they were used for the quantification of the phosphorimage using ImageQuant software (Amersham Biosciences). Values of output bands were divided by those of the corresponding input bands and were presented as arbitrary binding capacity units for Gata4.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay was performed using oligonucleotides representing a GATA-binding element (underlined) (5′-TCGAGGTAATTAACTGATAATGGTGC-3′) as described previously (39).

RESULTS

Hrt2 Inhibits Gata4-dependent ANF Gene Expression—To test whether HRT proteins modulate cardiac gene expression by interacting with other transcription factors, we examined the effects of Hrt2 on the transcriptional activity of a series of cardiac transcriptional activators. As depicted in Fig. 1*A*, Hrt2 significantly repressed Gata4-mediated transactivation of the *ANF* promoter, which contains two essential GATA binding sites (26). In contrast, transcriptional activity of various luciferase reporters controlled by MEF2C, NFAT3, or Hand2 was not significantly inhibited by co-expression of Hrt2 (data not shown).

GATA proteins are known to regulate the expression of various cardiac genes in normal and hypertrophic myocytes (7,22–25,38). We therefore analyzed the effects of Hrt2 expression upon endogenous cardiac gene expression. Adenovirus-mediated Hrt2 expression markedly decreased ANF mRNA expression in cultured neonatal rat cardiomyocytes (Fig. 1*B*). The relative ANF mRNA levels with control and Hrt2 adenovirus infection were 105 ± 11% and 26.5 ± 5.2% (mean ± S.D.), respectively, compared with the levels without adenovirus infection. Hrt2 also down-regulated other cardiac hypertrophy markers, *βMyHC* and α-skeletal actin. The *βMyHC* promoter contains a GATA binding site that is essential for its transcriptional activation in cardiac hypertrophy (40), suggesting that

