Genomes & Developmental Control

Prediction and characterisation of a highly conserved, remote and cAMP responsive enhancer that regulates \(Msx1\) gene expression in cardiac neural crest and outflow tract

Kerry Ann Miller, Scott Davidson, Angela Liaros, John Barrow, Marissa Lear, Danielle Heine, Stefan Hoppler, Alasdair MacKenzie *

School of Medical Sciences, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, UK

Received for publication 5 July 2007; revised 25 January 2008; accepted 9 February 2008

Available online 21 February 2008

Abstract

Double knockouts of the \(Msx1\) and \(Msx2\) genes in the mouse result in severe cardiac outflow tract malformations similar to those frequently found in newborn infants. Despite the known role of the \(Msx\) genes in cardiac formation little is known of the regulatory systems (ligand receptor, signal transduction and protein–DNA interactions) that regulate the tissue-specific expression of the \(Msx\) genes in mammals during the formation of the outflow tract. In the present study we have used a combination of multi-species comparative genomics, mouse transgenic analysis and in-situ hybridisation to predict and validate the existence of a remote ultra-conserved enhancer that supports the expression of the \(Msx1\) gene in migrating mouse cardiac neural crest and the outflow tract primordia. Furthermore, culturing of embryonic explants derived from transgenic lines with agonists of the PKC and PKA signal transduction systems demonstrates that this remote enhancer is influenced by PKA but not PKC dependent gene regulatory systems. These studies demonstrate the efficacy of combining comparative genomics and transgenic analyses and provide a platform for the study of the possible roles of \(Msx\) gene mis-regulation in the aetiology of congenital heart malformation.

© 2008 Elsevier Inc. All rights reserved.

Keywords: \(Msx1\) gene; Comparative genomics; Transfac; Enhancer element; Transgenic mouse; Embryo; Cardiac neural crest; Outflow tract; Transcription; PKA

Introduction

Proper morphogenesis of the outflow region relies on the ventral migration of a population of cells called the cardiac neural crest (cNC) (Jiang et al., 2000). Ablation of cNC cells derived from embryonic hindbrain rhombomeres 6, 7, and 8 in chick embryos resulted in a series of outflow tract defects that are frequently found in newborn human infants (Waldo et al., 1999, 2005). These cNC cells contribute to the formation of the endocardial cushions of the outflow tract that subsequently forms the conotruncal septum that, in turn, divides the aortic and pulmonary channels and contributes to the heart valves and the upper portions of the intraventricular septum (Kirby and Waldo, 1995). A class of homeobox genes called the muscle segment homeobox (\(Msx\)) genes are expressed in migrating cNC (Chan-Thomas et al., 1993; Houzelstein et al., 1997) and have recently been shown to be critical in normal morphogenesis of the outflow tract as deletions of the \(Msx1\) and \(Msx2\) genes cause extensive outflow tract defects that reflect those frequently seen in newborn human infants (Ishii et al., 2005; Lallemand et al., 2005; Ogi et al., 2005). Although these genes have been shown to be critical to formation of the outflow tract virtually nothing is known of the regulatory systems that coordinate and support the tissue-specific expression of these genes in cardiac neural crest or outflow tract. The reasons for this lack of knowledge stems from a previous inability to accurately predict the location, functional linkage and tissue-specific characteristics of key regulatory sequences that may possibly be located at some distance from the genes they regulate.

The current study describes the use of multi-species comparative genomics and Transfac bioinformatics to predict the location and tissue-specific characteristics of a novel enhancer. The status of this enhancer as a cNC specific enhancer of the
Msx1 gene was subsequently validated using a combination of transgenic embryo studies and in-situ hybridisation. In addition to predicting and validating many of its characteristics we also demonstrate, using transgenic embryonic explant culture, that this enhancer is responsive to PKA but not PKC signalling strongly suggesting the role of a PKA mediated signal transduction pathway in the regulation of Msx1 in cNC cells.

The prediction and validation of the tissue-specific properties of this highly conserved cNC specific enhancer demonstrates the power of using predictive multi-species comparative genomics in facilitating an understanding of the regulatory systems controlling the expression of clinically important genes. Furthermore, the discovery of this ultra-conserved functional enhancer element will serve as a platform for understanding the possible effects of gene mis-regulation in the production of cardiac outflow tract defects and lends greater weight to the assertion that, in addition to mutations of coding regions, regulatory regions should be examined when attempting to determine the roles of key genes in the production of developmental malformations and disease.

