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Origins of *Cdx1* regulatory elements suggest roles in vertebrate evolution

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ABSTRACT ***Cdx1*, an upstream regulator of Hox genes, is best characterized for its homeotic effects upon the developing axial skeleton, particularly in the neck. It responds to retinoic acid (RA) in both mouse embryos and embryonal carcinoma (EC) cells. By use of β-galactosidase chemiluminescence, we show that a mouse *Cdx1/lacZ* reporter expressed in P19 EC cells responds to RA by the combined activities of an intron retinoic acid response element (RARE) and an upstream RARE. In contrast, a chicken *Cdx1/lacZ* reporter responds only by activity of the intron RARE. Database analyses upon *Cdx1* from twenty three vertebrate species reveal that the intron RARE is structurally conserved in amniotes (eutherian mammals, marsupials, birds and Anole lizard), but not in *Xenopus* or fish. The upstream RARE is structurally conserved only in eutherian mammals. We conclude that the intron RARE originated at around the amphibian/amniote division, and the upstream RARE appeared around the marsupial/eutherian mammal division. In view of the site of action of *Cdx1,* we propose that acquisition of the intron RARE may have facilitated the substantial changes that occurred in the neck and anterior thorax at the advent of the amniotes. We present evidence that *Cdx1* is also a developmental regulator of the female urogenital system, and we suggest that acquisition of the upstream RARE may have contributed to morphological divergence of marsupial and eutherian mammals.**

KEY WORDS: *Cdx1, retinoic acid response element, evolution*

Cdx proteins (Cdx1, Cdx2 and Cdx4) are upstream regulators of vertebrate Hox genes (Deschamps and van Nes, 2005; Houle *et al*., 2003a). *Cdx1* knockout (Subramanian *et al*., 1995; van den Akker *et al*., 2002) or over-expresser (Gaunt *et al*., 2008) mice display homeotic mutations within the axial skeleton, particularly in cervical and anterior thoracic regions. The axial defects in *Cdx1* mutants extend forward to the skull/atlas level. The other reported anatomical defects in *Cdx1* mutants are in forelimb bud size (Gaunt *et al*., 2008) and, when combined with *Cdx2* mutation, in spinal ganglia (van den Akker *et al*., 2002).

Mouse *Cdx1* is activated by retinoic acid (RA) in both embryos (Houle *et al*., 2000) and P19 or F9 embryonal carcinoma cells (Beland and Lohnes, 2005; Houle *et al*., 2000). RA response reporter studies show that RA is present in the embryonic regions known to express *Cdx1*: the primitive streak of 7.5 day mouse embryos and, by 8.5 days, the developing somites (Rossant *et al*., 1991). RA administered to pregnant mothers induces homeotic mutations in the axial skeleton (Kessel and Gruss, 1991), and the range of these resembles those induced by Cdx1 protein over-expression (Gaunt *et al*., 2008).

RA activates *Cdx1* by a retinoic acid response element (RARE) located upstream of the coding region (Houle *et al*., 2000). Mutation of this RARE results in homeotic shifts in cervical vertebrae, indicating that this element plays a functional role (Houle *et al*., 2003b). In classical RA transactivation, all-*trans* RA first interacts with a protein dimer comprising a RA receptor and a retinoid X receptor. This complex regulates gene transcription by binding to a RARE, which typically consists of a pair of [T][G][A][C/A][C][T/C] hexamer repeats separated, most commonly, by either two (DR2-type RARE) or five (DR5) nucleotides, and these may lie with equal frequency on the coding or the non-coding strand (Balmer and Blomhoff, 2005). The *Cdx1* upstream RARE (Houle *et al*., 2000) is atypical in containing only one clear consensus element.

A DR-2 type RARE motif was subsequently found in the first

Abbreviations used in this paper: EC, embryonal carcinoma; RA, retinoic acid; RARE, retinoic acid response element.