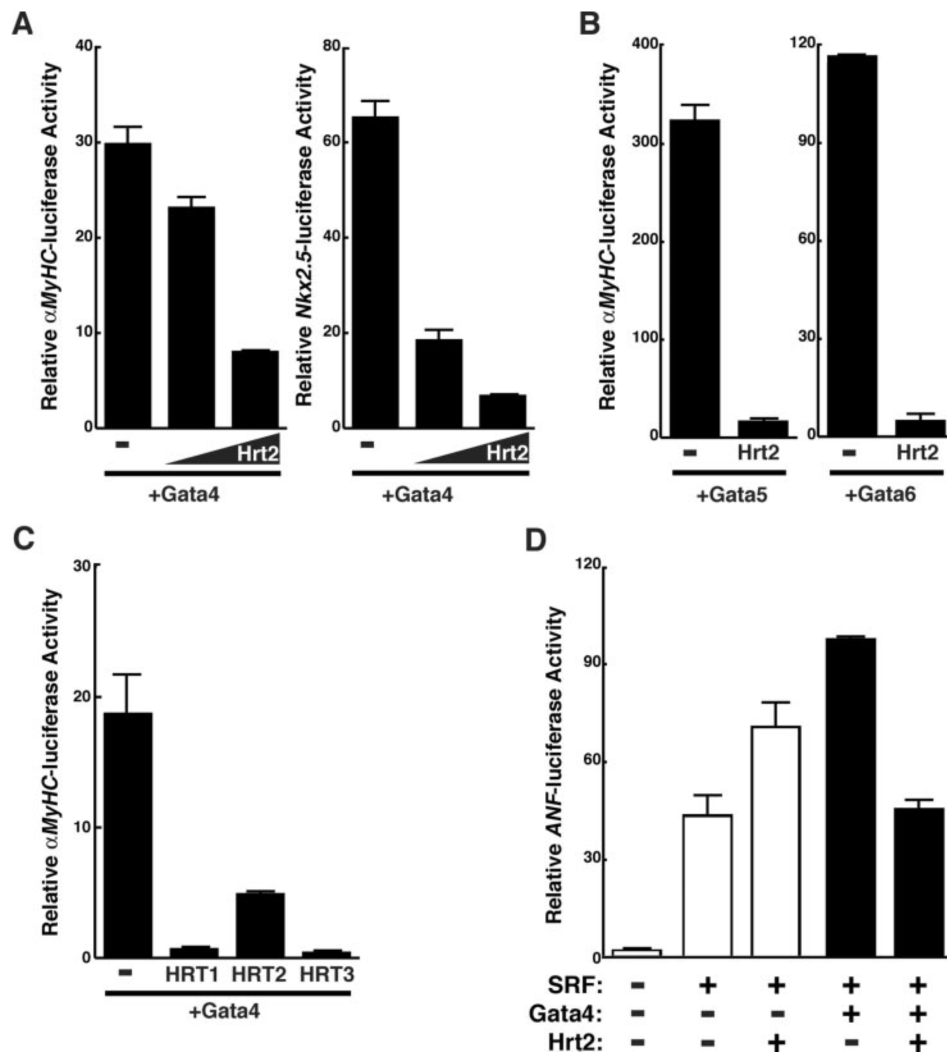

FIG. 2. **HRT1, -2, and -3 specifically suppress transcription driven by GATA family proteins.** *A*, Hrt2 suppressed Gata4-dependent transcription of *αMyHC*- or *Nkx2.5*-luciferase. Gata4, 300 ng; Hrt2, 300 or 600 ng. *B*, Hrt2 inhibited *αMyHC*-luciferase transcription driven by Gata5 and -6. Gata5/6, 300 ng; Hrt2, 600 ng. *C*, all of the human HRT proteins, HRT1, -2, and -3, showed repressive effects on Gata4-dependent transcription. Gata4, 300 ng; HRT1/2/3, 600 ng. *D*, Hrt2 specifically suppressed Gata4 activity and did not inhibit SRF-dependent *ANF*-luciferase transcription. Gata4, 300 ng; SRF, 300 ng: Hrt2, 600 ng. Basal luciferase activity without the expression of HRT, GATA, or SRF was given a value of 1. HRT proteins did not suppress basal luciferase activity without GATA or SRF expression (data not shown).

HRT proteins may generally suppress the GATA-dependent cardiac gene program by interfering with GATA proteins.

HRT1, -2, and -3 Specifically Suppress Transcription Driven by GATA Proteins—To further investigate the generality of the repressive effects of HRT on GATA proteins, we examined its effects on the transcription of other GATA-dependent cardiac regulatory elements. As shown in Fig. 2*A*, a similar pattern of Hrt2-mediated repression of Gata4-dependent transcription was observed for *αMyHC*- and *Nkx2.5*-luciferase reporters, both of which contain essential GATA sites (27, 28). Hrt2 also displayed repressive activity against transcription driven by other cardiac-enriched GATA proteins, Gata5 and Gata6 (Fig. 2*B*). In addition, all of the human HRT proteins, HRT1, -2, and -3, were able to repress Gata4-dependent transactivation (Fig. 2*C*). Because Gata4 cooperates with SRF to activate *ANF* transcription (41, 42), we asked whether Hrt2 could repress transactivation induced by co-expression of Gata4 and SRF. Hrt2 attenuated Gata4-SRF transactivation to levels similar to the contribution by SRF alone, whereas Hrt2 did not repress SRF-dependent transcription in the absence of Gata4 (Fig. 2*D*). These results indicate that HRT family proteins specifically inhibit GATA-dependent gene transcription.