Methods

Bioinformatic analysis

Comparative genomic analysis of non-coding DNA surrounding the Msx1 gene was carried out using the ECR browser (http://ecrbrowser.dcode.org) (Ovcharenko et al., 2004). Verification of transcription factor binding matrices was carried out using TRANSFAC professional (BIOBASE Biological Databases) (Matys et al., 2003, 2006).

Plasmid constructs

p1230/KE — Primers KE.for; TAT GTT TAG CCC ACC CTG GA and KE. rev; TGA GCC TGG CCT ATC TGA CT were used to amplify the Human KE element from human placental DNA (Sigma) using a high fidelity polymerase (Expand HiFi system, Roche) and annealing temperature of 57 °C. The PCR product was digested with enzymes EcoRI and SpeI, and ligated into compatible ends of plasmid pGEM-7zf (+) (Promega) to produce pGEMKE. The KE insert from pGEMKE was removed by digestion with SpeI and then with KpnI. This fragment was then ligated into the HindIII (made blunt end using Klenow enzyme) and then with KpnI restriction sites of the pBGZ40–1230 LacZ reporter plasmid (Yee and Rigby, 1993) to form p1230/KE. p1230/KE was linearised and released from the plasmid backbone using KpnI and NotI prior to pronuclear injection.

Pronuclear injection

Pronuclear microinjection of 1-cell mouse embryos was performed as previously described (Nagy et al., 2003). Briefly, linearised p1230/KE DNA at a concentration of 2 ng/μl was injected into the pronuclei of 1 cell embryos derived from superovulated and mated (CBA × c57BL/6)F1 females. Surviving eggs were transferred into the oviducts of CD1 pseudo pregnant mice.

Detection of transgene activity

After dissection from extra-embryonic tissue in room temperature PBS, embryos were washed briefly in standard wash (2 mM MgCl2, 0.1% sodium deoxycholate, 0.02% Nonidet P-40 and 0.05% BSA in 0.1 M phosphate buffer (pH 7.3) for 3 × 20 min at room temperature. Embryos were transferred to X-gal stain (0.085% β-galactosidase, 5 mM K3Fe (CN)6, 5 mM K4Fe (CN)6, and 0.1% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal); Melford Laboratories) and incubated at 37 °C for 2 h to overnight. After staining, embryos were transferred to 4% PFA at 4 °C overnight and stored in 100% methanol at −20 °C.

In-situ hybridisation

Plasmid pGEM7/Msx1 was linearised with NotI for production of antisense probe. Digoxigenin (DIG) labelled antisense probes were transcribed using components of the T7 RNA polymerase Maxscript In Vitro Transcription Kit (Ambion) as described in the manufacturer's instructions with the following modifications. DIG labelled antisense RNA probes were transcribed in the following reaction; 1× transcription buffer, 0.01 M DTT, 2 mM rNTP mix (0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.32 mM UTP and 0.18 mM dig-UTP, (Roche)), 5 μg linearised template plasmid DNA, 40 units RNAase inhibitor, RNAsin (Roche) and 90 units of the T7 RNA polymerase in nuclelease-free water to a final volume of 50 μl. Whole-mount in-situ hybridisation with fresh embryos was carried out as previously described (Lettice et al., 2002).

