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# The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors

**Daniel Durocher[1], Frédéric Charron[1], René Warren[1], Robert J.Schwartz[2] and Mona Nemer[1,3,4]**

[1]Laboratoire de Développement et Différenciation Cardiaques, Institut de Recherches Cliniques de Montréal, 110 des Pins Ouest, Montréal Québec, Canada H2W 1R7 and Department of Medicine, Division of Experimental Medicine, McGill University, Montréal Québec, Canada, [2]Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA and [3]Département de Pharmacologie, Université de Montréal, Canada

[4]Corresponding author
e-mail: nemerm@ircm.umontreal.ca

**The tissue-restricted GATA-4 transcription factor and Nkx2-5 homeodomain protein are two early markers of precardiac cells. Both are essential for heart formation, but neither can initiate cardiogenesis. Overexpression of GATA-4 or Nkx2-5 enhances cardiac development in committed precursors, suggesting each interacts with a cardiac cofactor. We tested whether GATA-4 and Nkx2-5 are cofactors for each other by using transcription and binding assays with the cardiac atrial natriuretic factor (ANF) promoter—the only known target for Nkx2-5. Co-expression of GATA-4 and Nkx2-5 resulted in synergistic activation of the ANF promoter in heterologous cells. The synergy involves physical Nkx2-5–GATA-4 interaction, seen *in vitro* and *in vivo*, which maps to the C-terminal zinc finger of GATA-4 and a C-terminus extension; similarly, a C-terminally extended homeodomain of Nkx2-5 is required for GATA-4 binding. Structure/function studies suggest that binding of GATA-4 to the C-terminus autorepressive domain of Nkx2-5 may induce a conformational change that unmasks Nkx2-5 activation domains. GATA-6 cannot substitute for GATA-4 for interaction with Nkx2-5. This interaction may impart functional specificity to GATA factors and provide cooperative crosstalk between two pathways critical for early cardiogenesis. Given the co-expression of GATA proteins and NK2 class members in other tissues, the GATA/Nkx partnership may represent a paradigm for transcription factor interaction during organogenesis.**

*Keywords*: ANF/cardiogenesis/GATA factors/homeodomain/transcription

## Introduction

The GATA family of transcription factors are key developmental regulators that have been conserved throughout evolution (Fu and Marzluf, 1990; Spieth *et al*., 1991; Winick *et al*., 1993; Stanbrough *et al*., 1995; Coffman *et al*., 1996; Platt *et al*., 1996). Various family members have been shown to alter transcription of target genes via binding to the consensus WGATAR sequence through a DNA-binding domain consisting of two adjacent zinc-fingers of the C2/C2 family. Sequence-specific DNA-binding requires the C-terminal zinc-finger and the N-terminal finger may stabilize the DNA–protein complex via electrostatic interactions with the phosphate backbone (Whyatt *et al*., 1993). This DNA-binding domain is the highest conserved region among the various GATA proteins. In vertebrates, six members have been identified so far and they can be divided into two subgroups based on sequence homology and tissue distribution. The first subgroup, which includes GATA-1, -2 and -3, is largely restricted to the hematopoietic system where all three GATA factors have been shown to play essential, non-redundant functions (Tsai *et al*., 1994; Pandolfi *et al*., 1995; Fujiwara *et al*., 1996; Ting *et al*., 1996). Remarkably, arrested proerythroblasts lacking GATA-1 express several GATA-1 target genes although they are unable to achieve terminal erythroid differentiation (Weiss *et al*., 1994), raising the possibility that GATA-2—which is co-expressed with GATA-1 in proerythroblasts—may partially substitute for GATA-1. Consistent with this, GATA factors appear to be functionally interchangeable in some (Blobel *et al*., 1995; Visvader *et al*., 1995) but not all (Briegel *et al*., 1993; Weiss *et al*., 1994) *in vitro* assays. Taken together with the *in vivo* data, these results suggest that functional specificity of GATA proteins likely involves interactions with other cell-restricted cofactors. Consistent with this hypothesis, GATA-1 was found to interact with the erythroid-specific LIM protein RBTN2 and to be present in complexes containing RBTN2 and the hematopoietic basic helix-loop-helix protein SCL/TAL1 (Osada *et al*., 1995). GATA-1 was also shown to cooperate with the ubiquitous SP1 protein and with two other erythroid factors, the basic leucine zipper NFE-2 (Walters and Martin, 1992; Gong and Dean, 1993) and the zinc finger EKLF (Merika and Orkin, 1995; Gregory *et al*., 1996) for transcriptional activation of erythroid promoters/enhancers. At least, in the case of SP1 and EKLF, the interaction was also observed with GATA-2 and involved direct contact through the DNA-binding domains (Merika and Orkin, 1995). Thus, the identity of the proteins that serve as cofactor(s) to impart functional specificity of GATA proteins in the hematopoietic system remains essentially unknown.

The other subclass of vertebrate GATA factors includes GATA-4, -5 and -6 whose expression is restricted to the heart and gut (Arceci *et al*., 1993; Kelley *et al*., 1993; Grépin *et al*., 1994; Laverriere *et al*., 1994; Jiang and Evans, 1996). All three genes are transcribed at very early stages of *Xenopus*, avian and mouse cardiac development (Kelley *et al*., 1993; Heikinheimo *et al*., 1994; Laverriere *et al*., 1994; Jiang and Evans, 1996; Morrisey *et al*., 1996).

Within the heart, transcripts for GATA-4, -5 and -6 are found in distinct cell types with GATA-5 mRNA predominantly found in endocardial cells while GATA-4 and -6 are present in the myocardium (Kelley *et al*., 1993; Grépin *et al*., 1994; Morrisey *et al*., 1996). The spatial and temporal expression of GATA-4 together with various functional studies are consistent with an important role of this GATA family member in cardiogenesis. Thus, GATA-4 was found to be a potent transactivator of several cardiac-specific promoters (Grépin *et al*., 1994; Ip *et al*., 1994; Molkentin *et al*., 1994); inhibition of GATA-4 expression in an *in vitro* model of cardiogenesis altered survival of precardiac cells and inhibited terminal cardiomyocyte differentiation (Grépin *et al*., 1995, 1997). Moreover, targeted inactivation of the GATA-4 gene blocks formation of the primitive heart tube, indicating a crucial role for GATA-4 in heart development (Kuo *et al*., 1997; Molkentin *et al*., 1997). However, ectopic expression of GATA-4 is not sufficient to initiate cardiac differentiation or to activate the cardiac genetic program, although it markedly potentiates cardiogenesis (Jiang and Evans, 1996; Grépin *et al*., 1997) suggesting cooperative interaction between GATA-4 and other cardiac transcription factors.