Akt1 Relieves HRT-mediated Repression of GATA-dependent Transcription—Because GATA and HRT proteins are co-expressed both in the embryonic and adult heart, we hypothesized that functional interactions between these proteins could be modulated by developmental and disease signals. To begin to search for such regulatory pathways, we examined whether any protein kinases or phosphatases implicated in cardiac growth and hypertrophy could affect Hrt2-mediated repression of Gata4-dependent transcription. Of the various proteins tested, only a constitutively active form of Akt1/protein kinase Bα, myr-Akt (32), significantly relieved Hrt2-mediated repression of Gata4-dependent transactivation (Fig. 3*A*). Calcineurin A, ribosomal S6 kinase 2, and protein kinase A have been implicated in cardiac growth (43, 44). Although they appeared to slightly increase GATA transcriptional activity, they did not relieve Hrt2-mediated repression (data not shown). As we showed previously (14), Hrt2 can also attenuate transactivation of its own promoter driven by the Notch/RBP-Jκ complex. However, myr-Akt did not antagonize Hrt2 repression in this system (Fig. 3*B*), suggesting that the effect of Akt1 is specific to Hrt2-mediated Gata4 inhibition. Various effects of Akt1 are mediated by the inhibition of GSK3β, a downstream protein kinase (45). However, because

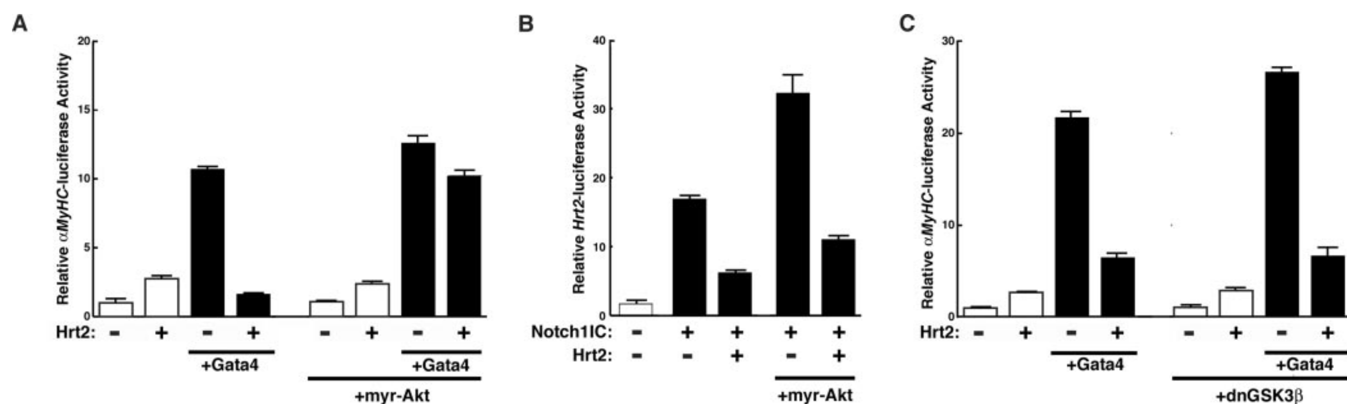

FIG. 3. **Akt1 relieves HRT-mediated repression of GATA-dependent transcription.** *A*, a constitutively active form of Akt1, myr-Akt, relieved Hrt2-mediated repression of Gata4-dependent *αMyHC*-luciferase transcription. Gata4, 100 ng; Hrt2, 900 ng; myr-Akt, 5 ng. *B*, Myr-Akt did not influence Hrt2 repressive effects on Notch1 intracellular domain (*Notch1IC*)-dependent *Hrt2*-luciferase transcription. Notch1 intracellular domain, 300 ng; Hrt2, 600 ng; myr-Akt, 5 ng. *C*, dominant negative GSK3β (*dnGSK3β*) did not affect Hrt2-mediated repression of Gata4-dependent transcription. Gata4, 100 ng; Hrt2, 900 ng; dnGSK3β, 5 ng. Basal luciferase activity was given a value of 1.