Immunohistochemistry

Embryonic tissue was fixed in 4%PFA for 1 h and left overnight in 20% OCT medium. Embryos were then orientated within pure OCT medium and frozen. Sections were cut at −30 °C using in a Hacker Bright Clinicut cryostat and sections were mounted on Polysine-coated slides (VWR). Sections were washed in Tris Buffered Saline (TBS) for 5 min then treated with 1% sodium dodecyl sulfate (SDS) solution in TBS for 5 min to facilitate antigen recovery. Slides were then washed four times for 5 min in TBS with gently rocking. The slides were then transferred to a humidified chamber and samples were pre-incubated with TBST (TBS plus 0.1% TritonX-100 at pH 7.5) containing 10% Fetal Calf Serum (FCS) for 10 min, at room temperature, to suppress non-specific binding of the antibody. The samples were washed in TBST 3 times for 5 min with gentle rocking. Consecutive sections were separately incubated with primary antibodies Anti-Msx1/2 (Hybridioma Bank) and 40-la (anti-β-galactosidase; Hybridioma Bank) at a dilution of 1:20 in TBST/10% FCS. After overnight incubation in a humidified chamber, unbound antibody was removed from samples by washing 3 × 5 min in TBST with gentle rocking. All samples, including control, were then incubated in the dark with secondary antibody (1:200, Alexa Fluor Rabbit anti-mouse IgG 594; Molecular Probes/Invitrogen) for 1 h at room temperature. The slides were then washed in TBST 3 × 5 min with gentle shaking. Slides were then mounted in VECTASHIELD® mounting medium with DAPI (Vector labs) and visualised immediately with an Axioplan microscope (Zeiss) with HBO100 Fluorescence lamp and GFP filters. Images were analysed using Axiovision Viewer 3.0 imaging software.

Vibratome sectioning

For vibratome sectioning embryos were fixed in 4% paraformaldehyde (PFA)/PBS from 1 h to overnight at 4 °C. Embryos were equilibrated through 4% and 20% sucrose/PBS solutions at 4 °C for several hours or until the embryos had sunk. Embryos were then transferred to BSA/gelatin mix (0.5% gelatin, 1% BSA and 5% sucrose in PBS) overnight or longer. Prior to embedding, embryos were removed from BSA/gelatin and excess mix removed by blotting. Embryos were fixed in 25% glutaraldehyde for no longer than 1 min and embedded in BSA/gelatin containing 0.25% glutaraldehyde. Blocks containing embryos were then prepared for sectioning by gluing the block to the cutting dish using cyanoacrylate based adhesives. Sections of 50 μm were taken at speed 2–10, amplitude 8 on a Lancer Vibratome series 1000. Sections were floated onto a SuperFrost microscope slide (VWR) and mounted in aqueous mountant (7% gelatin, 50% glycerol and 0.1% phenol in water).

Transgenic explant agonist studies

Embryonic day 10.5 (E10.5) transgenic embryos were recovered and divided in half transversely at a level posterior to the heart primordium in ice-cold PBS. Transgenic anterior halves were then divided sagittally using a sterile
scalpel. Transgenic explants were identified by 30 min X-gal staining of their posterior halves. AG-1X2-formate beads (a gift from Cheryl Tickle) were soaked for 1 h in either the adenylyle cyclase agonist forskolin (100 μM) or the PKC agonist phorbol dibutyrate (PDBu; 1 μg/ml) in dimethyl sulfoxide (DMSO). These beads were then implanted within the otic vesicles of transgenic explants. Head explants were cultured on Millicell Culture Plate Inserts (Millipore) with D-MEM medium supplemented with 2 mM GlutaMAX (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and 10% Fetal Bovine Serum (FBS; Biosera) for 24 h at 37 °C/5% CO2. Alternatively, either forskolin or PDBu were added to the medium at concentrations of 10 μM and 100 ng/ml respectively. In each case similar quantities of DMSO without the addition of agonists were added to control halves of the same head. Following incubation for 24 h agonist effects were assessed by X-gal staining for 6 h. X-gal staining was stopped by multiple washes in PBS and 1 h fixation in 4% paraformaldehyde (PFA)/PBS at 4 °C.

Results

Comparative genomic analysis reveals a highly conserved non-coding sequence preserved in-cis with Msx1 for 450 million years.

