SMAD-mediated modulation of YY1 activity regulates the BMP response and cardiac-specific expression of a GATA4/5/6-dependent chick *Nkx2.5* enhancer

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Accepted 8 July 2004

Development 131, 4709-4723
Published by The Company of Biologists 2004
doi:10.1242/dev.01344

Summary

Prior work has indicated that BMP signals act in concert with FGF8, WNT11 and WNT antagonists to induce the formation of cardiac tissue in the vertebrate embryo. In an effort to understand how these signaling pathways control the expression of key cardiac regulators, we have characterized the cis-regulatory elements of the chick *tinman* homolog chick *Nkx2.5*. We find that at least three distinct cardiac activating regions (CARs) of chick *Nkx2.5* cooperate to regulate early expression in the cardiac crescent and later segmental expression in the developing heart. In this report, we focus our attention on a 3′ BMP-responsive enhancer, termed CAR3, which directs robust cardiac transgene expression. By systematic mutagenesis and gel shift analysis of this enhancer, we demonstrate that GATA4/5/6, YY1 and SMAD1/4 are all necessary for BMP-mediated induction and heart-specific expression of CAR3. Adjacent YY1 and SMAD-binding sites within CAR3 constitute a minimal BMP response element, and interaction of SMAD1/4 with the N terminus of YY1 is required for BMP-mediated induction of CAR3. Our data suggest that BMP-mediated activation of this regulatory region reflects both the induction of GATA genes by BMP signals, as well as modulation of the transcriptional activity of YY1 by direct interaction of this transcription factor with BMP-activated SMADs.

Key words: Nkx2.5, BMP, YY1, GATA, SMADs, Cardiogenesis, Chick

Introduction

The heart is the first organ to develop during vertebrate embryogenesis and arises from cardiac progenitor cells that migrate into the anterior lateral mesoderm during gastrulation (DeHaan, 1965; Rawles, 1943; Rosenquist, 1966; Rosenquist and DeHaan, 1966). The tissues and signals that induce the specification of cardiac progenitors have been the subject of intense investigation. Signals from the anterior endoderm in both chick and amphibian embryos (Jacobson and Duncan, 1968; Sater and Jacobson, 1989; Schneider and Mercola, 2001; Schultheiss et al., 1995), and from the organizer region in *Xenopus* embryos (Schneider and Mercola, 2001) have been shown to promote the formation of cardiac tissue. Signals that promote the induction of cardiac mesoderm include BMP family members (Andree et al., 1998; Schlange et al., 2000; Schultheiss et al., 1997; Schultheiss and Lassar, 1997; Shi et al., 2000); FGF8 (Alsan and Schultheiss, 2002; Reifers et al., 2000); WNT11 (Eisenberg and Eisenberg, 1999; Pandur et al., 2002); and Crescent, FrzB and Dikkopf (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001), which act as antagonists of Wnt family members that are expressed in either nascent mesoderm of the primitive streak or in the neural tube at post-neurulation stages (Tzahor and Lassar, 2001).

One of the earliest genes to be induced in cardiac precursor cells in response to these various heart-inducing signals is the transcription factor *Nkx2.5*. *Nkx2.5* is a homolog of the *Drosophila tinman* gene, and is first expressed in the anterior lateral regions of gastrula stage vertebrate embryos termed the 'cardiac crescent', which contains both cardiac and foregut mesendoderm precursors. At later stages of development, *Nkx2.5* is expressed throughout the mature heart and in the pharyngeal arches, spleen, thyroid, stomach and tongue (Komuro and Izumo, 1993; Lints et al., 1993). *Nkx2.5* works in concert with other transcription factors to regulate early cardiac gene expression (Chen et al., 1996; Chen and Schwartz, 1996; Sepulveda et al., 1998; Sepulveda et al., 2002). Given the role of *Nkx2.5* in heart induction and development, identification of the regulatory sequences and transcription factors controlling the expression of this gene is of particular interest. Several activating regions (ARs) that flank the mouse *Nkx2.5* gene have been described that are capable of driving transgene expression in both the cardiac crescent and newly formed heart (Lien et al., 1999; Reecy et al., 1999; Schwartz and Olson, 1999; Searcy et al., 1998; Tanaka et al., 1999). Consistent with the finding that BMP signals promote *Nkx2.5* expression and cardiac induction (Schlange et al., 2000; Schultheiss et al., 1997), several consensus binding sites for SMAD4-containing complexes have been functionally

implicated in driving expression of the murine *Nkx2.5* AR2 cardiac crescent enhancer (Liberatore et al., 2002; Lien et al., 2002). In addition, consensus binding sites for the GATA zinc-finger transcription factors have been found to be necessary for the activity of both AR1 and AR2, and probably interact with GATA4, GATA5 or GATA6 (Lien et al., 1999; Searcy et al., 1998).

In this study, we have characterized genomic flanking regions of the chick *Nkx2.5* gene to identify three distinct cis-regulatory elements or cardiac activating regions (CAR 1, 2 and 3) that work in combination to drive transgene expression in both the cardiac crescent and in segment-specific compartments of the maturing heart. One enhancer, located 3′ to the coding exons of chick *Nkx2.5* (CAR3), confers BMP responsiveness to reporter genes in a heterologous assay system, and drives transgene expression in both the primary and secondary heart fields and throughout the outflow tract and right ventricle of the maturing heart. By a combination of deletion mapping and embryonic gel shift analysis, we have identified a 200 bp sequence capable of both conferring a BMP response and driving cardiac-specific transgene expression. This regulatory sequence contains a triad of functionally important binding sites for GATA4/GATA5/GATA6 and YY1 that act in conjunction with three closely associated SMAD-binding sites to regulate transgene expression in vivo and confer BMP responsiveness to CAR3 driven reporters in vitro. Detailed molecular analysis of this BMP response element (BMPRE) suggests that the transcriptional repressor, YY1, serves a primary role in both recruiting an activated SMAD complex to the BMPRE and becomes a transcriptional activator when bound adjacent to BMP-activated SMADs. These findings indicate that the combinatorial activity of both spatially restricted transcription factors such as GATA proteins work in concert with a more ubiquitously expressed transcription factor (i.e. YY1), the activity of which is modulated by SMAD association to drive a regionalized BMP response.

Materials and methods

Vectors and plasmids

Details regarding generation Nkx2.5-*lacZ* and luciferase constructs are available upon request. pCS2 MT-SMAD1 and pCS2 MT/Flag SMAD4 were a kind gift from M. Whitman. pGEX constructs for producing GST fusion proteins of SMAD MH1 domain proteins were a kind gift from M. Whitman and C. Yeo. pcDNA rat GATA4 was derived from pCG-GATA4, a kind gift of M. Nemer. pCS2 chick GATA5 was a kind gift from T. Schultheiss (unpublished). pcDNA mouse GATA6 was a kind gift from T. Collins. Flag-tagged YY1 full-length and deletion expression constructs were kind gifts from E. Seto (Yao et al., 2001), G. Sui and Y. Shi (unpublished). PCMV-p21E1b was a kind gift from O. Kranenburg (Kranenburg et al., 1995).

Transient and stable transgenic mouse assays

Nkx2.5-*lacZ* reporter constructs were introduced into a one-cell stage FVB mouse embryo using standard methods. In the case of the Nkx2.5-*lacZ*-BMPRE construct (Fig. 1R,S) an insulator sequence derived from the chick β-globin locus (Chung et al., 1993) was appended immediately 3′ to the reporter construct. F0 embryos were collected at 7.5-10.5 days post-injection following maternal sacrifice, and fixed and stained for β-galactosidase activity according to previously described methods (Zimmerman et al., 1994). Transgenic status of individual embryos was determined by PCR for the *lacZ* transgene from DNA derived from yolk sacs and embryo fragments (Wassarman, 1993). Stable lines were obtained by mating fully grown F0 to wild-type FVB mice.

BMP response assays

BMP response assays were performed as previously described (Chen et al., 1998). Cells were transfected with 0.1 μg luciferase or *lacZ* reporters, along with 20 ng TK-renilla luciferase (Promega) and the indicated quantities of expression vectors as per individual experiments. Individual wells were assayed after 18-24 hours of incubation with or without the addition of 50 ng/ml recombinant human BMP2 (a kind gift from Genetics Institute/Wyeth Pharmaceuticals) or conditioned medium from 293 cells programmed to overexpress mouse BMP4 (D.-W. Kim and A.B.L., unpublished) using either a dual luciferase assay system (Promega) or a chemiluminescent β-galactosidase assay (Galactolight; Tropix). Nkx2.5-driven luciferase activity was normalized to that of TK-renilla luciferase. All transfections are representative of three to five independent experiments with similar results.

Chick embryo extracts and gel shift assays

Anterior lateral plate, posterior primitive streak and heart explants were dissected from staged chick embryos. Explants from 50-100 embryos were pooled on ice in PBS, collected by mild centrifugation in a microfuge at 110 ***g***, then resuspended and homogenized in an extraction buffer containing 20 mM HEPES (pH 7.6), 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, protease inhibitors (‘C/Emplete, EDTA-free’ protease inhibitor cocktail from Roche), 2 mM $Na_4P_2O_7$, 1 mM $NaVO_3$, 10 mM NaF and 2.5 ng/ml calyculin B. Cellular debris was removed by centrifugation at 10,000 ***g*** for 10 minutes at 4°C. Shifts were performed with 1-2 μg total protein from the various extracts in 20 mM HEPES (pH 7.6), 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol and 0.5 μg dI/dC (Pharmacia), resolved on 5% acrylamide/0.25× TBE at 4°C and autoradiographed. Gel shifts were also performed using nuclear extracts prepared as above from COS-7 cells programmed to express either recombinant rat GATA4, chick GATA5, mouse GATA6 or flag-tagged full-length and deletion mutants of human YY1. GST-SMAD1 and SMAD4 MH1 domain fusion proteins and control GST proteins were produced in and purified from *E. coli* BL21 bacterial cells according to manufacturer’s instructions (Amersham). Gel shifts were performed as with chick embryo extracts, using 250 ng purified proteins and 1 μg dA/dT (Pharmacia). Supershifts were performed with the addition of 200 ng of control Ig, or rabbit polyclonal anti-mouse GATA4 and GATA6 (Santa Cruz Biotechnologies).

Co-immunoprecipitation and western blot analyses

For co-immunoprecipitation experiments, 5 μg of the indicated expression plasmids were used per 100 mm plate. pCS2 empty vector was used to adjust total DNA amounts where necessary. Total cell extracts were prepared in Co-IP buffer containing 50 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5% NP-40 and various protease inhibitors (‘Complete, EDTA-free’ protease inhibitor cocktail from Roche). The extracts were then centrifuged for 10 minutes at 10,000 ***g*** at 4°C and the supernatants were used for immunoprecipitation assays as previously described (Kim and Cochran, 2000). Anti-Myc rabbit polyclonal antibody and anti-Myc monoclonal (9E10) antibody were obtained from Upstate Biotechnology. Anti-YY1 polyclonal and monoclonal antibodies were obtained from Santa Cruz Biotechnology. Protein G-sepharose was obtained from Sigma.