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intron of both mouse and chicken *Cdx1* (Gaunt *et al.*, 2003). This was putative (Houle *et al.*, 2003b) since it had not been shown to respond to RA. The role of the intron RARE motif was examined using a chicken *Cdx1/lacZ* reporter construct expressed in transgenic mouse embryos (Gaunt *et al.*, 2003). Mutation in the motif resulted in a more posterior expression boundary in neural tube and greatly reduced expression in somites. Both chicken and mouse intron RARE motifs are adjacent to a Lef/Tcf motif, which is a response element usually used by the canonical Wnt pathway. Mutation of this in the *Cdx1/lacZ* reporter resulted in substantial loss of lacZ expression in transgenic embryos (Gaunt *et al.*, 2003).

We now provide direct evidence that the intron RARE motif in *Cdx1* is responsive to RA. Database analyses indicate that this element has a much earlier origin in vertebrate evolution (around the amphibian/amniote split) than the upstream RARE (around the marsupial/eutherian mammal split). We consider how origin of each RARE may have contributed to a different step in the morphological evolution of vertebrates.

Results

β-galactosidase chemiluminescence to detect Cdx1 reporter activity in P19 cells

Figs 1A, 2A show construct #1, the mouse *Cdx1/lacZ* reporter that provides a normal Cdx1-like pattern of expression in transgenic mouse embryos (Gaunt *et al.*, 2003). The construct contains the upstream RARE (Houle *et al.*, 2000) and also the putative intron RARE earlier identified as conserved in chicken *Cdx1* (*CdxA*) (Gaunt *et al.*, 2003). We first examined *luciferase (luc)* reporters since these are most commonly used in culture assays. Construct #2 contains *Cdx1* sequence upstream of the first exon while construct #3, designed to express a Cdx1/luc fusion protein, also includes parts of intron 1 and exon 2 (Fig. 1A). Construct #2 is stimulated by RA (Fig. 1B). However, construct #3 is inactive (Fig. 1B), showing that the Cdx1/luc fusion protein is non-functional. This raised the problem of how to test for activity in the intron RARE motif whilst maintaining its normal position in the gene.

We overcame this by using the *Cdx1/lacZ* reporter itself (construct #1), measuring lacZ (β-galactosidase) activity by a sensitive chemiluminescence technique. Fig. 1C shows that *Cdx1/lacZ* reporter is stimulated by RA, whereas *Cdx2/lacZ* and *Cdx4/lacZ*, both expressed in transgenic mice (Gaunt *et al.*, 2005), show little evidence of stimulation.

Functional assay of intron and upstream RAREs in mouse and chicken Cdx1/lacZ reporters

Following transfection of construct #1 into P19 cells, β-galactosidase activity is stimulated 20- to 30-fold by RA (Figs 1C, 2B). Mouse constructs #4, #5 and #6 are similar to construct #1 but contain, respectively, mutations within the intron RARE, upstream RARE, and both RAREs. Constructs #4 and #5 show only about 5- and 8-fold stimulations by RA, whereas construct #6 shows only 2-