In order to determine the location and identity of a possible cNC specific MSX1 enhancer we used the ECR browser to undertake a comparative genomic analysis of the MSX1 flanking regions covering 100 kb in both a 5′ and a 3′ direction with reference to the human MSX1 locus. We compared the flanking sequences of the MSX1 locus to 8 other vertebrate species spanning 450 million years of vertebrate evolution (Blair Hedges and Kumar, 2003) (Fig. 1A). Although comparison of human sequence with more diverged non-mammalian genomes such as that of chicken significantly reduced the numbers of these conserved sequences, it had the effect of greatly increasing the accuracy of prediction. This was highlighted by the accurate verification of previously characterised enhancer elements (PE and DE, see Fig. 1A) (MacKenzie et al., 1997; Miller et al., 2007). Furthermore, accurate prediction of an active and Wnt inducible TCF4 binding site within PE was previously possible using a combination of comparative genomics and Transfac analysis (Miller et al., 2007). In addition to the PE and DE elements the presence of a significant area of non-coding conservation over 40 kb 5′ of the Msx1 transcriptional start site was highlighted. This highly conserved sequence, that we called KE, continued to be highly conserved even in amphibians and fishes suggesting its critical role in some biological process common to all vertebrates. A clue to its possible purpose came from the observation that in all of the species analysed, KE was always maintained in cis with the Msx1 gene. Considering the known high rate of genomic rearrangement events known to occur during evolution, the continued retention of KE in synteny with the Msx1 gene over 450 million years of evolution strongly suggests that KE is functionally linked to the Msx1 gene (MacKenzie et al., 2004). Furthermore, recent studies have shown that over 75% of non-coding sequences demonstrating similar degrees of conservation consist of tissue-specific cis-regulatory elements such as enhancers (Pennacchio et al., 2006). We therefore explored the hypothesis that this sequence was a tissue-specific enhancer required for some aspect of the regulation of the MSX1 gene.

![Fig. 1. (A) A multispecies rVISTA plot from the ECR browser comparing 50 kb of human genomic flanking sequence 5′ of the Msx1 transcriptional start site to (from top to bottom) zebrafish (Zeb.), puffer fish (Fugu), Xenopus (Xen.), chicken (Chick), Marsupial (Mars.), rat, mouse and dog. The x-axis represents linear distance with reference to the human genome sequence. The scale bar represents 4 kb compared to the human genome sequence that has been used as the base sequence. The y-axis represents levels of sequence conservation between 50% and 100%. Red, blue, pink and yellow peaks represent areas of sequence conserved for more than 75% over 100 base pairs in intergenic non-coding, exonic, intronic and 5′ untranslated regions (UTR) respectively. Green bars and grey boxes represent areas of repetitive sequence including LINEs, SINES and Alu sequences. Previously described enhancers are highlighted in black boxes and include the proximal enhancer (PE) and the distal enhancer (DE; MacKenzie et al., 1997; Miller et al., 2007). KE is also highlighted within a black box. (B) A linear alignment of the human, dog, mouse, rat, marsupial (monodom), chicken, frog, fugu and tetrado (teta) KE enhancer sequences demonstrating levels of conservation (highlighted in pink for tetrapods and red for vertebrates). Putative transcription factor binding sites as predicted by Transfac professional (matrix similarity ≥0.90, core similarity = 1) are highlighted as areas of coloured sequence above each of the lines of sequences. A key for each of the transcription factor binding sites is displayed in the bottom right hand corner. S8 is also known as Prx2.]
The highly conserved KE contained a number of perfectly conserved binding sites for transcription factors specific to neural crest and heart cell lineages.

In order to predict the possible tissue-specific properties of KE we carried out a Transfac analysis on the sequence using high search stringencies (core = 1, matrix >0.9). We were able to detect the nearly perfectly conserved putative binding consensus of a number of neural crest (Sox5 and Sox9) (Perez-Alcala et al., 2004; Sakai et al., 2006) and heart specific (GATA, NKX2.5, S8 (Prx2)) (Durocher et al., 1997; Leussink et al., 1995) transcription factors (see Fig. 1B). This observation was consistent with the hypothesis that KE was an MSX1 cardiac neural crest specific enhancer.

KE is a tissue-specific enhancer and supports transgene expression within Msx1 expressing cNC

