

The Sperm Proteins from *Amphioxus* Mirror Its Basal Position among Chordates and Redefine the Origin of Vertebrate Protamines

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The sperm nuclear basic proteins (SNBPs) that participate in chromatin condensation in spermatozoa belong to 3 groups: histone (H), protamine-like (PL), and protamine (P) type. They share a common origin with histone H1 resulting from the segregation of PL components, corresponding to different regions of an H1 precursor molecule (N-terminal, winged-helix, C-terminal domains), becoming independent and following a subsequent process of parallel vertical evolution (H ↔ PL ↔ P). In the present work, we describe the sequence and primary structure of the main SNBP component in the sperm of the cephalochordate *Branchiostoma floridae* (amphioxus), revealing that it represents the deuterostome counterpart of the PL-III SNBP component from molluscs corresponding to the H1 N-terminal region. Until now, this has been a missing piece needed to complete the evolutionary history of SNBPs in metazoan genomes. The discovery of this PL lineage in deuterostomes definitively validates the parallel vertical evolution of SNBPs across metazoans, giving further support to the “basal” position of amphioxus among chordates, with respect to tunicates. Sequence analyses suggest that later on in evolution, the appearance of positively selected arginine-rich protamines, derived from the H1 C-terminal region, led to the extinction of this PL lineage in the genomes of early protostomes and deuterostomes. Given that tunicates are now viewed as a sister group of vertebrates, the lysine to arginine transition responsible for the origin of vertebrate protamines must be set a step back from tunicates.

Introduction

In somatic eukaryotic cells, the DNA is compacted within the nucleus by chromatin, a nucleoprotein complex that results from the association of DNA with basic chromosomal proteins known as histones (van Holde 1988). In contrast, sperm cells exhibit much more protein variability, allowing the extreme compaction of chromatin and the subsequent reduction in the volume of the sperm nucleus (Ausió et al. 2007). The sperm nuclear basic proteins (SNBPs) that participate in the condensation process have been shown to belong to 3 well-defined groups: histone (H), protamine-like (PL), and protamine (P) types (Ausió 1995, 1999). We have recently shown that PL proteins shared a common evolutionary origin with the histone H1 family early in metazoan evolution, resulting from the segregation of independent PL gene components corresponding to different regions of a precursor molecule (N-terminal, winged-helix, and C-terminal domains) (Eirín-López, Lewis, et al. 2006).

This observation represents an important starting point in assembling the pieces necessary to reconstruct the evolutionary histories of histone H1 and SNBPs. Piece one, we already evidenced that the long-term evolution of the histone H1 family has been subject to a birth-and-death process under strong purifying selection at the protein level, leading to the high levels of diversification and differentiation observed among the family members in higher eukaryotes (Eirín-López et al. 2004; Nei and Rooney 2006). Piece 2, the long-term evolution of SNBPs seems to be best described by a process of parallel vertical evolution (H ↔ PL ↔ P) subject to purifying selection at least in H-type and PL-type lysine-rich lineages in both protostomes and deuterostomes (Eirín-López, Lewis, et al. 2006). Accord-

ingly, only H- or PL-precursor SNBP types would be present in those taxa that arose early in metazoan evolution, whereas the more specialized PL and P types would represent a characteristic feature of those taxa located at the uppermost evolutionary branches of bilaterian evolution. Piece 3, the transition from lysine-rich PLs toward the highly specialized arginine-rich protamines was caused by a frameshift mutation in the PL component corresponding to the C-terminal region of H1 (Lewis et al. 2004) and involved a shift from negative selection to an adaptive selection process (Eirín-López, Lewis, et al. 2006).

However, an important piece is still needed in order to articulate and validate the whole evolutionary picture of these proteins. In one hand, functional PL components corresponding to the N-terminal region of an H1 precursor have been only identified in mollusc species (protostomes) (Rocchini et al. 1995). This observation is in contrast to the expectations made by the model of parallel vertical evolution of SNBPs, which suggests that the differentiation of the 3 PL components occurred before the split between protostomes and deuterostomes. On the other hand, and more importantly, very recent analyses have traded the position of tunicates with that occupied by cephalochordates (also known as amphioxus or lancelets) in chordate evolution (Bourlat et al. 2006), with the amphioxus now being viewed as the most “basal” chordate and the tunicates as the sister group of the vertebrates (Bourlat et al. 2006; Holland 2007).