Genetic studies in *Drosophila melanogaster* have identified the gene *Tinman* as a key regulator of heart differentiation. In *tin* embryos, flies lack the dorsal vessel, the fly structure homologous to the heart, as a result of defects in late mesoderm specification (Bodmer *et al*., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Presumptive homologues of *Tinman* have been cloned in vertebrates (Nkx2-3, Nkx2-5 and Nkx2-7) and are expressed in the myocardium (reviewed in Harvey, 1996; see also Lints *et al*., 1993; Tonissen *et al*., 1994; Evans *et al*., 1995; Schultheiss *et al*., 1995; Buchberger *et al*., 1996; Chen and Fishman, 1996; Lee *et al*., 1996). Targeted disruption of the Nkx2-5 gene in mice leads to embryonic death due to cardiac morphogenetic defects (Lyons *et al*., 1995). However, gain-of-function studies in zebrafish *Danio rerio* and *Xenopus laevis* indicate that ectopic expression of Nkx2-5 results in enhanced myocyte recruitment but is not sufficient to initiate cardiac gene expression or differentiation (Chen and Fishman, 1996; Cleaver *et al*., 1996), suggesting that Nkx2-5 acts in concert with other transcription factors to specify the cardiac phenotype.

Since the cardiac-specific atrial natriuretic factor (ANF) promoter is a transcriptional target for both GATA-4 and Nkx2-5 (Grépin *et al*., 1994; Durocher *et al*., 1996), we used it to investigate functional cooperation between Nkx2-5 and GATA-4 in the heart. We present data showing that Nkx2-5 and GATA-4 specifically cooperate in activating ANF and other cardiac promoters, and physically interact both *in vitro* and *in vivo*. This molecular interaction provides the interesting possibility that instead of being part of the same epistatic group, the two pathways collaborate in the early events of cardiogenesis.

## Results

### *GATA-4 and Nkx2-5 synergistically activate cardiac transcription*

Recent studies from our laboratory have demonstrated that two cardiac-specific transcriptional pathways converge on the ANF promoter (Grépin *et al*., 1994; Durocher *et al*., 1996), ANF being the major secretory product of embryonic and postnatal cardiomyocytes. The region of the ANF promoter which is essential for high basal cardiac activity (Argentin *et al*., 1994) harbors a GATA element located at –120 bp in the rat promoter which binds with high affinity all the members of the cardiac GATA subfamily (F.Charron *et al*., manuscript in preparation), and the NKE which binds Nkx2-5 and is required for ANF promoter and enhancer function (Durocher *et al*., 1996). As seen in Figure 1A, the nucleotide composition of these elements as well as their phasing are conserved across species, suggesting an evolutionary pressure to maintain important regulatory pathways. This led us to investigate whether GATA proteins and Nkx2-5 could functionally interact at the level of the ANF promoter. We tested this hypothesis by co-transfecting GATA-4 and Nkx2-5 expression vectors in non-cardiac cells (HeLa cells) at limiting DNA concentrations (Grépin *et al*., 1994; Durocher *et al*., 1996) in order to avoid squelching. Under these conditions, GATA-4 and Nkx2-5 were able to activate synergistically the ANF promoter but not control promoters lacking GATA and NKE sites such as TK109 (Figure 1B). This cooperative response was not caused by transactivation of the CMV promoter which drives Nkx2-5 and GATA-4 expression since the co-expression of both vectors does not alter the level of either Nkx2-5 or GATA-4 protein (see Figure 6B).

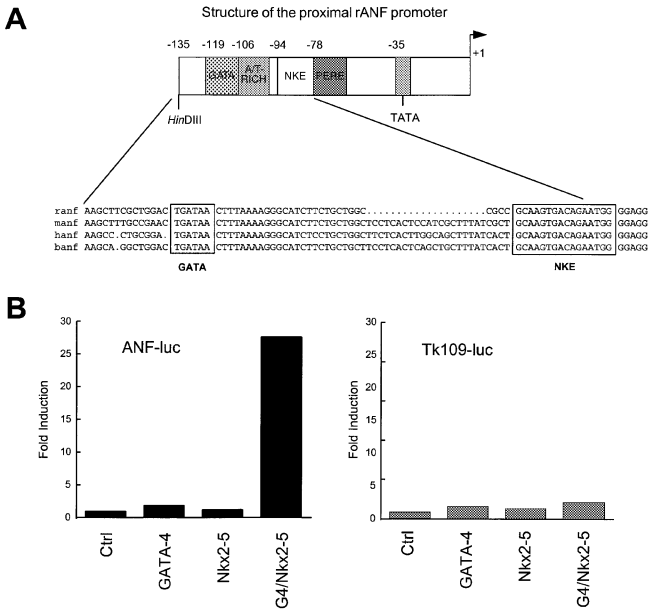


**Fig. 1.** Nkx2-5 and GATA-4 can cooperate transcriptionally. (**A**) Structural organization of the proximal ANF promoter. Regulatory elements of the ANF promoter are boxed, and their location relative to the transcription start are indicated. The PERE corresponds to the phenylephrine response element (Ardati and Nemer, 1993), the NKE to the NK2 response element (Durocher *et al*., 1996). The Nkx2-5 binding site and the GATA elements of the ANF promoter are conserved among species (rat, human, bovine and mouse promoters) and their spacing is conserved (20 bp, two turns of the DNA double helix). (**B**) GATA-4 and Nkx2-5 synergistically activate the ANF promoter. HeLa cells were transiently co-transfected as described in Materials and methods using CMV-driven expression vectors for GATA-4 and Nkx2-5 or the backbone vector as control (pCGN) in conjunction with either ANF-luciferase or Tk109-luciferase reporters. The results, expressed as fold induction of reporter constructs, are from one representative experiment (out of at least four) and represent the mean of a duplicate.

