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DNA variations within the sea urchin *Otx* gene enhancer

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Abstract **We performed both intra- and interspecific comparisons of the *Otx* gene in the sea urchin to investigate DNA variations within the enhancer elements. Intraspecific comparisons within *Hemicentrotus pulcherrimus* showed that indel variations were rare within the *Otx* enhancer, whereas SNP variations were found uniformly within the whole test region. A similar pattern of DNA variation was observed in comparisons between closely related species. On the other hand, both nucleotide substitution and indel variations were at high levels between distant species. Additionally, the regions corresponding to the *Otx* enhancer in two related species showed substantial activities during development. Our results suggest the possibility that a stabilizing selection has occurred during the evolution of the *Otx* gene enhancer in the sea urchin that maintains its expression pattern.**

Keywords: *cis*-Regulatory region; *Otx* gene; DNA variation; Indel; SNP; Sea urchin

1. Introduction

The gene regulatory networks (GRN) that are encoded by the genomes of different species comprise numerous *cis*-regulatory elements. These can be thought of as information processing units that receive multiple inputs in the form of the many transcription factors that bind to sites within them, and then generate output signals that control the expression of their target genes. The complex interactions between these *cis*-regulatory elements in the GRN control the morphogenetic processes that occur during development by generating a specific gene expression pattern in both space and time [1,2]. Hence, the analysis of the regions responsible for this control is particularly important for our further understanding of how the GRN functions during development.

One strategy to identify the *cis*-regulatory regions is to undertake interspecific sequence comparisons among related species [3]. This approach is based on the fact that the functional regions in a genome are more evolutionarily conserved relative to the non-functional regions. In the case of the sea urchin, the *cis*-regulatory regions have been inferred by the interspecific conservation between related species [4]. However, such analyses are not always efficient as much conservation can have been lost by extensive divergence in the rapidly evolving regions of the genome. Hence, for a more precise prediction of the *cis*-regulatory regions, it is necessary to also understand the mechanisms involved in evolutionary change by the use of information regarding the nature and dynamics of DNA variation. We have therefore focused our own efforts on intraspecific sequence comparisons that will assess the evolutionary changes within the *cis*-regulatory regions of a species in addition to performing interspecific sequence comparisons.

We have already shown that the orthodenticle-related protein (HpOtx) gene derived from sea urchin *Hemicentrotus pulcherrimus* encodes two distinct isoforms, HpOtxE and HpOtxL, which are differentially expressed and play important roles during the regulation of early development [5]. To investigate the regulatory mechanisms that control *HpOtx* gene expression, we have also previously described the structure of this gene [6], and identified the enhancer present in its first intronic region that is responsible for the temporal activation of *HpOtxL* transcription [7]. In our current study, we focus on the DNA variation and diversity that is present in this enhancer of the sea urchin *Otx* gene. To this end, we have analyzed single nucleotide polymorphisms (SNP), and insertions and deletions (indel) within the *H. pulcherrimus Otx* enhancer, and also nucleotide substitutions and indel levels between the related sea urchin species *Strongylocentrotus purpuratus* and *Lytechinus variegatus*. In addition, the enhancer activities of the corresponding regions of these related species were experimentally analyzed. Based on our present findings, we suggest the possibility that the *Otx* enhancer is functionally conserved by stabilizing selection events during the evolution of the sea urchin.

2. Materials and methods

2.1. Animals

Sea urchins (*H. pulcherrimus*) were harvested from the Seto Inland Sea, Japan.

2.2. Sequence analysis

Genomic DNA was extracted from the tube feet of *H. pulcherrimus* according to the method of Balhoff and Wray [8]. To amplify a 1.6 kb region of the *H. pulcherrimus Otx* (*HpOtx*) gene, PCR primers were designed based on previously determined sequence data (AB331398) [6] as follows: 5′-TGTTTGGCATGTAGCAGCCAGA-3′ within the first intron, and 5′-CCTTATGTGTGGCCGACGTAA-3′, which is located in the second exon. All PCR products were subcloned into the pBSK(−) vector (Stratagene, CA, USA) and sequenced using an ABI cycle sequencing kit (Applied Biosystems, CA, USA).

2.3. Data analysis

Twenty *HpOtx* sequences (AB331398–AB331417) and the *S. purpuratus Otx* (*SpOtx*) (AC131452) and *L. variegatus Otx* (*LvOtx*) (AC131493) sequences were used in the analyses. The DnaSP program

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[9] was used to analyze both intra- and interspecific variations via estimations of nucleotide diversity (π [10] and θ [11]). The averages of the SNP plus indel variations within species were measured by nucleotide diversity (π) and the number of indel differences (π). The length variations both between and within species were measured by the indel π as the average weighted number of indel differences between allele pairs [8], and indel θ as the weighted number of indels. In this study, each indel was counted as one difference plus the weight corresponding to the natural logarithm of the indel length, as beyond a certain length the local interactions between *cis*-regulatory regions will be so disrupted that different lengths will have similar effects [12]. The statistical significance of the differences in the nucleotide π and indel π values among the different parts of the test region was determined by the Tukey test using SPSS version 15.0.