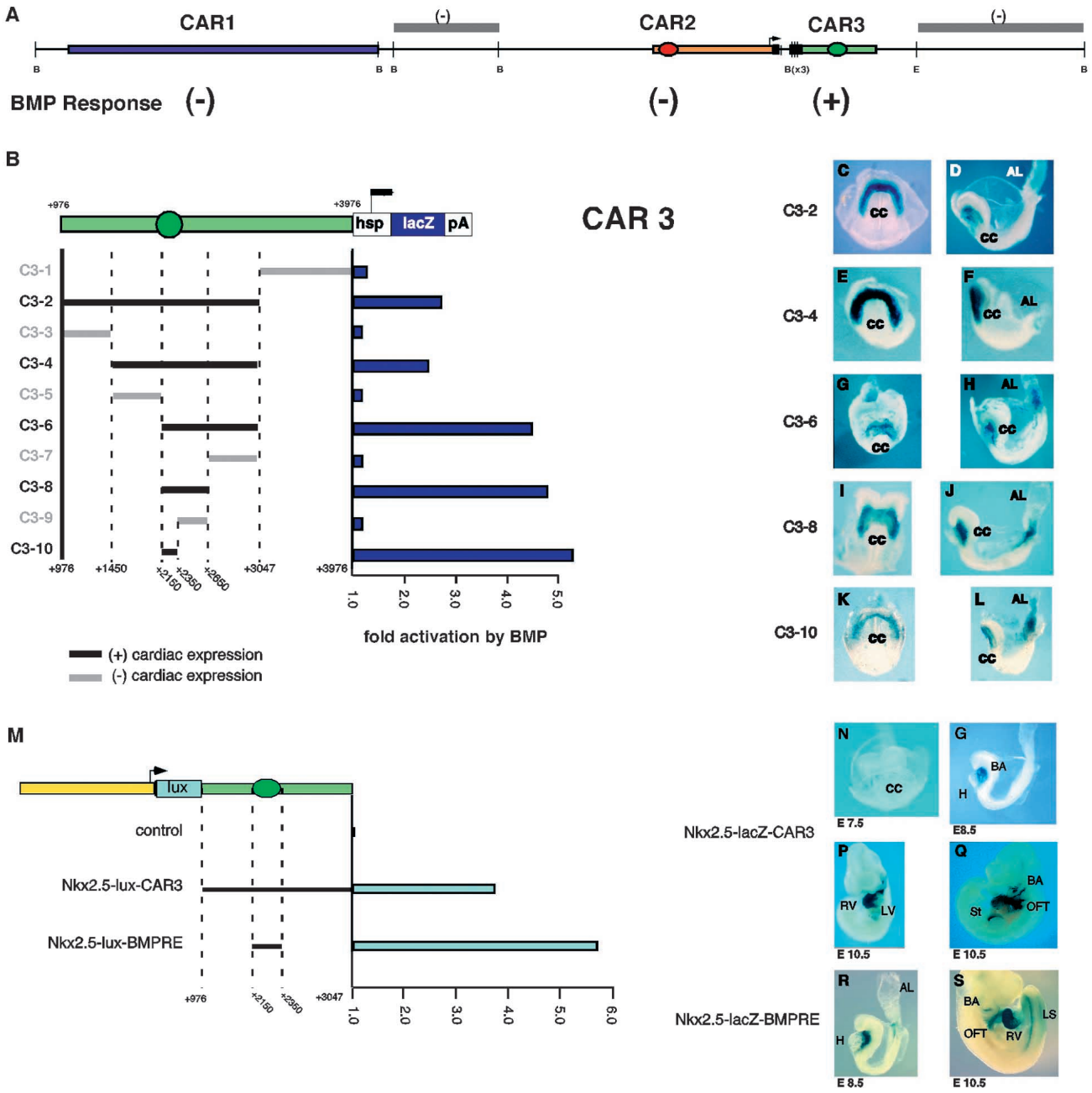

Fig. 1. BMP response and in vivo transgene expression maps to CAR3 enhancer. (A) Genomic flanking regions of chick *Nkx2.5* containing CAR enhancers as shown were assayed for BMP response in P19 cells in the context of both hsp68-*lacZ* and Nkx2.5-luciferase reporters (Nkx2.5-lux). Only the CAR3 enhancer is BMP responsive. (B) Deletion mapping of BMP response and transgenic expression for 3 kb 3′ genomic flanking region of chick *Nkx2.5*which contains CAR3, using hsp68-*lacZ* promoter/reporter. Nucleotide positions of 5′ and 3′ ends of deleted flanking regions are given relative to transcriptional start site of chick *Nkx2.5*. Extent of the genomic fragments yielding positive reporter *lacZ* expression in F0 transient transgenic mouse embryos are shown by black bars (C3-2, C3-4, C3-6, C3-8 and C3-10); negative reciprocal fragments are represented as gray bars (C3-1, C3-3, C3-5, C3-7 and C3-9). BMP response of these hsp-*lacZ*-CAR3 reporters is shown to immediate right of their representations, expressed as normalized fold induction by BMP. (C-L) Representative X-gal stained E7.5 F0 transient transgenic mice embryos injected with C3 reporters C3-1 to C3-10. (M) BMP response of 2 kb CAR3 (fragment C3-2) and 200 bp BMPRE (fragment C3-10) enhancers linked to Nkx2.5-lux reporters (Nkx2.5-lux-CAR3 and Nkx2.5-lux-BMPRE, respectively). Extents of CAR3 and BMPRE enhancers and nucleotide positions relative to transcriptional start site are shown as above. BMP response in P19 cell assay is shown to right as fold induction above basal. (N-S) Representative X-gal stained F0 transient transgenic mice embryos injected with Nkx2.5-*lacZ*-CAR3 (N-Q) and Nkx-*lacZ*-BMPRE (R,S). Embryonic stages are shown at bottom left of pictures. Frequency of transient or stable (C3-2) transgene expression in cardiac region (cardiac crescent): C3-1, 0/20 embryos; C3-2, 2/3 lines; C3-3, 0/18 embryos; C3-4, 4/5 embryos; C3-5, 1/19 embryos; C3-6, 1/1 embryos; C3-7, 1/12 embryos; C3-8, 12/12 embryos; C3-9, 0/5 embryos; C3-10, 12/16 embryos. CC, cardiac crescent; LS, lateral somite; OFT, outflow tract; AL, allantois; BA, branchial arches; St, stomach; RV, right ventricle; H, heart; LV, left ventricle.