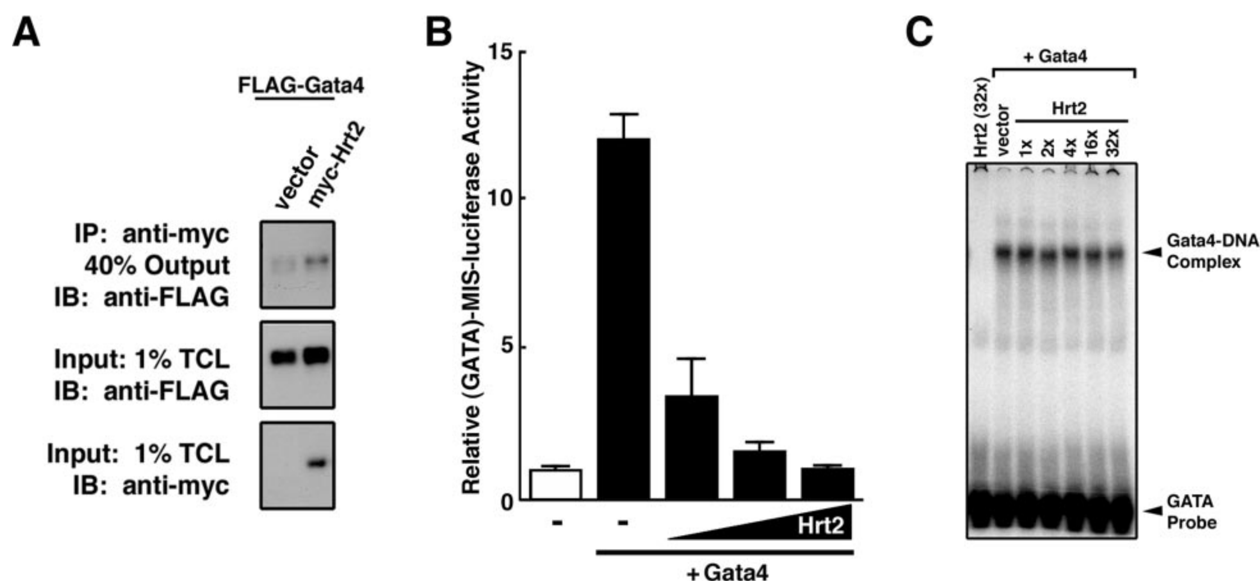

FIG. 4. **HRT physically associates with GATA and inhibits transcription through a GATA-binding DNA element.** *A*, Hrt2 showed physical interaction with Gata4 in co-immunoprecipitation assays. Myc-Hrt2 and FLAG-Gata4 were co-expressed in COS-1 cells, and immunoprecipitation (*IP*) was performed using anti-Myc antibody. *IB*, immunoblot; *TCL*, total cell lysate. *B*, Hrt2 specifically inhibited Gata4-dependent transcription of the luciferase reporter downstream of a single GATA binding site ((*GATA*)-*MIS-luciferase*). Hrt2 did not suppress basal luciferase activity without Gata4 expression (data not shown). Gata4, 20 ng; Hrt2, 20, 40, or 80 ng. *C*, Hrt2 did not inhibit the binding of Gata4 to GATA binding sites in an electrophoretic mobility shift assay. ^{32}P-labeled GATA site oligonucleotide fragments were incubated with 2 μl of TnT reticulocyte lysate expressing Gata4 and 2–64 μl of reticulocyte lysate expressing Hrt2 (*1–32X*).

dominant-negative GSK3β (33) did not mimic the action of Akt1 (Fig. 3*C*), Akt1 appeared to act independent of the GSK3β pathway, either by directly phosphorylating Hrt2 and/or Gata4 or via intermediate molecules upstream of HRT- and GATA-mediated transcriptional regulation.

HRT Physically Associates with GATA and Inhibits Transcription through a GATA-binding DNA Element—To clarify the mechanism underlying the repression of GATA by Hrt2, we tested whether Hrt2 could form a protein complex with GATA proteins. As shown in Fig. 4*A*, Hrt2 showed physical interaction with Gata4 in co-immunoprecipitation experiments. Association of human HRT1 and HRT3 with Gata4 was also detected (data not shown). Furthermore, Hrt2 significantly suppressed Gata4-dependent transcription of a luciferase reporter with a single GATA-binding element, (GATA)-MIS-luciferase (34) (Fig. 4*B*). However, Hrt2 did not inhibit the binding of Gata4 to the GATA-binding element in an electrophoretic mobility shift assay (Fig. 4*C*). These results suggested that direct interaction between HRT and GATA proteins on a GATA-binding DNA element is important for this repression.