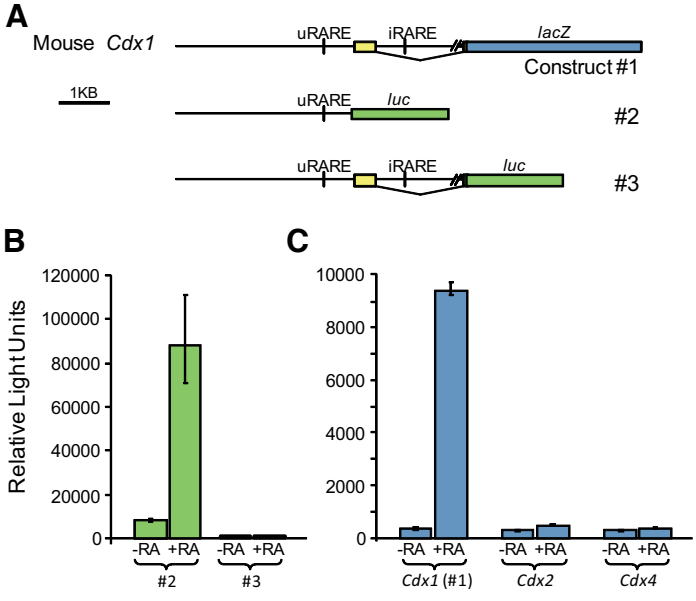

Fig. 1. Cdx1/lacZ fusion reporter responds to retinoic acid (RA) in P19 cells. (A) Cdx1/lacZ *and* Cdx1/luc *reporters. Yellow boxes,* Cdx1 *exons; uRARE, iRARE, upstream and intron RAREs;* lacZ, lacZ/SV40; luc, luciferase/SV40. **(B)** luc *reporter assays: only non-fused luc protein, as expressed by construct #2, is active and responds to RA.* **(C)** LacZ *fusion reporter assays:* Cdx1/lacZ *expression, but not* Cdx2/lacZ *or* Cdx4/lacZ, *is stimulated by RA. Each graph shows one experiment; bars show means of three replicate cultures, with ranges indicated.*

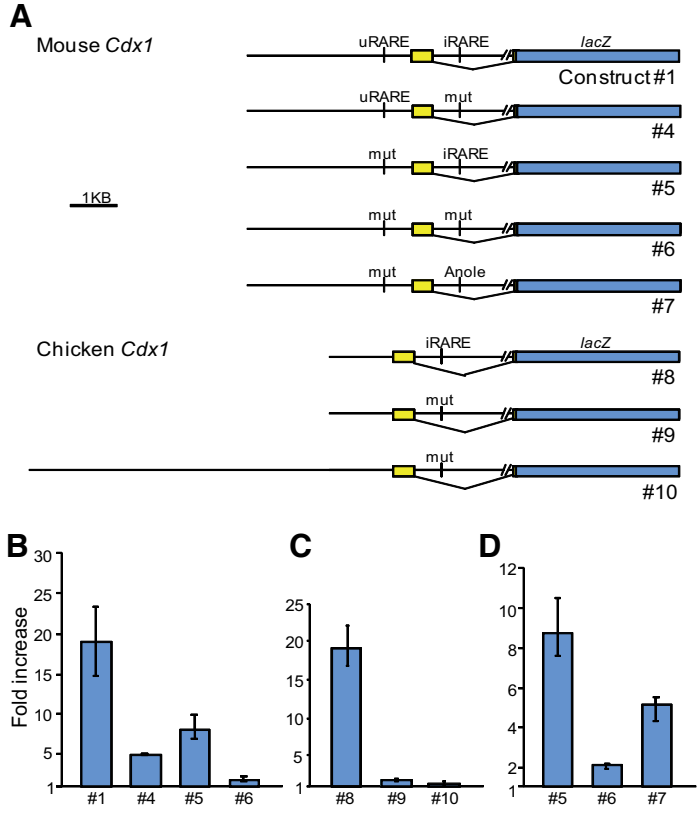

Fig. 2. Retinoic acid response element (RARE) activities of mouse and chicken *Cdx1/lacZ* reporters in P19 cells. (A) Cdx1/lacZ *fusion reporters. mut, mutation; Anole, Anole lizard-like iRARE; other labels as for Fig. 1.* **(B)** *Mouse* Cdx1*: RA response is shared between both RAREs,* **(C)** *Chicken* Cdx1*: RA response is mainly attributable to the intron RARE,* **(D)** *Anole lizard-like intron RARE has about 50% activity relative to mouse RARE. Fold increase is the effect of retinoic acid relative to no retinoic acid. Range bars as for Fig. 1.*