In the present work, we found that the SNBP component in the sperm of the cephalochordate *Branchiostoma floridae* is exclusively composed of the PL fraction corresponding to the N-terminal region of H1. The discovery of this PL lineage in deuterostomes definitively validates the parallel vertical evolution of SNBPs across metazoans, giving further support to the basal position of the amphioxus among chordates, with respect to tunicates. The appearance of arginine-rich protamines later on in evolution led to the extinction of this PL lineage due to its inability to undergo a lysine to arginine transition.

Key words: reproductive proteins, protamines, metazoans, long-term evolution, chromatin.

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Materials and Methods

SNBP Extraction and Fractionation

SNBPs were extracted from testes of *B. floridae* with 0.4 N HCl and precipitated with acetone as described in Wang and Ausió (2001). The protein extract thus obtained was fractionated by high-performance liquid chromatography (HPLC) on a reverse phase 300-Å Vydac C18 column (25 × 0.46 cm) eluted at 1 ml/min with a 0.1% Trifluoroacetic acid–acetonitrile gradient, and the absorbance was monitored at 230 nm (Ausió 1988). Proteins were analyzed by acetic acid–urea polyacrylamide gel electrophoresis (AU-PAGE) (15% acrylamide, 0.1% bisacrylamide, 5% acetic acid, and 2.5 M urea), and gels were then stained with 0.2% (w/v) Coomassie blue for 1 h in 25% (v/v) isopropanol 10% (v/v) acetic acid and destained overnight in 10% (v/v) isopropanol/10% (v/v) acetic acid.

In Silico Isolation of the Amphioxus PL Protein and Genomic DNA and cDNA Sequence Determination

Protein sequences corresponding to the PL component of the sperm of molluscs, tunicates and sea urchins were used in protein–protein basic local alignment and search tool (BLAST) searches against the complete genome sequence of *B. floridae* (unmasked v1.0, DOE Joint Genome Institute, <http://www.jgi.doe.gov>). The genomic region of the only SNBP candidate found (sequence ID 125082) was subsequently retrieved from the amphioxus database and used in the design of gene-specific primers. Total RNA was extracted from whole *B. floridae* animals using Trizol reagent (GibcoBRL, Burlington, ON), and mRNA was isolated using an mRNA purification kit (Amersham Bioscience, Piscataway, NJ). Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Mississauga, ON) following the manufacturers' instructions. Gene-specific primers were designed using the genomic sequence retrieved from the amphioxus genome as a reference, and they were subsequently used for polymerase chain reaction with cDNA and genomic DNA templates. In addition, rapid amplification of cDNA ends (RACE) was employed to obtain the complete cDNA sequence using the First Choice RACE Kit (Ambion, Austin, TX). Sequencing was done by the DNA Sequencing Facility, Centre for Biomedical Research at the University of Victoria.

Molecular Evolutionary Analyses

A total of 206 amino acid sequences (see supplementary table, Supplementary Material online) were used in the analyses, including 91 nonredundant histone H1 somatic sequences (68 replication dependent [RD] and 23 replication independent [RI]), 1 oocyte-specific H1, 16 testis-specific H1, 97 SNBP sequences (3 H type, 18 PL type, and 76 P type), and the PL protein from amphioxus. Multiple alignments of the amino acid sequences were conducted using the BIOEDIT (Hall 1999) program, and all molecular evolutionary analyses were conducted using the program MEGA version 3.1 (Kumar et al. 2004). The extent of the amino acid divergence was estimated