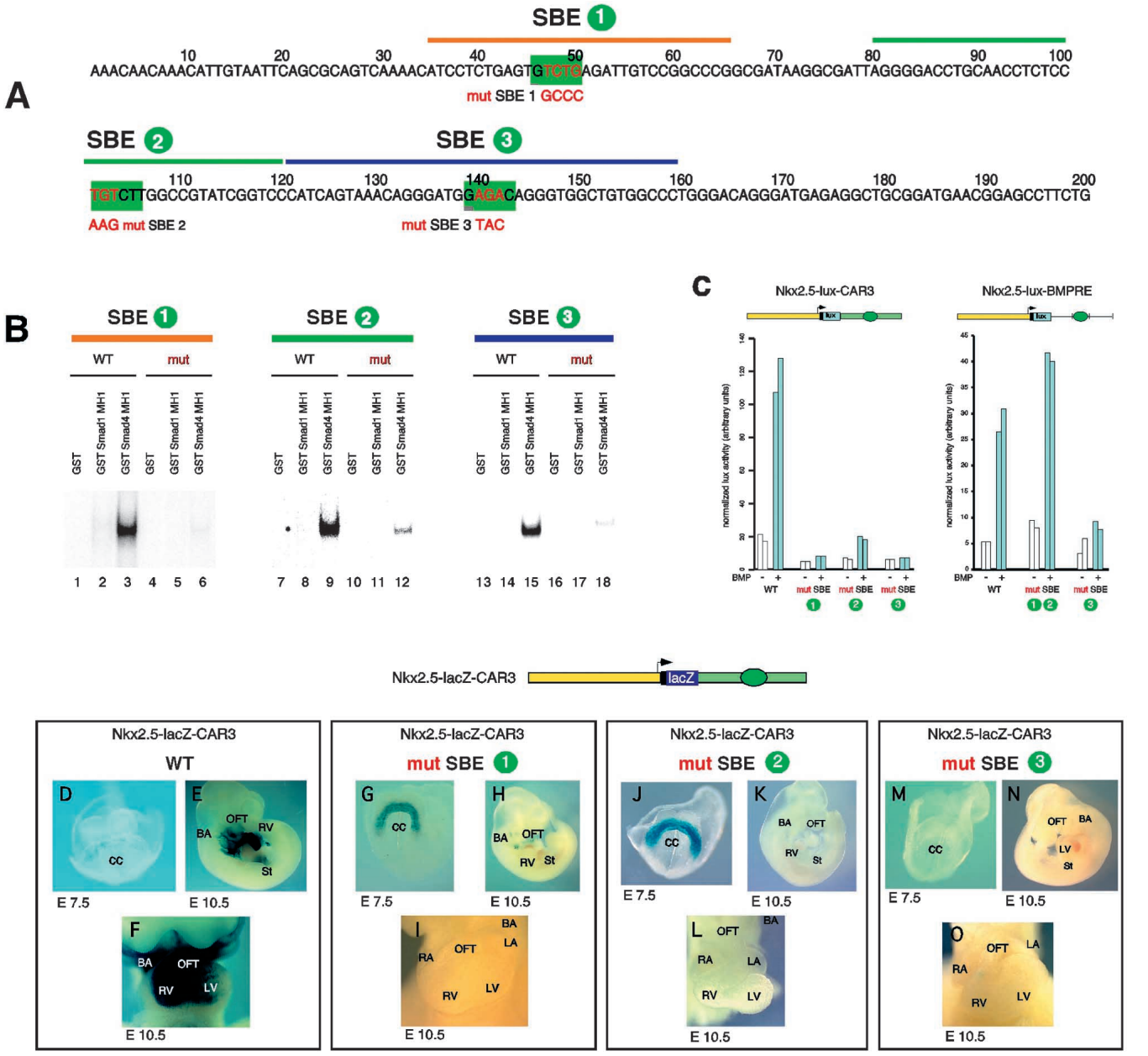

Fig. 2. SMAD consensus sites bind MH1 domain of SMAD4 and are required for BMP response of CAR3. (A) Oligonucleotides used for SMAD protein gel shifts are shown as colored horizontal lines over a sequence of 200 bp BMPRE, representing chick *Nkx2.5* genomic sequences from +2150 to +2350. Nucleotide positions in BMPRE are renumbered from 1-200 for convenience. Consensus SMAD-binding sites or SBEs are shown boxed in green; numerical designations are shown in green circles. Nucleotide substitutions made for mutant oligos and reporters are displayed in red under their cognate sites, as are correspondingly altered sequences within the BMPRE (SBE1, nucleotides 35-65; SBE2, nucleotides 80-120; SBE3, nucleotides 120-160). (B) Gel shifts performed with purified GST, GST-SMAD1 MH1 or GST-SMAD4 MH1 domain proteins on labeled wild-type (lanes 1-3, 7-9 and 13-15, respectively) or mutant (mut, lanes 4-6, 10-12 and 16-18, respectively) SBE oligomers. (C) P19 cell BMP response assays for wild-type Nkx2.5-lux-CAR3 (left) or Nkx2.5-lux-BMPRE (right) reporters, or cognate reporters bearing specific SBE mutations shown in A. Nkx2.5-lux constructs are shown schematically over bar graph. (D-O) Representative X-gal stained F0 transient transgenic mice embryos injected with wild-type or SBE mutant (mut) Nkx2.5-*lacZ*-CAR3 reporters. Embryonic stages are in the bottom left-hand corners. Whole-mount embryo at stages E7.5 (D,G,J,M) and E10.5 (E,H,K,N) and E10.5 hearts (F,I,L,O) are shown as designated for Nkx2.5-*lacZ*-CAR3 WT (D-F), mut SBE1 (G-I), mut SBE2 (J-L) and mut SBE3 (M-O) as shown in A. Cardiac staining shown was representative of the following numbers of transient transgenic embryos bearing Nkx2.5-*lacZ* constructs driven by CAR3 reporters: wild type, 3/5 E7.5 and 4/4 E10.5 embryos displayed expression in the cardiac crescent and forming heart; CAR3mut SBE1, 2/4 E7.5 embryos displayed robust transgene expression in the cardiac crescent and 9/9 E10.5 embryos either failed to express the transgene or displayed very weak transgene expression in the outflow tract; CAR3mut SBE2, 7/7 E7.5 embryos displayed robust transgene expression in the cardiac crescent and 2/2 E10.5 embryos failed to express the transgene; CAR3mut SBE3, 7/7 E7.5 embryos and 3/3 E10.5 embryos either failed to express the transgene or displayed residual transgene expression in the outflow tract. Abbreviations are as in previous figures. RA, right atrium; LA, left atrium; LV, left ventricle.

Results

Transgenic analysis with a heterologous promoter reveals three cardiac activating regions flanking the chick *Nkx2.5* gene

As the expression patterns of chick and mouse *Nkx2.5* appear highly conserved through embryonic development (Lints et al., 1993; Schultheiss et al., 1995), we investigated whether sequences flanking the chick gene were capable of driving cardiac-specific transgene expression in mouse embryos. Transient expression of the hsp68-*lacZ* reporter (Kothary et al., 1989) linked to three non-overlapping regions of the chick *Nkx2.5* gene drove cardiac-specific gene expression in transgenic mice (see Fig. S1C in the supplementary material). We termed these various regions of the chick *Nkx2.5* gene, cardiac activating regions (CARs) 1, 2 and 3. In an effort to better define their distinct roles, we performed further transgenic analysis of the various CARs in combination with the native *Nkx2.5* promoter. When fused to the minimal promoter for the *Nkx2.5* gene (see Fig. S2B in the supplementary material), the various above-described CARs drove several distinct patterns of cardiac-specific transgene expression in developing mouse embryos (see Fig. S2C-O in the supplementary material). CAR2, when appended to the Nkx2.5-*lacZ* reporter (see Fig. S2B in the supplementary material) drives transgene expression in the posterior regions of the early cardiac crescent at E7.5 (see Fig. S2C in the supplementary material) and in the outflow tract at later looping heart stages (E10.5) (see Fig. S2D,E in the supplementary material) (data not shown). CAR3, when positioned downstream of the minimal *Nkx2.5* promoter (as it is located in this position relative to the endogenous promoter in the native chick *Nkx2.5* gene), drives transgene expression in the anterior cardiac crescent at E7.5 (see Fig. S2F in the supplementary material) and in the mesoendoderm in the pharyngeal region adjacent to the outflow tract at E8.5 (see Fig. S2G in the supplementary material), in a pattern that is very reminiscent of the Isl1-positive progenitors of the secondary heart field (Cai et al., 2003). By E10.5, robust Nkx2.5-*lacZ*-CAR3 transgene expression was observed in the branchial arches and extended through the outflow tract and right ventricle up to the interventricular septum (see Fig. S2H in the supplementary material). Although CAR1 failed to drive transgene expression in the early cardiac crescent when linked to either the hsp68 or the minimal *Nkx2.5* promoter (data not shown), the combination of CAR1, CAR2 and CAR3 (see Fig. S2B in the supplementary material) drove exceptionally robust levels of *lacZ* expression in the branchial arch region and in the myocytes of the outflow tract, and both right and left ventricles at E10.5 (see Fig. S2M-O in the supplementary material).

CAR3 contains a BMP response element capable of driving transgene expression in the cardiac crescent, the branchial arches, the outflow tract and the right ventricle

Prior work by ourselves (Schultheiss et al., 1997) and others (Andree et al., 1998; Schlange et al., 2000) has indicated that BMP family members are necessary for inducing cardiac mesoderm and *Nkx2.5* gene expression in early chick embryos. We therefore tested the CARs flanking the chick *Nkx2.5* gene for responsiveness to BMP signaling by employing P19 embryonal carcinoma cells, which are known to be responsive to BMP signals (Chen et al., 1998; Hata et al., 2000). Administration of BMP2 or BMP4 (50 ng/ml) to transfected P19 cells induced the expression of reporter constructs containing CAR3 appended to either the hsp68 promoter or the minimal *Nkx2.5* promoter three- to fourfold relative to a co-transfected CMV-renilla luciferase control plasmid (Fig. 1A,B,M). By contrast, constructs containing these same promoters driven by either CAR1 or CAR2 failed to show any BMP response in transfected P19 cells (Fig. 1A). By sequential deletion analysis of the 2 kb CAR3 sequence, we were able to localize a 200 bp BMP response element (BMPRE) to nucleotides +2150-2350 in CAR3 (Fig. 1B). Interestingly, reporters driven by the 200 bp BMPRE displayed a greater response to BMP signals than did reporters driven by the original 2 kb fragment containing CAR3, suggesting that sequences outside the 200 bp BMPRE attenuate the BMP responsiveness of CAR3 in P19 cells. Transgenic analysis revealed that only reporter transgenes containing the 200 bp BMPRE from CAR3 were capable of driving *lacZ* expression in the cardiac crescent, branchial arches and outflow tract/right ventricle of the developing heart (Fig. 1C-L; data not shown). When appended to the endogenous chick *Nkx2.5* promoter, we found that either the 2 kb fragment containing CAR3 or the 200 bp subfragment containing the BMPRE drove BMP-responsive luciferase reporter expression in transfected P19 cells (Fig. 1M) and tissue-restricted *lacZ* expression in the branchial arches, outflow tract and right ventricle in transgenic mice (Fig. 1N-S).

SMAD binding elements are necessary for BMP induced activity of a CAR3 BMPRE-driven reporter in vitro and for CAR3-driven cardiac-specific transgene expression in vivo

Because SMADs are known to transduce BMP signals into the nucleus by both recognizing SMAD binding elements (SBEs) and associating with specific transcription factors, we evaluated whether SMAD1/4 would interact with sequences within the CAR3 BMPRE. The MH1 domain of SMAD proteins is known to bind weakly to the sequence GTCT/AGAC (Attisano and Wrana, 2000; Massague and Chen, 2000; Zawel et al., 1998). We found three such putative SMAD-binding elements (SBE1-3; boxed in green in Fig. 2A) located within the 200 bp BMPRE of CAR3. We found that the DNA-binding MH1 domain of SMAD4 could bind to oligomers containing either of these potential SBEs in vitro (Fig. 2B, lanes 3, 9 and 15), and that disruption of the GTCT/AGAC consensus binding sequence at each of these sites significantly diminished this interaction (Fig. 2B, lanes 6, 12 and 18).

We investigated whether these SBEs were necessary for either the BMP responsiveness of a CAR3-driven reporter or cardiac-specific expression of a CAR3-driven transgene. Mutation of either SBE1, SBE2 or SBE3 abrogated the BMP responsiveness of the Nkx2.5-lux-CAR3 construct in transfected P19 cells (Fig. 2C, left panel). Interestingly, the BMP responsiveness of a reporter driven by only the 200 bp CAR3 BMPRE (Nkx2.5-lux-BMPRE) required SBE3, but neither SBE1 nor SBE2 (Fig. 2C, right panel), suggesting that sequences flanking the 200 bp BMPRE impose the requirement

