Reporter Analyses Reveal Redundant Enhancers that Confer Robustness on *Cis*-Regulatory Mechanisms

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Abstract

Reporter analyses of *Hox1* and *Brachyury* (*Bra*) genes have revealed examples of redundant enhancers that provide regulatory robustness. Retinoic acid (RA) activates through an RA-response element the transcription of *Hox1* in the nerve cord of the ascidian *Ciona intestinalis*. We also found a weak RA-independent neural enhancer within the second intron of *Hox1*. The *Hox1* gene in the larvacean *Oikopleura dioica* is also expressed in the nerve cord. The *O. dioica* genome, however, does not contain the RA receptor-encoding gene, and the expression of *Hox1* has become independent of RA. We have found that the upstream sequence of the *O. dioica Hox1* was able to activate reporter gene expression in the nerve cord of the *C. intestinalis* embryo, suggesting that an RA-independent regulatory system in the nerve cord might be common in larvaceans and ascidians. This RA-independent redundant regulatory system may have facilitated the *Oikopleura* ancestor losing RA signaling without an apparent impact on *Hox1* expression domains. On the other hand, vertebrate *Bra* is expressed in the ventral mesoderm and notochord, whereas its ascidian ortholog is exclusively expressed in the notochord. Fibroblast growth factor (FGF) induces *Bra* in the ventral mesoderm in vertebrates, whereas it induces *Bra* in the notochord in ascidians. Disruption of the FGF signal does not completely silence *Bra* expression in ascidians, suggesting that

FGF-dependent and independent enhancers might comprise a redundant regulatory system in ascidians. The existence of redundant enhancers, therefore, provides regulatory robustness that may facilitate the acquisition of new expression domains.

Keywords

Reporter analysis
Retinoic acid
Hox1
Retinoic acid signaling
Brachyury
Fibroblast growth factor signaling
Ciona intestinalis
Larvacean *Oikopleura dioica*
Redundant enhancers
Shadow enhancers

7.1. Introduction

Reporter assay is a straightforward approach to analyzing transcriptional regulation. Embryos of urochordate ascidians provide a suitable experimental system for conducting reporter analyses. The compact genome of ascidians, with relatively short intergenic regions, facilitates the identification of *cis*-regulatory elements (Dehal et al. 2002). In addition, easy electroporation and rapid embryogenesis enable the production of hundreds of transgenic embryos within a single day (Corbo et al. 1997; Zeller 2004). A large number of embryos that can be obtained from a single electroporation procedure are sufficient to subdivide them into multiple groups that can be subjected to drug treatments at different doses during different developmental stages. Thus, we can compare patterns of reporter gene expression in a large number of drug-treated and control embryos from the same batch.

Another merit of transgenic technologies is the easy access to comparative studies of *cis*-regulatory mechanisms across animal taxa. Conservation of transcriptional regulatory mechanisms can be easily visualized by the activation of the enhancer in heterologous species. The similarities and differences of reporter gene expression patterns in different species may provide circumstantial evidence suggesting evolutionary events that might have contributed to the divergence of species.

In this review, we provide two examples in which reporter assays have facilitated the

identification of a robust *cis*-regulatory mechanism. First, we describe experiments in which we have identified response elements for retinoic acid (RA) within the regulatory regions of the *Hox1* gene in the ascidian *Ciona intestinalis*. Our reporter analyses identified RA-responsive and RA-independent mechanisms that activate *Hox1* expression in the nerve cord. Conservation and divergence were examined in two urochordate species, the ascidian *C. intestinalis* and the larvacean ~~Oikopleura dioica~~*Oikopleura dioica*. Second, we discuss the redundant regulatory pathways that activate notochord-specific expression of the *Brachyury* gene in ascidians in comparison to its vertebrate orthologs.

7.2. RA-Dependent Transcriptional Activation of *Hox1*

~~Reporter assay~~Retinoic acid regulates many aspects of chordate embryogenesis, mainly through transcriptional regulation of specific target genes (Mangelsdorf and Evans 1992). The nuclear RA receptor (RAR) is a transcription factor that recognizes specific DNA sequences, called the RA response element (RARE). Upon binding RA, RAR directly activates transcription of the target genes (Mangelsdorf and Evans 1992). The direct (primary) target genes include transcription factors, which in turn activate secondary target genes. The activation of the secondary targets depends on the translation of the direct targets, and is thus sensitive to translation inhibitors.

Genes orthologous to *RAR* have been identified in deuterostomes and lophotrochozoans (Cañestro et al. 2006; Albalat and Cañestro 2009). However, the contribution of RA to gene expression and organogenesis is still unclear in nonchordate animals. RA activates expression of the *Hox1* gene in the ascidian *Ciona intestinalis* (Ishibashi et al. 2003). The translation inhibitor, puromycin, does not affect the response of *Hox1* to RA (Ishibashi et al. 2005). This indicates that *Hox1* is a direct target, and that its enhancer contains RARE(s). Morpholino oligonucleotide-mediated knockdown of the RA synthase-encoding gene, *Raldh2*, suppressed the expression of *Hox1* (Imai et al. 2009; Sasakura et al. 2012). A *Hox1* null mutant did not form the atrial siphon placode, which in turn impaired the formation of atrial siphon muscle, body wall muscle and pharyngeal gill slits (Sasakura et al. 2012). Knockdown of *Raldh2* or *Hox1* resulted in similar phenotypes, suggesting that endogenous RA and *Hox1* might be required for organogenesis in ascidians (Sasakura et al. 2012).