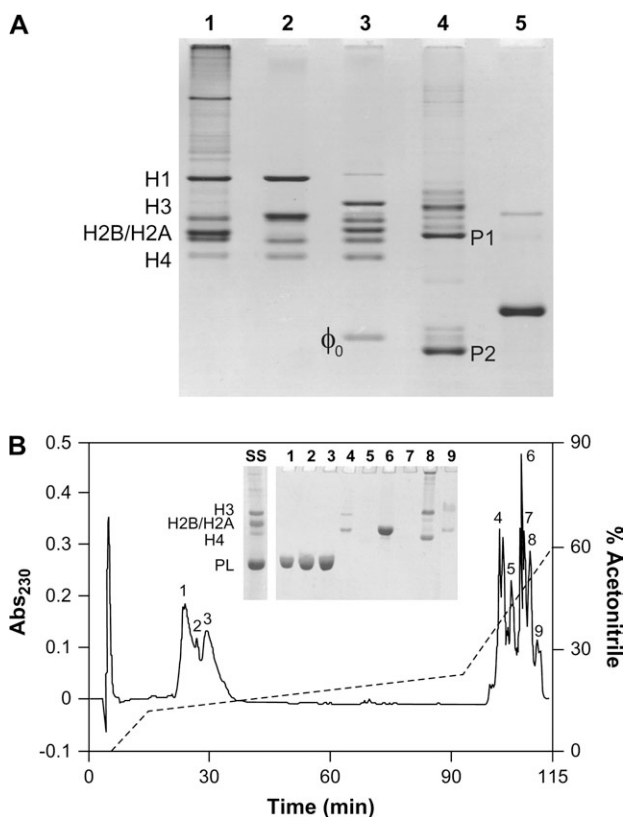


FIG. 1.—(A) AU-PAGE of acid extracted testes proteins from the sea urchin *Strongylocentrotus purpuratus* (1), the starfish *Pisaster ochraceus* (2), the sea cucumber *Thyone briareus* (3), the sea squirt *Styela montereyensis* (4), and the amphioxus *Branchiostoma floridae* (5). (B) Fractionation and purification of *B. floridae* SNBPs by reverse phase HPLC. Proteins were eluted with an acetonitrile gradient. The inset gel is an AU-PAGE of the different peaks (1–9), SS stands for a protein marker.

by means of the uncorrected differences (p distance), and the minimum evolution tree-building method (Saitou and Nei 1987) was used to reconstruct the phylogenetic trees. In order to assess that our results were not dependent on this method, the simplified phylogeny was compared with a maximum parsimony tree reconstructed using the close-neighbor-interchange tree search method. The reliability of the topologies was tested by the bootstrap method (Felsenstein 1985) and the interior branch test (confidence probability) method (Sitnikova 1996), based on 1,000 replicates. Codon usage analyses on PL, P, and H1 genes were performed by using the DnaSP version 3 program (Rozas et al. 2003), using the “effective number of codons” (ENC) to estimate the codon usage bias (Wright 1990).

Results and Discussion

The Main Sperm Chromosomal Protein of Amphioxus Corresponds to the N-terminal Region of a Histone H1 Precursor

Figure 1A shows the SNBP composition of *B. floridae* (lane 5) in comparison to that of representative species from different classes of echinoderms and a tunicate. As seen in this figure (lanes 1–3), echinoderm SNBPs belong to the H type with the exception of the class Holoturoidea, where

