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Distinct evolutionary rate in the eye field transcription factors found by estimation of ancestral protein structure

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ABSTRACT

Eye-field transcription factors (EFTFs) are a set of genes that compose a regulatory network for eye development in animals, which are highly conserved among various animal phyla. To investigate the processes of conservation and diversification of the transcription factors for eye development, we examined the structural changes in the EFTF proteins by estimating the ancestral sequences with the available genome information. Among the different types of EFTFs, we selected otx2, tbx3, rx1, pax6, six3/6, lhx2 and nr2e1 because they are highly conserved in bilaterian animals. We searched the genome sequences of representative animal phyla for EFTF protein sequences. With deduced ancestral sequences and three-dimensional structures of EFTFs, we traced the evolutionary changes in amino acid residues and found that the DNA-binding domains were always more conserved than other regions, and that the other regions showed distinct evolutionary rates. The EFTF rx1, which resides at the pivotal part of the EFTF network, had a faster evolutionary rate than the others. These results indicated that the evolutionary rates of each protein in the EFTF network, which were expected to be consistent with each other to maintain the interactions in the network, were not constant among or within the factors, but rather, varied to a significant extent.

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1. Introduction

Organisms with eyes appear sporadically on the tree of life, which suggests that the evolution of eyes occurred many times independently in various animals (Salvini-Plawen and Mayr, 1977). The differences in the morphology of eye types support the independent origins of those eyes. However, the acquisition of the eye might have occurred once in Cnidaria, which is one of the most primitive groups of animals that possess eyes, and for which the basic pattern for eye formation and development was established (Halder et al., 1995; Piatigorsky, 2003). Therefore, the evolution of the eye was a single event that originated from a prototype eye in an ancestral species. The present diversity of eye types was the result of modifications from the prototype eyes via differential activation of genes and changes in their regulatory mechanisms (Gehring and Ikeo, 1999; Rivera et al., 2010, Datta et al., 2011). The molecular evolution of the eye can be resolved once the fundamental genes for animal eye formation and a common type of photoreceptor cell are discovered in animals (Halder et al., 1995).

Eye-field transcription factors (EFTFs) are a set of genes (here, we considered the EFTFs otx2, tbx3, rx1, pax6, six3, six6, lhx2, and nr2e1) that are involved in the morphogenesis of eyes and that are extensively studied in frogs and mice (Oliver et al., 1995; Zuber, 2003). The EFTFs are synchronously expressed in the eye-field during its specification and form a transcription regulation cascade (Zuber, 2003; Sernagor et al., 2006). Previous studies characterized the transcription network among EFTFs in frogs and proposed a model for the interactions during eye-field specification (Fig. 1) (Zuber, 2003; Sernagor et al., 2006). These studies showed that the neural plate, which is formed in response to neural inducers such as noggin, generated the regions that developed into the eyes. A transcription factor, otx2 with a homeodomain, was required for forebrain and midbrain specifications. The early-expressed EFTFs, i.e., tbx3, rx1, pax6, six3, and lhx2 (the latter four contain a homeodomain DNA-binding region) in coordination specify the eye-field within the presumptive forebrain (Mathers et al., 1997; Kawakami et al., 2000; Gehring and Ikeo, 1999; Wilson, 2002; Pan et al., 2006; Srivastava et al., 2010). The EFTFs nr2e1, with a zinc-finger motif, and six6, with a homeodomain, play a role in the late specification of eye formation (Sernagor et al., 2006; Kawakami et al., 2000; Cheng et al., 2008). The EFTFs are also required for the induction of functional eyes at inauthentic locations (Mathers et al., 1997; Wawersik and Maas, 2000; Tucker et al., 2001).

Abbreviations: EFTF, eye field transcription factors; Md, the ratio of residue substitution in DNA-binding domains; Mn, the ratio of residue substitution in non-DNA-binding domains; PDB, Protein Data Bank.

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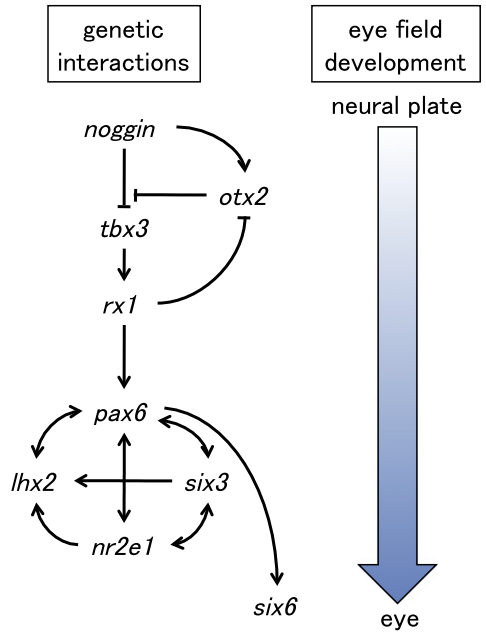

Fig. 1. The EFTF network. The proteins in the top half of the network (upper-stream) are involved in the spatiotemporal development of the eyes and the proteins in the bottom half (lower-stream) are involved in the development of the components of the eyes.