7.2.1. Identification of RA Response Elements Within the Hox1 Enhancers

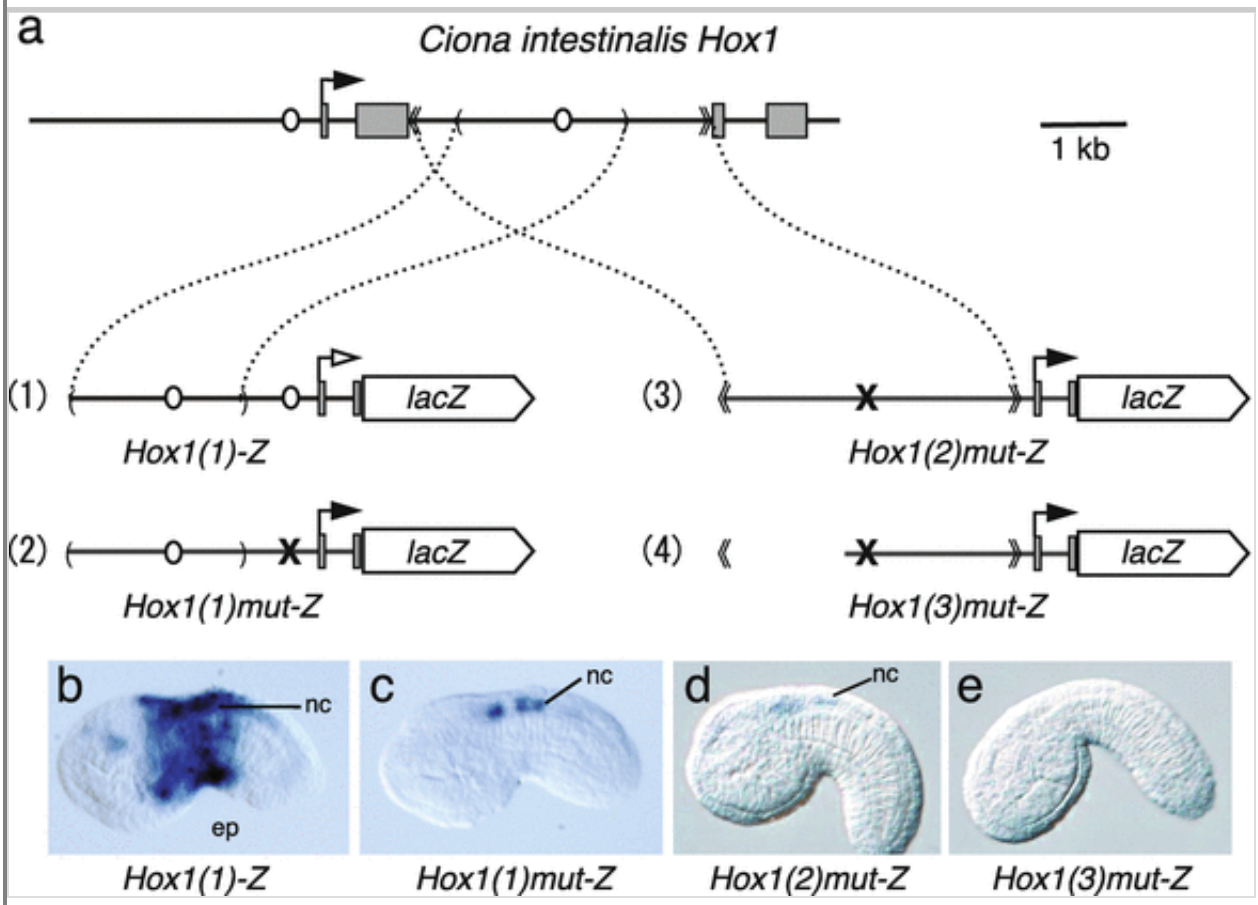
Ours and other reporter~~s'~~ analyses I would like to say that "Reporter analyses carried out

by ourselves and by others revealed that ...". revealed that the 5′ flanking region and the second intron contained enhancer elements required for transcriptional activation of *Hox1* in the epidermis and nerve cord respectively (Fig. 7.1) (Kanda et al. 2009, 2013; Natale et al. 2011). Both regions contain a single RARE (Fig. 7.1a) (Kanda et al. 2009, 2013). The binding of RAR to these RAREs was confirmed by electrophoretic mobility shift assays using in-vitro synthesized proteins (Kanda et al. 2009; Kanda, M. unpublished data). Disruption of the RARE sequence in the 5′ flanking region affected the enhancer activity only in the epidermis (Fig. 7.1b, c) (Kanda et al. 2009). On the other hand, transcriptional activation in the nerve cord required the RARE in the second intron (Fig. 7.1d, e) (Kanda et al. 2013). These results suggested that the tissue-specific expression of *Hox1* was not solely activated by RAR. Synergy between RAR and other tissue-specific transcription factors and/or the context of the enhancer sequences is necessary for activation in each tissue. The inhibitors of RA synthesis, citral and N,N-diethylaminobenzaldehyde, suppressed expression of the *Hox1* reporter genes in both tissues, supporting the requirement of RA for the transcriptional activation of the *Hox1* in normally developing embryos (Kanda et al. 2009).

