Int. J. Dev. Biol. 62: 717-722 (2018)
https://doi.org/10.1387/ijdb.180157sg

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Mouse embryo Hox gene enhancers assayed in cell culture: *Hoxb4, b8* and *a7* are activated by Cdx1 protein

STEPHEN J. GAUNT*

Department of Zoology, University of Cambridge, Cambridge, UK.

ABSTRACT Mouse Hox gene enhancer elements have typically been identified and characterized using *Hox/lacZ* transgenic mouse embryos. Such studies have, for example, identified Cdx responsive binding motifs in the enhancers of *Hoxb8* and *Hoxa7*. Production of transgenic mouse embryos involves issues of cost, welfare, and considerable technical skill. It would be of benefit if these studies could be performed, or advanced, in cell culture. It is shown here that Cdx1 activation of mouse *Hoxb4, b8* and *a7* embryo-active enhancers can be detected using a HepG2 cell culture model system. The technique employed uses co-transfection of an inducible Cdx1 expression construct together with a Hox enhancer/*luciferase* reporter construct. Cultures to be compared receive identical DNAs and differ only in whether or not they also receive inducer (doxycycline). Response of all three Hox enhancers to Cdx1 protein is inhibited by mutation of Cdx binding motifs which are conserved in sequence from fish or *Xenopus* to mammals. The magnitude of transfected chick *Hoxa7* activation by Cdx1 is increased by multiple copies of its enhancer, but for maximum effect these must contain intact Cdx binding motifs. Cdx1 protein was found not to activate *Hoxb4, b8* or *a7* enhancers in P19 mouse pluripotential cells.

KEY WORDS: *transgenic embryo, HepG2, Tet-On, transfection*

Introduction

Gene expression is regulated to a large extent by transcription factors that bind to specific DNA sequence motifs located within *cis*-regulatory elements (Cho, 2012, Gaunt and Paul, 2012, Long *et al.*, 2016). These elements are also known as enhancers. Enhancers of vertebrate Hox genes may be 1) local, positioned inside the gene cluster and usually regulating only nearby genes (Gould *et al.*, 1997, Sharpe *et al.*, 1998), or 2) long range, usually positioned outside the gene cluster and regulating multiple Hox genes over longer distances (Spitz *et al.*, 2003).

Analysis of Hox gene enhancers in transgenic mouse embryos

Enhancers regulating Hox gene expression during mouse embryogenesis have typically been identified in some or all of the following steps (Charite *et al.*, 1998, Marshall *et al.*, 1994, Tabaries *et al.*, 2005). 1) Identify a fragment of DNA that when positioned upstream of a promoter and *lacZ* reporter gene is able to confer a Hox-like pattern of lacZ expression in transgenic embryos. 2) Perform deletions and mutations upon the enhancer-active fragment to narrow-down the specific DNA sequences necessary for its function. 3) Check whether these sequences are conserved between different vertebrate species, as is commonly the case for essential regulatory regions. 4) Examine these sequences for presence of any known transcription factor binding motifs. 5) Test whether these candidate transcription factors do indeed bind to the sequences. 6) Test whether these transcription factors activate the gene. Use of these steps has, for example, identified gene-activating Cdx binding motifs within enhancers of both *Hoxb8* (Charite *et al.*, 1998) and *Hoxa7* (Gaunt *et al.*, 2004, Knittel *et al.*, 1995).

The above experiments can provide direct evidence about the sites and regulation of mouse Hox gene enhancers during embryo development. The approach does, however, involve substantial technical skill, cost, and animal welfare issues that are associated with the production of large numbers of transgenic mouse embryos. Furthermore, the extents of *lacZ* reporter activity detected in transgenic embryos are only weakly quantitative since many uncontrolled factors affect expression levels, such as number of transgene copies, sites of integration, and transgene methylation. Also problematic may be the testing of candidate transcription factors. In practice, this has been achieved *in vivo* in a few cases by electroporation of expression constructs into chick embryos main-

Abbreviations used in this paper: Dox, doxycycline.

***Address correspondence to:** Stephen J. Gaunt. Department of Zoology, University of Cambridge, Downing Street, Cambridge, U.K., CB2 3EJ. Tel: +44-1223-768917. Fax: +44-1223-336676. E-mail: sg397@cam.ac.uk - https://orcid.org/0000-0001-6038-2272

Submitted: 19 March, 2018; *Accepted:* 8 June, 2018.

ISSN: Online 1696-3547, Print 0214-6282

Fig. 1. Conservation between species in Cdx binding motifs within *Hoxb4, b8* and *a7* enhancers. *Sequences shown are the most highly conserved regions of enhancer fragments previously found to be active in* Hox/lacZ *transgenic embryos (Brend* et al.*, 2003; Charite* et al.*, 1995; Knittel* et al.*, 1995). Confirmed or putative Cdx binding motifs are boxed in green. The sequences, from Ensembl, are aligned by Clustal Omega. Asterisks show sequence identity.*

tained in culture. For example, a *Xenopus Xcad3* (*Cdx4*) construct electroporated into chick neural tube induced ectopic expression of *Hoxb4* (Bel-Vialar *et al.*, 2002). However, the electroporation procedure is itself technically demanding, poorly quantitative, and of low success rate.