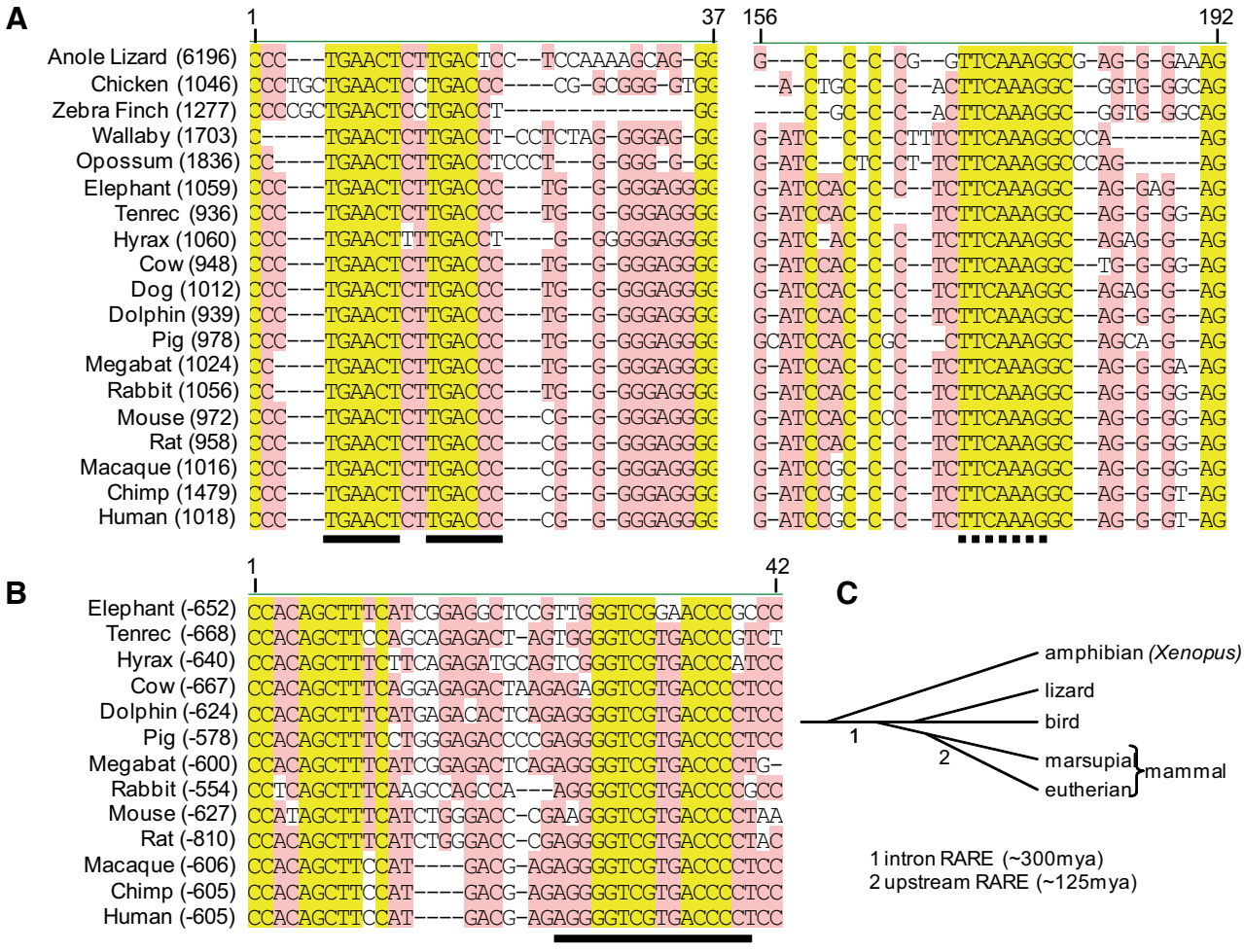

Fig. 3. *Cdx1* retinoic acid response elements (RAREs) in vertebrates. (A) *Alignment of* Cdx1 *intron sequences. Hexamer repeats of the RARE, beginning position 7, are underlined. Conserved Lef/Tcf motifs are also underlined (dotted).* **(B)** *Alignment of* Cdx1 *upstream RAREs. This RARE (underlined) is atypical, and its overall limits uncertain. Numbers in brackets in A and B indicate positions of nucleotide 1 relative to start of the coding sequence. Yellow highlight, fully conserved bases; red, highly conserved. Turkey and dog sequences are unavailable in regions of intron and upstream RAREs, respectively.* **(C)** *Origins of* Cdx1 *RAREs mapped on an evolutionary tree.*

fold stimulation (Fig. 2B). This shows that the putative intron RARE motif is indeed functional, and that the upstream and intron RAREs together account for almost all of the RA stimulation of mouse *Cdx1* reporter in P19 cells.

