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COUP-TF1 Antagonizes Nkx2.5-mediated Activation of the Calreticulin Gene during Cardiac Development*

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Calreticulin, a Ca^{2+} binding chaperone of the endoplasmic reticulum, is also highly expressed in the embryonic heart, and knockout of the calreticulin gene is lethal during embryogenesis because of impaired cardiac development. The protein is down-regulated after birth, and elevated expression of calreticulin in newborn hearts is associated with severe cardiac pathology and death. Here we show that the transcription factor Nkx2.5 activates expression of the calreticulin gene in the heart. Binding of chicken ovalbumin upstream promoter-transcription factor 1 to the Nkx2.5 binding site suppresses transcription from the calreticulin promoter. Nkx2.5 and chicken ovalbumin upstream promoter-transcription factor 1 play antagonistic roles in regulating the expression of calreticulin during cardiac development. These studies indicate that cardiac-specific transcription factor Nkx2.5 plays a central role in activating calreticulin expression and that there is a cooperation between chicken ovalbumin upstream promoter-transcription factor 1 and Nkx2.5 at the calreticulin promoter.

Cardiac development is an extremely complex process that progresses under strict transcriptional control (1). Several different transcription factors are critical for specific stages of vertebrate cardiac morphogenesis (1). One transcription factor implicated in regulating cardiac gene expression and differentiation in vertebrates is *tinman* (Nkx2.5) (2). Nkx2.5 was first identified as a key regulator of cardiac differentiation in *Drosophila* (3) and shown to be essential for heart formation in *Drosophila* and *Xenopus* (2). The protein is expressed in the developing myocardium (4–6), and in mice, disruption of the Nkx2.5 gene results in cardiac morphogenic defects, which are embryonic-lethal (7). So far, only the atrial natriuretic peptide and α-cardiac actin genes have been identified as cardiac-specific targets of Nkx2.5 (8–10).

Calreticulin is a major Ca^{2+} binding chaperone resident in the lumen of the endoplasmic reticulum (11). This protein is highly expressed in the embryonic heart and, similar to Nkx2.5, knockout of the calreticulin gene is lethal during embryogenesis (14.5–16.5-day-old embryos) because of impaired cardiac development (12). Conversely, calreticulin is significantly down-regulated after birth (12), which fits with the observation that elevated expression of calreticulin in newborn hearts is associated with severe cardiac pathology and death. These findings suggest the existence of well defined mechanism(s) for activation and repression of the calreticulin gene in the developing heart. The mechanisms that control the expression of calreticulin in the developing heart are not known. However, it is known that the promoter of the mouse calreticulin gene includes putative binding sites for NKx2.5 (13) suggesting that Nkx2.5 could be involved in regulating the expression of calreticulin during cardiomyogenesis. Here we show that the transcription factor Nkx2.5 activates expression of the calreticulin gene in the heart. We also show that binding of COUP-TF1 to the Nkx2.5 binding site suppresses transcription from the calreticulin promoter. Nkx2.5 and COUP-TF1 may play antagonistic roles in regulating the expression of calreticulin during cardiac development.

EXPERIMENTAL PROCEDURES

Plasmids, Transient Transfection, and Reporter Gene Assay—pLC1 and calreticulin promoter deletion/mutation plasmids were generated as described by Waser *et al.* (13). The 1 × CRT[1] site 2- and 4 × CRT site 2-luciferase reporter plasmids were constructed by inserting synthetic deoxyoligonucleotides upstream of the minimal calreticulin promoter (13). Plasmid DNA was purified by Qiagen column chromatography. Primary myocyte cultures were prepared from neonatal Harlan Sprague-Dawley rats as described previously (14). Primary cardiomyocytes or NIH3T3 cells were transfected with appropriate plasmid DNA, and luciferase and β-galactosidase activity assays were carried out as described previously (13). Site-directed mutagenesis of the CRT sites 2 and 3 was carried out using a mutagenesis kit from CLONTECH. The AAGTG nucleotide sequence in the CRT site 2 or site 3 was changed to a CCCCG sequence.