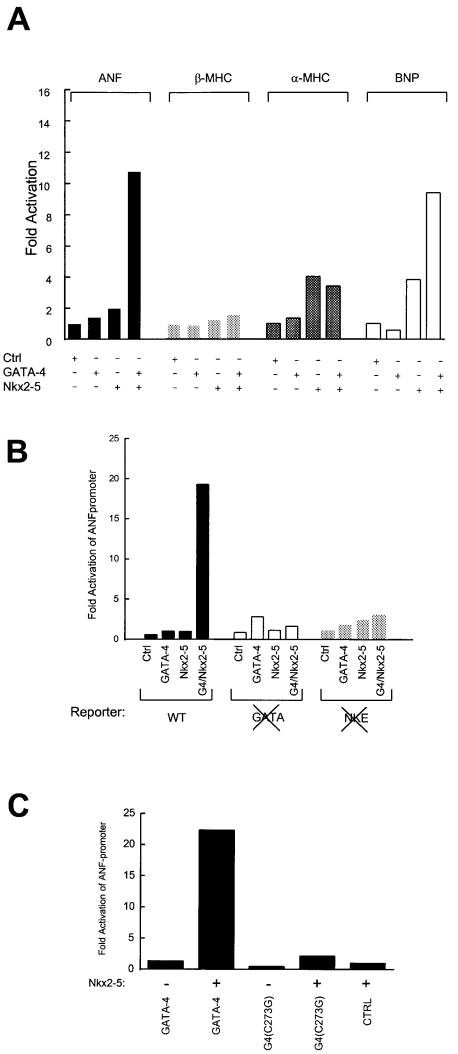


**Fig. 2.** A subset of cardiac promoters are synergistically activated by the Nkx2-5–GATA-4 combination. (**A**) HeLa cells were transiently co-transfected as described in Figure 1 using various cardiac promoters linked to the luciferase reporter. ANF represents the rat ANF –135 construct; β-MHC, the rat –667 bp promoter; α-MHC, the rat –613 bp promoter whereas BNP represents the rat –2 kbp promoter. (**B**) The synergy between GATA-4 and Nkx2-5 requires both binding sites in the context of the ANF promoter. Transient co-transfections in HeLa cells were carried out as described in the previous figures and the promoter described represents either the –135 bp promoter (WT), the Δ–106/–135 bp promoter which removes the GATA element and the Δ–57/–106 bp promoter which removes the NKE site. (**C**) GATA-4 binding to DNA is required for synergy. A point mutant of GATA-4 (C273G) which does not bind DNA *in vitro* and which cannot activate GATA-dependent promoters was used in a co-transfection assay with or without Nkx2-5.

The relevance of this synergy to cardiac transcription was further assessed by co-transfecting Nkx2-5 and GATA-4 with other cardiac promoters including ANF, β-MHC, α-MHC and the B-type natriuretic peptide (BNP) reporters. Under the conditions used in Figure 1, a subset of promoters that contain both NKE and GATA elements could be synergistically activated by the combination of Nkx2-5 and GATA-4 (Figure 2A). Thus, BNP promoter which is a GATA-4 target (Argentin *et al*., 1994) responds synergistically to Nkx2-5 and GATA-4. Interestingly, sequences with high homology to the NKE are present around –385 and –437 bp and are conserved across species; promoter fragments lacking these putative NKEs are no longer responsive to Nkx2-5/GATA synergy (data not shown). On the other hand, neither additive nor synergistic effects were observed on the α-MHC and the β-MHC promoters in response to Nkx2-5 and GATA-4 at all different DNA concentrations tested (Figure 2A and data not shown). These data suggest that only a subgroup of cardiac genes are targeted by both transcription factors and that both NKE and GATA sites are required for synergy. This hypothesis was further tested using ANF promoter mutants deleted of either the GATA or the NKE elements. As shown in Figure 2B, there appears to be an absolute requirement for both elements to achieve synergy. The same results were obtained using BNP promoters containing only GATA sites or heterologous promoters with multimerized GATA elements (data not shown). This suggests that, in natural promoters, both proteins have to be recruited at the promoter or require a conformational change induced upon DNA-binding. Indeed, a GATA-4 mutant that no longer binds DNA because one of the zinc-coordinating cysteines in the carboxy-terminal zinc finger was mutated, no longer supports Nkx2-5 synergy (Figure 2C).

Since multiple GATA and homeobox proteins are expressed in the heart, we investigated the specificity of the synergy. In co-transfection assays using the proximal −135 bp ANF promoter as reporter, we found that Nkx2-5 was able cooperatively to activate transcription of the ANF reporter only with GATA-4 and GATA-5 (Figure 3A). No synergy was observed with either GATA-1 or GATA-6. Since GATA-6 is as potent as GATA-4 in transactivating the ANF promoter (our unpublished data), the results suggest that transcriptional cooperativity between Nkx2-5 and GATA proteins requires specific molecular/structural determinants on the GATA-4 and -5 proteins. The same approach was used to identify homeoproteins that could cooperate with GATA-4, including other NK2 proteins (TTF-1/Nkx2-1; Guazzi *et al*., 1990; Lints *et al*., 1993), Hox proteins (HoxB3), Pou proteins (Oct1; Sturm *et al*., 1988) or *bicoid*-related homeoboxes (Ptx1; Lamonerie *et al*., 1996). Transcriptional cooperativity was observed with the members of the *Antp* superfamily Nkx2-5, TTF-1 and HoxB3, but not with Oct1 and Ptx1 (Figure 3B). These results suggest that the *Antp*-type homeodomain plays an important role in the specificity of the synergy.

We then used deletion mutants of both Nkx2-5 and GATA-4 to map the domains involved in synergy over the ANF promoter. All mutant proteins were tested for expression level and nuclear localization (data not shown). The analyses revealed that, in addition to the DNA-binding domain (Figure 2C), two GATA-4 domains are required for the synergy, one located within the N-terminal 119 amino acids of the protein, and the second in the C-terminal 121 amino acids (Figure 4). Both domains contain GATA-4 activation domains although the presence of either domain is sufficient for transactivation of GATA-dependent promoters in heterologous cells (Figure 4B). The observation that both domains are required for cooperative interaction with Nkx2-5, suggests that synergistic interaction between these two domains may be required for Nkx2-5-induced transcriptional activation of the ANF promoter or that each domain fulfills a distinct function. Mutational analysis