These lines of evidence suggest that EFTFs are candidates for being the fundamental genes of animal eye formation. Moreover, the regulatory network of EFTFs originated in the first organism with eyes. However, homologues for EFTFs are only reported in model organisms, such as *Drosophila melanogaster*, *Xenopus laevis*, *Oryzias latipes* and *Homo sapiens* (Zuber, 2003; Mathers et al., 1997; Wawersik and Maas, 2000; Quiring et al., 1994; Seo et al., 1998), and homologues in invertebrates are rarely reported. Examination of the distribution of the homologues among the animal phyla could be a source of information to address the evolution of the EFTF network. The existence of plausible orthologues of the set of known EFTF genes provides a clue to the foundation of the network, and the examination of the evolutionary rate of the proteins provides a clue to the stability of the interactions in the network. Unveiling these pieces of information will help to reveal the possible evolutionary path of the eye.

In this study, we addressed how the EFTFs and their network developed and evolved in animals, and we examined the evolution of EFTF protein structures and the EFTF network in various animal phyla. We searched for homologous proteins in animal genomes and tabulated the existence of EFTFs in different phyla. Computational analyses were performed on evolutionary rates and three-dimensional (3D) structures of EFTF proteins to trace the evolution of the EFTF network. We found that (i) the DNA-binding domains of the EFTFs were well conserved, and that the nonDNA-binding regions that interacted with other molecules often contained highly diversified residues, and that (ii) the evolutionary rates differed in an upper-stream and a lower-stream within the EFTF network. The results suggest that EFTFs emerged as a combination of, at least, two modules of networks, which managed a different set of roles in the development of the eye.

2. Results

2.1. The orthologue profile of EFTFs in various animal phyla

The EFTF sequences in different animal phyla are summarized in Table 1 and represent the orthologue profile. The orthologue profile showed that there were organisms without a complete set of EFTF genes. Nemertea was the phylum with the least EFTFs, with only the pax6 gene, although it has rhabdomeric and subepidermally situated eyes. This apparent inconsistency in the relation between the existence of the EFTF network and the eyes suggested that the eye formation in this group was regulated by a different set of genes, or that the genome sequencing was incomplete. By contrast, some invertebrate phyla without eyes, such as Placozoa, did have orthologous genes for part of the EFTF network. This suggested that the entire set of EFTFs was required for vertebrate eye formation.

The profile also showed that the duplication of six3/6 happened at the diversification of the vertebrates, and that this event evidently led to the completion of the EFTF gene set, which confirmed the results of Kawakami et al. (2000). The complete set of EFTF genes was established in the common ancestor of vertebrates. We, hereafter, lumped six3 and six6 as six3/6 in the current analyses.

2.2. Sequence conservation of the DNA-binding domain and diversification of the nonDNA-binding domain

With the sequences in the orthologue profile and a topology tree of the species, we inferred the ancestral sequences of each node of the tree, and we traced amino acid changes in the proteins of each site. The trace showed that the ratio of residue substitution in DNA-binding domains (*Md*) was almost always lower than that in nonDNA-binding domains (*Mn*) in every branch of the phylogenetic tree of EFTF proteins (Suppl. Table 1). For example, the *Mn* of Pax6 was five-fold higher than the *Md* of Pax6. To understand the balance between *Md* and *Mn* rates in animals, we calculated the mean of *Mn*/*Md* for all EFTFs for each branch on the tree and depicted the value in log-scaled color (Fig. 2). In Fig. 2, the deeper color of the branch indicates a larger *Mn*/*Md* ratio, and when the branch is white, *Mn* and *Md* are equal. After the bilaterian split, at the branch of deuterostomes, *Mn* was far higher than *Md* (Fig. 2). The *Mn*/*Md* of the vertebrate branch was not the highest, but the substitution rates of the nonDNA-binding domains accelerated before the divergence of vertebrates and the urochordata.

The differences in the substitution rates of DNA-binding and nonDNA-binding domains were substantiated with 3D protein structures. We obtained a reasonable model structure for each ancestral EFTF protein domain and pbx1, a reference protein, and located the functionally critical substitutions of amino acids in three dimensions. The mapping of the residue-wise substitution rates of EFTF proteins in 3D structures further emphasized the skewed distribution of the highly substituted residues in the different domains (Fig. 3, the other seven results are shown in Supplemental Fig. 2). The substituted sites were spatially clustered in nonDNA-binding domains in all EFTF proteins (red in Fig. 3), whereas the DNA-binding domains were well-conserved (blue in Fig. 3). The difference in substitution rates was evident particularly for nr2e1 in which the DNA-binding domain was more conserved than the activation (nonDNA-binding) domain.

2.3. Distinct substitution rate on the DNA-binding domain of Rx1

The comparison of *Mn* and *Md* among EFTFs showed notable differences (Fig. 4). The box plot of Mns showed a slight decrease from otx2 to six3/6 in the distribution range (Fig. 4a), though statistical significance was not found. The box plot of *Mds* showed a significant difference in the distributions ($p < 4.4 \times 10^{-5}$ by the Kruskal Wallis test), particularly of rx1 (Fig. 4b). Hence, the DNA-binding domain of rx1 experienced a significantly accelerated evolution. When the set of proteins in an EFTF network were divided into two at rx1, one group contained otx2, tbx3 and rx1, which corresponded to a subset of proteins in the upper-stream of the EFTF network, and the other group contained pax6, lhx2, nr2e1 and six3/6, which corresponded to a subset of proteins in the lower-stream of the network. The former was a group

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