Fig. 7.1

Identification of RA-responsive and RA-independent enhancer elements of the *C. intestinalis Hox1*. (**a**) Structure of the *Hox1* locus in the *C. intestinalis* genome is schematized at the top. *Boxes* indicate exons. The *arrow* indicates the transcription start site. *Circles* indicate RAREs. ~~(1)~~(1) A 1.9-kb central part of the second intron was placed upstream of the 0.9-kb 5′ flanking region of *Hox1*. The construct contains the first exon, first intron, and a part of the second exon. The *lacZ* translated region was placed downstream of the initiation codon of *Hox1* in the second exon. This construct was named *Hox1(1)-Z*. (2) The transgene *Hox1(1)mut-Z* carried a point mutation in the RARE within the 5′ flanking region of *Hox1(1)-Z*. The intronic RARE remained intact. (3) The transgene *Hox1(2)mut-Z* contained the entire second intron (3.6 kb), placed upstream of a 160-bp core promoter. *Hox1(2)mut-Z* contained a point mutation in the intronic RARE. (4) A 1.3-kb fragment of the second intron was deleted from *Hox1(2)mut-Z*. The construct was named *Hox1(3)mut-Z*. These constructs were described elsewhere (Kanda et al. 2009, 2013); however, they were renamed here for this review. (**b**–**e**) Expression of *lacZ* was visualized by in-situ hybridization in *C. intestinalis* tailbud embryos, carrying the reporter constructs (1) ~ (4) respectively. In all panels, the anterior side of the embryo is oriented to the left, with the dorsal side up. *ep* epidermis, *nc* nerve cord. (**b**) Embryo carrying *Hox1(1)-Z* expressed *lacZ* mRNA in the epidermis and nerve cord. (**c**) *Hox1(1)mut-Z* was activated only in the nerve cord. The epidermal expression was completely silenced by a mutation of the RARE within the 5′ flanking region. (**d**) *Hox1(2)mut-Z* was weakly activated in the nerve cord. (**e**) *Hox1(3)mut-Z*

was not activated in any tissue



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In the above experiments, we carried out deletion analyses of the intronic enhancer. For this purpose, we relocated the second intron upstream of the 5′ flanking region (Fig. 7.1a). We did not leave the intron in its original position because deletion of the intronic sequence would affect correct splicing. The relocated intronic enhancer drove reporter gene expression normally in the anterior nerve cord (Fig. 7.1b, c). This was not surprising, as many molecular biology dictionaries state that enhancers can stimulate gene transcription from far upstream or downstream of the transcription start site, and in both forward and reversed orientations. However, activities of relocated and/or reverse-oriented enhancers should be carefully evaluated, because some enhancers are position-dependent (Mocikat et al. 1993) or orientation-sensitive (Hozumi et al. 2013; Swamynathan and Piatigorsky 2002).

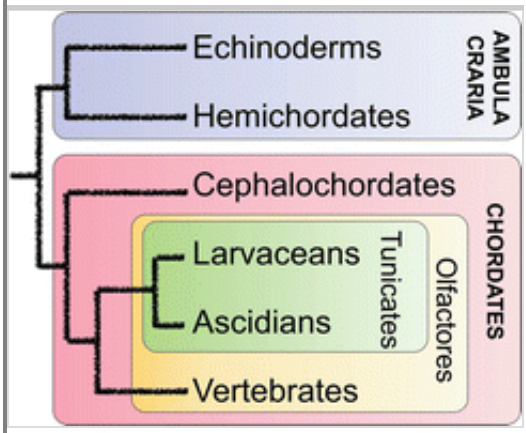
7.2.2. RA-Independent Expression of Hox1 in Larvaceans

In addition to the RA-driven *Hox1* expression in ascidians described above, RA also activates *Hox1* expression in the cephalochordate amphioxus (Schubert et al.

~~1995~~2005, ~~1996~~2006). An antagonist of RAR affected hindbrain patterning and pharyngeal morphogenesis, indicating the requirement of endogenous RA for normal cephalochordate embryogenesis (Schubert et al. ~~1995~~2005, ~~1996~~2006). These findings suggested an early origin of RA/*Hox1*-dependent embryogenesis in the common chordate ancestor, as cephalochordates are basally divergent within chordates, before the vertebrate/tunicate (also known as olfactores) split (Fig. 7.2) (Delsuc et al. 2006; Putnam et al. 2008).