Analysis of Hox gene promoters in cell culture

In attempt to find an alternative and more quantitative approach we have sought to obtain activation of Hox gene enhancers transfected into cultured cells. The enhancer fragments used are those which have already been reported in published studies to be expressed in a Hox-like pattern when tested in transgenic mouse embryos. Development of successful *in vitro* enhancer assays should facilitate future progress on the analysis of Hox gene regulations.

Candidate extracellular Hox activators such as Gdf11 can be tested simply by their addition to the culture medium after transfection of Hox enhancer/*luciferase* reporter plasmids (Gaunt *et al.*, 2013). However, to add candidate intracellular transcription factors it is necessary to co-transfect the cells with expression plasmid. A recent protocol (Gaunt, 2017) successfully demonstrated that the *Hoxc8* early enhancer (Shashikant and Ruddle, 1996) is activated in HepG2 cells by the combined (synergistic) action of Cdx proteins and Gdf11/Smad signalling. This protocol, which is also used in the current work, utilises the doxycycline-inducible Tet-On system (Clontech).

In the present study, it is shown that Cdx1 protein is able to activate embryo-active enhancers of *Hoxb4* (Brend *et al.*, 2003, Gilthorpe *et al.*, 2002), *Hoxb8* (Charite *et al.*, 1995) and *Hoxa7* (Knittel *et al.*, 1995) in HepG2 cells, though not in P19 mouse pluripotential (embryonal carcinoma) cells (McBurney, 1993). Cdx responsive motifs conserved from fish or *Xenopus* to mammals are identified in mutation studies.

Results and Discussion

Conserved Cdx binding motifs in Hoxb4, b8 *and* a7 *enhancers*

Cdx proteins bind optimally to the [A/T] [T] [A/T] [A] [T] [A/G] sequence motif, or its reverse complement (Margalit *et al.*, 1993). Fig. 1 shows such motifs found to be conserved from fish or *Xenopus*

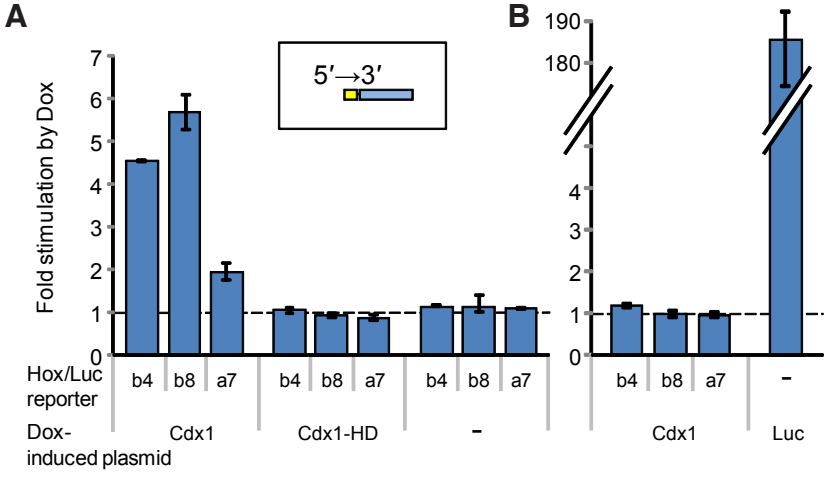


Fig. 2. Doxycycline-induced Cdx1 protein activates mouse *Hoxb4, b8* and *a7* enhancers transfected into HepG2 but not P19 cells. (A) *HepG2 Tet-On cells. All cultures were transfected with a* Hox/luciferase *reporter construct, either* Hoxb4, Hoxb8 *or* Hoxa7 *(insert: yellow box is* Hoxb4, b8, *or* a7 *enhancer; blue box is* SV40 *minimal promoter*/luciferase/SV40polyA). *Three bars at left show cultures co-transfected with doxycycline-inducible Cdx1 expression construct. Three bars in middle show cultures co-transfected with inducible Cdx1-minus-homeodomain expression construct. Three bars at right show cultures not given Cdx1 expression construct.* **(B)** *P19 Tet-On cells. Three bars at left show cultures co-transfected with* Hox/luciferase *reporter construct and doxycycline-inducible Cdx1 expression construct. Bar at right shows cultures transfected only with an inducible* luciferase *control vector. Data are plotted as fold stimulation relative to replicate cultures not given doxycycline (dotted baseline). Throughout, each bar shows average values for three replicate cultures, and range bars are shown. Dox, doxycycline; Luc, luciferase; Cdx1-HD, Cdx1 minus homeodomain.*

to mice within the embryo-active enhancers of *Hoxb4, b8* and *a7*. These sequences in the *Hoxb8* enhancer have already been identified as Cdx binding sites by electrophoretic mobility shift assays (Charite *et al.*, 1998). For *Hoxb8* (Charite *et al.*, 1998) and *Hoxa7* (Gaunt *et al.*, 2004) they have also been shown by mutation studies to regulate position of *Hox/lacZ* transgene expression boundaries in mouse embryos. However, it is not yet clear whether Cdx proteins operate alone, or must co-operate with other factors to activate the *Hoxb8* and *Hoxa7* enhancers. For *Hoxb4*, the conserved Cdx motifs shown in Fig. 1 have not, apparently, been previously noted.