Yeast One-hybrid System—An 11-day mouse embryo Matchmaker cDNA library (CLONTECH) was screened for interactions with the CRT site 2 response element. Three tandem repeats of the CRT site 2

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[1] The abbreviations used are: CRT, calreticulin; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1, EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis.

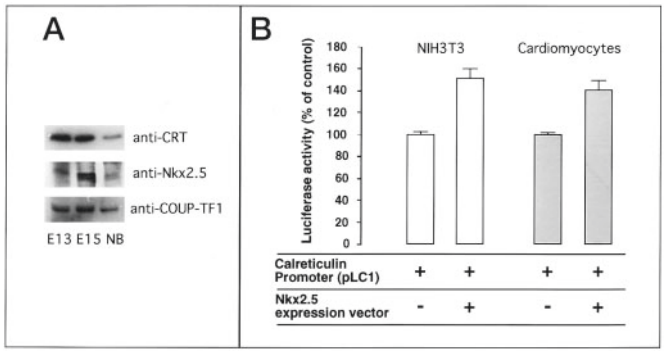

FIG. 1. **Activation of the calreticulin promoter by Nkx2.5.** *A*, the level of expression of CRT, Nkx2.5, and COUP-TF1 in embryonic and newborn hearts. Hearts were harvested from 13-day-old embryos (*E13*), 15-day-old embryos (*E15*), and 1-week-old mice (*NB*). Cardiac proteins were separated by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and probed with anti-CRT, anti-Nkx2.5, or anti-COUP-TF1 antibodies. *B*, NIH3T3 cells (*open bars*) or rat primary cardiomyocytes (*shaded bars*) were transiently cotransfected with a luciferase reporter construct containing 1.8 kilobases of the calreticulin promoter (*pLC1*) and with the Nkx2.5 expression vector. Luciferase activity was normalized against a β-galactosidase expression vector. Data are means ± S.D. of three independent experiments.

element (5′-CTCAAGTGT-3′) were cloned into *Eco*RI and *Xba*I restriction sites of pHISi, and into *Eco*RI and *Sal*I sites of pLacZi, generating pHIS-Nkx and pLacZ-Nkx, respectively. Screening of the library was carried out as recommended by the manufacturer.

Electrophoretic Mobility Shift Assay (EMSA)—Full-length COUP-TF1, Nkx2.5, and luciferase proteins were synthesized using a coupled transcription and translation reticulolysate system. Plasmids pRC-CMV/COUP-TF1, encoding recombinant COUP-TF1 (15), and pSPUTK/Nkx2.5, encoding recombinant Nkx2.5, were used. The two complementary deoxyoligonucleotides were labeled with [γ^{32}P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase. EMSA was carried out as described (16).

SDS-PAGE and Immunoblotting—Mouse heart tissue was homogenized, and solubilized proteins were separated by SDS-PAGE followed by immunoblotting with goat anti-calreticulin or mouse anti-Nkx2.5 antibodies (17). Antibody binding was detected with peroxidase-conjugated secondary antibodies followed by a standard ECL development reaction.