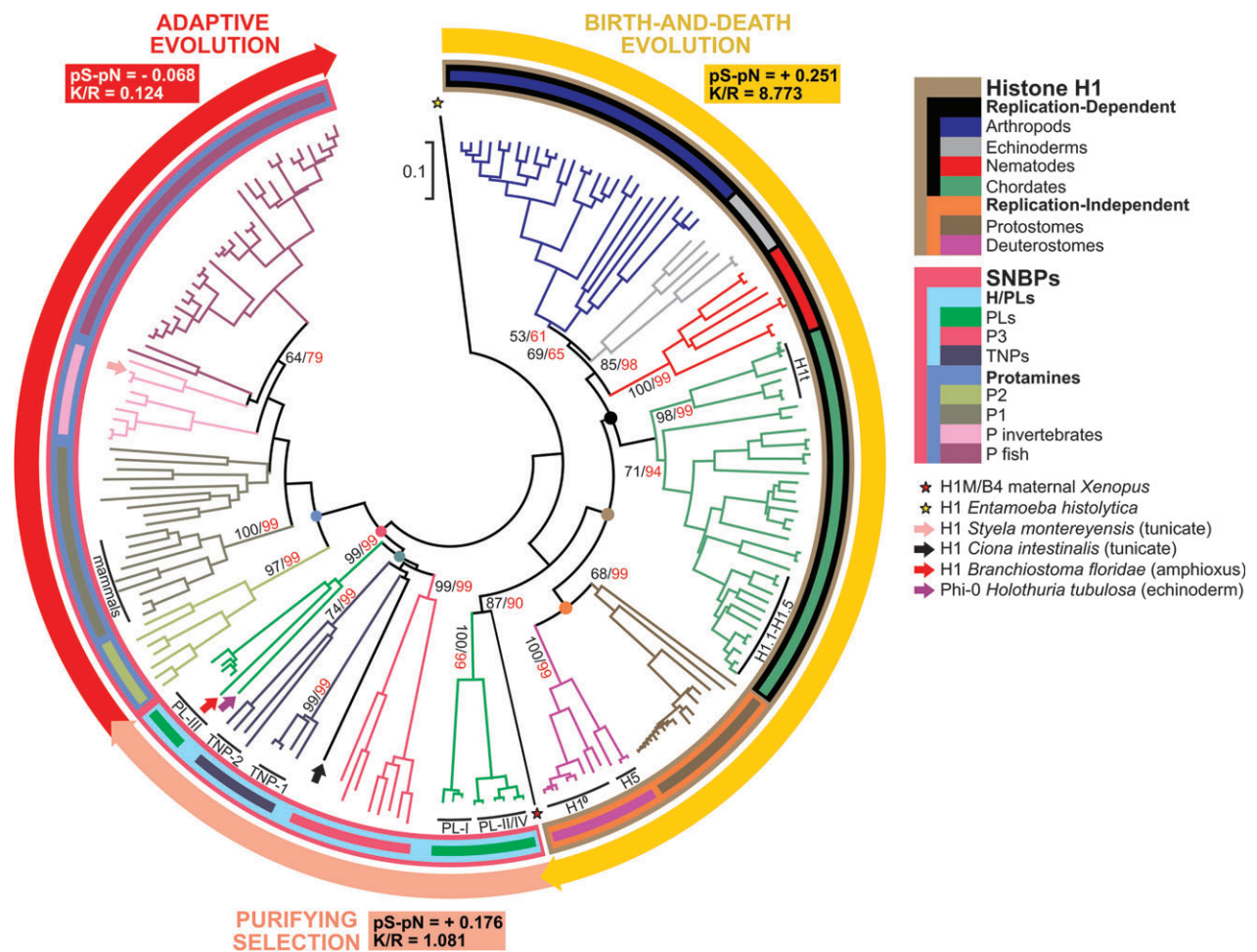


FIG. 3.—Phylogenetic relationships between SNBPs and histone H1 reconstructed from 206 protein sequences, showing the progressive diversification of the members of the H1, PL, and P groups. Whereas the long-term evolution of H1 members is subject to birth-and-death evolution under a strong purifying selection, SNBPs of the PL type are subject to a slightly less constrained selection process that is concomitant with the decrease in the lysine/arginine (K/R) ratio. The replacement of lysines by arginines in PL proteins eventually results in a shift toward an adaptive selection process ($pN > pS$) favoring a high arginine content in specialized protamines. Numbers for interior branches indicate bootstrap (black) and interior branch test (red) confidence values based on 1,000 replications (only shown if greater than 50%) for the groups defined by the topology.

2 clearly differentiated groups, with PLs occupying an intermediate position. The occurrence of RD and RI H1 histones, as well as PL- and P-type SNBPs in protostome and deuterostome representatives, provides support that their evolutionary origin occurred prior to the split between these 2 groups of organisms.

The topology shown in figure 3 is in good agreement with the taxonomic relationships among species, indicating a monophyletic origin for the group of RI H1s and a functional clustering pattern for the different mammalian somatic H1 isoforms (Eirín-López, Lewis, et al. 2006). The major SNBP component of *B. floridae* locates in the PL region of the topology and shares a monophyletic origin with the PL-III SNBP component from molluscs, supporting the biochemical and molecular analyses presented in the previous section. Given that amphioxus represents the only deuterostome in which the main sperm chromosomal protein corresponds to the N-terminal region of a histone H1 precursor, this result definitively validates the hypothesis of

their parallel vertical evolution (Ausió 1999). Accordingly, the products of the segregation of the different SNBP components from a histone H1 molecule should be present in both protostomes and deuterostomes, in agreement with their common evolutionary origin (Eirín-López, Lewis, et al. 2006). However, only components corresponding to the C-terminal region of H1 were found in deuterostomes, which eventually led to the differentiation of highly specialized arginine-rich protamines (Lewis et al. 2004). The reconstruction of a simplified tree (fig. 4) underscores the close relationship of *B. floridae* PL with SNBPs of the PL type in molluscs, especially PL-I and PL-III. Furthermore, the PL component from *B. floridae* appears to be somewhat more closely related to molluscan PL-I, PL-III, and Phi-0 SNBPs, suggesting a more primitive origin and a closer relationship to echinoderms, as supported by recent phylogenetic evidence (Jutglar et al. 1991).