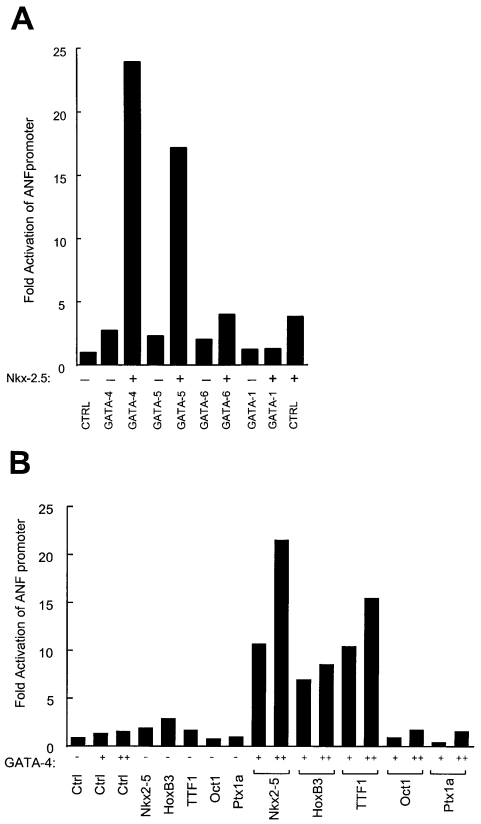


**Fig. 3.** The synergy is specific for a subset of cardiac GATA proteins and *Antp*-type homeoproteins. (**A**) Co-transfection assays in HeLa cells using various GATA expression vectors were done in presence (+) or absence (–) of the Nkx2-5 expression vector. Ctrl represents the backbone vector for most of the GATA constructs (pCGN). (**B**) Co-transfection assays in HeLa cells were performed in the presence of various homeodomain protein expression vectors in absence (–) or in presence (+) of GATA-4 (+, 0.1 μg; ++, 0.25 μg).

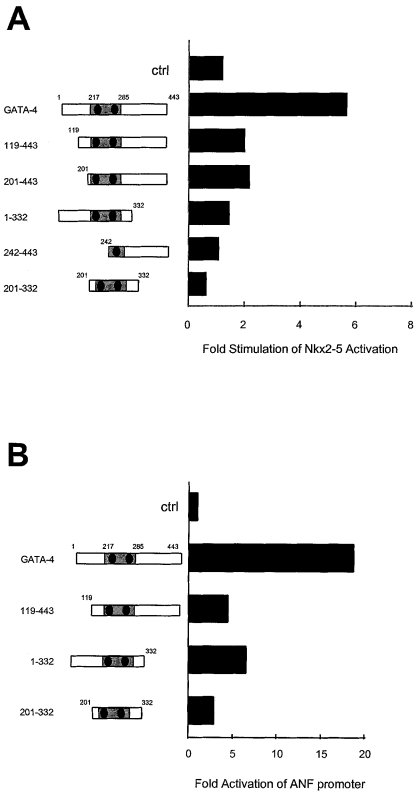


**Fig. 4.** The synergy requires both activation domains of GATA-4. (**A**) GATA-4 vectors (50 ng/35 mm dish) expressing truncated GATA-4 proteins able to translocate to the nucleus were used in co-transfection assays with or without the Nkx2-5 expression vector. ctrl represents the backbone vector. The results are expressed as fold stimulation of Nkx2-5 activation (equivalent to fold synergy where the value of 1 represents no synergy, i.e. the ratio between the activity of the reporter in the presence of the GATA deletion mutant plus Nkx2-5 over the activity of the reporter only in the presence of the GATA deletion mutant). (**B**) GATA-4 activation domains are located both at the C- and N-termini. GATA-4 vectors were transfected in HeLa cells at the dose of 0.2 μg/dish with the ANF –135 bp luciferase reporter.

of Nkx2-5 showed that, while the homeodomain is critical for Nkx–GATA synergy, domains outside the homeobox, particularly sequences C-terminus of the homeodomain, are also important (Figure 5A). Thus, neither the homeodomain (122–203), nor in fusion with the N-terminal regions of Nkx2-5 (1–203) is able to stimulate GATA-4 activity. Deletion of the entire C-terminal region totally impairs the ability of Nkx2-5 to stimulate GATA-4 transcription while partial deletions of the C-terminus (1–246 and Δ204–246) reduce consistently the extent of synergy observed without completely abolishing it. This result suggests that these two regions of the C-terminus are only partially redundant or that the functional interaction between Nkx2-5 and GATA-4 requires an 'extended' homeodomain in the C-terminus. The C-terminus is not known as a transcriptional activation domain, in fact; it appears to be an autorepression domain since its deletion leads to superactivation (Figure 5B). Thus, the requirement for the C-terminus suggests that GATA-4 physically interacts with Nkx2-5 to cause a conformational change and derepress (or unmask) Nkx2-5 activation domains.

### *GATA-4 and Nkx2-5 physically interact in vitro and in vivo*

We first assessed possible physical interaction between Nkx2-5 and GATA-4 using pull-down assays with immobilized MBP–Nkx2-5 and *in vitro*-translated, $^{35}$S-labeled GATA-4 (Figure 6A). MBP–Nkx2-5 was able to retain GATA-4 specifically while a MBP–LacZ control fusion could not retain GATA-4 on the column and the immobilized Nkx2-5 could not interact with labeled control luciferase (Figure 6A). The interaction between GATA-4 and Nkx2-5 was also observed in the presence of ethidium bromide, suggesting that this interaction occurs without DNA bridging (data not shown). In order to ascertain the *in vivo* relevance of this interaction, co-immunoprecipitations were performed on nuclear extracts from 293 cells transfected with expression vectors for wild-type GATA-4 or HA-tagged Nkx2-5 alone or in combination. Nuclear extracts from these transfected cells were then incubated with the monoclonal antibody 12CA5 which recognizes the HA epitope. Immunocomplexes were separated on SDS–PAGE, subjected to Western blotting and visualized with the anti-GATA-4 antibody. As seen in Figure 6B, GATA-4 protein was precipitated by the 12CA5 mAb solely when both proteins were expressed, implying either a direct or indirect contact with Nkx2-5.