RESULTS AND DISCUSSION

Fig. 1 shows that Nkx2.5 (18) and calreticulin (12) are both highly expressed in embryonic hearts (day 13 (*E13*) and 15 (*E15*)) and that the expression of both proteins decreases dramatically after birth (Fig. 1*A*). We did note, however, that in embryonic hearts from 13-day-old embryos (Fig. 1, *E13*) the expression of calreticulin was significantly higher than that of the Nkx2.5 transcription factor. Western blot analysis also shows that COUP-TF1 was expressed in both embryonic and postnatal hearts, and the relative level of COUP-TF1 did not change significantly during cardiogenesis (Fig. 1*A*). Relatively high expression of Nkx2.5 and calreticulin in embryonic hearts is consistent with suggestions that these proteins play a role in cardiac development (7, 12). These finding also indicate that Nkx2.5 might be responsible for activation of the calreticulin gene during development of the embryonic heart. To test this, we cotransfected NIH3T3 cells or rat primary cardiomyocytes with an Nkx2.5 expression vector and a vector (pLC1) containing the luciferase reporter gene. In pLC1, expression of the luciferase reporter gene is controlled by 1.8 kilobases of the calreticulin promoter region. pSVβ-galactosidase was used as an internal control. Fig. 1*B* shows that this cotransfection resulted in significant induction of luciferase activity in both NIH3T3 cells and in cardiomyocytes. Cells transfected with promoterless control plasmids showed no detectable luciferase activity (data not shown). Thus, it appears that Nkx2.5 can activate transcription from the calreticulin promoter, *in vivo*, in both NIH3T3 cells and in cardiomyocytes. Calreticulin has not previously been described as a gene target for the Nkx2.5 transcription factor. The expression of Nkx2.5 is restricted to the heart and heart progenitor cells from very early on in embryonic development (18). Therefore, Nkx2.5 is one transcription factor that probably plays a critical role in activating the calreticulin gene in the embryonic heart.

Comparison between the Nkx2.5 response element in the calreticulin promoter (5′-CTCAAGTGT-3′ (antisense 5′-GAGTTCACA-3′)) and the consensus sequence for COUP-TF1 binding to DNA (5′-(A/G)G(G/T)TCA-3′) (Fig. 2*A*) (15) indicates that the Nkx2.5 response element is also likely a COUP-TF1 binding site. In the heart, the expression of calreticulin is downregulated after birth (12), but the mechanisms responsible for this repression are not known. It has been shown previously that COUP-TF1 can inhibit the action of nuclear receptors by binding competitively to their DNA binding sites (15, 19–23). To test whether COUP-TF1 affects transcriptional activity of the calreticulin promoter, we cotransfected NIH3T3 cells with the pLC1 vector (the luciferase gene controlled by the calreticulin promoter) and with increasing amounts of a COUP-TF1 expression vector. Fig. 2*B* shows that COUP-TF1 repressed transcription from the calreticulin promoter, and this repression was concentration-dependent. When cells were transfected with 1 or 3 μg of the COUP-TF1 expression vector there was greater than 70% repression of calreticulin promoter activity (Fig. 2*B*).

Because the COUP-TF1 and Nkx2.5 binding sites in the calreticulin promoter overlap, we investigated whether COUP-TF1 and Nkx2.5 exhibit cooperation in regulating transcription of the calreticulin gene. First, we cotransfected cells with the pLC1 vector and the COUP-TF1 expression vector. Fig. 2*C* shows that, as before, coexpression of COUP-TF1 decreased the activity of the calreticulin promoter. However, when we transfected cells with the pLC1 vector, the COUP-TF1 expression vector, and the Nkx2.5 expression vector, it was evident that Nkx2.5 abolished the COUP-TF1-dependent suppression of calreticulin promoter activity (Fig. 2*C*). We also carried out a converse experiment. Cells were transfected with pLC1 vector, a constant amount of the Nkx.25 expression vector, and increasing amounts of COUP-TF1 expression vector. Fig. 2*C* shows that under these conditions Nkx2.5 also exhibited a dominant effect on the calreticulin promoter.

Fig. 3 shows a schematic representation of the calreticulin promoter and the position of 3 putative Nkx2.5 elements, designated CRT sites 1, 2, and 3, respectively. Deletion analysis revealed that COUP-TF1 effectively repressed transcriptional activity when all three sites (1, 2, and 3) were present in the promoter (Fig. 3). Deletion of sites 1 and 3 did not affect the repression, whereas the deletion of site 2 effectively abolished it (Fig. 3). The highest activation of the calreticulin promoter by Nkx2.5 was also dependent on the presence of the site 2 (not shown). We conclude that COUP-TF1 represses transcription from the calreticulin promoter and that it does this via interaction with the calreticulin promoter at a putative binding site termed CRT site 2.