Mouse Hoxb8, b4 *and* a7 *enhancer activation by Cdx1 in HepG2 but not P19 cells*

In preliminary studies (not shown), experimental cultures were co-transfected with Hox enhancer/*luciferase* reporter together with constitutive promoter/Cdx expression plasmids. Luciferase levels were compared with those from control cultures that received only the reporter plasmid. The comparison was difficult to interpret since experimental cultures acquired not only Cdx expression but also twice the overall amount of transfecting DNA. It was concluded that two cultures could only be validly compared if transfected with identical DNAs. To enable this, the doxycycline-inducible Tet-On system (Clontech) is used. Cultures to be compared receive replicate DNAs and differ only in whether or not they receive doxycycline. Data are expressed as 'fold stimulation' (luciferase value for doxycycline-induced cultures divided by value for replicate non- induced cultures). This gives results that are consistent between different experiments and DNA preparations.

Fig. 2A (three bars at left) shows activation of mouse *Hoxb4*, *b8* and *a7* enhancer/*luciferase* reporters in HepG2 Tet-On cells by Cdx1 protein produced from a *pTRE3G-Cdx1* doxycycline-inducible expression construct. As controls, doxycycline given in the presence of a *pTRE3G-Cdx1*-minus-homeobox construct (three bars in middle), or in absence of *pTRE3G-Cdx1* (three bars at right) does not activate the *Hox/luciferase* reporters in HepG2 Tet-On cells.

Fig. 2B, in contrast, shows that there is little or no activation of the Hox enhancer/*luciferase* reporters when Cdx1 is induced in P19 Tet-On cells. As a positive control, a transfected *pTRE3G-Luc* plasmid is abundantly activated in these cells, showing that they are fully able to host doxycycline-activation of *pTRE3G* vectors (Fig. 2B, right). The reason why P19 Tet-On cells fail to show Cdx1 activation of transfected Hox enhancers is not clear, and is currently under investigation. Possible explanations include lack of an essential co-factor or presence of an inhibitor. P19 pluripotential cells represent a cell type in embryogenesis (inner cell mass, about 3-4 days) that appears earlier in time than those that first express Cdx1 and Hox genes (about 7.5 days).

Mutations in Cdx binding motifs inhibit response to Cdx1

Functional transcription factor binding motifs within enhancers are commonly, though not always, conserved between species (Cho, 2012). Mutations were therefore introduced into the species-conserved (Fig. 1), putative Cdx binding motifs of the mouse Hox enhancers as shown in Fig. 3A. Fig. 3B shows how, for all three Hox enhancers, the mutations result in inhibitions of their responses to Cdx1 protein, suggesting that Cdx1 protein exerts a direct stimulatory effect via these motifs. The inhibitions are, however, incomplete, raising the following possibilities. First, Cdx1 protein might activate canonical binding motifs (Margalit *et al.*, 1993) which are not conserved between species but which are located in the mouse Hox enhancer DNAs outside the conserved regions shown in Fig. 3. Second, Cdx1 protein might activate non-canonical binding motifs in the Hox enhancers. Third, the possibility is not excluded of an indirect effect, where Cdx1 may induce some other transcription factor(s) which then, in turn, activate the Hox enhancers via alternative sequence motifs.

Hoxa7 response to Cdx1 is increased by multiple copies of the enhancer

The level of expression from an enhancer/promoter/reporter construct is typically increased, with retained tissue specificity,