The differentiation of the amphioxus SNBP lineage reported in figure 3 agrees with the evidence provided by

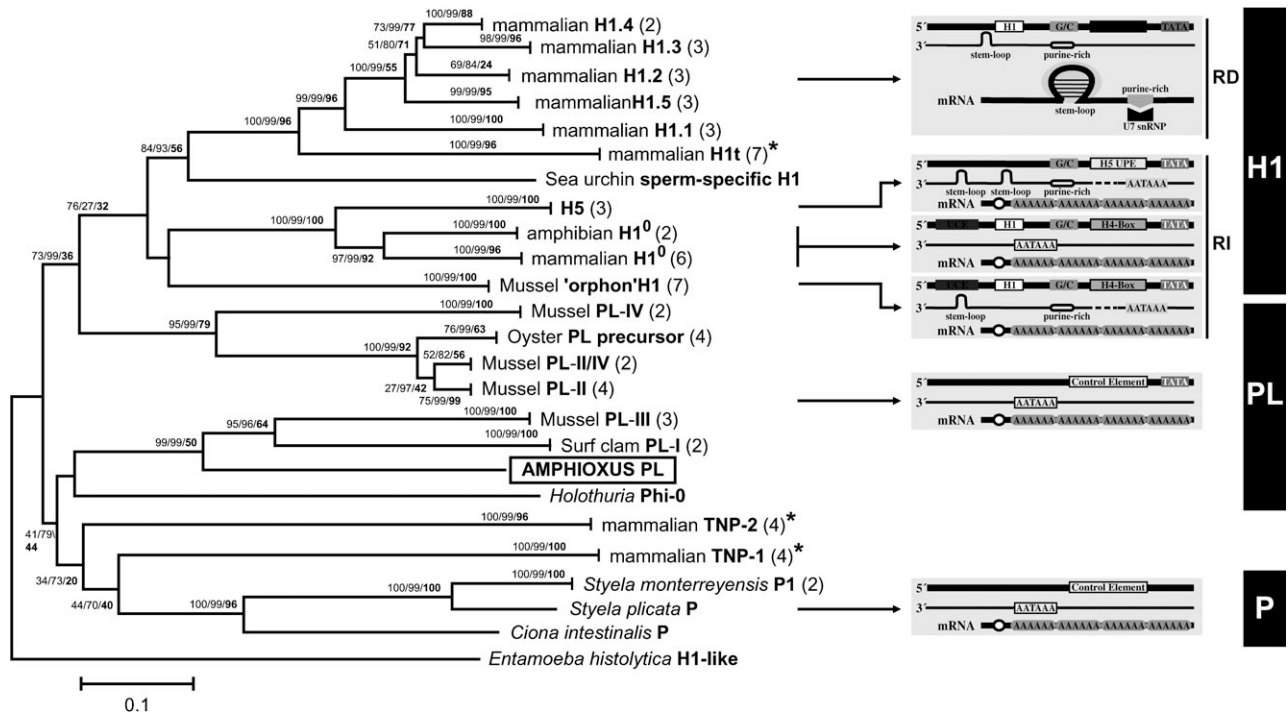


FIG. 4.—The tree was rooted with the H1-like sequence from the protist *Entamoeba histolytica* as it represents one of the most primitive eukaryotes for which a H1-related protein has been characterized (Kasinsky et al. 2001). The different lineages of H1 (RD/RI) as well as PL-type and P-type SNBPs are indicated in the right margin of the topology together with the mechanisms of 3' mRNA processing specific for each group. Transition proteins and testis-specific H1 histones (H1t) are marked as (*) to indicate that their expression pattern is intermediate between RD and RI H1 histones. Numbers for interior branches are representative of bootstrap and interior branch test, followed by the bootstrap values obtained in the reconstruction of the maximum parsimony tree (in boldface). Numbers in parentheses by the species names indicate the number of sequences used.

recent reports supporting the status of the amphioxus as the most basal chordate, in exchange of tunicates (Bourlat et al. 2006). In such scenario, the SNBP component from amphioxus (whose protostome counterpart is represented by the PL-III protein from molluscs) would represent the oldest deuterostome PL lineage, prior to the differentiation of PLs corresponding to the C-terminal region of H1.