The Basic Domain of Hrt2 Is Essential for Transcriptional Repression—To determine the Hrt2 domains that are necessary for repression of GATA-dependent transcription, we tested the effects of various Hrt2 mutations (Fig. 5*A*) on Gata4-dependent transactivation of the *ANF* promoter. In contrast to most mutations, a deletion of the basic domain, Hrt2$^{\Delta(33–37)}$, failed to repress GATA-mediated transcription (Fig. 5*B*). Lack of transcriptional repression by Hrt2$^{\Delta(33–37)}$ was also observed using the reporter with a GATA-binding element (Fig. 5*C*). Because the basic domain of Hrt2 can also act as a nuclear localization signal, mislocalization of Hrt2$^{\Delta(33–37)}$ to the cytoplasm might contribute to the loss of repressive activity. However, Hrt2$^{\Delta(33–37)}$ was observed in the nucleus as well as the cytoplasm in HeLa cells, and increasing amounts of Hrt2$^{\Delta(33–37)}$ did not result in any repressive activity (data not shown).

The Basic Domain of Hrt2 Is Necessary for Physical Interaction with Gata4—We next tested the effects of Hrt2 mutations on Hrt2-Gata4 association. Because the expression levels of

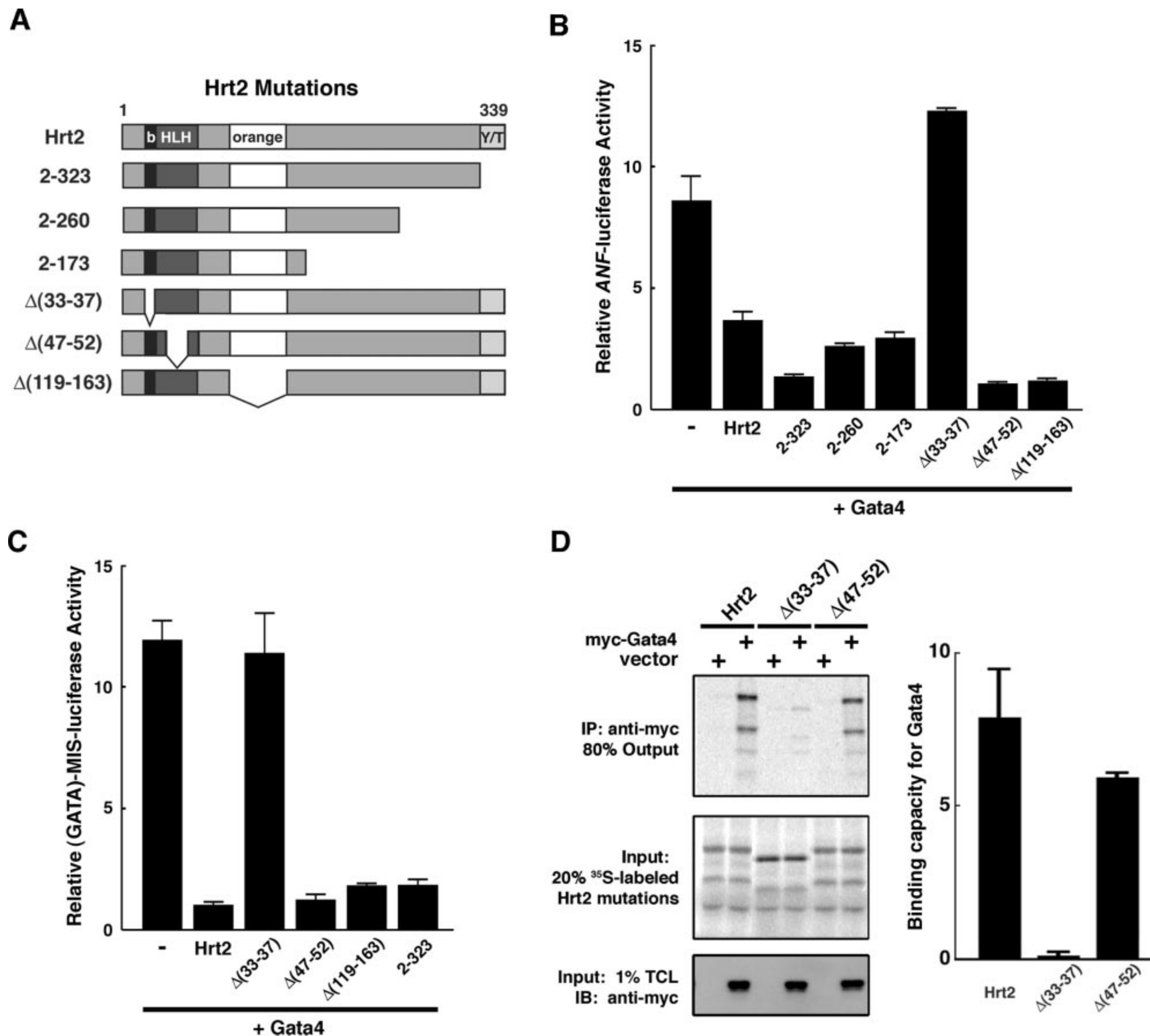

FIG. 5. **The basic domain of Hrt2 is essential for transcriptional repression and physical interaction with Gata4.** *A*, schematic of Hrt2 deletion mutants. *b*, basic domain; *HLH*, helix-loop-helix domain; *orange*, orange domain; *Y/T*, C-terminal YXPW-TEIGAF motif. *Numbers* of amino acids are used for nomenclature of the mutants. *B*, a deletion mutant of the basic domain, $Hrt2^{\Delta(33-37)}$, failed to repress Gata4-mediated *ANF*-luciferase transcription. Gata4, 300 ng; Hrt2, 600 ng. Different amounts of the plasmids for Hrt2 mutants (600–1200 ng) were used to achieve similar expression levels to