In order to validate our hypothesis that KE represented a cNC specific enhancer sequence we amplified and isolated the human KE from human placental DNA. The KE sequence was then cloned into a lacZ reporter construct containing a LacZ marker gene (pBGZ-1230) fused to a human β-globin minimal promoter to form the p30KE plasmid. pBGZ-1230 has been used as a reporter plasmid several times in the past and is incapable of supporting any degree of consistent tissue-specific expression (Yee and Rigby, 1993). We linearised the p30KE plasmid and produced 3 different transgenic mouse lines by pronuclear injection of 1-cell mouse embryos. In later development, several differences were observed between these lines in terms of their staining within limb buds and the developing nervous system (not shown). These differences were attributed to integration effects. However, all lines demonstrated strong and reproducible expression in presumptive neural crest cells migrating between the embryonic hindbrain region and the developing heart field in cells surrounding the otic vesicle. Because of its reproducibility this expression was directly attributable to the activity of the KE element. Using Xgal histological staining we analysed the expression of the transgene throughout the early stages of embryonic development from embryonic day 8 (E8) to E12.5. At E8 expression of the p30KE transgene was strongest in an area of the neural head fold lip destined to form the hindbrain. Expression of the transgene also extended ventrally and laterally within the epiblast (Figs. 2A and B). At E9.5 expression of the transgene persisted within the dorsal lip of rhombomeres 4 and 6 of the hindbrain (Figs. 2C and D). In addition LacZ expression was obvious in 2 different streams of presumptive neural crest cells migrating from rhombomeres 4 and 6 migrating either side of the otic vesicle (Fig. 2D). These observations are entirely consistent with the reported expression of a LacZ based marker gene used to target the second exon of the Msx1 gene in mice.

![Fig. 2. (A–F) Whole mount analysis of E8 (A and B), E9.5 (C and D) and E10.5 (E) p30KE transgenic mouse embryos stained with X-Gal to detect expression of the p30KE transgene construct. hb; hindbrain, hf; head fold, dl; dorsal lip, ov; otic vesicle, 1,2,3; brachial arches 1, 2 and 3, ey; optic vesicle, r4, r5 and r6; rhombomeres 4–6, pf; pontine flexure. White arrowheads (E) highlight the positions of cells between the epithelium and neuroepithelium and caudal of the otic vesicle that express the transgene.](image-url)
(Houzelstein et al., 1997). In the study, expression of the lacZ marker can clearly be seen in cells migrating from rhombo meres 4 and 6 either side of the otic vesicle (Houzelstein et al., 1997). By E10.5 expression of the transgene was still obvious within migrating neural crest cells either side of the otic vesicle (Fig. 2E) that also expressed Msx1 transcripts (Figs. 3A and B). These observations were further verified using monoclonal antibodies specific to the Msx1 protein (Figs. 3D and G) and LacZ protein (Figs. 3E and H) that detected both Msx1 and LacZ within presumptive neural crest derived mesenchyme cells immediately caudal of the otic vesicle (Figs. 3D and E) and in cells migrating next to the 3rd branchial arch artery (Figs. 3G and H), a known conduit of cardiac neural crest migration (Sieber-Blum, 2004). We also examined the expression of the transgene in the developing heart and were able to detect LacZ expression within cells lining the outflow tract previously shown to express Msx1 (Chan-Thomas et al., 1993) (Figs. 3J and K). However, the expression of the transgene in the outflow tract was detected at least a day later than the expression of the Msx1 gene (Figs. 3I–K) and was not as widespread throughout the outflow tract as previously reported for Msx1 transcripts.

Fig. 3. (A) Vibratome section of an E10.5 mouse embryo sectioned coronally through the hindbrain region at the level of the otic vesicle and analysed by whole mount in-situ hybridisation using a DIG-labelled Msx1 specific anti-sense probe (purple staining). (B) Vibratome section of an E10.5 p30KE embryo stained using X-gal to detect expression from the transgene and sectioned coronally through the hindbrain. In both panels A and B tissue layers are delimited using broken white lines to clarify the positions of each cell layer. In both panels A and B the rostro-caudal axis runs from left (rostral; Rost.) to right (caudal; Caud.). Although the sections are taken at the same level it should be noted that morphological differences between A and B reflect the different methods used for LacZ staining and for in-situ hybridisation. DAPI stained (C and F), and fluorescent immunohistochemical analysis using monoclonal antibodies raised against the Msx1 protein (D and G) and the bacterial β-galactosidase protein (LacZ; E and H) in adjacent 8 μm cryostat sections of an E10.5 mouse embryo transgenic for the p1230/KE plasmid. Cellular expression of Msx1 and LacZ is highlighted using white arrow heads and can be seen in mesenchyme cells caudal to (D and E) the otic vesicle (ov), and adjacent to the 3rd branchial artery (3ba; G and H) in which neural crest are known to migrate into the heart field. (I) Whole mount in-situ hybridisation analysis of Msx1 mRNA expression (purple stain) within the outflow tract (off) of an E10.5 wild type embryo heart where the extent of expression is highlighted using black arrow heads. (J) X-gal stained whole mount preparation of an E11.5 embryo heart transgenic for the p1230/KE construct demonstrating transgene expression within the outflow tract, highlighted using white arrow heads. (K) Sectional analysis of X-Gal stained E11.5 embryonic heart transgenic for the p1230/KE construct demonstrating LacZ expression within cells lining the outflow tract (off). The extent of transgene expression is highlighted using white arrow heads. at, atrium; NT, neural tube.
Tissue-specific activity of the KE element is responsive to cAMP mediated but not PKC mediated signal transduction pathways