The Amphioxus PL Lineage Is Subject to Different Evolutionary Constraints than the Protamines or the PL-I Lineage from Tunicates

The progressive specialization of SNBPs has followed a parallel vertical pattern of evolution defined as H ↔ PL ↔ P (Ausió 1999; Eirín-López, Frehlick, and Ausió 2006), encompassing a progressive replacement of lysines (K) by arginines (R) leading to a more efficient DNA packaging in the sperm as well as in the activation of enzymatic cascades after fertilization in vertebrates (Ausió et al. 1984; Ohtsuki et al. 1996; Rooney and Zhang 1999). The gradual variation in the K/R ratio across H1/SNBP evolution is evident in the phylogeny shown in figure 3, ranging from around 8.7 in the case of H1 to 0.1 in the case of protamines. Furthermore, the increase in arginine is concomitant with the increase in the numbers of nonsynonymous substitutions among P lineages (see fig. 3), suggesting that this trait is positively selected.

It has been recently shown that a frameshift mutation involving only 2 nt would be enough to duplicate the per-

cent of arginine in the PL component of the tunicate *Ciona intestinalis*, due to the high frequency of AAG and AGA triplets encoding polylysine and polyarginine tracts, respectively (Lewis et al. 2004). By studying the relative codon usage for lysine and arginine residues (fig. 5A), it is evident that, although the SNBP from *B. floridae* presents similar amounts of lysine and arginine (as does PL-III from molluscs), all lysines are encoded by the same codon (AAA) with a higher proportion of arginine residues encoded by CGU and CGC. This codon preference is in sharp contrast to that shown by histone H1 and the PL components from molluscs, where arginines are mainly encoded by AGA and AGG triplets, and would ultimately represent an insurmountable barrier for a lysine to arginine transition based on a frameshift mutation.

Although molluscan PLs and the amphioxus SNBP are very biased in terms of their codon usage, somatic histone H1s and tunicate PLs exhibit intermediate and very low codon bias, respectively. This may be explained by the fact that although PL proteins rely equally on lysines and arginines for DNA packaging, constraints acting on protamines are only dependent on the overall arginine content of the molecule, rather than on the specific position of the arginines or the nature of their encoding triplets (Rooney et al. 2000; Lewis et al. 2003). This unusual form of purifying selection, together with the lysine to arginine transition, would be consistent with the low codon bias values observed in the arginine-rich PLs from tunicates in comparison with lysine-rich PLs.