To demonstrate specific binding of COUP-TF1 to the Nkx2.5 response element, we carried out an EMSA. We used recombinant COUP-TF1 and a [^{32}P]-labeled synthetic deoxyoligonucleotide corresponding to the 3 tandem repeats of the CRT site 2. Fig. 4*A* shows that COUP-TF1 bound to the [^{32}P]-labeled deoxyoligonucleotide (*lanes 1–3*). It also shows that this binding was competed out in the presence of a 50-fold excess of unlabeled the CRT site 2 deoxyoligonucleotide (Fig. 4*A*, *lane 4*). Luciferase, which was synthesized *in vitro* with a coupled transcription and translation system, did not bind to the element.

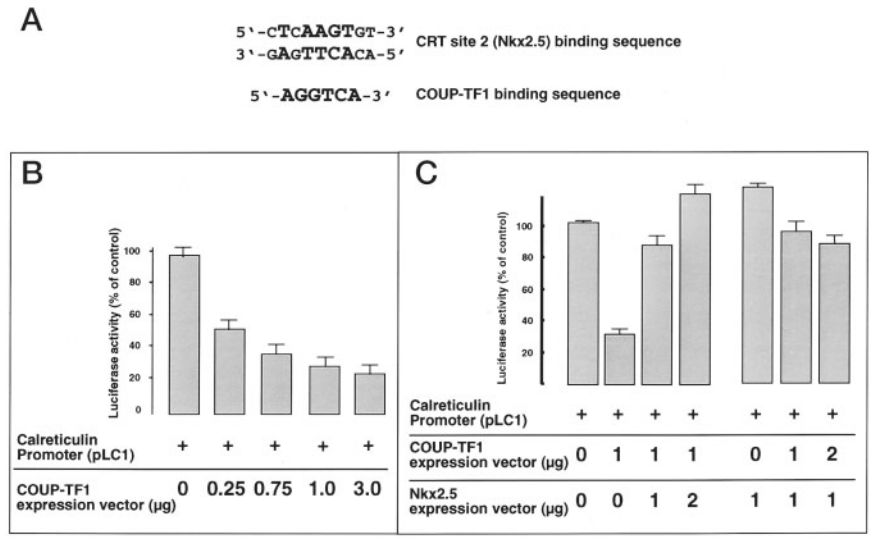

FIG. 2. **Repression of the calreticulin promoter by COUP-TF1.** *A*, the nucleotide sequences of the CRT site 2 (Nkx2.5) binding site in the calreticulin promoter and of a consensus COUP-TF1 binding site. *B* and *C*, NIH3T3 cells were cotransfected with a luciferase reporter gene controlled by the calreticulin promoter (*pLC1*) and with the indicated amounts of both Nkx2.5 and COUP-TF1 expression vectors. Luciferase activity was normalized against β-galactosidase. Data are means ± S.D. of three independent experiments.

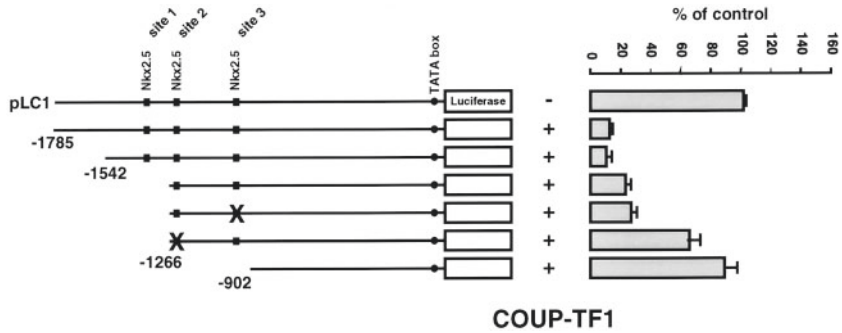

FIG. 3. **Suppression by COUP-TF1 specifically requires the CRT site 2 in the calreticulin promoter.** NIH3T3 cells were cotransfected with luciferase reporter constructs and with the COUP-TF1 expression vector. Luciferase activity was normalized against β-galactosidase. Three putative Nkx2.5 binding sites (CRT sites 1, 2, and 3) in the calreticulin promoter are indicated. *X* depicts deletion of a specific CRT site. Data are means ± S.D. of three independent experiments.

