The Early ANTP Gene Repertoire: Insights from the Placozoan Genome

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Abstract

The evolution of ANTP genes in the Metazoa has been the subject of conflicting hypotheses derived from full or partial gene sequences and genomic organization in higher animals. Whole genome sequences have recently filled in some crucial gaps for the basal metazoan phyla Cnidaria and Porifera. Here we analyze the complete genome of *Trichoplax adhaerens*, representing the basal metazoan phylum Placozoa, for its set of ANTP class genes. The *Trichoplax* genome encodes representatives of Hox/ParaHox-like, NKL, and extended Hox genes. This repertoire possibly mirrors the condition of a hypothetical cnidarian-bilaterian ancestor. The evolution of the cnidarian and bilaterian ANTP gene repertoires can be deduced by a limited number of cis-duplications of NKL and “extended Hox” genes and the presence of a single ancestral “ProtoHox” gene.

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Introduction

Transcription factors of the homeodomain family fulfill important roles during development, cell differentiation and cell proliferation in animals, plants, and fungi [1,2]. They are characterized by the presence of a conserved DNA binding motif - the 60 amino acids of the homeobox [3]. In the Metazoa they can be mainly divided into the superfamilies ANTP, PRD, POU, LIM, SIX and TALE, most of which seem to be restricted to the animal kingdom [1,2].

By far the highest gene diversity can be observed in the ANTP superclass which seems to have been a key factor for the evolution of metazoan bauplans (reviewed in [4]), fueling the radiation of metazoan phyla and regulating the development of body axes, symmetry, and the nervous system. The ANTP superclass consists of the Hox/ParaHox, extended Hox, and NKL genes [4,5] which fund the instrumentation. Genome sequencing was performed under the auspices of the US Department of Energy’s Office of Science, Biological and Environmental Research Program, and supported by the University of California, Lawrence Livermore National Laboratory, Lawrence Berkeley National Laboratory, and Los Alamos National Laboratory. The Center for Integrative Genomics is supported by a grant from the Gordon and Betty Moore Foundation.

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The genome of *Trichoplax adhaerens*, representing the early-branishing metazoan phylum Placozoa, can give valuable insights into the origin of the ANTP superclass even though its specific phylogenetic position is still debated: While analyses of the mitochondrial genome place the phylum Placozoa at the root of Metazoa [17], genes from the nuclear genome support the divergence of Placozoa from other animals after the divergence of sponges but before (or close to) the cnidarian-bilaterian split [18]. A previous analysis of the ANTP gene content in *Trichoplax adhaerens* suggested that the early bifurcation of bilaterians and cnidarians might not represent the most ancestral ANTP gene repertoire and may rather lie in true NKL genes because the sponge *Amphimedon* lacks any Hox/ParaHox-like or extended Hox genes but possesses several clustered NKL genes [16].
revealed a surprisingly low diversity [19]. With the draft genome of Trichoplax in hand [18] we are now able to investigate its ANTP and homeobox gene repertoire in detail. Our analysis of the placozoan genome revealed a greater diversity of ANTP class genes than found in sponges, but less gene complexity than found in other metazoans. The data are consistent with either gene loss in the Porifera from an ancestral metazoan state resembling other metazoans. The data are consistent with either gene loss genes than found in sponges, but less gene complexity than found in other metazoans. The data are consistent with either gene loss in the Porifera from an ancestral metazoan state resembling other metazoans. The data are consistent with either gene loss genes than found in sponges, but less gene complexity than found in other metazoans.

**Results and Discussion**

Seven homeobox classes comprised of 37 homeobox genes (Table 1, Table S1), including 14 from the ANTP superclass, were identified in the genome of Trichoplax adhaerens [18]. Phylogenetic analyses were conducted to infer relationships of these 14 genes to known ANTP families. Bayesian Inference and Maximum Likelihood analyses identify robust grouping for 11 of the 14 genes, representing a primitive eumetazoan branch. These findings have strong implications for understanding the genomic complexity of the earliest animals and the genetic mechanisms underlying the radiation of metazoan body plans.

Comparing the homeobox gene composition of Trichoplax to genomes of the poriferan Amphimedon and the cnidarian Nematostella reveals that the Placozoa possess significantly less homeobox diversity than Cnidaria but a similar diversity as found in Porifera (Table 1). While no significant differences in diversity are found for PRD, POU, LIM, SIX, and TALE class genes, the most striking difference is the absence of any extended Hox or any Hox/ParaHox-like genes in the Porifera (as represented by Amphimedon [16]). Furthermore, Amphimedon has the smallest ANTP-class gene repertoire with 0 (all NK related) genes [16], while Trichoplax harbors 14 and Nematostella close to 80 ANTP class genes [13]. Nearly all ANTP genes in Trichoplax adhaerens show a clear relationship to known homologs from bilaterians (Figure S1 & S2) and are organized in two tight NK clusters, with the exception of the unclustered extended Hox genes and the Gsx type gene, Trox-2 (Figure 1).