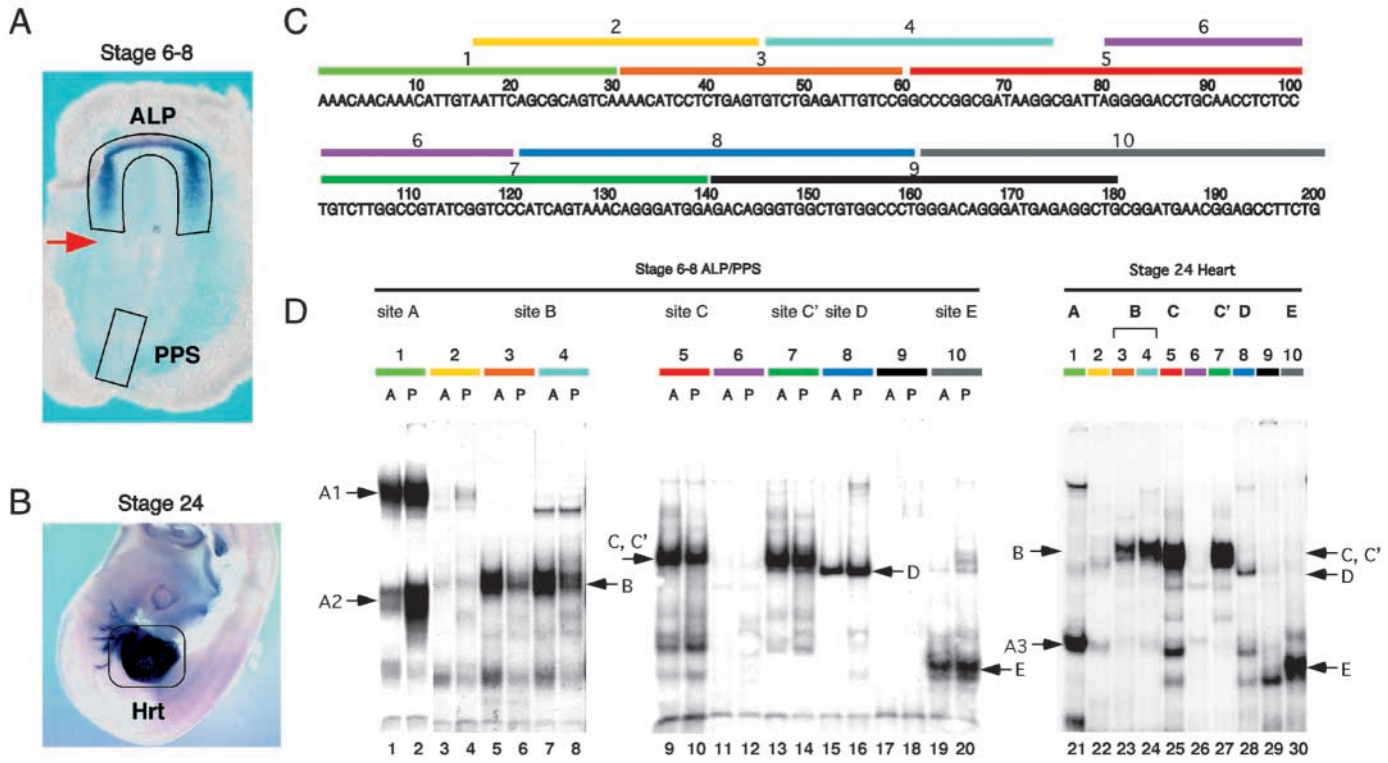

Fig. 3. Embryonic gel shift analysis of BMPRE. (A,B) Dissected regions of HH stage 6-8 and 24 (day 3) chick embryos are depicted as boxed regions of whole mount chick embryos stained by in situ hybridization for Nkx2.5 mRNA. Whole-cell extracts were made from either anterior lateral plate (ALP) or posterior primitive streaks (PPS) dissected from stage 6-8 chick embryos (A), or from hearts (Hrt) from stage 24 (day 3) chick embryos (B). (C) EMSA oligomers derived from 200 bp BMPRE enhancer sequence (see Fig. 2). Numbered and colored horizontal lines show extent of 30 or 40 bp double stranded oligomers used for gel shift assays. (D) Gel shifts obtained with anterior lateral plate (A), posterior primitive streak (P) or 3 day heart whole cell extracts. Discrete shifts were found for eight sites labeled A-E. Sites A1-A3 (nucleotides 1-30), lanes 1, 2, 21; site B (nucleotides 30-60 and 45-75), lanes 5-8, 23, 24; site C (nucleotides 60-100), lanes 9, 10, 25; site C′ (nucleotides 100-140), lanes 13, 14, 27; site D (nucleotides 120-160), lanes 15, 16, 28; site E (nucleotides 160-200), lanes 19, 20, 30. Multiple shifts seen with Site A oligo in different extracts are labeled separately A1-3 to the left of the autoradiogram, and are as discussed in the text.

for these additional SMAD binding elements. Although mutation of SBE1, SBE2 or SBE3 significantly reduced or eliminated CAR3-driven transgene expression in day 10.5 hearts (Fig. 2H,I,K,L,N,O), mutation of only SBE3, but not SBE1 or SBE2, reduced expression of this reporter in the cardiac crescent of day 7.5 embryos (Fig. 2G,J,M). Indeed, mutation of SBE1 and SBE2 potentiated transgene expression in the cardiac crescent of day 7.5 embryos (Fig. 2G,J). In summary, all three SBEs present in the CAR3 BMPRE seem to be required for both BMP induction and high level expression of a CAR3-driven transgene in the day 10.5 heart; however, SBE1 and SBE2 are dispensable for both CAR3-driven transgene expression in the cardiac crescent (at day 7.5) and BMP responsiveness of a reporter driven by the isolated CAR3 BMPRE.

Factors binding to sequences in the BMPRE are present in extracts made from the embryonic chick heart

To identify transcription factors that interact with the 200 bp CAR3 BMPRE (shown in Fig. 3C), we employed an electrophoretic mobility shift assay (EMSA) to determine whether factors in whole cell extracts made from various embryonic chick tissues interact with the BMPRE. As shown in Fig. 3A,B, endogenous Nkx2.5 is expressed in both stage 6-8 anterior lateral plate (ALP) tissue and in hearts of day 3 (HH stage 24) chick embryos, and is not expressed in posterior primitive streak (PPS) tissue from stage 6-8 chick embryos (Fig. 3A,B). We systematically assayed the ability of components in extracts derived from these dissected tissues (outlined in Fig. 3A,B) to bind to double stranded 30-40 bp oligomers representing consecutive overlapping portions of the 200 bp BMPRE (diagrammed by numbered lines in Fig. 3C). As shown in Fig. 3D, we were able to detect distinct DNA binding activities interacting with six different regions within the BMPRE. Five of these binding activities (A1, C, C′, D, E) were found at approximately equal levels in extracts made from either stage 6-8 ALP or PPS (Fig. 3D, lanes 1, 2, 9, 10, 13, 14, 15, 16, 19 and 20). By contrast, activity A2, although present to some degree in ALP extracts, was relatively enriched in PPS extracts (Fig. 3, lanes 1 and 2). Conversely, activity B was relatively enriched in ALP extracts (Fig. 3D, lanes 5, 6, 7, and 8). Whereas complexes B, C, C′, D and E were all observed in extracts made from 3-day-old hearts (Fig. 3D, lanes 21-30), a factor (A3) that bound to site A with a distinct mobility and binding specificity compared with A1 and A2 (data not shown) was additionally observed in 3-day-old heart extracts (Fig. 3D, compare lane 21 with lanes 1 and 2).

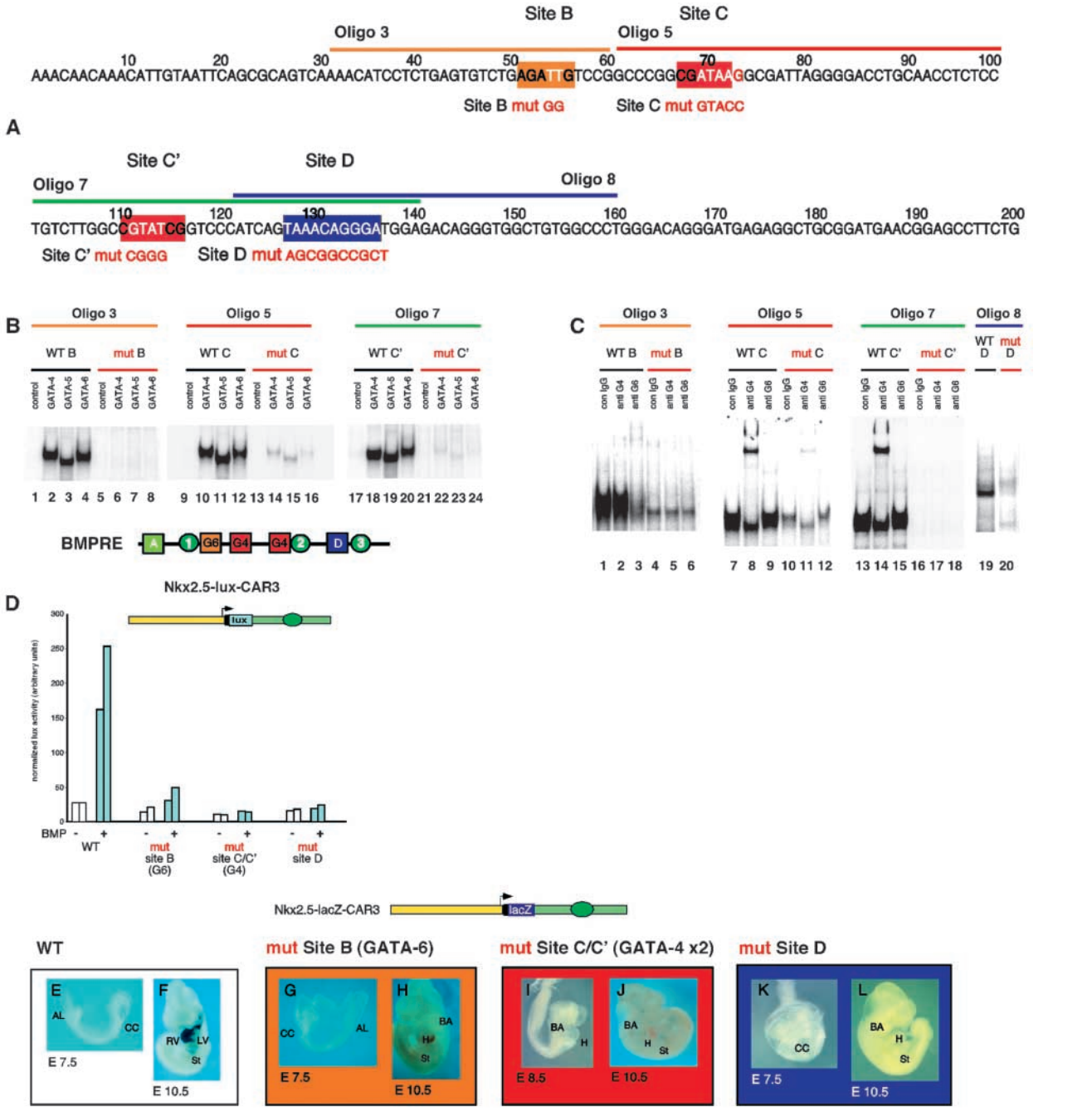

Fig. 4. Characterization of BMPRE gel shift binding sites. (A) 200 bp BMPRE is shown with numbered horizontal lines of selected double-stranded oligonucleotides shifted by embryonic extracts (see Fig. 3 and text). Likely binding sites for embryonic gel shifts are boxed (orange for Site B, red for C and C′, blue for Site D. Nucleotide changes made in mutant oligos are shown in red under their cognate site and are also highlighted in either white or red on the 200 bp BMPRE sequence. (B) Gel shifts of putative GATA-binding sites. Oligomers 3, 5 and 7, containing Sites B, C and C′, respectively, were used in EMSA experiments with nuclear extracts prepared from control COS cells, or COS cells expressing GATA4, GATA5 and GATA6. All three oligomers bind all cardiac GATAs (lanes 2-4, site B; lanes 10-12, site C; lanes 18-20, site C′) and binding is greatly reduced or eliminated upon mutation of consensus GATA-binding sites within the oligomers (lanes 6-8, mutant site B; lanes 14-16, mutant site C; lanes 22-24, mutant site C′). (C) Gel shifts and antibody supershifts of either wild-type (WT) (lanes 1-3, 7-9, 13-15 and 19) or mutant (mut) (lanes 4-6, 10-12, 16-18 and 20) site B, C, C′ and D oligomers with day 3 heart whole cell extracts. Supershifts for sites B (lanes 1-6), C (lanes 7-12) and C′ (lanes 13-18) were performed with either control mouse Ig (lanes 1, 4, 7, 10, 13 and 16), anti-GATA4 (lanes 2, 5, 8, 11, 14 and 17) or anti-GATA-6 (lanes 3, 6, 9, 12, 15 and 18) rabbit polyclonal antibodies as indicated. (D) BMP responsiveness of either wild-type Nkx2.5-lux-CAR3 reporter or similar reporters bearing mutations in either sites B, C/C′ or D (as diagrammed in A). (E-L) Representative X-gal stained F0 transient transgenic mice embryos containing either wild type or mutant Nkx2.5-*lacZ*-CAR3 reporters. Embryonic stages are shown in the bottom left-hand corner. Results shown are representative of the following numbers of transgenic embryos: mut Site B, 11/11 embryos; mut site C/C′, 14/15 embryos (1/15 E8.5 embryos displayed residual heart staining); mut Site D, 7/7 embryos. Abbreviations are as in previous figures.