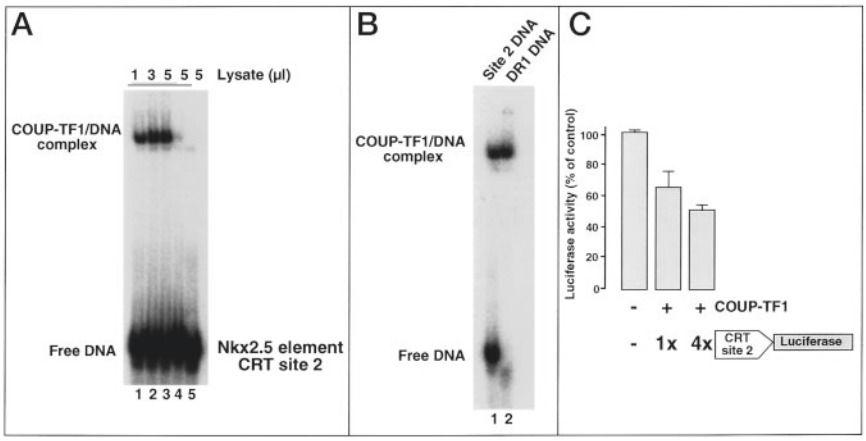

FIG. 4. **COUP-TF1 binds to the Nkx2.5 response element.** EMSA was performed with COUP-TF1, Nkx2.5, and luciferase (control) that were translated *in vitro*. A synthetic calreticulin promoter CRT site 2 element and a synthetic DR1 element were used as probes for binding activity. *A*, COUP-TF1 binds to the CRT site 2. *Lanes 1*, *2*, and *3* contain increasing amounts of lysate containing COUP-TF1 protein. *Lane 4* contains COUP-TF1 and a 50-fold excess of unlabeled, synthetic CRT site 2 DNA. *Lane 5*, 5 μl of luciferase control protein. *B*, COUP-TF1 binds to the DR1 element and to the CRT site 2 element as a homodimer. *C*, NIH3T3 cells were cotransfected with a COUP-TF1 expression vector and with a luciferase reporter gene controlled by either one or four CRT site 2 elements and a minimum calreticulin promoter. Luciferase activity was normalized against β-galactosidase. Data are means ± S.D. of three independent experiments.

We conclude that COUP-TF1 binds specifically to the CRT site 2 in the calreticulin promoter.

COUP-TF1 recognizes the consensus sequence (5′-(A/G)G(G/T)TCA-3′). It binds with highest affinity as a homodimer to a direct repeat of 5′-AGGTCA-3′ separated by a single base space, termed the DR1 element (15). To determine whether COUP-TF1 binds to the CRT site 2 element as a homodimer we carried out an EMSA. We compared binding of COUP-TF1 to the CRT site 2 (Fig. 4*B*, *lane 1*) and to DR1 (Fig. 4*B*, *lane 2*). We expected that if COUP-TF1 binds to the CRT site 2 element as a homodimer, then the COUP-TF1·CRT site 2 complex would have a similar electrophoretic mobility to the COUP-TF1·DR1 complex. Fig. 4*B* shows that the electrophoretic mobility of the COUP-TF1·CRT site 2 complex (*lane 1*) was identical to that of