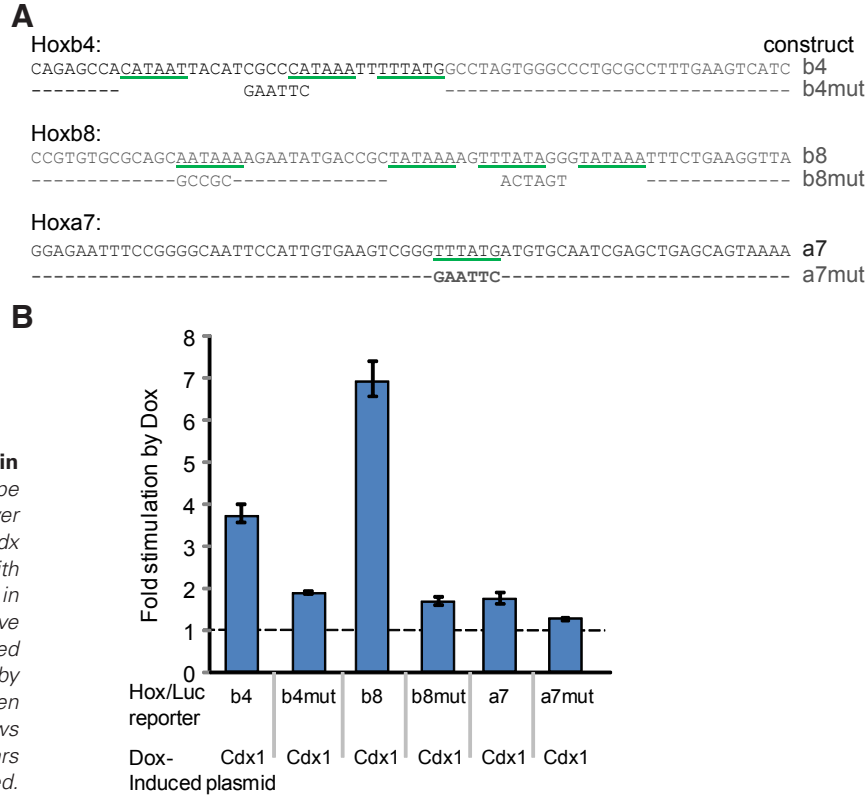


Fig. 3. Cdx1 stimulatory effects inhibited by mutations in Cdx binding motifs. (A) *For each mouse Hox gene, wild-type enhancer DNA (upper sequence) was mutated (mut; lower sequence) in its species-conserved (Fig. 1), putative Cdx binding motifs (green underline). Dashes indicate identity with wild-type sequence.* **(B)**. *Mutated constructs are inhibited in their response to doxycycline-induced Cdx1 protein relative to their corresponding wild-type constructs. Transfected plasmids are as indicated. Bars show fold stimulation by doxycycline relative to results for replicate cultures not given doxycycline (shown as dotted baseline). Each bar shows average values for three replicate cultures, and range bars are shown. Dox, doxycycline; Luc, luciferase; mut, mutated.*

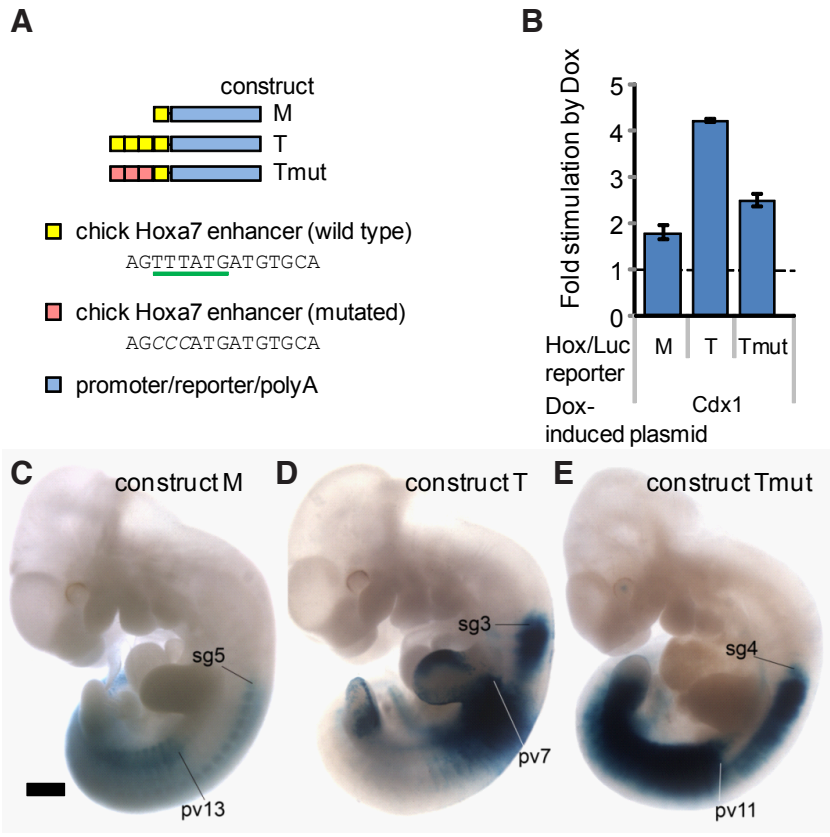


Fig. 4. *Hoxa7* response to Cdx1 is increased by multiple copies of the enhancer. (A) *Chick* Hoxa7 *reporter constructs. The promoter/reporter (blue bar) is either* SV40 *promoter/* luciferase *for cell transfection, or chick* Hoxa7 *promoter/*lacZ *for transgenic embryos (Gaunt* et al.*, 2004).* **(B)** *Expression of* luciferase *reporter constructs in HepG2 cells. Transfected plasmids are as indicated.* **(C-E)** *Expression of* lacZ *reporter constructs in 10.5 day transgenic mouse embryos. Four copies of the enhancer results in higher fold-stimulation by Cdx1 (Doxycycline) in HepG2 cells* **(B)**, *and more anterior embryonic expression* **(D)** *than does only one copy* **(B,C)**. *These effects are partially inhibited when three copies of the enhancer tetramer are mutated in their species-conserved (Fig. 1) Cdx binding motifs (green underline in A)* **(B,E)**. *Embryos shown in* **(C,D)** *are transient transgenic embryos derived independently from embryos shown previously (Gaunt* et al.*, 2004). Bars in (B) show fold stimulation by doxycycline relative to results for replicate cultures not given doxycycline (shown as dotted baseline). Abbreviations: Dox, doxycycline; Luc, luciferase; M, monomer; pv, prevertebra; sg, spinal ganglion; T, tetramer; Tmut, tetramer mutated in three copies. Bar, 0.5 mm.*

by incorporating multiple tandem copies of the enhancer element (Blain *et al.*, 2010, Wang *et al.*, 2008).