Fig. 7.2

Phylogenetic relationships of major deuterostome groups, reconstructed from Putnam et al. (2008) with a slight modification. The tree topology was supported by Bayesian and maximum likelihood methods, based on a concentrated alignment of 1,090 genes (Putnam et al. 2008). Branch length is not considered in this figure

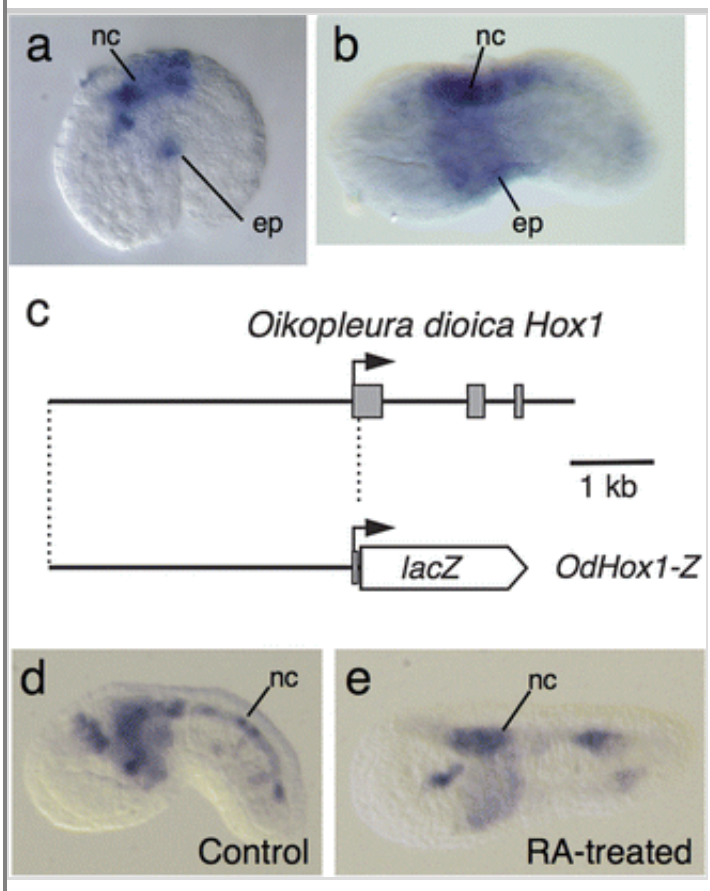


Larvaceans (appendicularians) are a sister group of ascidians within the tunicates (Fig. 7.2) (Tsagkogeorga et al. 2009). The pattern of *Hox1* expression in the larvacean ~~Oikopleura dioica~~*Oikopleura dioica* is similar to that in ascidians (Fig. 7.3a, b) (Cañestro et al. 2005). However, an excess or lack of the RA signal did not affect the expression of *O. dioica Hox1* (Cañestro and Postlethwait 2007). In fact, the *O. dioica* genome does not contain genes encoding RAR and none of the metabolic enzymes that regulate the synthesis and degradation of RA (Cañestro et al. 2006; Martí-Solans et al. 2016). This finding prompted us to test whether the enhancer of *O. dioica Hox1* was activated in *C. intestinalis* embryos, and in such a case, to test if this expression was responsive to RA.

Fig. 7.3

The 5′ upstream region of the *O. dioica Hox1* shows enhancer activity in the nerve cord of the *C. intestinalis* embryo. (**a**) Expression of endogenous *Hox1* mRNA in the

O. dioica tailbud embryo. (**b**) Expression of endogenous *Hox1* mRNA in the *C. intestinalis* tailbud embryo. (**c**) Structure of the *Hox1* locus in the *O. dioica* genome is indicated at the top. The reporter construct, *OdHox1-Z*, contained a 3.6-kb 5′ upstream region. The construct also contained the 5′ untranslated region (5′ UTR) and the initiation codon of the *O. dioica Hox1*. (**d**) Expression of *OdHox1-Z* in the nerve cord of the *C. intestinalis* embryo. This embryo was reared in artificial seawater containing 0.1% dimethylsulfoxide, which was used as a solvent for preparation of the concentrated stock solution of RA. A similar pattern of expression was also observed in untreated normal embryos. The result indicated that transcription factors in the *C. intestinalis* nerve cord can activate the upstream enhancer of the *O. dioica Hox1*. (**e**) Expression of *OdHox1-Z* in *C. intestinalis* embryo, treated with 1 μM RA. RA did not up-regulate the expression of *lacZ*, suggesting that RAR might not have been involved in transcriptional activation. In all panels, the anterior side of the embryo is oriented to the left, with the dorsal side up. *ep* epidermis, *nc* nerve cord



A 5′ flanking region of *O. dioica Hox1* was fused in-frame with the translated region of *lacZ* (Fig. 7.3c). This transgene was expressed in the nerve cord of the *C. intestinalis* tailbud embryo (Fig. 7.3d) (Kanda, M., Rodriguez-Marí, A., Tagawa, M., Cañestro, C., Fujiwara, S., unpublished data). Therefore, it is clear that the *O. dioica* upstream

enhancer can be activated by transcription factors expressed in the nerve cord of *C. intestinalis*. Treatment with RA did not up-regulate the enhancer activity (Fig. 7.3e), suggesting that the 5′ flanking region of *O. dioica Hox1* might not contain any functional RARE, but it still preserved conserved enhancers able to drive the expression in the nerve cord in ascidians.