Chicken construct #8 (Fig. 2A), encoding a chicken Cdx1/lacZ fusion protein, is expressed in transgenic mouse embryos with a similar pattern to construct #1 although the level of expression in somites is reduced (Gaunt *et al.*, 2003). Construct #8 in P19 cells is stimulated about 20-fold by RA (Fig. 2C). Construct #9, which differs from construct #8 only by mutation in the intron RARE, is stimulated only 1.5-fold. Addition of a further 6.8kb of upstream sequence (construct #10) does not increase response to RA (Fig. 2C). We conclude that the chicken *Cdx1* reporter responds to RA mainly through the intron RARE.

Structural survey of Cdx1 RAREs in vertebrate genome databases

We examined sequences from fourteen eutherian (placental) mammals, two marsupials (wallaby and opossum), three birds (chicken, zebra finch and turkey), one lizard (Green Anole), one amphibian (*Xenopus tropicalis*) and two fish (Fugu and Tetraodon). Homeodomain amino acid sequences were aligned to facilitate identification of genes as *Cdx1* (supplementary Fig. 1). Amino acids consistently conserved in Cdx1 are Asp at position 2, Tyr at 22, Ser at 23, and Asn at 60.

Fig. 3 A,B shows alignments of *Cdx1* nucleotide sequences around the two RAREs. The *Cdx1* intron RARE (repeats underlined in Fig. 3A) is conserved in all mammals and birds but we were unable to detect it in *Xenopus* or fish. The Anole lizard has a RARE-like motif but this differs in one base that is usually, although not invariably, conserved (Balmer and Blomhoff, 2005). Moreover, the lizard RARE is located much further from exon1 and lies within an intron that is much greater in length than in other species examined. We consider, however, that this is the homologous RARE sequence in the lizard since (i) surrounding regions show sequence similarities with birds and mammals, including the adjacent Lef/Tcf binding motif (dotted underline in Fig. 3A) (Gaunt *et al.*, 2003), (ii) the mouse sequence retains RARE activity even after its mutation to the Anole sequence (construct #7), although activity is reduced by about 50% (Fig 2D), (iii) it is

known that non-coding DNA in Anole may be extended by insertion of retrotransposable elements (Di-Poi *et al.*, 2009), (iv) lizards are commonly considered as a sister group to birds (Fig. 3C) (Eernisse and Kluge, 1993) and would therefore be expected to possess the intron RARE, at least in their ancestry.

The upstream RARE (underlined in Fig. 3B) is atypical, containing only one clear consensus hexamer (Houle *et al.*, 2000). It is conserved in all eutherian mammals, although it appears to be diverging in the Afrotherian sub-group (elephant, lesser hedgehog tenrec and hyrax) (Kriegs *et al.*, 2006; Murphy *et al.*, 2001). We were unable to detect the upstream RARE in marsupials, birds, Anole lizard, *Xenopus* or fish. Fig. 3C shows the inferred origins of the intron and upstream RAREs marked on a commonly adopted vertebrate evolutionary tree (Eernisse and Kluge, 1993).

Defects in the female urogenital systems of Cdx1 over-expresser mice

Fig. 3C raises the question of what are the anatomical changes occurring at these points in evolutionary history that might be relevant to a change in *Cdx1* regulation? The main defects so far recorded in *Cdx1* mutant mice lie within the cervical and anterior thoracic vertebrae and the ribs. We now present evidence that *Cdx1* is also involved in development of the female urogenital system.