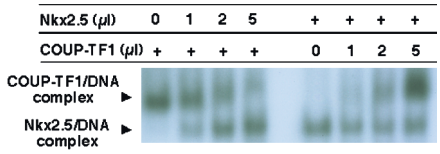

FIG. 5. **COUP-TF1 and Nkx2.5 compete for binding to the CRT site 2 response element.** EMSA was performed with COUP-TF1 and Nkx2.5 that were translated *in vitro*. The indicated amounts of COUP-TF1 and Nkx2.5 were incubated with the CRT site 2 synthetic oligonucleotide followed by ESMA. *Arrows* indicate the positions of the COUP-TF1·DNA complex and the Nkx2.5·DNA complex.

the COUP-TF1·DR1 (*lane 2*). This result indicates that the COUP-TF1 protein binds to the CRT site 2 response element as a homodimer.

To further investigate the role of the CRT site 2 in COUP-TF1-dependent repression of calreticulin promoter activity, we constructed plasmids with the luciferase reporter gene under the control of either 1 or 4 copies of the CRT site 2 response element (5′-CTCAAGTGT-3′). In both cases, the element(s) were placed upstream of a minimal calreticulin promoter (−173 to +40 encompassing the TATA and SP1 sites) (13). We then cotransfected NIH3T3 cells with a COUP-TF1 expression vector and one of the luciferase reporter constructs. When COUP-TF1 was cotransfected with a reporter construct containing 1 copy of the CRT site 2 element, 36% inhibition of calreticulin promoter activity was observed (Fig. 4*C*). When COUP-TF1 was cotransfected with a reporter construct containing 4 copies of the element, 50% inhibition of calreticulin promoter activity was observed (Fig. 4*C*).

To demonstrate that COUP-TF1 binds to the CRT site 2 in the calreticulin promoter *in vivo,* we carried out a yeast one-hybrid screen. We screened an embryonic (11-day) mouse cDNA library, fused to a GAL4 activation domain, using the CRT site 2 response element (5′-CTCAAGTGT-3′) as bait. A total of 1.2×10^6 yeast transformants were screened. We isolated 18 positive clones, which grew on His^-/Leu^- plus 15 mM 3-amino-1,2,4-triazole over 5 days at 30 °C. Two of the 18 positive clones encoded the N-terminal region of COUP-TF1, indicating that COUP-TF1 bound to the CRT site 2 response element *in vivo*.

Our results suggest that cooperation may occur, between COUP-TF1 and Nkx2.5, at the calreticulin promoter. To study this further, we used an EMSA to measure the binding of COUP-TF1 to the synthetic CRT site 2 in the presence of Nkx2.5. As expected, both COUP-TF1 (Fig. 5, *lane 1*) and Nkx2.5 (Fig. 5, *lane 5*) bound to the CRT site 2. However, the CRT site 2·COUP-TF1 complex was significantly larger than the CRT site 2·Nkx2.5 complex (Fig. 5, compare *lanes 1* and *5*). Recombinant Nkx2.5, in increasing amounts, efficiently competed with COUP-TF1 for the DNA binding site, favoring formation of the faster migrating CRT site 2·Nkx2.5 complex (Fig. 5, *lanes 2–4).* In contrast, although COUP-TF1 was able to compete with Nkx2.5 to some extent, it did so with a much lower affinity (Fig. 5, *lanes 6–7*).

COUP-TF1 can form heterodimers with other nuclear receptors and inhibit their activity (19). To determine whether COUP-TF1 and Nkx2.5 interact directly with each other, *in vivo*, we performed coimmunprecipitation experiments. We cotransfected NIH3T3 cells with HA-tagged Nkx2.5 and with COUP-TF1. We then immunoprecipitated nuclear extracts with anti-HA antibodies and immunoblotted the precipitates. Our Western blot analysis, with anti COUP-TF1 antibodies, revealed that Nkx2.5 and COUP-TF1 did not coimmunoprecipitate (data not shown), indicating that these two transcription factors do not form a heterodimer.