Transfac analysis of the KE element demonstrated the presence of several highly conserved putative binding sites of the Sox9 transcription factor that is known to be expressed during, and required for, neural crest differentiation and migration (Kordes et al., 2005). Furthermore, previous studies have shown that the expression of Sox9 in these neural crest cells is responsive to PKA signalling (Sakai et al., 2006). In order to explore the hypothesis that the KE enhancer was responsive to cAMP mediated PKA signalling, we recovered E10.5 embryos transgenic for the p30KE transgene and divided their heads sagittally. These halves were then cultured in the presence or absence of the adenylyl cyclase agonist forskolin. In parallel, we also cultured sagittally divided transgenic embryo heads in the presence of the PKC agonist phorbol 12,13-dibutyrate (PDBu). Both agonists were applied either to the medium or were soaked in AG-1X2-formate beads that were then placed within the otic vesicle of the transgenic embryonic explant. Following overnight culture the embryo head explants were fixed and stained for equal periods of time in equal concentrations of X-gal. Comparisons were made between PDBu exposed transgenic explants and those not exposed to PDBu. No discernable difference could be detected in the staining intensity of either transgenic explant half exposed to PDBu placed either in the medium or applied using formate beads (data not shown). However, we were able to discern a clear and significant change in both the intensity and distribution of LacZ staining cells in head explants cultured with forskolin (see Fig. 4). In the case of the implanted beads elevated expression of the transgene was clearly evident in cells surrounding the bead implantation site to the extent that discerning the location of the bead was difficult following LacZ staining (Figs. 4A–D). Furthermore, expression of the transgene was greatly enhanced throughout the explant and extended into regions surrounding the developing eyes and pharyngeal arches by addition of forskolin to the medium. These are areas of expression not previously associated with the activity of the transgene in the untreated explants (Figs. 4E–H) and clearly demonstrate that the KE element is responsive to cAMP/PKA mediated pathways.

Discussion

The high degrees of conservation of regulatory regions that modulate the expression of developmentally important genes suggests that control of the expression of these genes has been at least as important as the function of the proteins they produced during evolution (Davidson et al., 2006a,b; Mackenzie and Quinn, 2004; Mackenzie et al., 2004). It has been realised for a number of years that comparative genomics is an important tool in the search for sequences within the genome critical to normal human development and health (Loots and Ovcharenko, 2005; Loots et al., 2002; Ovcharenko and Loots, 2003; Wasserman et al., 2000; Wei et al., 2002). Although the primary reason for the sequencing of the human genome, as well as a number of other vertebrate genomes, was to find novel genes, it is now obvious that these genome sequences are also extremely useful in detecting the regulatory elements responsible for controlling the expression of these genes. In the present study we have used comparative genomics to locate, isolate and characterise a highly conserved enhancer element and provide evidence for the regulatory systems that modulate its activity.
**Clues to the regulatory systems supporting Msx1 expression in dorsal neural tube lip and migrating cNC**

The Msx genes have been shown to be critical components of the mechanisms required for normal formation of the outflow tract of the heart (Ishii et al., 2005; Lallemand et al., 2005; Ogi et al., 2005). The current study describes the discovery of a highly conserved cardiac neural crest specific enhancer, KE, that has been conserved in cis with the Msx1 gene for over 450 million years and supports the activity of a marker gene in dorsal neural tube lip and presumptive cNC cells migrating from the fourth and sixth hindbrain rhombomeres that also express MSXI transcripts (Houzelstein et al., 1997). In addition, the regulatory systems that support the tissue-specific properties of this enhancer are cAMP responsive, suggesting a role for a PKA dependent signal transduction pathway that may have been conserved from our fish ancestors. The role of cAMP mediated PKA pathways in the specification, migration and differentiation of neural crest is well documented (Benjamin et al., 2006; Chen et al., 2005; Huber et al., 2003; Sakai et al., 2006). Furthermore, many of these PKA mediated regulatory systems include the involvement of transcription factors such as Sox 5 and 9 (Perez-Alcala et al., 2004; Sakai et al., 2006). Therefore, our observation that the KE element is not only cAMP inducible but also contains a number of perfectly conserved putative Sox transcription factor binding sites is consistent with our current understanding with regard to the SoxE/PKA dependent regulatory systems known to modulate the specification of cardiac neural crest (Perez-Alcala et al., 2004; Sakai et al., 2006). Thus, our data, in combination with the published literature, suggests a role for the KE element in the proper morphogenesis of the heart.