From examination of the thoracic and abdominal organs of heterozygous female OE1, OE2 and OE4 mice (which over-express, respectively, *Cdx1*, *Cdx2* and *Cdx4* under control of their own promoter elements) (Gaunt *et al.*, 2008) only the urogenital systems of OE1 and OE2 mice show obvious defects. About 16% of both OE1 (10/62) and OE2 (4/25) heterozygous mice show defects in their reproductive tracts. The range of defects is apparently identical for two independently-derived OE1 lines and one OE2 line. Four different phenotypes are seen. First, there is absence of one uterine horn, with persistence of an undeveloped Müllerian duct-like structure which can be traced from the uterine bifurcation to the ovary (Fig. 4A). Second, there is absence of one uterine horn with no detected remnants of the Müllerian duct (Fig. 4B). Third, there is absence of the posterior region of one uterine horn with the anterior part swollen due to retained uterine fluid; the extent of the retained anterior part is variable (Fig. 4 C-E,H). Fourth, there is posterior shift in the level of the uterotubal junction, resulting in elongation of the oviduct (Fig. 4F). In addition, about 8% of OE1 and OE2 mice show defects in their urinary system. This usually appears as hydronephrosis due to an apparent absence/blockage of the ureter (not shown). The hydronephrotic kidney is vesicular and appears swollen (Fig. 4G), shrunken (Fig. 4D), or haemorrhagic (Fig 4H). In other cases there is no kidney (Fig. 4 B,E) or a small kidney (Fig. 4A). A blocked ureter may be swollen (hydroureter) and haemorrhagic (Fig. 4H). Where reproductive and urinary defects occur together they are usually located on the same side (Fig. 4 B,D,E,H). Because gain of Cdx1 and Cdx2 function may lead to these anomalies, we propose a role for these genes in the network that normally regulates development of the female reproductive and urinary systems.

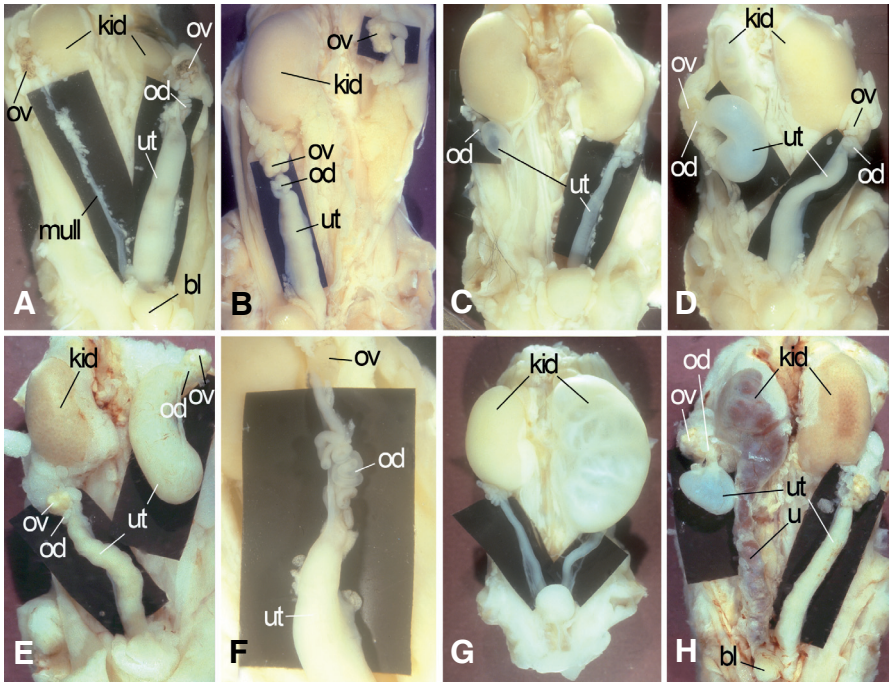

Fig. 4. Defects in the female urogenital systems of *Cdx1* over-expresser mice. *The reproductive tracts of one-to-twelve week old OE1 heterozygous mice are shown underlain with black plastic film to provide contrast.* **(A,B)** *Absence of one uterine horn with* **(A)** *or without* **(B)** *persistence of an undeveloped Müllerian duct-like structure.* **(C,D,E)** *Absence of the posterior region of one uterine horn with the anterior part swollen with fluid.* **(F)** *Posterior shift in the uterotubal junction results in elongation of the oviduct.* **(G)** *Hydronephrotic kidney swollen in size.* **(H)** *Haemorrhagic hydronephrosis and hydroureter. kid, kidney; ov, ovary; od, oviduct; ut, uterus; mull, Müllerian duct; bl, bladder; u, ureter.*

Discussion

lacZ fusion constructs assayed in P19 cells

In our study, we found Cdx1/lacZ fusion protein to be active while Cdx1/luc fusion protein was not. This allowed, in conjunction with use of a sensitive β-galactosidase chemiluminescence assay, lacZ reporters to be readily examined for presence of regulatory elements within *Cdx1* introns. lacZ fusion reporters have been a commonly used tool in transgenic mouse studies and so we suggest that the methods now described might have more general applicability for *in vitro* analysis of constructs already proven *in vivo*. We show that mouse *Cdx1* has both intron and upstream functional RAREs, while chicken *Cdx1* has only the intron RARE activity.