**Table 1.** The homeobox gene complement of Trichoplax adhaerens shows a slightly higher diversity of the homeobox classes ANTP, PRD and LIM.

| ANTP | Nematostella | Amphimedon |
|------|--------------|-------------|
| 14   | 78           | 8           |

| NKL | Ext. Hox | NKL | Ext. Hox |
|-----|----------|-----|----------|
| NK2, NK5, NK6, Hex, Dlx, Dbx/Hlx+2 NK-related genes | Not, Mnx | all bilaterian families except Tlx | NK2(2/3), NK6, Mnx, BarH-related, Ext. Hox-related, NK-related |
| Gsx+1 gene with some affinity to ext. Hox | Gsx, anterior Hox, posterior Hox/Cdx-like+several genes with unclear relation to bilaterian Hox/Parahox genes | - | - |
| 9 (Arx, Ebx/Arx-like, Pax3-like, PaxB, Prd/Parahox-like, Pitx, Gsc, Otpr) | 33 (PaxA, B, C, Pax6-like, Arx, Rx, Pitx, Otpr, Gsc & several unassigned) | 9 (Arx, PaxB, Rx, OG12) | |
| 2 (Pou3, 4) | 5 (Pou1, 3, 4, 6) | 4 (Pou1, 6, 2–5) | |
| 5 (Lim1, 1/5, 3/4, 2/9, Isf) | 6 (Lim1, 2, 3/4 Awh, Isf) | 3 (LIM3, Lin-11, Isf) | |
| 2 (Six1/2, Six3/6) | 5 (Six2, 3, 4, 4/5) | 1 (Six1/2) | |
| 4 (Pbx/BFC, Pknx, Lnx, Meis) | 7 (Pbc, Tgf, Meis, Lnx) | 6 (Meis, Pbc, Lnx) | |
| 1 | 1 | - | |
| 37 | 134 | 31 | |

Ref. This study [13] [16]

Orthology assignment of the Trichoplax homeobox genes is based upon BLAST hits in the case of non-ANTP genes. ANTP genes have been classified according to phylogenetic analyses shown in Figure S1 & S2.

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gene which founded the Hox/ParaHox genes, although we cannot completely exclude the possibility that *Trichoplax adhaerens*, which harbors several clustered NKL genes, two genes of the extended Hox and a single Hox/ParaHox-like gene which is a true Gsx ortholog and may be the founder of the Hox/ParaHox-like subclass of ANTP genes. [IIb] Based on the assumption that Placozoa are basal within extant metazoan phyla, Porifera must have lost extended Hox, Hox/ParaHox-like and some NKL genes. An alternative scenario that Porifera are most basal would in turn require loss of some NKL genes in Placozoa (e.g. Msx and Bar/Bsh related genes). [III] Further expansion of ANTP genes in a cnidarian-bilaterian ancestor leading to almost complete repertoires of NKL and extended Hox genes. Occurrence of anterior Hox and posterior Hox/Cdx-like genes. The assumption that a Gsx type gene was the founder of Hox/ParaHox-like genes requires that Gsx and a posterior Hox/Cdx-like gene were part of an ancestral ANTP mega-array for which we still lack any evidence in extant species (question mark in III). In urochordates, however, we find one example of a ParaHox gene (Cdx) which is tightly linked to a Hox gene [23]. [IV] occurrence of a true canonical Hox System and complete ParaHox cluster in Bilateria. Linkages other than for *Trichoplax adhaerens* are based upon [11,13,15,16]. In the case of Nematostella some linkages have been deduced from the current JGI genome assembly. The proposed ANTP mega-array in a cnidarian-bilaterian ancestor has been deduced from [7,8,10,12,15]. Remark: For completeness the black square in the *Trichoplax* genome represents the PRD class gene Gsc which is tightly linked to NKL genes. [B] A Porifera basal scenario (simplified) for the Evolution of ANTP genes leading to crown group diploblasts and a putative protostome deuterostome ancestor: starts with a single (proto)NK gene and an initial expansion of the NKL subclass. Hox/ParaHox-like and extended Hox genes are an invention of the lineage leading to Placozoa, Cnidaria and Bilateria.