**A role for KE in heart morphogenesis**

Understanding where KE is important in heart development necessitates an understanding of the origins and destination of the cNC. The role of the cardiac neural crest in morphogenesis of the outflow tract of the heart can be broken down into 3 main steps: specification, migration and differentiation (Hutson and Kirby, 2003). Defects in the molecular pathways controlling each of these steps have been shown to result in outflow tract defects. Specification of the neural crest from the dorsal lip of the neural tube involves a morphological change from an epithelial cell type to that of a migratory cell type (Firulli and Conway, 2004; Jung et al., 2005). A number of different studies have implicated the Msxl gene as being a major player in neural crest cell specification (Burstyn-Cohen et al., 2004; Monsoro-Burg et al., 2005; Tribulo et al., 2003) along with members of the SoxE family (Sox 8, 9 and 10 (Kordes et al., 2005)), the Snail family, FoxD3, Lhx1, c-Myc and Pax3 (Barembaum and Bronner-Fraser, 2005). In the current study we demonstrate that KE is active in the neural tube dorsal lip region at E8 which is where Msx1 would need to be expressed in order to play a role in neural crest specification. Thus, the high degrees of evolutionary conservation of the KE element, the putative transcription factor binding sites contained within it and its activity in the early dorsal neural tube lip supports a role for Msx1 in the specification of cardiac neural crest.

In addition to being expressed in the neural plate dorsal lip during neural crest specification, Msx1 continues to be expressed in neural crest cells undergoing migration in chick embryos (Taneyhill and Bronner-Fraser, 2005) an observation confirmed in mammalian embryos in the current study. Further evidence comes from previous examinations of the expression of a LacZ marker gene used to target the Msx1 locus where it was shown that LacZ activity was present within migrating neural crest cells derived from rhombomeres 4 and 6 (Houzelstein et al., 1997). Based on this existing literature and the findings described here we propose that the KE element is required to support the expression of the Msx1 gene within cardiac neural crest cells during their specification and migration where Msx1, in its role as a potent antagonist of cell differentiation (Odelberg et al., 2000; Thompson-Jaeger and Raghow, 2000), may be required to act as a brake to differentiation thus supporting cell migration. The mechanism through which Msx1 achieves this might involve a direct interaction with the Pax3 protein that is also expressed in dorsal neural tube lip and migrating cNC and is also known to be required for cNC specification (Epstein et al., 2000). Msx1 and Pax3 have been shown to cooperate in the specification of neural crest (Monsoro-Burq et al., 2005). In addition, previous studies have suggested that within other organ systems the Msx1 protein prevents cellular differentiation by directly binding to the Pax3 protein (Bendall et al., 1999; Bendall et al., 1998). Such a relationship between Pax3 and Msx1 may also exist within migrating cardiac neural crest cells where Pax3 may be required to specify cNC (Epstein et al., 2000) but where Msx1 is required to modulate this activity of the Pax3 protein thus allowing cell migration.