Origins and possible early roles of the Cdx1 RAREs

Our database searches on the *Cdx1* genes of twenty three different vertebrates provide evidence that the intron RARE originated at around the division between amphibians and amniotes, whereas the upstream RARE originated later, at around the split between marsupial and eutherian mammals. We now consider anatomical changes occurring at these points in evolutionary history that may have been directed by changes in *Cdx1* regulation.

Living amphibians (frogs/toads, salamanders and caecilians) have three features in their neck and anterior thorax that distinguish them from amniotes (Wake, 1979). First, they have a single neck vertebra (atlas). Second, there is absence of an odontoid peg/axis, which in amniotes permits rotational movement at the atlas/axis joint. Third, their sternum is joined to the pectoral girdle but not to the ribs. The extensively reported involvement of both *Cdx1* (Gaunt *et al*., 2008; Subramanian *et al*., 1995; van den Akker *et al*., 2002) and RA signalling (Kessel and Gruss, 1991; Lohnes *et al*., 1994) in development of the vertebrae and ribs in mice leads us to propose that origin of the *Cdx1* intron RARE at around the amphibian/amniote split may have contributed to evolutionary changes in the neck and thorax, including an enhanced ability to lift and/or rotate the head. *Xenopus Cdx1 (Xcad2)* does not respond to retinoid signalling (Shiotsugu *et al*., 2004). It is not possible to predict whether the *Cdx1* intron RARE arose within the early amniotes or in their amphibian ancestors. Multiple neck vertebrae were already present in some Carboniferous amphibians, while the odontoid peg first appeared at around the transition from amphibian to amniote, also in the Carboniferous (Carroll, 2001; Evans, 1939).

Instead, or in addition, it is possible that some other role of *Cdx1* may have become facilitated by acquisition of the intron RARE at the amphibian/amniote split. The adult metanephric kidney, for example, first appeared with the amniotes and, as we have shown, this structure may be developmentally regulated by *Cdx1*. However, the amphibian opisthonephric kidney does already include a metanephric-like region (Nelson, 1953). There is no evidence that *Cdx1* regulates development of extra-embryonic membranes, a defining feature of the amniotes.

At the marsupial/eutherian split there is no consistent accompanying change in the arrangement and numbers of vertebrae (Narita and Kuratani, 2005). The most obvious difference between these mammalian groups lies in the structure and function of their reproductive tracts. In particular, the paired uteri of a marsupial remain unfused and lead separately into a single vaginal sinus. This structure connects laterally with paired vaginae, and ventrally with a separate birth canal site. The differences between the split-marsupial and the fused-eutherian female anatomies may be due to the Müllerian ducts originating lateral to the ureters in marsupial embryos, but medial in eutherians (Lombardi, 1998; Nowak, 1999). We have proposed, based upon the phenotype of *Cdx1* overexpression, that *Cdx1* normally affects development of the uterus and ureters in mice. This leads us to suggest that acquisition of the *Cdx1* upstream RARE may have contributed to morphological divergence of marsupial and eutherian mammals.

Many of the urogenital defects that we find in *Cdx1* overexpressers (partial or complete agenesis of the uterus and ureter, renal agenesis, hypoplasia and hydronephrosis, and hydroureter) are also seen in mice with double mutations in RA receptor genes (Ghyselinck *et al*., 1997; Mendelsohn *et al*., 1994). It might be expected that *Cdx1* expression is reduced in these mice, although this is not established. Together, the mouse studies might then indicate that correct dosage of Cdx1 protein is crucial for normal urogenital development, with over- or under-expression both generating similar phenotypes, a finding also made for Pax6 protein (Manuel *et al*., 2008). In this scenario it is not clear why urogenital defects are not reported in *Cdx1* knockout mice. One possibility is overlapping function of *Cdx2* (Gaunt *et al*., 2008; van den Akker *et al*., 2002), a gene which our over-expresser mice also implicate in urogenital development. However, it would then not be clear why *Cdx2* cannot compensate in RA receptor mutants. These points might be elucidated by re-examination of *Cdx1* knockout mice for urogenital defects, and by measuring Cdx1 protein levels in RA receptor mutants. Over-expression of *Cdx4* was not associated with urogenital defects. This might be due to a different pattern of *Cdx4* expression in urogenital progenitors, or to a different property of the protein.