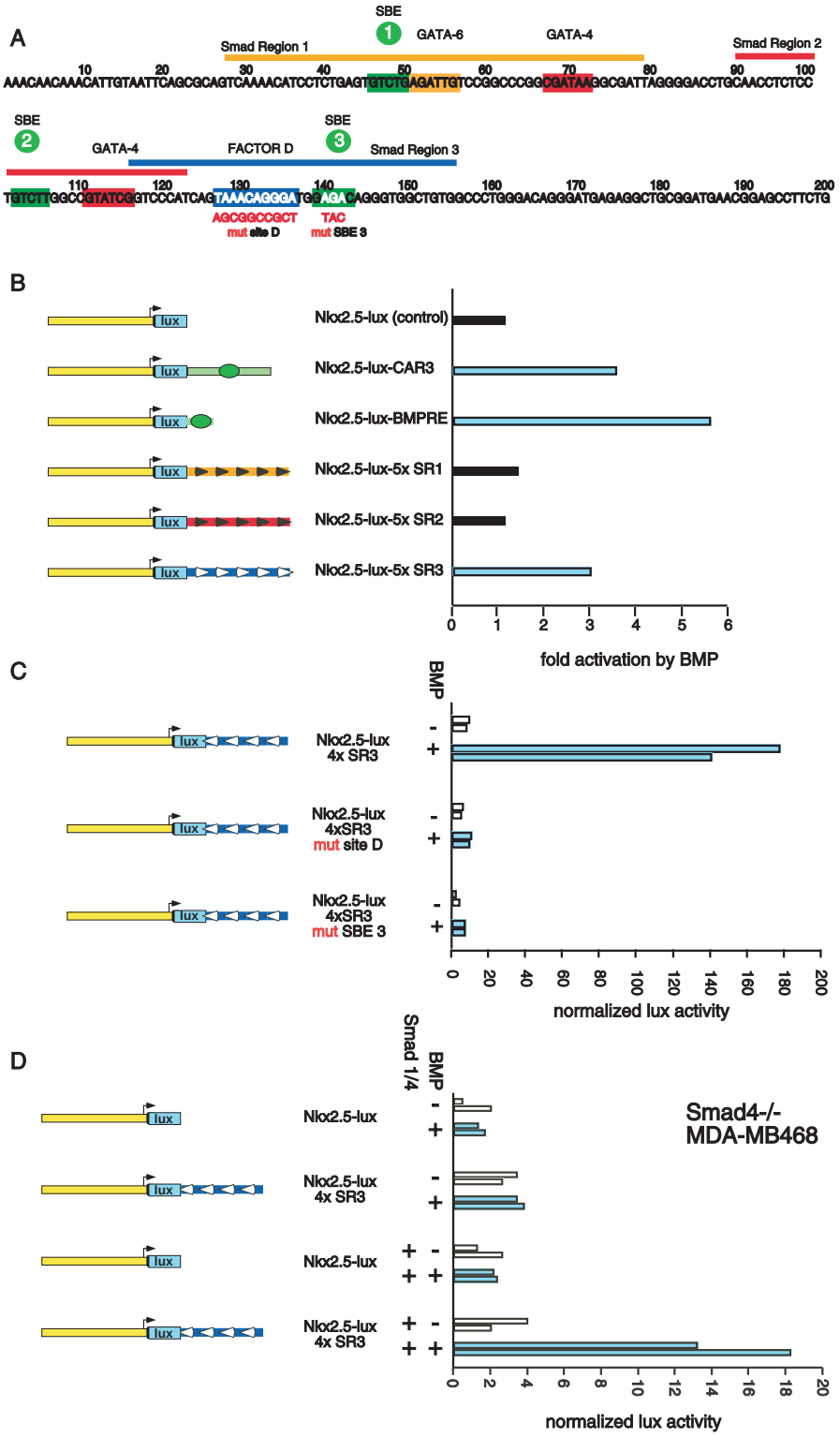

Fig. 5. SMAD-dependent BMP response is associated with site D activity. (A) BMPRE sequence is displayed. Colored horizontal lines show extent of double-stranded oligonucleotides used to create multimerized enhancers encompassing SBE1 plus GATA6 and GATA4/5 binding sites (SMAD region 1 or SR1, orange line), SBE2 plus GATA4-binding sites (SMAD region 2 or SR2, red line) and SBE3 plus binding site D (SMAD region 3 or SR3, blue line). Site D and SBE3 mutations are displayed below sequence of BMPRE in red, and corresponding altered nucleotides are displayed as white. (B) BMP response of Nkx2.5-lux driven by either the 2 kb CAR3, the 200 bp BMPRE, or 5×-multimerized repeats of SR1, 2 or 3. Reporters are shown on the left, with colored arrowheads representing each repeat and its orientation. Cognate BMP response is shown to the immediate right of the construct schematic, and is expressed as fold activation over basal activity. (C) BMP induction of SR3 requires both binding site D and SBE3. BMP response of Nkx2.5-lux driven by either 4×-multimerized repeats of SR3-WT, or multimers of SR3 bearing mutations in either binding site D or associated SMAD-binding element 3 (SBE3) (shown in A). (D) SMAD4 dependence of SR3-mediated BMP response. BMP4 response of Nkx2.5-lux-4xSR3 was assayed in the SMAD4-deficient MDA-MB468 cell line in the absence or presence of co-transfected SMAD1 and SMAD4 expression vehicles. Activation is seen only in the presence of both co-expressed SMAD proteins and BMP4 (bottom lanes).

BMPRE, we found that substitution of 10 nucleotide blocks through either nucleotides 1-30 or 160-200 of the BMPRE failed to consistently downregulate the BMP response in transfected P19 cells by greater than 50% (data not shown). By contrast, linker scan mutations in the central region of the BMPRE (extending from nucleotides 30 to 160) could strongly reduce and in some cases completely abolish this response (data not shown). As the binding sites for complexes B, C, C′ and D all lie within this critical region, we focused our attention on identifying these binding activities.

GATA factor interactions with sites B and C/C′ are necessary for BMP responsiveness of CAR3 and cardiac-specific transgene expression

To prioritize our analysis of these various BMPRE-binding activities, we focused on those binding activities that bind to regions of the BMPRE required for BMP responsiveness. By performing linker scan mutational analysis through the 200 bp

We noted that complex B interacts with oligomers (#3 and #4, Fig. 3C) containing a variant GATA-binding site (AGATTG) (Molkentin, 2000) (boxed in orange, Fig. 4A), while complexes C and C′ interact with oligomers [5 (C) and 7 (C′), Fig. 3C] containing sequences similar to the consensus GATA-binding site WGATAR (Molkentin, 2000) (boxed in red in Fig. 4A). Because GATA4, GATA5 and GATA6 are all expressed in lateral plate mesoderm

and embryonic cardiac tissue (Arceci et al., 1993; Heikenheimo et al., 1994; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997), we examined whether these GATA factors could interact with binding sites B, C or C′. As shown in Fig. 4B, nuclear extracts from COS cells programmed to express either GATA4, GATA5 or GATA6 specifically shifted oligomers 3 (site B), 5 (site C) and 7 (site C′) by EMSA (Fig. 4B, lanes 2-4, 10-12 and 18-20). These shifts were significantly diminished by specific mutation of the GATA consensus binding sites (Fig. 4A,B, lanes 6-8, 14-16 and 22-24).

To examine if endogenous GATA proteins were also capable of interacting with putative binding sites B, C or C′, we examined whether the gel shifts of oligonucleotides containing these sites observed with three day heart extracts could be supershifted with anti-GATA antibodies. Indeed we found that addition of anti-GATA6 antibody could specifically supershift complex B (Fig. 4C, lane 3) but not complexes C and C′ (Fig. 4C, lanes 9 and 15), while anti-GATA4 antisera could supershift approximately half of complexes C and C′ (Fig. 4C, lanes 8 and 14) but failed to interact with complex B (Fig. 4C, lane 2). Because GATA4, GATA5 and GATA6 can all bind to oligonucleotides containing site C or C′ (see Fig. 4B) we speculate that the remainder of complex C or C′ not supershifted by either anti-GATA4 or anti-GATA6 antisera may contain GATA5. Mutation of the putative GATA-binding sites within the oligonucleotides used for EMSA either abolished or significantly decreased the formation of complexes B, C and C′ with 3 day heart extracts (Fig. 4C, lanes 4-6, 10-12, 16-18). Together, these findings suggest that complex B contains GATA6, while complexes C and C′ contain GATA4 and GATA5.

Site D binds an activity common to ALP, PPS and day 3 heart extracts. We observed that a DNA-binding complex on site D was abrogated by a linker scan mutation at nucleotides 127-137 in the 200 bp BMPRE that strongly inhibited the BMP responsiveness of a BMPRE-driven reporter in P19 cells (Fig. 4A; data not shown). Mutation of these residues abrogated interaction of a protein present in both day 3 heart extracts and P19 cells with oligo #8 spanning residues 120 to 160 of the BMPRE (Fig. 4C, lanes 19 and 20; data not shown).

To ascertain the importance of either GATA-binding sites (B, C or C′) or site D for BMP responsiveness in vitro or cardiac gene expression in vivo, mutations that blocked protein-DNA complex formation in vitro (described above) were built into the 2 kb CAR3. Mutations that eliminate either the binding of GATA6 to site B, the binding of GATA4/GATA5 to both sites C and C′, or the binding of factor D to site D, each blocked BMP-mediated induction of a luciferase reporter driven by the *Nkx2.5* minimal promoter appended to CAR3 (Nkx2.5-lux-CAR3) (Fig. 4D). In addition, mutation of each of these binding sites significantly diminished *lacZ* expression of cognate Nkx2.5-*lacZ*-CAR3 constructs in transient transgenic mouse embryos (Fig. 4E-L). Thus, binding sites for GATA4, GATA5, GATA6 and site D are all necessary for BMP-mediated induction and cardiac-specific gene expression of CAR3-driven transgenes.