gene which founded the Hox/ParaHox genes, although we cannot completely exclude the possibility that *Trichoplax adhaerens* has lost other Hox/ParaHox genes. Unfortunately gene trees may not be able to resolve this problem because relationships (clades) beyond the family level of ANTP genes (e.g. the Gsx, NK6, Evx etc. family) are, in most cases, weakly supported (e.g. Figure S1 & S2; cf. [4,5]). In other words, there is no real confidence which Hox/ParaHox clade is basal to the others. Understanding the evolution of ANTP genes requires interpreting the data in a phylogenetic context. Unfortunately, the relationships between the four extant lower metazoan phyla, Placozoa, Porifera, Cnidaria, and Ctenophora, remain contentious. Total evidence analyses as well as mitochondrial genome data suggest a basal branch for Placozoa relative to all other diploblast phyla [17,24–26], while extensive nuclear gene phylogenies [18] place Placozoa as the sister taxon to the Cnidaria and Bilateria. Depending on the placement of Placozoa, two scenarios for ANTP gene evolution come to mind: (1) Porifera branching first would support the hypothesis of an Urmetazoan harboring a set of NKL genes only, which later in eumetazoans (all non-poriferan metazoans) diverged into the different ANTP gene families (cf.[7,10]) (Figure 1). (2) Placozoa branching first among extant phyla supports a scenario in which the ancestral Urmetazoan harbored a set of NKL, Hox/ParaHox-like and
extended Hox genes (Figure 1) (cf.[7,10]). In this view Porifera have lost Hox/ParaHox related and extended Hox genes. This is congruent with a recent hypothesis based on the alternative interpretation of gene trees [27]. Both scenarios as well as all recent phylogenetic analyses, are congruent with the hypothesis that Placozoa mirrors the closest condition for an ancestral eumetazoan genome from which the situation in Cnidaria and that Placozoa mirrors the closest condition for an ancestral interpretation of gene trees [27]. Both scenarios as well as all have lost Hox/ParaHox related and extended Hox genes. This is extended Hox genes (Figure 1) (cf.[7,10]). In this view Porifera rise to all Hox/ParaHox and related genes.

While the final answer to the origin of Metazoa, the basal metazoan bauplan and (homeobox) gene content, might never be found [28], overall evidence is congruent with the view that an ancestral metazoan ANTP array harbored at least one ortholog each of Hox/ParaHox-like, NK, and extended Hox genes. During anagenetic evolution into crown-group placozoans, cnidarians and bilaterians, gene duplications within each ANTP family then gave rise to a wide array of NKL genes in all extant metazoans, most extended Hox genes in the cnidarian-bilaterian lineage, Hox-like genes in the Cnidaria, and true Hox and ParaHox gene clusters in Bilateria (Figure 1). Independent of the above models, our data support the view that ANTP gene evolution was driven by three major events: (A) an initial expansion of the NKL subclass, (B) the expansion of the extended Hox, and (C) a Gsx type gene giving rise to all Hox/ParaHox and related genes.

Materials and Methods

Genome sequencing

The so-called “Grell” strain has been in culture in Hannover as clonal lineage since 1999 [25], and at Yale University since 2001. From this clone, which is the same used in earlier reports on placozoan genomics [17,19,25,29], genomic DNA was prepared for genomic library construction at JGI. For details on shotgun sequencing and bioinformatic analyses see [18].

Identification of Trichoplax homeodomain genes

A database of the 8× coverage of assembled scaffolds of the Trichoplax adhaerens genome was queried locally with a list of 1,063 known homeodomain protein motifs (available upon request), using the translated blast algorithm (tblastn) filtered at a low-stringency e-value of 0.001. At this stage of the analysis, some false positive hits were accepted in favor of detecting all true positives. These false hits would later be sifted out by a second reciprocal blast search as described below. The tblastn hits were sorted by genomic coordinates yielding 403 unique HSPs (blast High Scoring Pair) representing putative Trichoplax homeodomain homologs.

For the reverse blast analysis, four kilobases of genomic DNA sequence encompassing each of the tblastn HSPs was retrieved from the Trichoplax genome database using custom perl scripts (available upon request). These 4 kb subcontigs were individually queried against a local blastn database using the translated blastx program, filtered at a more stringent e-value of 10^-5. The blastx output for each contig was parsed using custom scripts along with the identity of its top blast hit and e-value (available upon request). Forty-seven loci in the T. adhaerens genome were confirmed to have strong blast similarity to known homeodomain containing proteins, of which 37 were confirmed to harbor a homeobox. Gene linkage and clustering were identified based on scaffold coordinates.