Cdx1 enhancer activation in HepG2 Tet-On cells is modest for mouse *Hoxa7*: up to about two-fold (Fig. 2A, 3B). It is now shown that a tetramer (T) of the chick *Hoxa7* enhancer provides a more substantial quantitative response to Cdx1 (doxycycline) than does monomer (M) (Fig. 4A,B), and much of this is overcome when three copies of the enhancer are mutated in their species-conserved (Fig. 1) Cdx binding motifs (Fig. 4B).

In earlier published work (Gaunt *et al.*, 2004) we compared these monomers (M) and tetramers (T) of the chick *Hoxa7* enhancer (Fig. 4A) in their ability to drive *lacZ* transgene expression in mouse embryos. Tetramer produces a forward shift in the anterior boundaries of lacZ expression (Fig. 4D) when compared with monomer (Fig. 4C). It may also shift forward the posterior boundaries, though this is not seen consistently in all independently-derived transgenic embryos: compare Fig. 4D with our earlier Fig. 2C (Gaunt *et al.*, 2004). Much, but not all, of the tetramer effect is overcome if three copies of the enhancer are mutated in their Cdx binding motif (Tmut) (Fig. 4A,E). The full tetramer effect therefore depends upon an overall increase in the number of Cdx binding motifs, both *in vitro* (Fig. 4B) and *in vivo* (Fig. 4 C-E).

Role of Cdx proteins in positioning of Hox gene expression boundaries

It is suggested that Cdx proteins provide activation, and are essential for opening the chromatin structure, of the central group of Hox genes (*Hox4* to *Hox9*) (Neijts *et al.*, 2017, Neijts and Deschamps, 2017). Cdx binding sites are reported within the central Hox cluster region but not around more posteriorly-expressed Hox genes. Anteriorly-expressed Hox genes (*Hox1* to *Hox3*) are activated independently of Cdx (Neijts *et al.*, 2017, Neijts and Deschamps, 2017).

It has been proposed that Cdx proteins in the vertebrate embryo may be instructive in the positioning of Hox gene expression boundaries (Bel-Vialar *et al.*, 2002, Charite *et al.*, 1998, Schyr *et al.*, 2012), and that this may be by their acting as graded morphogens (Gaunt *et al.*, 2004, Gaunt *et al.*, 2008). This is consistent with observations that Cdx proteins are expressed in posterior-to-anterior gradients along the tail-to-head axis (Gamer and Wright, 1993, Gaunt *et al.*, 2008, Marom *et al.*, 1997) and that, as indicated below, Hox expression boundaries are regulated by Cdx proteins in a dose-dependent way.

Dose-dependency in Hox activation by Cdx proteins is shown by the following. 1) Knockout of some or most Cdx gene activity results in posterior shifts in the anterior limits of Hox expressions in embryos (Subramanian *et al.*, 1995, van den Akker *et al.*, 2002). 2) Increased Cdx protein dosage causes forward shift in Hox expressions and homeotic activities in embryos (Gaunt *et al.*, 2008). 3) Increase in the number of enhancer elements in both *Hoxb8/lacZ* and *Hoxa7/lacZ* transgenes causes forward shifts in their embryo expressions, and this depends upon the increased number of Cdx binding motifs (Charite *et al.*, 1998, Gaunt *et al.*, 2004). In the latter report, forward shift in *Hoxa7/lacZ* expression is associated with an earlier time of initial expression (Gaunt *et al.*, 2004). Similarly, manipulating Cdx1 protein concentrations in *Xenopus* embryos can change the timing of *Hoxc8/lacZ* first expressions (Schyr *et al.*, 2012).

Hoxa7/lacZ and *Hoxb4/lacZ* expressions in transgenic mouse embryos show some caudal regression in mid-gestation stages (Brend *et al.*, 2003, Gaunt *et al.*, 2004). This coincides in time with caudal regression of the Cdx protein gradient (Bel-Vialar *et al.*, 2002, Gamer and Wright, 1993). A similar regression is not reported for expression of endogenous *Hoxb4* (Bel-Vialar *et al.*, 2002). This suggests a two-step mechanism: Cdx gradients may regulate the early position of a Hox expression boundary, and then

this is subsequently maintained by Cdx-independent mechanisms (Gaunt *et al.*, 2008, Schyr *et al.*, 2012), such as autoregulation in the case of *Hoxb4* (Gould *et al.*, 1997).