We have succeeded in detecting the activity of the KE element in presumptive dorsal neural tube lip cells and in migrating cardiac neural crest cells, that we show also express Msx1 transcripts at E10.5, consistent with a role for KE in supporting Msx1 expression during cNC specification and migration. However, the observed activity of KE in the developing endocardiac cushions during formation of the outflow tract was less conclusive. For example, Msx1 is strongly expressed in the endocardiac cushions from E10.5 onwards but we were unable to detect activity of KE within the outflow tract until E11.5. These observations suggest that, although important for Msx1 expression in the specification and migration of cNC, KE may not play such an important role in the expression of Msx1 in differentiating endocardiac cushions. A number of studies have demonstrated that the expression of the Msx1 gene during the morphogenesis of the limb buds and neural tube roof plate is associated with, and is supported by, the expression of bone morphogenic proteins (BMPs) (Lallemand et al., 2005). In addition, there have been a number of studies which suggest that the expression of the Msx genes in the endocardiac cushions of the differentiating heart outflow tract is supported by BMP mediated regulatory systems (Abdelwahid et al., 2001; Angello et al., 2006; Eisenberg and Markwald, 1995). Thus, we hypothesise that at least two enhancers are required for the expression of Msx1 during the development of the OFT. We believe that we have identified one of these enhancers in the form of the highly conserved and cAMP dependent KE element that may be required for expression of...
Msx1 during early specification and migration of cNC cells. Thus, the requirement for expression of Msx1 in migrating cNC is an ancient one and is reflected by the exceptional degrees of KE conservation. However, we believe that a second enhancer, that supports Msx1 in the endocardiac cushions during their differentiation, remains to be identified. We hypothesise that this second enhancer is likely to be BMP dependent and is required for Msx1 expression in the endocardiac cushions. We would further venture that this second enhancer will not be so easy to identify as it will not be as highly conserved as KE. This lack of conservation will reflect its role in the later stages of the morphogenesis of the mammalian outflow tract that is a comparatively recent adaptation within the hearts of birds and mammals from an evolutionary perspective. We are currently extending our analysis to determine the possible location of this element.

Although Msx1 has been shown to be expressed in cardiac neural crest during their specification, migration and differentiation and is, together with Msx2, critical for normal morphogenesis of OFT, the precise stage at which these genes are most crucial to OFT development remains to be determined. The requirement for Msx1 in the specification and migration of cNC cells is suggested by the extreme conservation of the KE element which our studies suggest supports expression of Msx1 in cNC cells during these stages. This observation is not without precedence as although it has been shown that the Pax3 gene is essential for the normal formation of the OFT migrating cNC cells stop expressing Pax3 before their arrival in the heart (Epstein et al., 2000). Thus it is entirely feasible that because the requirement for the expression of Msx1 in the dorsal neural tube lip and in migrating cardiac neural crest cells precludes the expression of Msx1 in cardiac outflow tract that expression of Msx1 in dorsal lip and migrating cNC represent the most important stages of Msx1 expression required for normal heart development. Therefore, concentrating exclusively on the regulatory mechanisms governing the expression of Msx1 in the endocardiac cushions may be a mistake as many outflow tract defects might result from problems in the specification and migration of cNC rather than the formation of endocardiac cushions.

It has been largely accepted that the Msx1 protein is a classic transcription factor that influences gene activity through DNA binding. The two mouse Msx1 gene deletion models previously produced reflected this belief and, in both cases, only sequence 3' and including the third helix of the homeodomain were deleted leaving the rest of the homeodomain intact (Houzelstein et al., 1997; Satokata and Maas, 1994). More recent evidence now suggests that another mode of action of the Msx1 protein includes protein–protein interactions with other transcription factor proteins such as the Pax, Dlx and Lhx genes via the N-terminus and the first helix of the homeodomain (Bendall et al., 1998, 1999; Bryan and Morasso, 2000; Zhang et al., 1997). Thus, existing Msx1 targeted deletion models may not fully reflect the developmental significance of the Msx1 gene in the inhibition of gene expression through protein–protein interaction with known cNC specification factors such as Lhx1 and Pax3. This possibility warrants further analysis through the production of a complete Msx1 null mouse model.

Conclusions

OFT defects make up one of the most frequent class of birth defects affecting newborn infants. Understanding the causes of susceptibility to OFT defects through genetic analysis of patient DNA through linkage and association studies of DNA polymorphisms is one of the most important directions we can take towards understanding how outflow tract defects occur. Until recently, however, much of the effort into understanding the causes of OFT defects has been directed against the coding regions of genes. The current study highlights the extreme conservation of a regulatory element responsible for supporting the expression of a gene known to be involved in OFT formation and underlines the need to look at regulatory regions, as well as coding regions when looking for the polymorphisms that affect susceptibility to OFT defects.

Acknowledgments

KAM and SD were supported by BBSRC studentships. This project was funded by Tenvous Scotland and the BBSRC.

References