Phylogenetic Analysis

Blast searches identified 14 of the 37 loci as ANTP class homeobox genes. The remaining 23 putative homeobox genes were roughly classified based on Blast results. The 14 putative ANTP class genes were further analyzed to infer clear relationships to known ANTP gene families. In the cases where the homeodomain is encoded by more than one exon, the homeodomain amino acid sequences were predicted by Genscan and GenomeScan [30,31].

The 60 amino acids of the homeodomain were aligned with ClustalW implemented in MEGA 3.1 [32]. Bayesian Inference implemented by Mr.Bayes v3.1.2 [33], and maximum-likelihood analyses were conducted to infer phylogenetic relationships to known ANTP families. Mr.Bayes employed the JTT model of amino acid substitution assuming the presence of invariant sites and a gamma distribution approximated by four different rate categories to model rate variation between sites. MrBayes was run with four chains for 5 million generations sampling every 1000 generations. The likelihoods of the generations were examined to estimate the beginning of stationary phase and trees after the first 1.25 million generations were used to create a consensus. Maximum Likelihood analyses were performed using PHYML v.2.4.4 [34] with the JTT model and a gamma distribution approximated by four rate categories. Tree robustness was assessed by 1000 bootstrap resamplings. The resulting trees of Bayesian and Maximum Likelihood analyses were rooted on midpoint. The dataset comprised the 14 putative ANTP genes from Trichoplax together with representatives of known ANTP families (cf. [2,4,7,10,12,14,16]) of Bilateria, Cnidaria and Porifera from public databases (166 homeodomain in total).

Nematostella sequences were taken from a published dataset [11,12] except for NvBarx (ABG67788), NvHd065 (ABG66441) and NvHlxB (ABG67796). Amphimedon sequences were taken from [16]. Accession numbers for the remaining sequences are available as supplemental data (Text S1). Trichoplax sequences have been deposited to GenBank (EU700377-EU700590 for the ANTP genes; EU700377-EU700413 for all homeobox genes) and are also available as supplemental data (Table S1), together with genomic scaffold coordinates.

Supporting Information

Figure S1 Bayesian analysis reveals robust grouping for 11 of 14 placozoan ANTP genes to known ANTP genes from Cnidaria, Porifera, and Bilateria. Genes included in the analysis represent Placozoa (Trichoplax adhaerens Ta), Cnidaria (Nematostella vectensis Nv, Eleutheria dichtoma Ed, Aplysia Formosa Af), Porifera (Amphimedon queenslandica Aq), protostomes (Drosophila melanogaster Dm, Anopheles gambiae Ag, Drosocelis tigrina Dt, Schistocerca gregaria Sg), and deuterostomes (Branchiostoma floridae Bf, Danio rerio Dr, Homo sapiens Hs, Oikopleura dioica Od, Stronglylocentrotus purpuratus Sp, Xenopus laevis Xl). Genes of the NKL subclass are framed in yellow, extended Hox genes in blue and Hox/ParaHox-like genes in red. Amphimedon, Nematostella and Trichoplax genes are color coded. Tree rooted on midpoint. Found at: doi:10.1371/journal.pone.0002457.s001 (0.09 MB PDF)

Figure S2 Maximum Likelihood analysis with the same dataset as above. Tree rooted on midpoint. Found at: doi:10.1371/journal.pone.0002457.s002 (0.07 MB PDF)

Figure S3 Linkage of non-ANTP homeobox genes in Trichoplax adhaerens. In the current assembly of the Trichoplax genome it is evident that the genes of the homeobox classes ANTP, PRD, LIM, TALE and Hnf are reciprocally linked. This supports the view that at least the founders of most metazoan homeobox classes have been ancestrally clustered (cf.[21,22]). Only distances between two genes in the megabase range are given. All other distances are ≤300 kb. Found at: doi:10.1371/journal.pone.0002457.s003 (0.05 MB PDF)
Table S1  Sequences of the *Trichoplax* homeodomains/homeoboxes as well as other conserved motifs and genomic scaffold coordinates.  
Found at: doi:10.1371/journal.pone.0002457.s004 (0.04 MB XLS)  

Text S1  Accession numbers of the sequences used for phylogenetic analyses.  
Found at: doi:10.1371/journal.pone.0002457.s005 (0.06 MB DOC)  

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Author Contributions

Analyzed the data: SD BS KK RR MS. Wrote the paper: SD BS KK. Other: Coordination of genome sequencing figures: SD. Coordination of genome sequencing: DR BS. Designed figures and illustrations: KK.