Site D and its associated SMAD binding site comprise a minimal BMP responsive module

Examination of the 200 bp BMPRE in CAR3 revealed that binding sites for SMAD4 MH1 were located adjacent to a combination of GATA6 and GATA4/GATA5-binding sites [located in SMAD region 1 (SR1)], a single GATA4/GATA5-binding site (located in SR2) or site D (located in SR3) (SR1, SR2 and SR3 are shown in Fig. 5A). As SMADs often bind to DNA adjacent to transcription factors whose activity they modulate (Attisano and Wrana, 2000; Whitman, 1998), we evaluated whether reiterated versions of the various SMAD regions would constitute a minimal BMP inducible regulatory element. We constructed Nkx2.5 promoter-luciferase reporters driven by either five copies of SR1 (5×SR1, containing SBE1 and the adjacent GATA6 and GATA4/GATA5-binding sites), five copies of SR2 (5×SR2, containing SBE2 and the adjacent GATA4/GATA5-binding site), or five copies of SR3 (5×SR3, containing SBE3 and the adjacent factor D binding site) (Fig. 5A,B). Although reiteration of either SBE1 with its associated GATA4/GATA5 and GATA6-binding sites (5×SR1), or SBE2 with its associated GATA4/GATA5-binding site (5×SR2) failed to constitute a BMP inducible regulatory element, reiteration of SBE3 with the adjacent factor D-binding site (5×SR3 or 4×SR3) resulted in a BMP inducible regulatory element (Fig. 5B,C).

SR3 contains adjacent binding sites for factor D and SMAD4. To evaluate the necessity of these binding sites in SR3 to mediate a BMP response, these sites were independently mutated in parallel Nkx2.5-lux reporter constructs. Mutation of either the factor D binding site or the adjacent SBE3 completely abolished the ability of an SR3-driven reporter to respond to BMP signals (Fig. 5C). In addition, BMP4 administration was unable to induce the activity of the SR3 reporter in MDA-MB-468 epithelial cells, which lack endogenous SMAD4 expression (de Winter et al., 1997), unless these cells were supplemented with exogenous SMAD1 and SMAD4 (Fig. 5D). Together, these results suggest that BMP signaling activates the CAR3 response element, at least in part, by regulating the transcriptional activity of the site D-binding factor, and that this modulation requires the presence of a BMP-activated SMAD complex on the adjacent SBE3 site.

Site D-binding factor is the zinc-finger transcription factor YY1

A detailed examination of nucleotides 115-155 of the BMPRE encompassed by the SR3 construct revealed the presence of two similar motifs of the sequence CCATC, present as inverted repeats in nucleotides 120-124 and 135-139. Comparison of this sequence to known transcription factor binding consensus sites revealed a similarity between this motif and the binding site for the Gli-Kruppel zinc-finger transcription factor YY1, CCATNT(A/T) (shown schematically in Fig. 6A). YY1 is a ubiquitously expressed, multifunctional transcription factor (Shi et al., 1991), and has been implicated in the positive or negative regulation of cardiac genes, including BNP (Bhalla et al., 2001) and cardiac myofibrillar genes (Chen and Schwartz, 1997; Latinkic et al., 2004; MacLellan et al., 1994; Sucharov et al., 2003). To evaluate if YY1 binds to either of the CCATC repeats, we compared the SR3-binding activities in extracts made from either day 3 chick heart extracts or P19 cells with that of purified YY1. Mutation of the most 5′ putative YY1 binding site (mutation A) in SR3 failed to significantly affect the interaction of the SR3 oligomer with either purified YY1

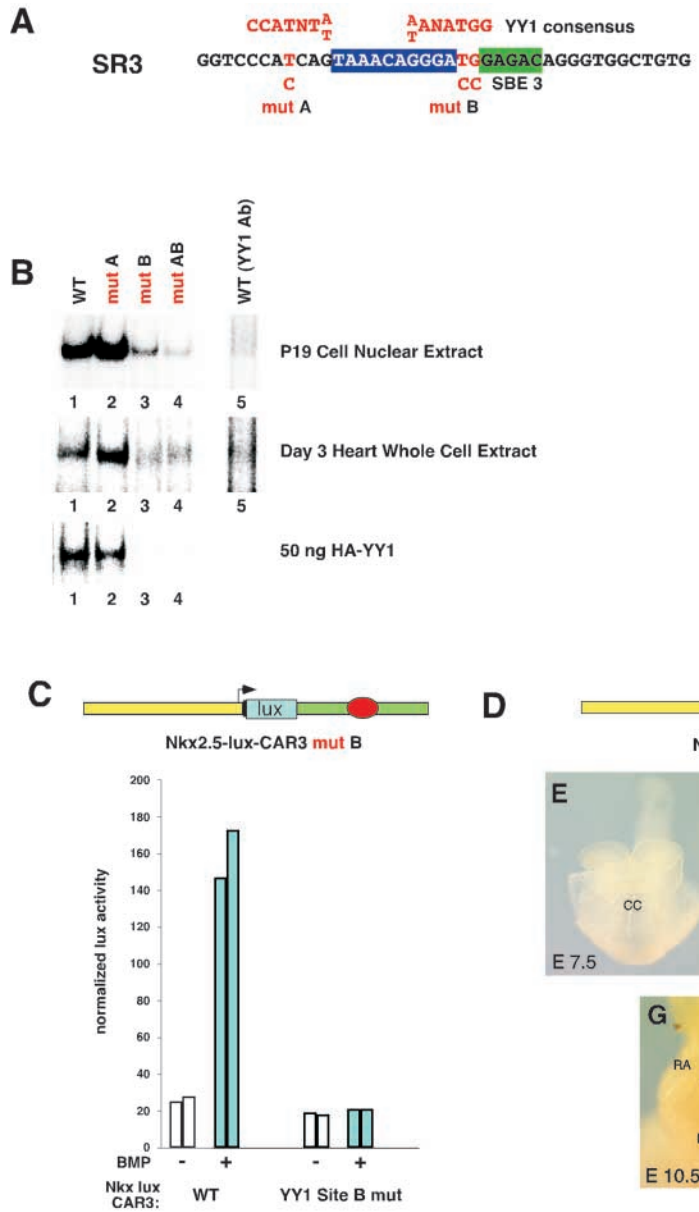

Fig. 6. BMP responsiveness of SR3/CAR3 and cardiac expression of a CAR3-driven transgene requires intact binding sites for transcription factor YY1. (A) Sequence of SR3 region encompassing site D (blue box) and SBE3 (green box). Sense and antisense YY1 consensus binding sites are shown above putative YY1-binding sites within SR3. Point mutations in putative YY1 binding sites A and B are shown in red below their cognate nucleotides. (B) YY1 binds to SR3. EMSA with oligos encoding either WT SR3 (lanes 1 and 5) or SR3 with mutations in putative YY1-binding site A (lane 2), site B (lane 3) or sites A+B (lane 4) with either P19 cell nuclear extract (top panel), whole cell extracts from day3 (HH Stage 24) chick hearts (middle panel) or purified HA-tagged YY1 protein (lower panel). Point mutation of the YY1 consensus binding site B in the SR3 oligomer significantly decreased or abolished DNA-binding activities in all samples (lane 3), as did mutations of both sites A and B (lane 4). Anti-YY1 polyclonal antibody added to the EMSA disrupted the interaction of factors in both P19 cells and day 3 chick hearts with the SR3 oligomer (lane 5). (C) Point mutation of the YY1 consensus site B in the context of an Nkx2.5-lux-CAR3 reporter (schematized at top) results in loss of BMP response in P19 cells. Results are shown in duplicate for wild-type Nkx2.5-lux-CAR3 (left) and Nkx2.5-lux-CAR3 mutB (right). (D-G) YY1-binding sites are required for CAR3-driven transgene expression in the heart. Representative X-gal staining patterns are shown for transgenic mice embryos containing Nkx2.5-*lacZ*-CAR3 mut B (shown in D). *lacZ* expression is abrogated at all stages assayed, indicating a requirement for YY1-binding sites in CAR3 to drive transgene expression in the heart (compare with the robust cardiac expression of the parental Nkx2.5-*lacZ*-CAR3 construct in Fig. 2D-F). Embryonic stages are shown in the lower left-hand corner. Results are representative of 3/3 E7.5 and 5/5 E10.5 transgenic embryos. Abbreviations are as in previous figures.

or the binding activities in either P19 cells or day 3 heart extracts (Fig. 6B, lane 2). By contrast, mutation of the 3′ most putative YY1-binding site (mutation B) in SR3 abrogated the interaction of the SR3 oligomer with purified YY1 and significantly decreased interaction of this oligomer with binding activities present in both embryonic chick heart or P19 cell extracts (Fig. 6B, compare lanes 1 and 3). In addition, the interaction of the SR3 oligomer with factors present in either embryonic chick heart extracts or P19 cells was extinguished by incubation with an anti-YY1 antisera (Fig. 6B, lane 5), supporting the notion that YY1 in such extracts is a component of the site D binding complex. Consistent with the gel shift analyses, we found that mutation of YY1 site B eliminated BMP induction of CAR3 driven reporters in P19 cells (Fig. 6C) and cardiac-specific expression of such reporters in transgenic mice at both days 7.5 and 10.5 (Fig. 6D,E-G). Because mutation of the YY1-binding site (mut B) did not alter interaction of the SR3 oligomer with the SMAD4 MH1 domain in vitro (data not shown), we think it is most likely that the loss of both BMP responsiveness and heart-specific transgene expression following mutation of the YY1-binding site (mut B) in CAR3-driven reporters reflects the loss of YY1 interaction with these sequences. Conversely, because mutation of SMAD-binding element 3 (SBE3), which lies adjacent to the YY1 binding site did not alter the interaction of YY1 with the SR3 oligomer in vitro (data not shown), it seems most likely that the loss of both BMP responsiveness and heart-specific transgene expression following mutation of SBE3 in CAR3-driven reporters reflects the loss of SMAD interaction with these sequences.