Next, deletion mutants of GATA-4 were generated in order to map the region(s) of GATA-4 protein involved in physical interaction with Nkx2-5. Figure 7A displays

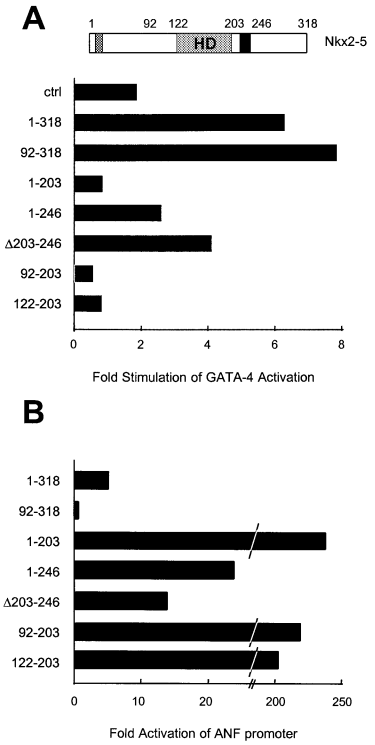


**Fig. 5.** The synergy requires the C-terminus of Nkx2-5. (**A**) CMV-driven vectors, expressing various deletions of Nkx2-5, were used in co-transfection assays as described in Figure 4A, where Nkx2-5 concentration was kept at 0.5 μg/35 mm dish. ctrl represents the backbone vector without insert. The data are expressed as fold stimulation of GATA-4 activation which is calculated by the ratio of the reporter activation when GATA-4 and Nkx2-5 expression vectors are present over the reporter activation when GATA-4 alone is present. (**B**) The C-terminus domain of Nkx2-5 is an auto-inhibitory domain that masks an activation domain located N-terminal of the homeodomain. Co-transfections in HeLa cells were carried out with an optimal dose of pCGN–Nkx2-5 constructs (2 μg/dish) on the ANF –135 bp promoter. The results depict the mean of six independent experiments.

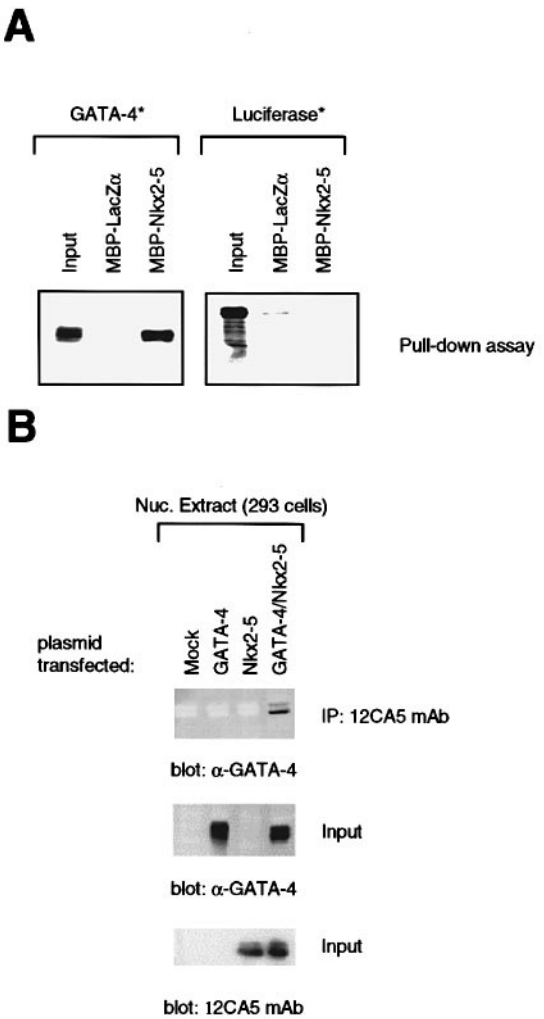


**Fig. 6.** GATA-4 and Nkx2-5 physically interact *in vitro* and *in vivo*. (**A**) GATA-4 and Nkx2-5 interact *in vitro*. Pull-down protein–protein binding assays were performed using immobilized, bacterially produced MBP fusions (MBP–Nkx2-5 and MBP–LacZ as control) and either $^{35}$S-labeled GATA-4 or luciferase protein. After incubation, the protein complexes were spun, extensively washed and separated on a 10% SDS–PAGE. Labeled proteins were visualized and quantified by autoradiography on phosphor plates. (**B**) GATA-4 and Nkx2-5 interact *in vivo*. Nuclear extracts from the simian kidney cell line 293 transfected with either the backbone vectors (mock), GATA-4 expression vector alone (GATA-4), HA-tagged Nkx2-5 (Nkx2-5), or a combination of GATA-4 and HA–Nkx2-5 (GATA-4/Nkx2-5) were used for immunoprecipitation. 60 μg of nuclear extract were incubated with the mAb 12CA5 and precipitated with protein-G–agarose. The top panel represents the immunocomplex separated by electrophoresis and blotted with an anti-GATA-4 polyclonal antibody. The bottom two panels represent Western blots on the transfected nuclear extracts (20 μg) using either the anti-GATA-4 Ab (middle panel) or the anti-HA (12CA5) mAb. The white ghost bands are produced by the immuglobulin heavy chains that co-migrate with GATA-4 on SDS–PAGE.

the results of the binding studies and the left panel of Figure 8A shows the structure of the deletion mutants and summarizes their behavior in pull-down and transfection assays. The Nkx2-5 interaction domain seems to map to the second zinc-finger and a C-terminal basic region that is not part of any known activation domain of GATA-4. This localization is consistent with the observation that the physical interaction requires zinc ions, since pull-down assays in the presence of chelating agents (EDTA and phenanthroline) abolish the interaction (data not shown). Unfortunately the requirement of the Nkx2-5 binding domain for the synergy could not be assessed since it is part of a basic region essential for the nuclear targeting of GATA-4 (F.Charron *et al.*, unpublished results). Interestingly, neither GATA-1 nor GATA-6, which do not transcriptionally synergize with Nkx2-5, could be retained on the MBP–Nkx2-5 column, suggesting that physical interaction is required for functional cooperativity.

The same approach was also used to map the GATA-4 interaction domain on Nkx2-5. A series of Nkx2-5 deletion mutants were bacterially produced in fusion with MBP, quantified and assayed for their ability to interact with $^{35}$S-labeled GATA-4. The results of these binding assays revealed that both the homeodomain and its C-terminal region are required for physical interaction (Figures 7B and 8A). The homeodomain by itself (122–203) or extended to contain the N-terminal domain (1–203) were insufficient for physical interaction. However, when the homeodomain was fused to parts of the C-terminal (1–246 and Δ204–246) the fusion proteins regained the ability to bind GATA-4, suggesting that the C-terminal extension provided an essential docking site for the GATA protein or was required for the proper folding of the homeodomain.