7.2.3. Redundant Enhancers Confer Robustness on the Cis-Regulatory Mechanism

A close look at the activation of the *C. intestinalis Hox1* neural enhancer carrying a mutation at the intronic RARE showed that the intronic neural enhancer was not completely silenced by the mutation (Fig. 7.1d) (Kanda et al. 2013). Similarly, knockdown of *RAR* using morpholino ~~oligos~~oligonucleotides did not completely suppress the neural expression of endogenous *Hox1* (Kanda et al. 2013). By contrast, mutation at the 5′ RARE almost completely silenced the epidermal enhancer (Fig. 7.1c) (Kanda et al. 2009). These observations implied that RA-independent transcription factors contributed to the transcriptional activation of *Ciona Hox1* in the nerve cord, and that plausibly, these neural transcription factors might also be responsible for the activation of the *O. dioica Hox1* enhancer in the nerve cord of both *C. intestinalis and O. dioica* embryos. The enhancer element responsible for the RA-independent activation of *Ciona Hox1* was located within the second intron (Fig. 7.1a, e) (Kanda et al. 2013). The 5′ flanking region also activated reporter gene expression in the nerve cord at later larval stages (Natale et al. 2011). This may be a maintenance element that contributes to maintaining the activated state after the initial activation. However, it is also possible that this 5′ element is another genuine redundant neural enhancer. This 5′ neural enhancer may correspond to the *O. dioica* 5′ neural enhancer.

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Two or more parallel pathways contributing to the expression of *Hox1* in the same tissue may explain how the *Hox1* expression domain remained apparently unaltered in larvaceans, despite the loss of *RAR* and RA-metabolic genes during the evolution of this lineage (Albalat and Cañestro 2016; Martí-Solans et al. 2016). In the common chordate ancestor, these redundant enhancers may have activated the neural expression of *Hox1*. One is RA-dependent, and the other is RA-independent. When RA signaling was lost during the evolution of larvaceans, neural expression of *Hox1* was able to persist thanks to the robustness provided by the RA-independent enhancer. Recently, Yoshida et al. (2017) revealed that the expression of *Hox1* in the posterior endostyle of *C. intestinalis* juvenile adults also requires RA. Expression of *Hox1* was also observed in the endostyle of *O. dioica*, which was, however, not activated by RA

(Cañestro et al. 2008). It is therefore plausible that the expression of *Hox1* in the endostyle has also been regulated by redundant RA-dependent and RA-independent enhancers in the common tunicate ancestor.

Redundant enhancers are similar to shadow enhancers. In *Drosophila* embryos, gene expression in the neurogenic ectoderm is controlled by a set of transcription factors, Dorsal, Twist, and Snail (Ip et al. 1992). Hong et al. (2008) found that many of the Dorsal target genes contained two clusters of binding sites for these transcription factors. Either one of the clusters was able to activate reporter gene expression in the neurogenic ectoderm (Hong et al. 2008). In such cases, the promoter proximal cluster is called “primary enhancer” and the distal one is called “shadow enhancer” (Barolo 2011). Shadow enhancers provide the *cis*-regulatory mechanisms with the robustness required to keep a normal pattern of gene expression under some environmental pressure, such as high temperatures (Perry et al. 2010). Redundant enhancers found in the *Hox1*, however, differ from shadow/primary enhancers as the latter is composed of the same set of transcription factor-binding sites (Hong et al. 2008), whereas in the case of *Hox1* they are different. Shadow enhancers can retain normal gene expression upon a sudden loss of the primary enhancer. However, a loss of function of Dorsal or Twist may severely affect both enhancers. In contrast, each of the redundant enhancers may be activated by a distinct set of transcription factors. They provide a tough regulatory framework for normal gene expression, because neither deletion of one of the enhancers nor loss of function of a transcription factor causes a critical effect on normal gene expression, which utilizes redundant enhancers. In addition, redundant enhancers may provide an opportunity to acquire new expression domains, as described below.