A major challenge is to identify the genetic changes responsible for appearance of new animal groups during the course of evolution. To our knowledge, we now provide the first evidence that origins of regulatory elements in a homeotic gene, *Cdx1*, correspond with evolutionary transitions between vertebrate groups. The Cdx genes, as upstream regulators of Hox genes, seem ideally suited for effecting large scale evolutionary changes in body plan. It is unlikely that modifications in expression of *Cdx1* alone could have resulted in the morphological changes that we have discussed. *Cdx1* modification may have been one of several changes, each acquired step-wise and providing some selective advantage. Sexual reproduction will have accelerated the accumulation of changes necessary for large scale morphological evolution.

Materials and Methods

Plasmids

Cdx1/ luciferase reporters are shown in Fig. 1A compared with construct #1, the *Cdx1/lacZ* reporter previously named construct 7 (Gaunt *et al*., 2003). Construct #2 contains the upstream sequence of *Cdx1*, extending to 21 bases 5' of ATG, subcloned into pGL3-Basic (promoterless *luciferase* vector; Promega). Construct #3 has the *Cdx1* component of construct #1 spliced in-frame to *luciferase*. *Cdx2/lacZ* and *Cdx4/lacZ* reporters are constructs 5 and 1 described earlier (Gaunt *et al*., 2005).

Cdx1/lacZ reporters (Fig. 2A) have the second exon of *Cdx1* spliced in-frame to *lacZ/SV40* polyA and express Cdx1/lacZ fusion proteins. Construct #4 resembles construct #1 but with mutation of the intron RARE from TGAACTCTTGACCC to CATATGCCGACTAG. Construct #5 resembles construct #1 but with mutation of the upstream RARE from GAAGGGTCGTGACCCCTAA to GAAGGGGTCGACCCCCTAA. Construct #6 is similar but with the mutations in both RAREs. Construct #7 resembles construct #5 but the intron RARE is mutated to TGAACTCTTGACTC, like that in Anole lizard. Constructs #8 and #9 are chicken *Cdx1/lacZ* reporters previously named constructs 1 and 5 (Gaunt *et al*., 2003); construct #9 has the intron RARE mutated from TGAACTCCTGACCC to CTCGAGCCGACTAG. Construct #10 is similar but with the 6.8kb *Xho*I/*Hin*dIII fragment (Gaunt *et al*., 2003) extending the upstream sequence. Mutations were introduced by PCR or by QuikChange *in vitro* mutagenesis (Stratagene).

Cdx1 reporter expression

P19 cells, from ATCC, were cultured on gelatinized surfaces in Dulbecco's MEM with glutamine and 10% foetal bovine serum. For reporter assays, cells were grown in 24-well plates. Plasmid DNA was transfected uncut into monolayers using Lipofectamine 2000 (Invitrogen) and following manufacturer's instructions. Medium was replaced after 5 hours, with or without 2.5μM all-*trans* retinoic acid (RA) (Sigma). After a further 20 hours, luciferase or β-galactosidase activities were assayed by chemiluminescence. The Luciferase Assay System (Promega) or the Galacto-Light Plus System (Applied Biosystems) were used, respectively. Methods were as instructed by manufacturers. Light levels were

assayed using a LB 96V MicrolumatPlus luminometer.

Sequence analysis

RARE and other conserved motifs were identified in database sequences (Accession numbers in supplementary Table 1) visually or by use of the EMBOSS fuzznuc tool. We searched, in both orientations, up to 10kb of upstream sequence and the entire first intron. Sequence alignments were made using Vector NTI (Invitrogen).

Acknowledgements

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