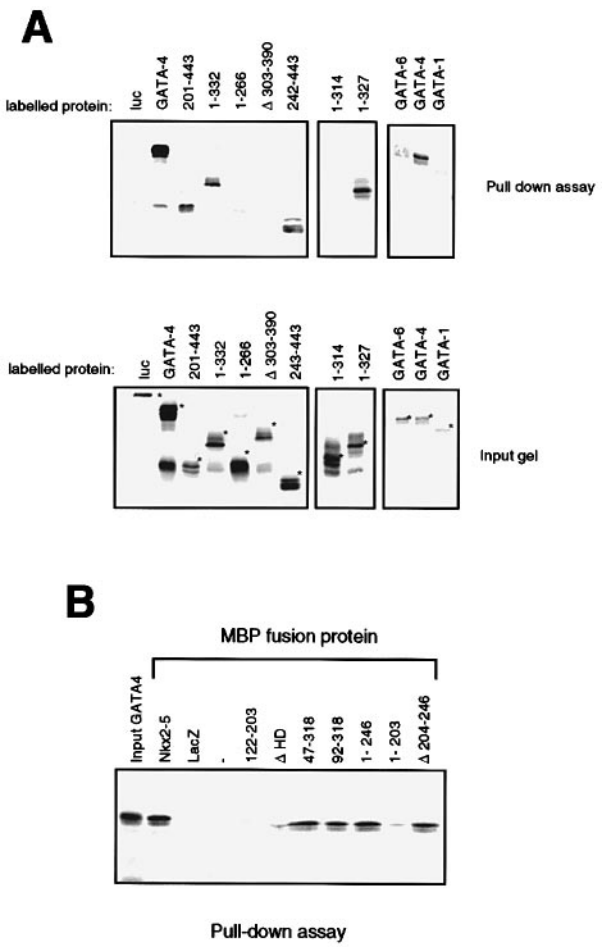


**Fig. 7.** The physical interaction maps near the C-terminal zinc-finger of GATA-4 and to the C-terminally extended homeodomain of Nkx2-5. (**A**) Luciferase (luc) or deletion mutants of GATA-4 were translated and labeled with [$^{35}$S]methionine to be subsequently used in pull-down assays with full-length MBP–Nkx2-5 as described in Figure 6. (**B**) A series of immobilized deletion mutants of Nkx2-5, in fusion with the maltose binding protein (MBP), were produced in bacteria, quantified on gel, and used in pull-down assays with *in vitro* translated GATA-4. ΔHD represents Nkx2-5 Δ122–203. Protein complexes were separated by electrophoresis and GATA-4 protein was visualized by autoradiography on phosphor plates.

It is noteworthy that these results are in complete agreement with the transfection data and indicate that the determinants of Nkx2-5 and GATA-4 interaction reside mostly in the homeodomain and a C-terminal extension. Collectively, the results also suggest that functional synergy between Nkx2-5 and GATA-4 requires physical interactions of the two proteins.

## Discussion

Transcription factors GATA-4 and Nkx2-5 are two of the earliest markers of precardiac cells and, as evidenced by gene inactivation studies (Lyons *et al*., 1995; Kuo *et al*., 1997; Molkentin *et al*., 1997), both play critical roles in cardiogenesis. The data presented here show that GATA-4 and Nkx2-5 interact physically and synergistically to activate cardiac transcription, suggesting functional convergence of two critical cardiac pathways.

### *Modulation of Nkx2-5 activity by GATA-4*

Members of the GATA family of transcription factors (GATA-1, -2 and -3) have been shown to interact with other classes of nuclear proteins containing Lim domain (Osada *et al*., 1995), zinc finger (Merika and Orkin, 1995; Gregory *et al*., 1996), and basic leucine zipper (Walters and Martin, 1992; Gong and Dean, 1993; Kawana *et al*., 1995) motifs. The present work demonstrates that GATA factors are also able to interact with homeodomain-containing proteins of the NK2 and *Antp* type. This GATA–Nkx interaction is so far the first example of zinc finger–homeodomain interaction in vertebrates. The only other known zinc finger–homeodomain cooperation is in *Drosophila*, where it was recently shown that the orphan nuclear receptor αFtz-F1 is a cofactor for the homeodomain protein Ftz (Guichet *et al*., 1997; Yu *et al*., 1997); in this case, the physical association between αFtz-F1 and Ftz is thought to enhance the binding of the Ftz to its lower-affinity target sequences (Guichet *et al*., 1997; Yu *et al*., 1997), much in the same way that Extradenticle and Pbx modulate the DNA binding activity of Hox proteins (Phelan *et al*., 1995; Lu and Kamps, 1996; Peltenburg and Murre, 1997). The interaction of GATA-4 with Nkx2-5 does not appear to result in cooperative DNA binding since neither protein appears to alter the affinity or sequence specificity of the other; moreover, the presence of both GATA and NKE sites does not enhance either GATA-4 or Nkx2-5 binding to their sites, as evidenced by gel shift assays using nuclear extracts containing both proteins or each one separate (data not shown). Instead, the data suggest that GATA-4 interaction with Nkx2-5 serves to unmask the activation domains of Nkx2-5 as illustrated in Figure 8B; this would be reminiscent of the Extradenticle-induced conformational change, that switches Hox proteins from repressors to activators (Chan *et al*., 1996; Peltenburg and Murre, 1997).

The region of GATA-4 that contacts Nkx2-5 spans the second zinc finger and a ~40 amino acid C-terminal extension (Figure 8A, left panel). This represents a highly conserved segment among the cardiac GATA-4, -5 and -6 proteins with an overall 85–95% homology; notable differences between GATA-4 and -6 (but not GATA-4 and -5) that may account for the differential interaction with Nkx2-5 are found in the hinge region (aa 243–270) preceding the second zinc finger and three non-conservative changes that affect phosphorylatable residues (H244S, N250S, S262P). The differential interaction of GATA proteins with Nkx2-5 reveals for the first time differences between GATA proteins in an *in vitro* assay.