that of wild type Hrt2. *C*, lack of repressive activity by $Hrt2^{\Delta(33-37)}$ was also observed using (GATA)-MIS-luciferase. Gata4, 20 ng; Hrt2 and its mutants, 80 ng. Basal luciferase activity without Hrt2 and Gata4 protein expression was given a value of 1; fold activation by Gata4 is shown for *B* and *C*. Hrt2 mutants did not suppress basal luciferase activity without Gata4 expression (data not shown). *D*, $Hrt2^{\Delta(33-37)}$ showed a marked decrease in binding capacity for Gata4. Hrt2 and its mutant proteins were synthesized by *in vitro* transcription/translation, and Myc-Gata4 was expressed in COS-1 cells. An *in vitro* pull-down assay was performed using anti-Myc antibody. Arbitrary binding capacity units for Gata4 were calculated as described under "Materials and Methods." *IP*, immunoprecipitate; *IB*, immunoblot.

these Hrt2 mutations were variable in COS-1 cells, we synthesized ^{35}S-labeled Hrt2 proteins using rabbit reticulocyte lysate and performed *in vitro* association assays with Gata4 expressed in COS-1 cells. Hrt2 fragments containing the bHLH and orange domains were sufficient for the interaction with Gata4 *in vitro* (data not shown). Although a deletion of the loop segment in the HLH domain ($Hrt2^{\Delta(47-52)}$) did not affect the association significantly, $Hrt2^{\Delta(33-37)}$ showed a marked decrease in binding capacity for Gata4 (Fig. 5*D*). This result is consistent with the results of the luciferase assays and suggests that Hrt2 associates with Gata4 mainly through its basic domain to inhibit Gata4-dependent transcription.

DISCUSSION

In this study, we demonstrated that the HRT family of bHLH transcriptional repressors forms a protein complex with the GATA family of transcriptional activators and inhibits cardiac gene transcription.

