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

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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.

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

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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 GGC 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 *Eco*RI and *Spe*I, and ligated into compatible ends of plasmid pGEM-7Zf (+) (Promega) to produce pGEMKE. The KE insert from pGEMKE was removed by digestion with *Sph*I (made blunt end using Klenow enzyme) and then with *Kpn*I. This fragment was then ligated into the *Hin*dIII (made blunt end using Klenow enzyme) and *Kpn*I 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 *Kpn*I and *Not*I 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 pronucleii 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 $MgCl_2$ in PBS) and fixed in 4% paraformaldehyde (PFA) at 4 °C for 1 h. Embryos were washed using detergent wash (2 mM $MgCl_2$, 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% $NaCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ 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 *Nsi*I for production of antisense probe. Digoxygenin (DIG) labelled antisense probes were transcribed using components of the T7 RNA polymerase Maxiscript In Vitro Transcription Kit (Ambion) as described in the manufacturers 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, RNAasin (Roche) and 90 units of the T7 RNA polymerase in nuclease-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 gentle 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 (Hybridoma Bank) and 40-1a (anti-β-galactosidase; Hybridoma 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 mins with gentle shaking. Slides were then mounted in VECTASHIELD® mounting medium with DAPI (Vector labs) and visualised immediately with an Axioskop 2 plus microscope (Zeiss) with HBO100 FluoArc mercury 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 sagitally using a sterile