2.4. Fusion gene constructs and luciferase reporter assay

The *pOtxL293-Luc* reporter construct, in which the *HpOtxL* promoter drives the firefly luciferase gene, has been previously described [7]. The genomic fragment corresponding to *HpOtx* gene, which includes the *Otx* enhancer, and the corresponding region of the *SpOtx* gene, were amplified from the subcloned *HpOtx* gene previously described by Kiyama et al. [6] and from genomic DNA extracts of *S. purpuratus* [provided by Takae Kiyama, The University of Texas, MD, Anderson Cancer Center, TX], respectively. A common primer set (5′-GGTGAATGACCATTTGAAAAGAAG-3′ and 5′-GATGAGTCCATTCAACTCATTC-3′) was used for the amplification of both genes. The corresponding region of the *LvOtx* gene was artificially synthesized based on sequence data (AC131493) and then amplified by PCR. The primer sequences used in this case were 5′-CCCGGGGGTGAATGACCGTCAGAAAAGAAG-3′ and 5′-GGTACCGTGATCTCGTCATTCATTCATTC-3′. These three genomic fragments from the related sea urchin species were subsequently inserted into *pOtxL293-Luc* within the multicloning region upstream of the *HpOtxL* promoter to generate *HpE-OtxL293-Luc, SpE-OtxL293-Luc* and *LvE-OtxL293-Luc*, respectively.

Introduction of the DNA reporter constructs into sea urchin embryos was carried out using a particle gun according to the method described previously [7]. Particle gun bombardment was performed three times for each construct. The bombarded embryos were then cultured in dishes and incubated at a constant temperature of 16 °C. Luciferase assay was measured at 24 h after fertilization, according to the manufacturer's protocol (Dual-luciferase Reporter Assay System, Promega, WI, USA).

3. Results and discussion

3.1. DNA variations within the enhancer sequence of the H. pulcherrimus Otx gene

We previously identified the enhancer element in the first intron of the *HpOtx* gene (Fig. 1A). To examine the DNA variations in this *Otx* enhancer, we isolated and sequenced a 1.6 kb genomic region that contained a portion of the first intron and second exon of the *HpOtx* gene from 19 individual sea urchin samples. Using these and previously determined *HpOtx* sequence data, we then performed intraspecific sequence comparisons. As shown in Fig. 1B, 87 SNPs and 20 indels could be detected within these 1.6 kb *HpOtx* regions. The SNPs were detected uniformly throughout this test region, whereas most of the indels were found within the 5′ flanking region of the *Otx* enhancer (5′ FRE). In one clone, a SNP within the coding sequence (CDS) that resulted in a stop codon was evident. The average of the SNP plus indel variations between allele pairs was determined to be 1.2% within this 1.6 kb region of the *HpOtx* gene.

We next estimated the level of SNP variation among different parts of our 1.6 kb test region of the *HpOtx* gene using nucleotide diversity as the measure of this (Table 1 and Fig. 2A). SNP variations within the *Otx* enhancer, the 3′ flanking region of the *Otx* enhancer (3′ FRE) and the CDS in the second exon of the gene were found to be at relatively low levels compared to the 5′ FRE and the untranslated region (UTR) in the second exon. However, by the use of sliding window analysis, no clear differences in the levels of SNP variations could be observed throughout the test regions (Fig. 2B). Only the region surrounding the Otx consensus sequence, which has been established as the *cis*-regulatory element within the *Otx* enhancer [7], exhibited any SNP variation, although this was at a low levels in this instance. In addition, no SNP variation was observed in the Otx consensus sequence (Fig. 1B) suggesting that a functional selection has occurred in the region surrounding the Otx consensus sequence within the *Otx* enhancer.

The polymorphism spectrum, as summarized by Tajima's *D* statistics [13], was then examined among the different regions of the *HpOtx* gene under study (Table 1). Tajima's *D* values were negative in all parts of our test region. We then performed sliding window analysis, and found that the 200 bp region in the middle of 5′ FRE and the *Otx* enhancer showed lower *D* values (Fig. 2C), suggesting the possibility that there are selective constraints in both regions.

Length variation was next evaluated among the different parts of our *HpOtx* gene (Table 1 and Fig. 2D). In these analyses, we measured the indel π, the average weighted number of indels between allele pairs that takes into account of the size of the indels, and the indel θ, the weighted number of sites containing an indel. In general, the level of the indel variation in the coding region would be lower than in the non-coding region since most indels in coding regions are considered deleterious. As expected, no indel variation was evident in the second exon of the *HpOtx* gene in our current analysis. Furthermore, within the *Otx* enhancer and 3′ FRE, the level of indel variation was found to be very low. On the other hand, however, by sliding window analysis, we observed a high level of indel variation that produced several peaks within the 5′ FRE region (Fig. 2E). These results suggested that there are evolutionary constraints upon the occurrence of indel variations within the *Otx* enhancer as well as within the exon regions. Consistent with this, it has been reported previously that indels are mostly absent in a functionally important region within the *Endo16* promoter of another sea urchin species, *S. purpuratus* [8]. In this *S. purpuratus Endo 16* promoter region, the binding sites for transcription factors are also well conserved within *S. purpuratus* [8]. Taken together therefore, our current results and earlier findings suggest the possibility that indels may impair the maintenance of the spacing between specific transcription factor binding sites in *cis*-regulatory regions.

3.2. Divergence of the Otx enhancer among related species

Our 1.6 kb test region of the *HpOtx* gene was compared with the corresponding *Otx* gene regions in the closely related species *S. purpuratus* (*SpOtx*) and in the more distant species *L. variegatus* (*LvOtx*) (Fig. 3). The nucleotide substitution and indel levels were estimated between 20 *HpOtx* sequences and one sequence each for *SpOtx* and *LvOtx*. In the *H. pulcherrimus–S. purpuratus* comparison, nucleotide substitutions were found to be present in whole test region with the highest levels observed in the 5′ FRE (Fig. 3A). The level of indel variation was seen to be very low in the *Otx* enhancer regions as observed also in our intraspecific comparisons (Fig. 3C). Sliding window analyses further showed that the patterns of nucleotide substitution