### *Is Nkx2-5 a specificity cofactor for GATA-4?*

Two GATA proteins, GATA-4 and -6, are present in the myocardium and both are potent activators of cardiac transcription. However, inactivation of the GATA-4 gene arrests cardiac development at a very early stage, despite marked up-regulation of GATA-6 arguing for specificity of GATA-4 and -6 function (Kuo *et al*., 1997; Molkentin *et al*., 1997). The up-regulation of GATA-6 might, at least partially, account for ANF expression in presumed cardiogenic cells of the GATA-4$^{-/-}$ embryos (Molkentin *et al*., 1997), much like up-regulation of GATA-2 in GATA-1$^{-/-}$ pre-erythroblasts might explain globin gene expression in the absence of GATA-1. However, it should

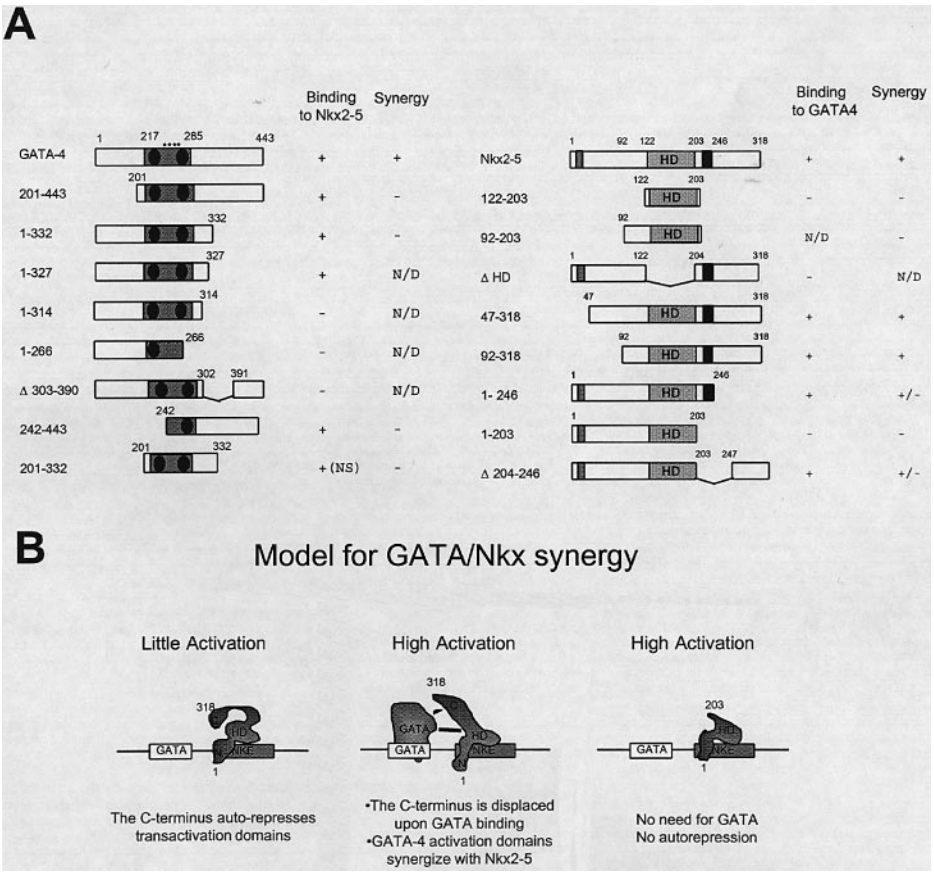


**Fig. 8.** (**A**) Schematic summary of the Nkx2-5–GATA-4 interaction. The left panel represents the activity of GATA-4 deletions both in transfection experiments and in pull-down assays. N/D represents constructs that were not tested. Constructs deleting the 266–332 region cannot be used in co-transfections since they do not translocate into the nucleus. The right panel summarizes the activities of Nkx2-5 deletion mutants in co-transfections with GATA-4 or in pull-down assays. The asterisks (*) on the GATA-4 molecule represents the amino acids that are conserved between GATA-4 and -5 but not in GATA-6, they are: H244S, N250D, L261V, S262P where the second amino acid represents the residue present on GATA-6 at the equivalent position. (**B**) Model of Nkx/GATA synergy. Synergistic interactions between Nkx2-5 and GATA-4 require the binding of GATA-4 and Nkx2-5 to their cognate binding sites. GATA-4 displaces the C-terminal auto-inhibitory region of Nkx2-5 and liberates the Nkx2-5 activation domain. The GATA-4 activation domains can then synergize with Nkx2-5 activation domains.

be pointed out that ANF transcription is controlled by multiple pathways in complex spatiotemporal manner (Argentin *et al*., 1994; Durocher *et al*., 1996); thus, the presence of ANF transcripts in GATA-4$^{-/-}$ cells may reflect activation or maintenance of more complex compensatory pathways; moreover, GATA-5—which can also cooperate with Nkx2-5 in the heart—does not seem to be restricted to the endocardial cells before the primitive heart tube stage (Morrisey *et al*., 1997). The exact reason for which GATA-4 is obviously essential for primitive heart development must await further biochemical and genetic studies. Nevertheless, the available evidence clearly indicates that GATA-6 is unable to substitute fully for GATA-4 with respect to cardiogenesis. Similarly, despite their seemingly interchangeable role in some *in vitro* assays (Blobel *et al*., 1995; Visvader *et al*., 1995), the hematopoietic members of the GATA family are clearly non-redundant (Tsai *et al*., 1994; Pandolfi *et al*., 1995; Fujiwara *et al*., 1996; Ting *et al*., 1996). Unfortunately, the molecular basis for GATA factor specificity has yet to be unraveled. The data presented suggest that interaction of GATA proteins with other tissue-restricted transcription factors might be the underlying mechanism for functional specificity of the GATA family members. Thus, Nkx2-5 may be the specificity cofactor for GATA-4 while other homeodomain proteins of the NK2 or *antennapedia* class may fulfill a similar function for GATA-6 in the myocardium.

The presence of a cofactor for GATA proteins is likely the case for the hematopoietic system. Indeed, in a recent publication, Weiss *et al*. demonstrated that the presence of the GATA-1 zinc fingers was essential for erythroid differentiation. Interestingly, the homologous region of GATA-3 (which is not co-expressed with GATA-1) but not the entire GATA-2 (which is up-regulated in GATA-1$^{-/-}$ pre-erythroblasts) could functionally substitute for GATA-1 zinc fingers, suggesting that interaction of zinc fingers with an as yet unidentified nuclear factor may be an important determinant for definitive erythropoiesis (Weiss *et al*., 1997). Since GATA proteins and other members of the NK2 family are also co-expressed in other tissues such as spleen (GATA-5 and Nkx2-5) and gut (GATA-5, -6 and Xbap) (Lints *et al*., 1993; Morrisey *et al*., 1996, 1997; Newman *et al*., 1997), it is tempting to speculate whether the GATA–Nkx partnership may represent a paradigm for transcription factor interaction during cell fate determination.