7.3. Transcriptional Activation of *Brachyury* in the Notochord

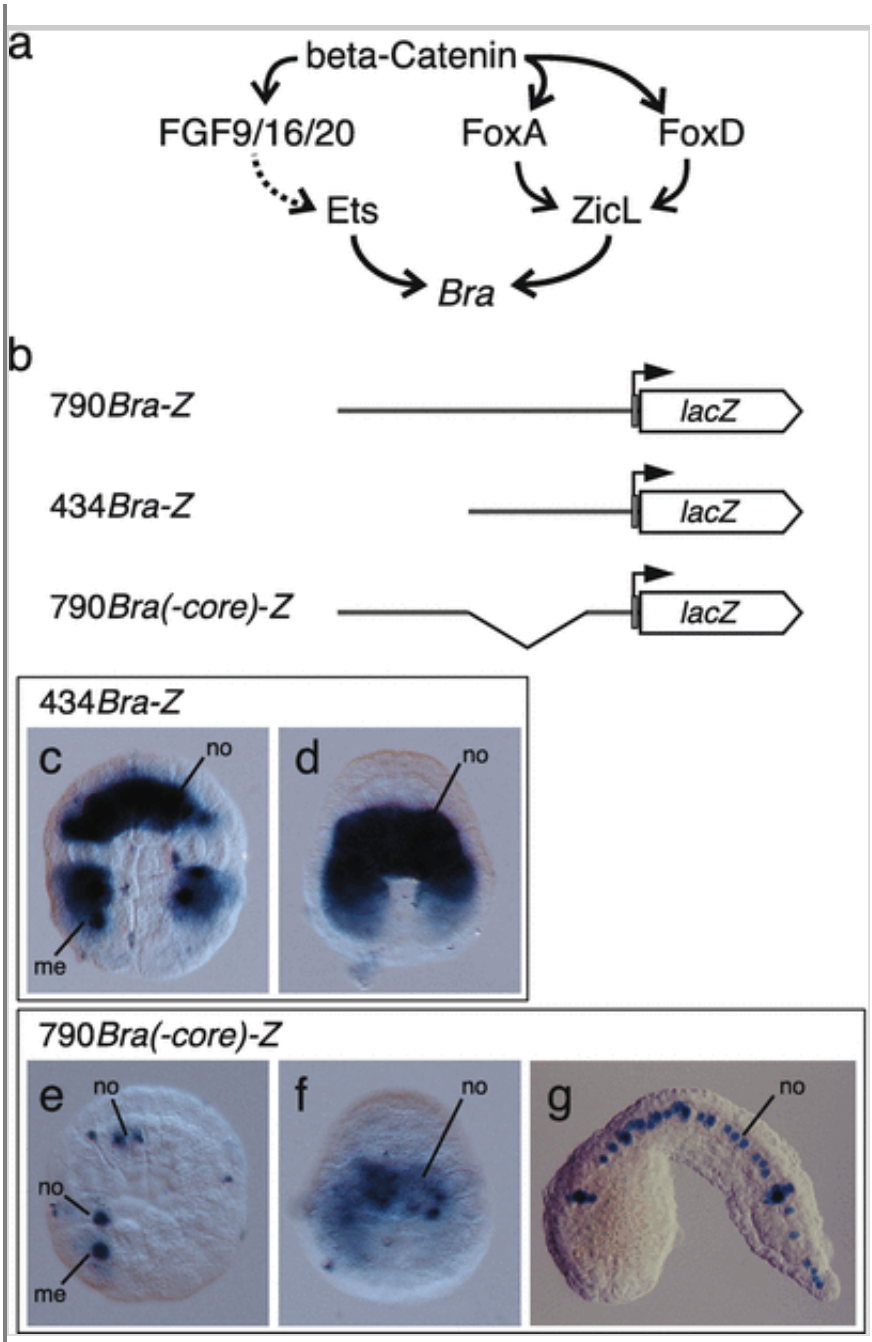
The *Brachyury* gene (*Bra*) in ascidians is expressed exclusively in the developing notochord (Yasuo and Satoh 1993). This pattern of expression is different from that of vertebrates, in which *Brachyury* genes are expressed in the notochord and ventral mesoderm (Herrmann and Kispert 1994). During ascidian gastrulation, fibroblast growth factor (FGF) activates *Bra* expression in the notochord (Nakatani et al. 1996; Imai et al. 2002a). Many transcription factors have been identified as responsible for the activation of *Bra* expression, including Suppressor-of-Hairless [Su(H)] (Corbo et al. 1998; Imai et al. 2002b), ZicL (Imai et al. 2002c; Yagi et al. 2004), and FoxA (Kumano et al. 2006).

7.3.1. Redundant Pathways Activate Bra Expression in Ascidians

Knockdown of *FGF9/16/20* (formerly called *FGF4/6/9*) severely affected initial activation of the *Brachyury* gene in another *Ciona* species, *C. savignyi* (Imai et al. 2002a). However, the FGF-depleted embryos finally expressed *Bra* in the notochord by the tailbud stage (Imai et al. 2002a). Similarly, suppression of the FGF signaling by a MEK inhibitor did not completely suppress the expression of *Bra* reporter genes in another ascidian species *Halocynthia roretzi* (Matsumoto et al. 2007). This was not surprising because the expression of ZicL and FoxA does not require the FGF signal (Fig. 7.4a) (Shimauchi et al. 1997; Imai et al. 2006). Nuclear localization of β-catenin activated the expression of the transcription factors FoxA and FoxD, which in turn activates the expression of ZicL (Fig. 7.4a) (Imai et al. 2002c, 2006). In contrast, the downstream effector of the FGF signaling is the transcription factor Ets (Fig. 7.4a; Miya and Nishida 2003; Matsumoto et al. 2007). These pathways independently activate the transcription of *Bra*.

Fig. 7.4

Activation of upstream enhancers of the *C. intestinalis Bra* gene. (**a**) Redundant pathways activating the transcription of *Bra* (Imai et al., 2006). *Dashed line* indicates multiple steps of the mitogen-activated protein kinase pathway. (**b**) The 790-bp 5′ flanking region was placed upstream of the *lacZ* translated region. The construct contained the 5′ UTR and 50-bp coding region of *Bra*. This construct was named 790*Bra-Z*. The construct, 434*Bra-Z*, contained 434-bp proximal enhancer. The region between –434 ~ –61 was deleted from 790*Bra-Z*. This construct was named 790*Bra(-core)-Z*. Corbo et al. (1997) constructed 790*Bra-Z* and 434*Bra-Z*, which were re-named here for this review. (**c**) 434*Bra-Z* was activated in the notochord lineage blastomeres (*no*) at the early gastrula stage. Expression of *lacZ* mRNA was also observed in the mesenchyme lineage cells (*me*). (**d**) 434*Bra-Z* was strongly expressed at the middle gastrula stage. (**e**) 790*Bra(-core)-Z* was weakly activated in the notochord lineage (*no*) and mesenchyme lineage (*me*). (**f**) The expression of 790*Bra(-core)-Z* was also observed at the middle gastrula stage. (**g**) In this tailbud embryo, the expression of 790*Bra(-core)-Z* was visualized by enzymatic histochemical staining of the β-galactosidase activity. The expression was observed in the notochord (~~no~~*no*)