Concluding remarks

An inducible expression system in HepG2 cells allows Cdx1 activation of *Hoxb4, b8 and a7* enhancers that previously had only been shown to function in transgenic embryos. Compared with the embryo approach, the *in vitro* technique now described offers the relative advantages of requiring low cost and skill, of avoiding animal welfare issues, of allowing greater quantitation, and of potentially identifying enhancers active in both embryonic and adult tissues. However, it has the relative disadvantages of not specifically showing that an enhancer is embryo-active, and of not providing information about spatial patterns of embryonic expression. The *in vitro* approach cannot therefore fully replace *in vivo* work. However, it may provide a useful early screen for enhancer-active DNA regions that can subsequently be tested in transgenic embryos. It can also permit rapid deletion analyses to narrow-down enhancer fragment size. The essential binding motifs within an enhancer, once identified, can more easily be analysed by the *in vitro* approach. To maximise the numbers of Hox enhancers detectable, it will be of value to conduct screens on additional cell lines.

Materials and Methods

DNA constructs

Luciferase reporter constructs were prepared using enhancers known to regulate Hox-like patterns of reporter (lacZ) expression in transgenic mouse embryos. Each of the enhancers was inserted, in 5′ to 3′ orientation, upstream of the minimal *SV40* promoter and *luciferase* reporter gene in *pGL3-promoter* (Promega) (Fig. 2A insert). Mouse *Hoxb4* intron enhancer was the 1.4kb *Sal*I/*Bgl*II fragment (Brend *et al.*, 2003, Gilthorpe *et al.*, 2002). Mouse *Hoxb8* upstream enhancer was 555bp of DNA cloned by PCR using oligos GCGAAGGAAGTCCCAGTTTC (5′) and CCAGCTGCTAGCTTCTT-TAG (3′). This includes the essential EcMs79 fragment within the larger BH1100 fragment, both of which fragments regulate lacZ expression in at least part of the *Hoxb8*-like expression pattern (Charite *et al.*, 1998, Charite *et al.*, 1995). Mouse *Hoxa7* upstream enhancer was 469bp of DNA cloned by PCR using oligos CTATTTTAGAATTTTATTTCTC (5′) and AGGCCATGCTGGAAGACTGGCGAC (3′) (Knittel *et al.*, 1995). Various mutations were introduced by PCR into putative Cdx binding motifs of the Hox enhancers as shown in Fig. 3A. The chick *Hoxa7* enhancer (271bp) and its tetramers (Fig. 4A) were described earlier (Gaunt *et al.*, 2004). For luciferase assays these were inserted into the *pGL3-promoter* vector.

Cdx1 protein expression construct *(pTRE3G-Cdx1)* and a control derivative expressing Cdx1 protein without the homeodomain (*pTRE3G-Cdx1*-minus-homeobox), both inducible by doxycycline, were prepared in *pTRE3G-IRES* plasmid (Clontech) and were described earlier (Gaunt, 2017). A control plasmid *pTRE3G-Luc* (Clontech) was used to test the responsiveness of transgenic Tet-On cell lines to doxycycline.

Cell culture and luminometry

HepG2 is an established line of hepatocellular carcinoma (HCC) cells (Aden *et al.*, 1979). HCC cells, unlike normal liver cells, typically express a wide variety of Hox genes (Kanai *et al.*, 2010), though this is not specifically documented for HepG2 cells. HepG2 cells do not normally express Cdx genes (Gautier-Stein *et al.*, 2003), but do express Gdf11 receptors making them a useful model system for induction of posterior Hox genes (Gaunt, 2017, Gaunt *et al.*, 2013). The HepG2 Tet-On Advanced transgenic cell line (Clontech, cat. 631150) is designed for use with the doxycycline-inducible *pTRE3G* plasmids. A stable P19 Tet-On cell line was prepared by transfection of P19 cells (McBurney, 1993) (obtained from ATCL) with linearized *pCMV-Tet3G* plasmid (Clontech) followed by selection for a transgenic clone in 1.6 mg/ml G418.

Cell culture conditions, transfections using Lipofectamine 2000 (Invitrogen), treatment with doxycycline (10 μM), luciferase assays (Promega cat. E1500) and luminometry were all as described earlier (Gaunt, 2017, Gaunt and Paul, 2011), and were in accordance with manufacturers' instructions. Cultures were transfected for 5 hours, followed by change of medium with or without doxycycline. After a further 18 hours monolayers were rinsed in phosphate buffered saline then lysed for luminometry. As is usual in cell culture transfection assays, plasmids were transfected as closed circular DNA, including vector sequences. Plasmids for transfections were prepared using Sigma GenElute HP Plasmid Midiprep kits.

Each bar on each bar chart shows the mean value obtained from three replicate cultures (n=3). Range bars show the values obtained from the highest and lowest of these three biological replicates. Range bars are preferred to statistical error bars where n is small, including n=3 (Krzywinski and Altman, 2013).

Acknowledgements

I thank Rob Asher for provision of laboratory space, Adrian Kelly for use of the luminometer, and Debbie Drage for production of transgenic mice.

References

ADEN, D.P., FOGEL, A., PLOTKIN, S., DAMJANOV, I. and KNOWLES, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 282: 615-616.

BEL-VIALAR, S., ITASAKI, N. and KRUMLAUF, R. (2002). Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. *Development* 129: 5103-5115.

BLAIN, M., ZENG, Y., BENDJELLOUL, M., HALLAUER, P.L., KUMAR, A., HASTINGS, K.E., KARPATI, G., MASSIE, B. and GILBERT, R. (2010). Strong muscle-specific regulatory cassettes based on multiple copies of the human slow troponin I gene upstream enhancer. *Hum Gene Ther* 21: 127-134.