It is noteworthy that, at least in cardiac muscle, such paradigm appears to have been evolutionarily conserved. Indeed, in *Drosophila*, the cardiac promoter of the transcription factor D-mef which is a target for Tinman,

**Table I.** Evolutionary conservation of the GATA–NK2 interaction on muscle promoters

| Gene | Species | Promoter sequence | GATA | NK2 | Reference |
|---|---|---|---|---|---|
| ANF | vertebrates | **TGATAA**CTT($N_{20}$)CGCC**GCAAGTG** | GATA-4 | Nkx2-5 | this study |
| Myo-2 | *C.elegans* | **TAAAGTG**GTTGTGT**GGATAA** | elt-2 (?) | Ceh-22 | Okkema and Fire (1994) |
| D-Mef | *Drosophila* | **GGATAA**GGGGC**TCAAGTGG**<br>**CACTTGA**GACCGGGGCTCG**CTATCG** | pannier (?) | Tinman | Gajewski *et al.* (1997) |

Conserved GATA or NKE motifs are depicted in bold letters.

contains juxtaposed GATA and NKE sites (Table I); while the NKEs are necessary, they are not sufficient for cardiac expression, thus raising the possibility of an interaction with other factors (Gajewski *et al*., 1997). Moreover, in *Caenorhabditis elegans*, two members of the GATA family have been described (elt-1 and -2; Spieth *et al*., 1991; Hawkins and McGhee, 1995) whose expression is found in gut and perhaps pharyngeal muscles, and GATA elements are necessary for tissue-specific transcription in those tissues (Okkema and Fire, 1994; Egan *et al*., 1995). Moreover, at least one member of the NK2 family, CEH-22, is also expressed in *C.elegans* pharyngeal muscle and has been implicated in activation of the muscle-specific myosin heavy chain (Myo-2) enhancer (Okkema and Fire, 1994). Interestingly, the Myo-2 enhancer requires the closely linked GATA and NKE sites (Table I) for muscle expression (Okkema and Fire, 1994). Thus, at least in muscle cells the GATA and Nkx interactions appear to have been evolutionarily conserved.

## Materials and methods

### *Cell cultures and transfections*

HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out using calcium phosphate precipitation 24 h after plating. At 36 h post-transfection, cells were harvested and luciferase activity was assayed with an LKB luminometer and the data were recorded automatically. In all experiments, RSV-hGH was used as internal control and the amount of reporter was kept at 3 μg per dish; the total amount of DNA was kept constant (usually 8 μg). Unless otherwise stated, the results reported were obtained from at least four independent experiments with at least two different DNA preparations for each plasmid. Primary cardiocyte cultures were prepared from 1- or 4-day-old Sprague-Dawley rats and kept in serum-free medium as described previously (Argentin *et al*., 1994).

### *Plasmids*

ANF-luciferase promoter constructs were cloned in the PXP-2 vector as described previously (Argentin *et al*., 1994; Durocher *et al*., 1996). The construction of the various pCG–GATA-4 vectors was based on the original rat GATA-4 cDNA described by Grépin *et al*. (1994). The position of the mutation/deletion is indicated on the figures. All constructs were sequenced and functionally tested for nuclear translocation and DNA-binding activity following transfection in L cells as previously described (Grépin *et al*., 1994). pRSET–GATA-4 derivatives for *in vitro* translation were constructed by insertion of the *Xba*I–*Bam*HI fragment of the corresponding pCG–GATA-4 construct into the *Nhe*I–*Bam*HI sites or *Nhe*I–*Bgl*II sites of pRSETA (Invitrogen Corp.). MBP–Nkx2-5 (Δ203–246) was obtained by the insertion of an oligonucleotide corresponding to aa 198–203 in the *Pfl*MI–*Sac*II sites of Nkx2-5. The *Sph*I–*Sac*II fragment corresponding to the deletion was then transferred in MBP–Nkx2-5. The other MBP–Nkx2-5 deletions were described by Chen and Schwartz (1995).

### *Recombinant protein production*

After transformation of BL21(DE3) *Escherichia coli* strain with the MBP fusion vectors derived from pMalc-2 (New England Biolabs), individual colonies were picked and grown in 50 ml 2XYT up to an OD of 0.6 at 600 nm. Induction of the recombinant proteins and their purification were carried out as previously described (Durocher *et al*., 1996). *In vitro* translation of GATA-4 and Nkx2-5 derivatives were performed with rabbit reticulocyte lysates using the TNT-coupled *in vitro* transcription/translation system (Promega Corp., Madison, WI)

### *Protein–protein binding assays*

*In vitro* binding studies were performed with MBP–Nkx2-5 derivatives purified from bacteria and coupled to amylose–Sepharose beads (New England Biolabs). GATA-4 derivatives were labeled with [$^{35}$S]methionine during *in vitro* translation and typically 2–8 μl of labeled GATA proteins were incubated in the presence of 300 ng of immobilized Nkx2-5 fusion proteins in 400 μl of 1× binding buffer (150 mM NaCl, 50 mM Tris–Cl, pH 7.5, 0.3% Nonidet P-40, 10 mM $ZnCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.25% BSA) for 2 h at 4°C with agitation and then centrifuged for 2 min at 13 000 r.p.m. at room temperature. Beads were washed three times by vortexing in 500 ml of binding buffer at room temperature, the protein complexes were released after boiling in Laemmli buffer and resolved by SDS–PAGE. Labeled proteins were visualized and quantified by autoradiography on phosphor storage plates (PhosphorImager, Molecular Dynamics).

### *Immunoprecipitations and immunoblots*

Immunoprecipitations on nuclear extracts of transfected 293 cells were done using 60 μg of nuclear extract. Extracts were pre-cleared with 20 μl of normal rabbit serum and 15 μl of agarose–protein G beads (Sigma Chemicals) for 2 h at 4°C. Binding reactions were carried out with 40 μl of 12CA5 antibody in 500 μl of 1× binding buffer without BSA as described in the protein–protein binding assays paragraph for 2 h at 4°C, with agitation without protein G beads and for an additional 2 h with 15 μl of protein G beads. Bound immunocomplexes were washed four times in 1× binding buffer and were resuspended in 20 μl of 1× Laemmli buffer, boiled and subjected to SDS–PAGE electrophoresis. Proteins were transferred on Hybond-PVDF membrane and subjected to immunoblotting. GATA-4 antibody (Santa-Cruz Biotechnolgy) was used at a dilution of 1/1000 and was revealed with biotinylated anti-goat antibody (dilution 1/12 000) and avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain). The 12CA5 (anti-Ha) antibody was used at a dilution of 1/500 and was a generous gift of Benoit Grondin and Muriel Aubry (Grondin *et al*., 1996). The secondary antibody was anti-mouse-HRP and the antigens were visualized with chemiluminescence (Kodak).

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