The amino terminus of YY1 interacts with the SMAD1/4 complex

Because juxtaposition of adjacent YY1 and SMAD-binding sites are necessary and sufficient to constitute a BMP response element, we investigated whether YY1 and SMADs 1/4

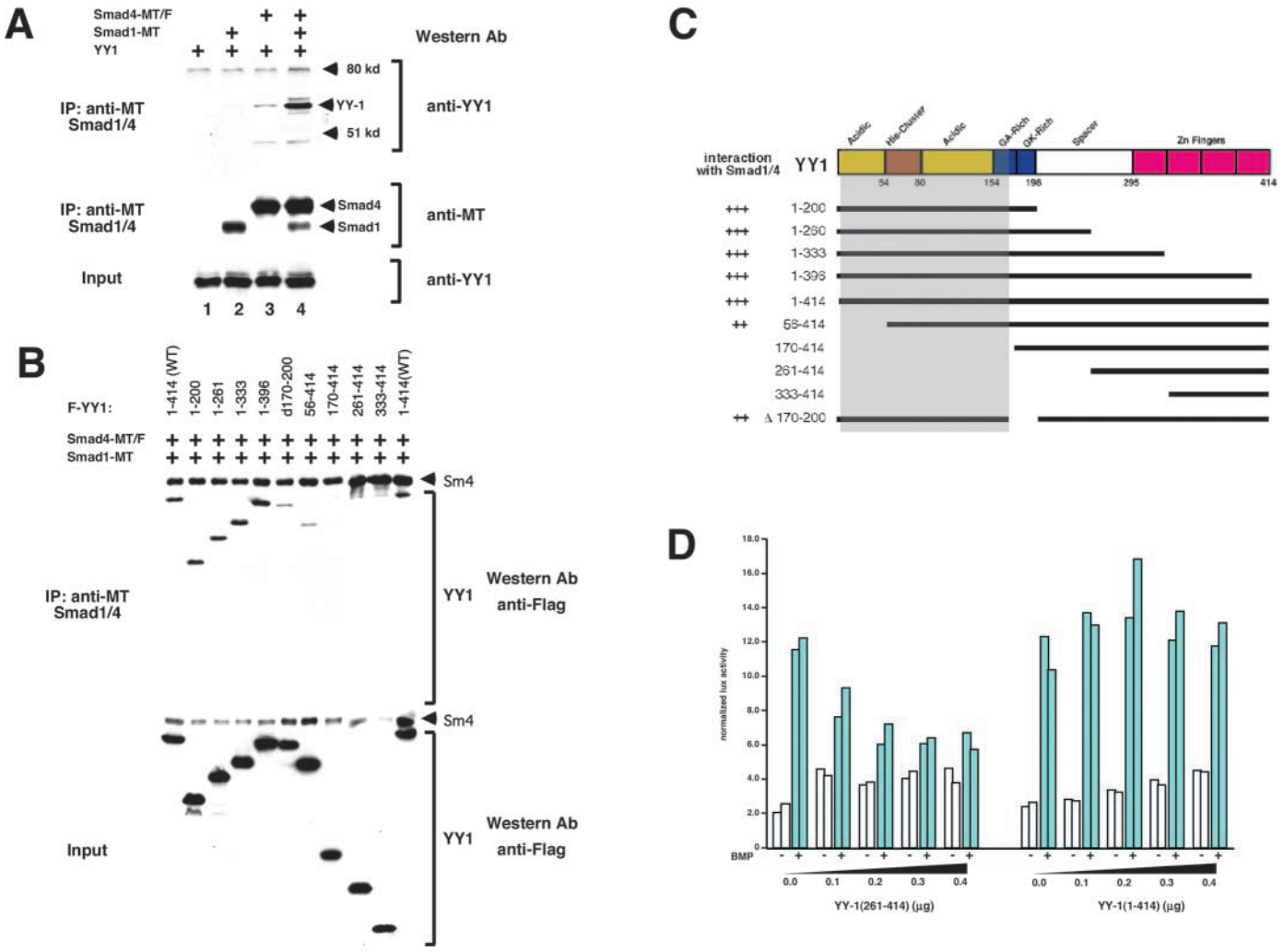

Fig. 7. BMP SMADs associate with the N terminus of YY1: (A) Co-immunoprecipitation of YY1 with the SMAD1/4 complex. Whole-cell extracts from either BMP-stimulated COS cells transfected with expression vehicles encoding either human YY1 (lanes 1-4), Myc-tagged (MT) SMAD1 (lanes 2 and 4) or myc/Flag-tagged (MT/F)-SMAD4 (lanes 3 and 4). SMAD-associated proteins were immunoprecipitated from cell extracts with an anti-myc monoclonal antibody, subjected to polyacrylamide gel eletrophoresis (PAGE) and detected following western blot with either anti-myc or anti-YY1 polyclonal antibodies. Levels of YY1 present in the input extract used for immunoprecipitation are shown in bottom panel. Bands corresponding to YY1, SMAD1 and SMAD4 are indicated by arrowheads on the right, as are molecular mobility markers. (B) Mapping the SMAD1/4 interaction domain of YY1 by co-immunoprecipitation. COS cells were transfected with expression vehicles encoding either wild-type (WT) Flag-tagged (F)-YY1(1-414) or various Flag-tagged deletion mutants of YY1, plus expression vehicles encoding MT/F-SMAD4 and MT-SMAD1, as indicated. SMAD-associated proteins were immunoprecipitated from cell extracts with an anti-Myc monoclonal antibody, subjected to polyacrylamide gel eletrophoresis (PAGE) and detected following western blot with anti-Flag monoclonal antibody (upper panel). Anti-Flag western blot of ~2.5% of input used in co-immunoprecipitation confirms substantial expression of all tagged deletion mutants of YY1 and SMAD4 in the various cell extracts (lower panel); in addition, equivalent levels of MT-SMAD1 expression was detected in the various cell extracts (data not shown). Deletion mutant used in each sample is shown above the lanes; band representing Flag/MT SMAD 4 (Sm4) is shown by arrowheads on the right, as are those of YY1 isoforms, bracketed on the right. Results shown are representative of three independent co-immunoprecipitation experiments. (C) The SMAD1/4 interaction domain of YY1 maps between residues 1-170. Schematic representation of YY1 structural regions is shown at top [adapted, with permission, from Thomas and Seto (Thomas and Seto, 1999)]; solid bars showing extent of various YY deletion mutants assayed in B. Gray box identifies SMAD1/4-interacting region of YY1 based upon amino acids shared by all YY1 deletion mutants that associate with SMAD1/4. Relative strength of SMAD1/4 association of various YY1 deletion mutants is indicated on extreme left by either +++ (strong association), ++ (weak but detectable association), no mark (no detectable association). (D) Co-expression of YY1(261-414), which lacks the SMAD1/4 interaction domain, inhibits BMP induction of a CAR3-driven reporter. P19 cells were transfected with Nkx2.5-lux-CAR3, Tk-renilla-luciferase (for normalization) and expression vehicles encoding either SMADs1 and 4 and p21E1b (to inhibit apoptosis due to loss of YY1 activity), plus increasing amounts (0-100 ng/well) of expression vectors encoding either wild-type YY1(1-414) (right lanes) or N-terminally truncated YY1(261-414) (left lanes). YY1(261-414) is able to bind to Site D in SR3 (data not shown) but is incapable of SMAD1/4 interaction (see above), and blocks the ability of BMP signals to induce the expression of a CAR3-driven reporter.

associate with one another. Cell extracts from COS cells expressing epitope-tagged SMAD1, SMAD4 and YY1 were harvested for co-immunoprecipitation experiments following transient (30 minutes) BMP4 stimulation. We observed that approximately 2% of transfected YY1 was associated with Myc-tagged SMAD1/SMAD4 complexes under these conditions (Fig. 7A, lane 4, top panel). By employing a series of truncation mutants of Flag tagged-YY1 in the co-immunoprecipitation assay, we mapped the SMAD interaction domain of this protein to lie between amino acids 1 to 170 near the N terminus of YY1 (Fig. 7B, summarized in Fig. 7C). By contrast, the DNA-binding domain of YY1 maps to the zinc fingers located between amino acids 295 to 414 in the C-terminal region of the protein (Fig. 7C).

A mutant form of YY1 lacking the SMAD interaction domain blocks BMP induction of a CAR3 reporter

Because an N-terminal truncation mutant of YY1 containing amino acids 261-414 retains an intact zinc-finger domain, can bind to YY1-binding sites [including those in the CAR3 BMPRE (data not shown)] but lacks the SMAD1/4 interaction domain (Fig. 7B,C), we employed this mutant version of YY1 to investigate whether a YY1/SMAD interaction was necessary for BMP signals to modulate expression of a CAR3-driven reporter. P19 cells were co-transfected with the Nkx2.5-lux-CAR3 reporter plus expression vehicles encoding SMAD1/SMAD4 and increasing amounts of expression vehicles encoding either wild-type YY1(1-414) or YY1(261-414), and assayed for luciferase expression following 18 hours of treatment with BMP4. In addition, we included an expression vehicle encoding the p21E1b protein to block apoptosis (Kranenburg et al., 1995), which could potentially result following loss of YY1 activity (Y. Shi, personal communication). Although co-expression of increasing amounts of wild-type YY1(1-414) did not significantly affect the ability of BMP4 to induce the expression of this CAR3-driven reporter construct (Fig. 7D, right lanes), co-transfection with increasing amounts of YY1(261-414) markedly attenuated induction of this reporter by BMP signals (Fig. 7D, left lanes). Together, these finding suggest that YY1 recruitment of SMAD1/4, which is mediated by interaction with the N terminus of YY1, is necessary to coordinate a BMP-mediated induction of the chick *Nkx2.5* CAR3 enhancer.

Discussion

Identification of three cardiac activating regions flanking the chick *Nkx2.5* gene that combinatorially drive transgene expression in the developing heart

In this work, we have identified three cardiac-activating regions (CAR1, CAR2 and CAR3), which surround the chick *Nkx2.5* gene, that in combination can drive transgene expression in a pattern which recapitulates many aspects of endogenous Nkx2.5 expression. One of these regulatory sequences (CAR3), located 3′ to Nkx2.5-coding sequences, was able to drive robust transgene expression in both the primary and secondary heart fields, maintain expression in the outflow tract and right ventricle, and direct reporter gene expression in a BMP-responsive fashion. Chick *Nkx2.5* CAR2 contains a highly conserved region that is present in both mouse and human *Nkx2.5* loci (Searcy et al., 1998), and like its murine counterpart [termed AR2 (Schwartz and Olson, 1999)] is capable of driving transgene expression in both the cardiac crescent and in the outflow tract. Although the more 5′ chick *Nkx2.5* CAR1 does not drive transgene expression in the primary heart field, when linked to CAR2 and CAR3 it extends the maintenance of transgene expression in the maturing heart to include the left ventricle. Notably, CAR1, CAR2 and CAR3 alone or in combination fail to drive transgene expression in the atria, where endogenous Nkx2.5 is also expressed.