During cardiogenesis there is a requirement for high expression of Nkx2.5, to activate cardiac-specific genes. In this study we demonstrate that, in the heart, the calreticulin gene is also a target for the transcription factor Nkx2.5. We also show that COUP-TF1 suppresses transcription of the calreticulin gene and that Nkx2.5 and COUP-TF1 bind to a common element in the calreticulin promoter. Apparently, these transcription factors exhibit cooperation in regulating transcription of the calreticulin gene. Calreticulin gene knockout is embryonic-lethal, resulting from impaired cardiac development (12, 24). In contrast, overexpression of calreticulin in the newborn heart leads to severe pathology and death.[2] Together, these observations indicate that cardiomyocytes are sensitive to changes in the abundance of calreticulin. Therefore, transcription of the calreticulin gene in cardiac cells is presumably tightly and specifically controlled. In particular, the evidence indicates a need for elevated expression of calreticulin during the early stages of cardiac development. However, this high level of expression must be reversed, because postnatal pathology is observed in mice overexpressing this protein.[2] The present study indicates that the COUP-TF1 may be one transcription factor responsible for a postnatal repression of the calreticulin gene. Similar to calreticulin cardiac overexpresser mice, COUP-TF1-deficient mice die postnatally, indicating that COUP-TF1 is essential for postnatal survival (25). These animals display defects in development of the ninth cranial ganglion and nerve (25). Presently, there is no information on cardiac function and development and/or the expression pattern of calreticulin in COUP-TF1-deficient mice.

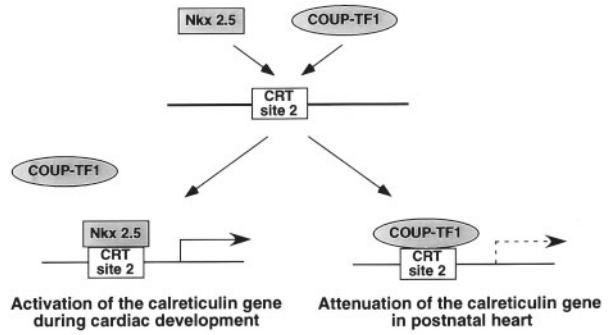

FIG. 6. **A model for transcriptional cooperation between Nkx2.5 and COUP-TF1.** The figure shows a putative model for the role of Nkx.25 and COUP-TF1 in transcriptional activation of the calreticulin gene, although other transcription factors (not shown) may also play a role in transactivation of the gene. Nkx2.5 and COUP-TF1 can bind to the CRT site 2 element in the calreticulin promoter. During embryonic development Nkx2.5 plays a central role in activating the expression of calreticulin. In embryonic hearts Nkx2.5 expression is high, and COUP-TF1 cannot bind to the CRT site 2 in the calreticulin promoter. Down-regulation of Nkx2.5 expression, which occurs as cardiac development progresses and in newborns, permits association between COUP-TF1 and the calreticulin promoter, resulting in repression of the calreticulin gene.

We propose that Nkx2.5 plays a central role in activating calreticulin expression in the developing heart (Fig. 6) by binding to CRT site 2. As we show in this study, binding of COUP-TF1 to the calreticulin promoter results in suppression of promoter activity. The presence of elevated Nkx2.5, during early cardiac development, minimizes the interaction of calreticulin promoter to COUP-TF1. Progressive down-regulation of Nkx2.5 expression, which occurs as cardiac development progresses and following birth, exposes the CRT site 2 binding site in the calreticulin promoter, permitting the interaction with COUP-TF1 (Fig. 6). It is likely that other transcription factors may play an important role in the activation and sup-

[2] K. Nakamura and M. Michalak, submitted for publication.

pression of the calreticulin gene during cardiogenesis. This work, however, implicates COUP-TF1 as one transcription factor contributing to the decline in calreticulin levels seen in the newborn/mature heart compared with those seen in the developing heart.

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