The 434-bp upstream region of the *C. intestinalis Bra* gene activated reporter gene expression in the notochord cells (Corbo et al. 1997). This upstream enhancer drove detectable expression of *lacZ* mRNA in gastrulating embryos (Fig. 7.4b–d). The 434-bp upstream sequence contained the binding sites for ZicL, Su(H), and Ets (Yagi et al. 2004; Farley et al. 2015). When the region –434 ~ –61 was deleted from the 790-bp upstream sequence, the resultant upstream enhancer activated *lacZ* expression in the notochord lineage cells (Fig. 7.4b, e–g) (Farley et al. 2015). The distal region (–790 ~ –435) also contained the binding sites for ZicL and Ets; therefore, the distal enhancer is regarded as a shadow enhancer (Farley et al. 2015). In the 5′ flanking region of the *C. intestinalis Bra* gene, binding sites for Ets and ZicL are intermingled (Farley et al.

2015). On the other hand, three Ets-binding sites, two Fox-binding sites, and a single ZicL-binding site appeared to be separately located in the 5′ flanking region of the *H. roretzi Bra* gene (Matsumoto et al. 2007). In such a case, these binding sites may be independent, both functionally and structurally, and may be regarded as redundant enhancers.

7.3.2. Redundant Enhancers May Provide an Opportunity to Acquire New Expression Domains

Fibroblast growth factor is an important factor that induces mesoderm in amphibians (Amaya et al. 1991). The expression of *Bra* is activated by FGF in the amphibian ventral mesoderm (Smith et al. 1991). Latinkić et al. (1997) identified an upstream enhancer of ~~Xenopus laevis Brachyury~~*Xenopus laevis Brachyury* that was only activated in the ventral mesoderm, but not in the notochord. The results suggested that separate enhancer elements activated *Brachyury* expression in different mesodermal tissues. In zebrafish, ~~nodal~~Nodal activates the expression of *no tail* (a zebrafish homolog of *Bra*), mainly in the dorsal mesoderm, including the notochord (Harvey et al. 2010). On the other hand, bone morphogenetic proteins (BMPs) and Wnt activate *no tail* in the ventral mesoderm (Harvey et al. 2010). Two separate enhancer elements mediate the inputs from the ~~nodal~~Nodal and BMP/Wnt signaling pathways (Harvey et al. 2010).

Pan-mesodermal expression of the *Bra* ortholog is observed in amphioxus (Holland et al. 1995). Parsimonious speculation can assert that the ancestral *Bra* gene contained two separate enhancers. One was an FGF-responsive ventral mesoderm enhancer, and the other an FGF-independent notochord enhancer. During the evolution of ascidians, the role of FGF may have changed to induce the notochord. Thus, the FGF-responsive enhancer has become an additional notochord enhancer. The ascidian *Bra* contains two (at least partially) redundant notochord enhancer elements. Conversely, one of the redundant enhancers may acquire a new expression domain without compromising the original expression pattern. According to circumstances, redundant enhancers may provide a situation similar to a transitional state of neo-functionalization after the duplication of genomic DNA segments.

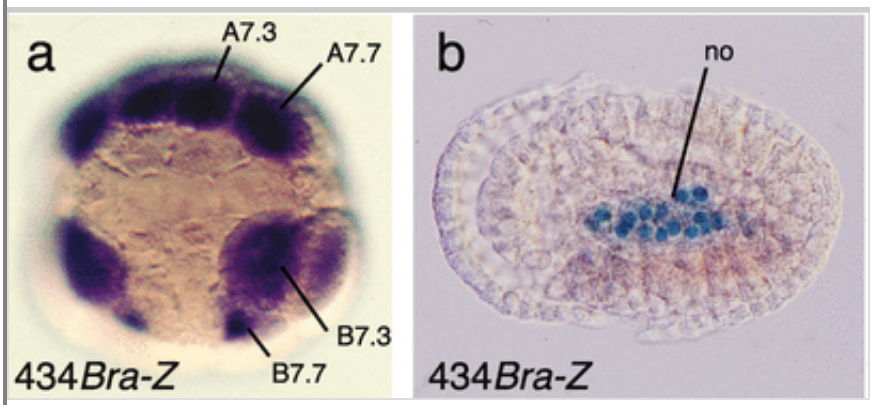
7.4. Lessons from the *Bra* Reporter Analyses (Technical Tips)

The reporter analysis appears as a powerful method to analyze the mechanism of transcriptional regulation; however, some weaknesses should be kept in mind. Widely used reporter genes, such as *lacZ* and *GFP*, are easily visualized by using their enzymatic activity or fluorescence. However, there is a time lag between the initial

activation of transcription and the emergence of their protein activity. This could not be overlooked when using ascidian embryos, as their embryogenesis is very rapid. For instance, the expression of the *Bra* mRNA starts at the 64-cell stage, whereas we are only able to detect the *β*-galactosidase activity of *Bra > lacZ* constructs about 2~3 h later at the late gastrula or early neurula stage (Fig. 7.5c) (Corbo et al. 1997). During this period, the notochord lineage cells divide two or three times. We can overcome the time lag problem by detecting the reporter gene expression by in situ hybridization. For instance, we can detect *lacZ* mRNA, driven by the *Bra* enhancer, at the 64-cell stage (Fig. 7.5b). With this early detection, we can be confident that the proximal *Bra* enhancer is not a maintenance element, but is responsible for initial activation.