BREND, T., GILTHORPE, J., SUMMERBELL, D. and RIGBY, P.W. (2003). Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of Hoxb4 expression. *Development* 130: 2717-2728.

CHARITE, J., DE GRAAFF, W., CONSTEN, D., REIJNEN, M.J., KORVING, J. and DESCHAMPS, J. (1998). Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development* 125: 4349-4358.

CHARITE, J., DE GRAAFF, W., VOGELS, R., MEIJLINK, F. and DESCHAMPS, J. (1995). Regulation of the Hoxb-8 gene: synergism between multimerized cis-acting elements increases responsiveness to positional information. *Dev Biol* 171: 294-305.

CHO, K.W. (2012). Enhancers. *Wiley Interdiscip Rev Dev Biol* 1: 469-478.

GAMER, L.W. and WRIGHT, C.V. (1993). Murine Cdx-4 bears striking similarities to the *Drosophila* caudal gene in its homeodomain sequence and early expression pattern. *Mech Dev* 43: 71-81.

GAUNT, S.J. (2017). Gdf11/Smad signalling and Cdx proteins cooperate to activate the Hoxc8 early enhancer in HepG2 cells. *Int J Dev Biol* 61: 427-432.

GAUNT, S.J., COCKLEY, A. and DRAGE, D. (2004). Additional enhancer copies, with intact cdx binding sites, anteriorize Hoxa-7/lacZ expression in mouse embryos: evidence in keeping with an instructional cdx gradient. *Int J Dev Biol* 48: 613-622.

GAUNT, S.J., DRAGE, D. and TRUBSHAW, R.C. (2008). Increased Cdx protein dose effects upon axial patterning in transgenic lines of mice. *Development* 135: 2511-2520.

GAUNT, S.J., GEORGE, M. and PAUL, Y.L. (2013). Direct activation of a mouse Hoxd11 axial expression enhancer by Gdf11/Smad signalling. *Dev Biol* 383: 52-60.

GAUNT, S.J. and PAUL, Y.-L. (2011). Origins of Cdx1 regulatory elements suggest roles in vertebrate evolution. *Int J Dev Biol* 55: 93-98.

GAUNT, S.J. and PAUL, Y.-L. (2012). Changes in Cis-regulatory Elements during Morphological Evolution. *Biology* 1: 557-574.

GAUTIER-STEIN, A., DOMON-DELL, C., CALON, A., BADY, I., FREUND, J.N., MITHIEUX, G. and RAJAS, F. (2003). Differential regulation of the glucose-6-phosphatase TATA box by intestine-specific homeodomain proteins CDX1 and CDX2. *Nucleic Acids Res* 31: 5238-5246.

GILTHORPE, J., VANDROMME, M., BREND, T., GUTMAN, A., SUMMERBELL, D., TOTTY, N. and RIGBY, P.W. (2002). Spatially specific expression of Hoxb4 is dependent on the ubiquitous transcription factor NFY. *Development* 129: 3887-3899.

GOULD, A., MORRISON, A., SPROAT, G., WHITE, R.A. and KRUMLAUF, R. (1997). Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping Hox expression patterns. *Genes Dev* 11: 900-913.

KANAI, M., HAMADA, J., TAKADA, M., ASANO, T., MURAKAWA, K., TAKAHASHI, Y., MURAI, T., TADA, M., MIYAMOTO, M., KONDO, S. *et al.,* (2010). Aberrant expressions of HOX genes in colorectal and hepatocellular carcinomas. *Oncol Rep* 23: 843-851.

KNITTEL, T., KESSEL, M., KIM, M.H. and GRUSS, P. (1995). A conserved enhancer of the human and murine Hoxa-7 gene specifies the anterior boundary of expression during embryonal development. *Development* 121: 1077-1088.

KRZYWINSKI, M. and ALTMAN, N. (2013). Points of significance: error bars. *Nat Methods* 10: 921-922.

LONG, H.K., PRESCOTT, S.L. and WYSOCKA, J. (2016). Ever-Changing Landscapes: Transcriptional Enhancers in Development and Evolution. *Cell* 167: 1170-1187.

MARGALIT, Y., YARUS, S., SHAPIRA, E., GRUENBAUM, Y. and FAINSOD, A. (1993). Isolation and characterization of target sequences of the chicken CdxA homeobox gene. *Nucleic Acids Res* 21: 4915-4922.

MAROM, K., SHAPIRA, E. and FAINSOD, A. (1997). The chicken caudal genes establish an anterior-posterior gradient by partially overlapping temporal and spatial patterns of expression. *Mech Dev* 64: 41-52.

MARSHALL, H., STUDER, M., POPPERL, H., APARICIO, S., KUROIWA, A., BRENNER, S. and KRUMLAUF, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* 370: 567-571.

MCBURNEY, M.W. (1993). P19 embryonal carcinoma cells. *Int J Dev Biol* 37: 135-140.