Although these enhancers drive accurate cardiac expression of β-galactosidase expression in transgenic mouse assays, only CAR2 is well conserved between mammalian and avian species (Liberatore et al., 2002; Lien et al., 2002). The absence of a similarly conserved CAR3-like element in genomic regions flanking the mouse or human *Nkx2.5* genes suggests that different species employ a variety of regulatory sequences to drive expression of *Nkx2.5* homologs in the heart. However, in all cases examined to date, GATA- and SMAD-binding sites play an essential role in the activity of the regulatory sequences that drive Nkx2.5 expression in the forming heart. Indeed, Bob Schwartz and colleagues have recently characterized a regulatory element (termed G-S) located ~6 kb upstream of the mouse *Nkx2.5* gene, which contains 27 consensus and five interspersed non-consensus GATA-binding sites followed by nine consensus SMAD-binding sites, that responds to BMP signals in transfected P19 cells and is capable of driving transgene expression in both the cardiac crescent and lateral plate mesoderm (Brown et al., 2003). Thus, it is possible that BMP-mediated induction of Nkx2.5 expression relies upon regulatory sequences that either contain a minimal number of GATA- and SMAD-binding sites positioned adjacent to another SMAD-regulated transcription factor, such as YY1 (as in chick *Nkx2.5* CAR3), or relies upon a highly reiterated number of GATA-and SMAD-binding sites (as in the murine *Nkx2.5* G-S sequence), which can respond to BMP signals in the absence of other associated SMAD-regulated transcription factors.

BMP signals modulate Nkx2.5 expression by several synergistic pathways

Detailed mutagenesis of a 200 bp BMPRE within CAR3 revealed that binding sites for GATA4, GATA5, GATA6, SMAD1/4 and YY1 are all necessary for both BMP-mediated activation and cardiac-specific expression of reporter constructs driven by this regulatory region. Our findings indicate that BMP signaling engages several pathways to induce the expression of the *Nkx2.5* gene in cardiac progenitor tissue (Fig. 8). BMP signals are known to be transduced via BMP receptor-activated SMAD1, SMAD5 or SMAD8, which bind to DNA in complex with SMAD4. SMAD proteins have been documented to bind to consensus SBEs in both distal and proximal regulatory elements in the murine *Nkx2.5* gene [i.e. AR1 and AR2 (Liberatore et al., 2002; Lien et al., 2002)], and in the chick *Nkx2.5 CAR3* enhancer (this work). Interestingly, while SBE1, SBE2 and SBE3 in CAR3 are all required to maintain expression of transgenes in day 10.5 mouse hearts, the initiation of CAR3-driven transgene expression in the cardiac crescent of day 7.5 mouse embryos requires only SBE3, suggesting that the transcription factors required to

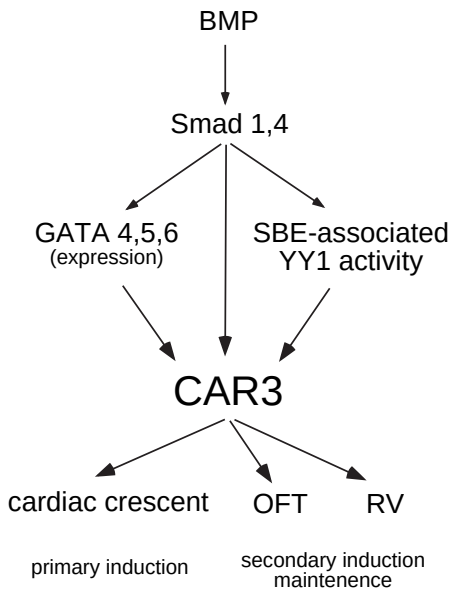

Fig. 8. CAR3-mediated cardiac expression requires the collaboration of multiple factors. Diagram outlines three arms of BMP signaling that synergistically activate CAR3-driven cardiac expression: SMAD nuclear localization and direct interaction with SBE sites located in CAR3; induction of cardiac GATA proteins; and SMAD-mediated modulation of the activity of the ubiquitously expressed YY1 repressor/activator.

induce versus maintain CAR3-driven *Nkx2.5* gene expression may be distinct.

BMP signals are also necessary for GATA4, GATA5 and GATA6 gene expression in cardiac precursor cells (Andree et al., 1998; Schlange et al., 2000; Schultheiss and Lassar, 1997) and are sufficient to induce expression of GATA4, GATA5 and GATA6 in explants of chick paraxial mesoderm (R. Reshef, H. Kempf and A.L., unpublished). Interaction of these GATA factors with both distal and proximal regulatory elements in the mouse *Nkx2.5* enhancer and with the chicken CAR3 enhancer are also essential for cardiac-specific activity of these regulatory elements. It is interesting that all of the well-characterized murine and chick *Nkx2.5* sequences which drive expression in the primary heart field contain essential GATA-binding sites (Brown et al., 2003; Lien et al., 1999; Searcy et al., 1998), consistent with the finding in zebrafish that GATA5/Faust is essential for Nkx2.5 expression in this species (Reiter et al., 1999) and underline the importance of GATA proteins in either the induction or maintenance of *Nkx2.5* gene expression.

Finally, we have documented that SMAD1/SMAD4 interaction with YY1 modulates the activity of this transcription factor when bound to an adjacent SMAD-binding site in the chick *Nkx2.5* CAR3 enhancer and have found that this interaction is essential for the BMP-responsiveness of this regulatory element. Thus, BMP signals modulate the activity of the chick *Nkx2.5* CAR3 enhancer by: (1) enabling SMAD complexes to directly bind to this regulatory element; (2) inducing the expression of GATA4, GATA5 and GATA6, which also bind to this regulatory region; and (3) modulating the activity of YY1 when bound to an adjacent SMAD-binding site in CAR3 (see Fig. 8).

BMP-activated SMADs modulate YY1 transcriptional activity

A SMAD1/SMAD4-binding site that lies immediately adjacent to the YY1-binding site in CAR3 was found to be crucial for both BMP-mediated activation of this regulatory sequence and expression of CAR3-driven transgenes in the early cardiac crescent. Reiteration of this YY1 binding site and the adjacent SMAD1/SMAD4-binding site was sufficient to constitute a BMP-responsive element. We think it is most likely that BMP signals are modulating the transcriptional activity of YY1 bound to CAR3 as opposed to modulating YY1 expression or DNA interaction as we have not observed a change in YY1-binding affinity for the CAR3 YY1-binding site in gel shift experiments using nuclear extracts from either SMAD overexpressing or BMP-stimulated P19 cells, nor do these conditions result in a change in YY1 protein expression levels, as assayed by western blot (data not shown). However, we observed by co-immunoprecipitation assay that the N-terminal region of YY1 interacts with the SMAD1/SMAD4 complex. Furthermore, we found that an N-terminal truncation mutant of YY1 lacking this SMAD-interacting domain but capable of binding to DNA acts in a dominant-negative fashion to inhibit BMP-mediated induction of the chick *Nkx2.5* CAR3 enhancer. Although we mapped the SMAD1/SMAD4 interaction domain of YY1 to the N terminus, others have documented interaction of the C-terminal zinc-finger domain of YY1 with SMAD4 (Kurisaki et al., 2003), which was not evident in our analysis. At present, we cannot account for this difference; however, this discrepancy may reflect the different experimental assays employed to map the SMAD interaction domains of YY1 in these two studies, co-immunoprecipitation (present study) versus GST-pull down (Kurisaki et al., 2003). Interestingly, Kurisaki and colleagues have implicated YY1 as a repressor of SMAD-mediated TGFβ responses in fibroblast cell lines (Kurisaki et al., 2003), and presented evidence that YY1 overexpression correspondingly attenuated the association of activated SMAD complexes with multiply reiterated SBEs. These results are not inconsistent with our finding that YY1 and SMADs synergistically activate the *Nkx2.5* CAR3 enhancer, as it is possible that YY1 recruits SMAD complexes to regions of the genome containing both YY1 and adjacent SMAD-binding sites at the expense of other SMAD targets that lack adjacent YY1-binding sites.

How might the interaction of SMADs with YY1 modulate the activity of this transcription factor when bound to CAR3? Because YY1 can function as either a transcriptional activator or repressor (Shi et al., 1991; Thomas and Seto, 1999), SMAD association with YY1 may serve to recruit co-activators that modulate the activity of this transcription factor to become an efficient transcriptional activator. Indeed, recruitment of co-activators such as p300 by TGFβ activated SMADs is a well-characterized mechanism for SMAD target gene activation (Attisano and Wrana, 2000; Whitman, 1998). Similarly, known interacting partners of YY1 also include several members of the histone deacetylase family (Galvin and Shi, 1997; Thomas and Seto, 1999; Yao et al., 2001) as well as a histone H4 methylase (Rezai-Zadeh et al., 2003), which have been implicated in either transcriptional repression or activation of YY1 regulated target genes, respectively. It will be interesting to determine if SMAD association with YY1 alters the interaction of this transcription factor with either of these

families of histone modifying enzymes, and to what extent chromatin modification is responsible for appropriate regulation of *Nkx2.5*.

SMAD-mediated modulation of YY1 activity adds an interesting new facet to the repertoire of functions of YY1 during heart development, which also includes direct recruitment of transcriptional co-activators to promote the expression of cardiac B-type natriuretic peptide (Bhalla et al., 2001), inhibition of the expression of the cardiac α-actin gene (Chen and Schwartz, 1997), and both activation and inhibition of the expression of the cardiac-specific *Mlc2* gene (Latinkic et al., 2004). Clearly, the context within which YY1 functions is of great importance, and it is likely that transcription factors such as GATA and SMAD proteins, when bound to neighboring cognate binding sites, modulate either the association of co-factors with adjacently bound YY1 or the activity of such co-factors. In addition to the GATA, YY1- and SMAD-binding sites, linker scanning mutational analysis of the chick *Nkx2.5* CAR3 BMPRE has revealed other sites yet to be characterized that also have a significant impact on the BMP response of this regulatory element (K.-H.L. and A.B.L., unpublished). A complete understanding of complex enhancers such as *Nkx2.5* CAR3 will require not only the identification of the transcription factors that regulate their expression but also elucidation of the transcriptional co-factors that are recruited to such regulatory elements in a combinatorial fashion.

We thank Tucker Collins, Gary Felsenfeld, Mona Nemer, Stuart Orkin, Ed Seto, Tom Schultheiss, Yang Shi, Guangchao Sui, Sergei Tevosian and Malcolm Whitman for kindly supplying us with plasmids; Genetics Institute/Wyeth Pharmaceuticals for supplying us with recombinant human BMP2; and Lena Du and Arlene Sharpe at the Brigham and Women's Hospital transgenic core facility for superb assistance with production of transgenic mice. Particular thanks to Yang Shi and Guangchao Sui for both purified YY1 and for their very helpful suggestions; and to Malcolm Whitman for his excellent advice and generous contribution of salary support for K.-H.L. This work was supported by grants to A.B.L. (RO1-HL58862) and K.-H.L. (K08HL003371) from the National Heart, Lung and Blood Institute of the NIH; and to Malcolm Whitman (RO1-HD-29468).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/19/4709/DC1

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