Fig. 7.5

Initial activation of 434*Bra-Z*; early detection of *lacZ* mRNA and late emergence of the *β*-galactosidase activity. (**a**) Expression of *lacZ* mRNA in the 64-cell embryo, carrying 434*Bra-Z*. The embryo is oriented to show the vegetal hemisphere (future dorsal side), with the anterior to the top. As the ascidian embryos are bilaterally symmetrical, names of blastomeres are only indicated on the right side. The *lacZ* mRNA was observed in A7.3, A7.7, B7.3, and B7.7 blastomeres. A7.3 and A7.7 have already been determined to give rise to the notochord. The developmental fate of B7.3 is to be determined after the next cell division. B7.3 expressed both endogenous *Bra* and 434*Bra-Z*. The expression of the *Bra* mRNA was detectable only in the notochord lineage daughter cell (B8.6). In contrast, the *lacZ* mRNA was observed in many (if not all) of the daughter cells. B7.7 has already been determined to give rise to the mesenchyme. 434*Bra-Z* was expressed in B7.7, unlike endogenous *Bra* mRNA. This was because the vector contains a sequence that mimics mesenchyme enhancers (Robert W. Zeller, personal communications). (**b**) The neurula, carrying 434*Bra-Z*. The activity of *β*-galactosidase was observed in the differentiating notochord cells. The enzymatic activity could not be detected until the late gastrula stage (Corbo et al. 1997)



The detection of mRNA should still be carried out carefully because the *lacZ* or *GFP* mRNAs are not subject to post-transcriptional regulations, as are endogenous genes. The *C. intestinalis Bra* mRNA is expressed in B7.3 blastomeres at the 64-cell stage (Fig. 7.5b) (Corbo et al. 1997). B7.3 undergoes asymmetric cell division to produce B8.6 (notochord-lineage) and B8.5 (mesenchyme-lineage) daughter cells. The *Bra* mRNA is observed only in B8.6. There are several possibilities to explain how this occurs. (1) The *Bra* mRNA produced in B7.3 may be selectively degraded in B8.5. (2) The mRNA may be exclusively inherited by B8.6 during cell division. (3) The *Bra* mRNA has an extremely short half-life and soon disappears unless constantly produced. In this case, *Bra* mRNA is detectable only in B8.6, provided that only B8.6 continues to express *Bra*. The mRNA of *lacZ* can hardly recapitulate any of these regulations. Therefore, the *Bra* reporter genes are also expressed in the mesenchyme lineage blastomeres (Fig. 7.4c, e and Fig. 7.5a) (Corbo et al. 1997). Some aspects of the post-transcriptional regulations may be recapitulated by adding the 3′ untranslated region of the gene of interest to the translated region of reporter genes.

7.5. Conclusions

Reporter analysis is a powerful method for understanding the *cis*-regulatory network during embryogenesis. This method also allows cross-taxa experimental approaches to be made to empirically test for the conservation of *cis*-regulatory regions during the evolution of species, even from distant ones such as those between larvaceans and ascidians, which show poor sequence conservation in regulatory regions. Although developmental gene expression is regulated by diverse extracellular signals and maternally inherited factors, most of these inputs are concentrated on the enhancer elements. Reporter analyses revealed redundant enhancers that activate tissue-specific expression of developmental regulatory genes. Redundant enhancers are activated by a distinct set of transcription factors, but activate the transcription of genes in the same tissue. Redundant enhancers confer robustness on the *cis*-regulatory system, as mutation in either one of the enhancers or loss-of-function of one of the transcription factors does not severely affect normal gene expression. This provides a plausible explanation for the so-called “inverse paradox” of EvoDevo, in which apparently similar structures (phenotypic unity) show significant differences in the underlying genetic mechanisms that regulate their development (genetic diversity) (Cañestro et al. 2007). In addition, one of the redundant enhancers may also change where and/or when the regulated gene is activated, thereby contributing to the evolution of transcriptional regulatory networks.

Acknowledgments

We thank Chikako Imaizumi, Reiko Yoshida, Yutaka Satou, Megumi Koutsuka, and Kazuko Hirayama (NBRP) for the animals. As this review includes our unpublished results, we thank members of our group, Miyuki Kanda, Minami Tagawa, and Adriana Rodriguez-Marí. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to SF, and by the grant BFU2016-80601-P from the Ministerio de Economía y Competitividad (Spain) and SGR2014-290 from Generalitat de Catalunya to CC. Our collaborative research was supported by the Heiwa Nakajima Foundation. We also thank Zenji Imoto, Kouki Tanaka, and other members of the Usa Marine Biological Institute of Kochi University for maintenance of the aquarium.

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