HRT was identified as a novel subfamily of bHLH repressors similar to *Drosophila* Hairy, Enhancer of split, and mammalian HES (8–13). Although members of the HRT family can bind to DNA via an E-box element (14, 15), no target genes of HRT proteins have been identified to be regulated via direct DNA binding. HRT can repress Notch-dependent transactivation by a mechanism independent of its binding to DNA (14), and HRT/CHF proteins also repress transcription by associating with transcriptional activator complexes such as HIF1/ARNT and MyoD/E12 (11, 21). We found that HRT proteins physically associate with GATA proteins and inhibit GATA-dependent transcription and that the basic domain of Hrt2 is important for both transcriptional repression and physical association. A single GATA-binding site is sufficient for HRT-

mediated GATA inhibition, suggesting that HRT proteins might associate with GATA proteins on GATA-binding DNA elements. However, Hrt2 does not appear to alter GATA DNA-binding affinity, at least in electrophoretic mobility shift assays *in vitro*. One mechanism for HRT-mediated repression may be via recruitment of its co-repressors, including class 1 and class 3 histone deacetylases (15, 46), to the GATA complex. The bHLH domain of HRT family proteins is important for association with these co-factors (15, 46). The deletion of the basic domain may result in inefficient association with GATA proteins as well as impairment of co-repressor recruitment. Alternatively, the interaction with HRT proteins might interfere with the association of GATA with its co-activators. Transcriptional activity of GATA is modulated via interactions with various co-factors such as histone acetyltransferase p300 and DNA-binding transcriptional activators SRF, Nkx2.5, Tbx5, MEF2C, Hand2 and NFAT3 (7, 47–53). Future studies will determine whether HRT can affect the formation of GATA-dependent transcription factor complexes.

Null mutations in the *Drosophila Gata4* ortholog *pannier*, zebrafish *Gata5*, or mouse *Gata4* result in early defects in cardiac development (22–25), and mutations of *GATA4* in humans result in congenital heart disease (7). In addition, expression of Gata4 or Gata6 induces cardiomyocyte hypertrophy *in vitro* and *in vivo* (54). Hrt2 can suppress cardiac gene expression in cardiomyocytes, but it remains to be determined whether the effects of Hrt2 on endogenous gene expression occur via repression of GATA-mediated transcription. It will be intriguing to examine whether HRT proteins also modulate GATA-dependent cardiac gene expression during the course of cardiac development and hypertrophy.

The present study implicates Akt1 as a potential regulator of HRT-mediated repression of GATA-dependent transactivation. Akt1 is a critical regulator of cell growth and survival in many tissues including the heart (55–57). Through inhibition of GSK3β (45), Akt1 activates GATA- and NFAT-mediated cardiac gene expression (45, 58). The attenuation of HRT-mediated repression of GATA-dependent gene expression might serve as an additional mechanism by which Akt1-mediated signaling provokes cardiac growth and hypertrophy. Akt1 did not have strong effects on the transactivation by Gata4 in luciferase assays, and Akt1 did not influence the repressive activity of Hrt2 in a Notch-related system, suggesting that the effects of Akt1 may be specific to HRT-GATA functional interaction. Akt1 is known to regulate the sublocalization and/or transcriptional activity of various transcription factors either by direct phosphorylation or via intermediate signaling molecules (59, 60). Although Akt1 can phosphorylate Hrt2 and Gata4 fragments *in vitro*,[2] it is still unknown whether Akt1 influences Hrt2-mediated Gata4 inhibition by phosphorylation of Hrt2 and/or Gata4. We await further studies to elucidate how Akt1 modulates functional interactions between HRT and GATA.

In summary, we have demonstrated that HRT proteins physically associate with GATA proteins and inhibit GATA-dependent cardiac gene expression in an Akt1-sensitive manner. An understanding of such functional interactions among cardiac transcription factors and their responsiveness to cellular signals should provide further insight into the mechanisms of cardiac development and disease.

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[2] M. Murakami, O. Nakagawa, and E. N. Olson, unpublished observation.

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