NEIJTS, R., AMIN, S., VAN ROOIJEN, C. and DESCHAMPS, J. (2017). Cdx is crucial for the timing mechanism driving colinear Hox activation and defines a trunk segment in the Hox cluster topology. *Dev Biol* 422: 146-154.

NEIJTS, R. and DESCHAMPS, J. (2017). At the base of colinear Hox gene expression: cis-features and trans-factors orchestrating the initial phase of Hox cluster activation. *Dev Biol* 428: 293-299.

SCHYR, R.B., SHABTAI, Y., SHASHIKANT, C.S. and FAINSOD, A. (2012). Cdx1 is essential for the initiation of HoxC8 expression during early embryogenesis. *FASEB J* 26: 2674-2684.

SHARPE, J., NONCHEV, S., GOULD, A., WHITING, J. and KRUMLAUF, R. (1998). Selectivity, sharing and competitive interactions in the regulation of Hoxb genes. *EMBO J* 17: 1788-1798.

SHASHIKANT, C.S. and RUDDLE, F.H. (1996). Combinations of closely situated cis-acting elements determine tissue-specific patterns and anterior extent of early Hoxc8 expression. *Proc Natl Acad Sci USA* 93: 12364-12369.

SPITZ, F., GONZALEZ, F. and DUBOULE, D. (2003). A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell* 113: 405-417.

SUBRAMANIAN, V., MEYER, B.I. and GRUSS, P. (1995). Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* 83: 641-653.

TABARIES, S., LAPOINTE, J., BESCH, T., CARTER, M., WOOLLARD, J., TUGGLE, C.K. and JEANNOTTE, L. (2005). Cdx protein interaction with Hoxa5 regulatory sequences contributes to Hoxa5 regional expression along the axial skeleton. *Mol Cell Biol* 25: 1389-1401.

VAN DEN AKKER, E., FORLANI, S., CHAWENGSAKSOPHAK, K., DE GRAAFF, W., BECK, F., MEYER, B.I. and DESCHAMPS, J. (2002). Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* 129: 2181-2193.

WANG, B., LI, J., FU, F.H., CHEN, C., ZHU, X., ZHOU, L., JIANG, X. and XIAO, X. (2008). Construction and analysis of compact muscle-specific promoters for AAV vectors. *Gene Ther* 15: 1489-1499.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Gdf11/Smad signalling and Cdx proteins cooperate to activate the Hoxc8 early enhancer in HepG2 cells
Stephen J. Gaunt
Int. J. Dev. Biol. (2017) 61: 427-432
https://doi.org/10.1387/ijdb.170066sg

The significance of Hox gene collinearity
Stephen J. Gaunt
Int. J. Dev. Biol. (2015) 59: 159-170
https://doi.org/10.1387/ijdb.150223sg

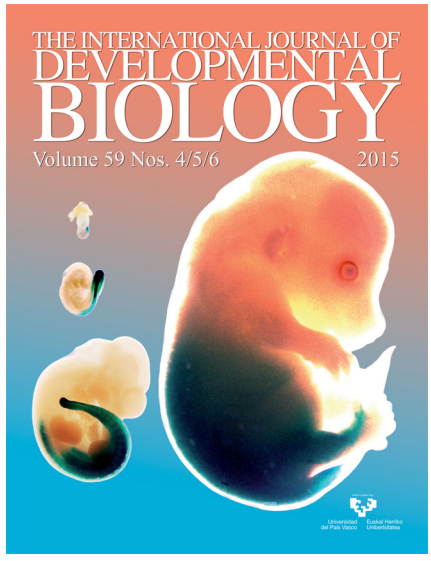
Synergistic action in P19 pluripotential cells of retinoic acid and Wnt3a on Cdx1 enhancer elements
Stephen J. Gaunt and Yu-Lee Paul
Int. J. Dev. Biol. (2014) 58: 307-314
https://doi.org/10.1387/ijdb.140003sg

Origins of Cdx1 regulatory elements suggest roles in vertebrate evolution
Stephen J. Gaunt and Yu-Lee Paul
Int. J. Dev. Biol. (2011) 55: 93-98
https://doi.org/10.1387/ijdb.103252sg

cdx4/lacZ and cdx2/lacZ protein gradients formed by decay during gastrulation in the mouse
Stephen J. Gaunt, Deborah Drage and Richard C. Trubshaw
Int. J. Dev. Biol. (2005) 49: 901-908
http://www.intjdevbiol.com/web/paper/052021sg

Additional enhancer copies, with intact cdx binding sites, anteriorize Hoxa-7/lacZ expression in mouse embryos: evidence in keeping with an instructional cdx gradient
Stephen J. Gaunt, Adam Cockley and Deborah Drage
Int. J. Dev. Biol. (2004) 48: 613-622
http://www.intjdevbiol.com/web/paper/041829sg

Initiation, establishment and maintenance of Hox gene expression patterns in the mouse.
J Deschamps, E van den Akker, S Forlani, W De Graaff, T Oosterveen, B Roelen and J Roelfsema
Int. J. Dev. Biol. (1999) 43: 635-650
http://www.intjdevbiol.com/web/paper/10668974



5 yr ISI Impact Factor (2016) = 2.421