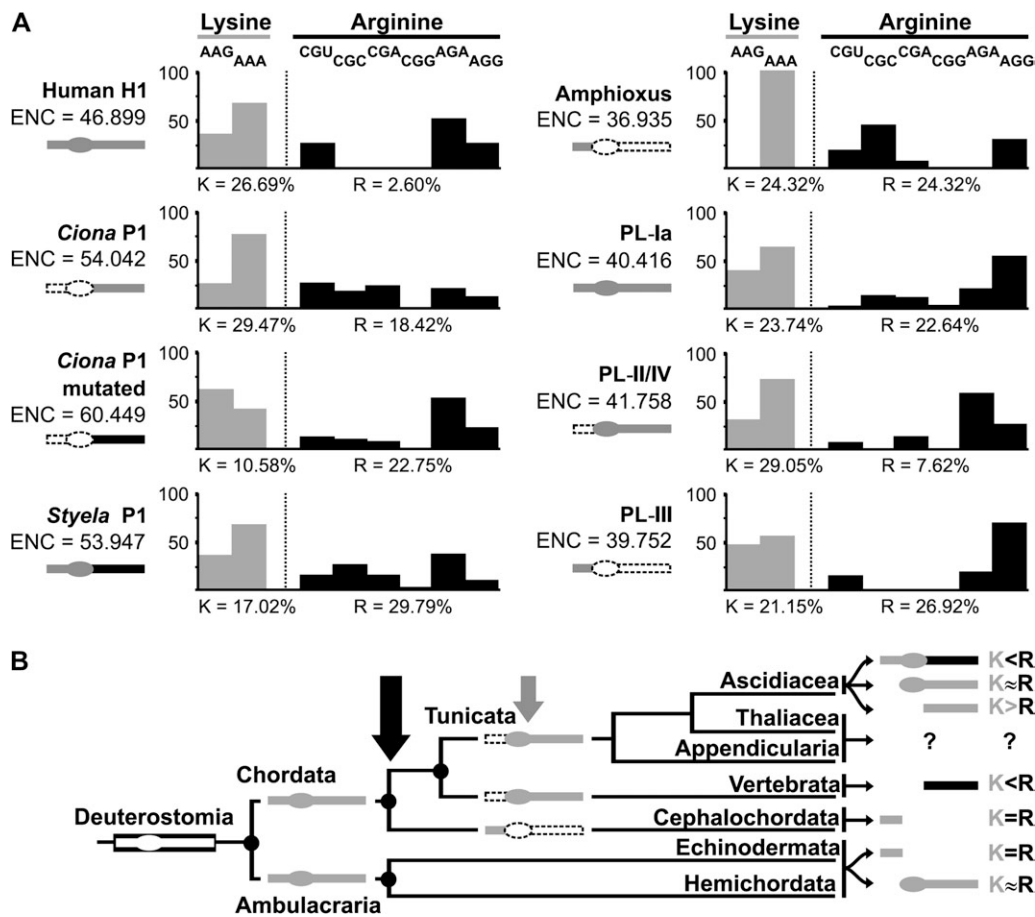


FIG. 5.—(A) Codon bias (ENC) and relative codon usage (%) of basic residues in protamine-like (PL-type) SNBPs from different protostome and deuterostome taxonomic groups corresponding to different regions of a histone H1 precursor. The lysine (K) and arginine (R) content is indicated in comparison with human histone H1. (B) Schematic evolution of SNBP segregation and specialization in the currently understood phylogenetic context for deuterostomes (Holland 2007). The lysine-rich SNBP component corresponding to the C-terminal region of H1 (indicated in grey) was the preferred choice of tunicates and vertebrates. This region was subject to a process of positive selection, resulting in a lysine to arginine transition, which gave rise to specialized protamines (represented in black). Such a fast adaptive process, together with the inability of the SNBP component corresponding to the H1 N-terminal region to undergo a lysine to arginine transition, led to the extinction of this later lineage (indicated in grey), which represented the choice of cephalochordates and possibly of some echinoderms. The small grey arrow represents the point at which the lysine to arginine transition was believed to occur (Lewis et al. 2004), in comparison with the point at which this transition must have occurred (big black arrow) in order to account for the presence of arginine-rich protamines in both vertebrates and tunicates, as indicated in the present work. Question marks in the class Thaliacea and in the class Appendicularia indicate that SNBP composition in the sperm of these organisms is yet unknown. The overall content of lysine (K) respect to arginine (R) in each SNBP lineage is indicated in the right hand margin (>, higher; <, lower; =, similar; ≈ approximate).

The PL component from *Amphioxus* Provides a Footprint in the Process of Parallel Evolution Observed in SNBPs across Metazoan Genomes

There is now plenty of evidence indicating that the exclusion of specialized H1 genes from the main repetitive histone units to a solitary location in the genome resulted in the generation of an “orphan” group of H1 genes early in the evolution of metazoans (Eirín-López et al. 2002, 2004). The independent evolution of this group eventually led to the differentiation of RD and RI H1 variants, followed by the subsequent differentiation of SNBPs within the second lineage (Eirín-López, Lewis, et al. 2006). During the differentiation and diversification of these chromosomal proteins, the transition toward arginine-rich protamines appears to have involved in the progressive specialization of a PL protein corresponding to the C-terminal region of H1 (Lewis et al. 2004), which eventually suffered a shift from a puri-

fying selection process (present in PLs) to adaptive selection favoring high arginine contents in protamines (fig. 5B) (Balhorn 2007). However, the presence and fate of the SNBP component corresponding to the N-terminal part of H1 have remained elusive until now.

Conclusions

The identification of a deuterostome counterpart (represented by the *amphioxus* PL reported here) to the protostome PL component corresponding to the N-terminal region of a precursor histone H1 (represented by the PL-III protein from molluscs) provides a footprint that definitively validates the parallel vertical evolution of SNBPs (summarized in fig. 6). This result is even more critically relevant in light of recent studies placing the *amphioxus* in the basal position in chordate evolution (Bourlat et al. 2006), in place

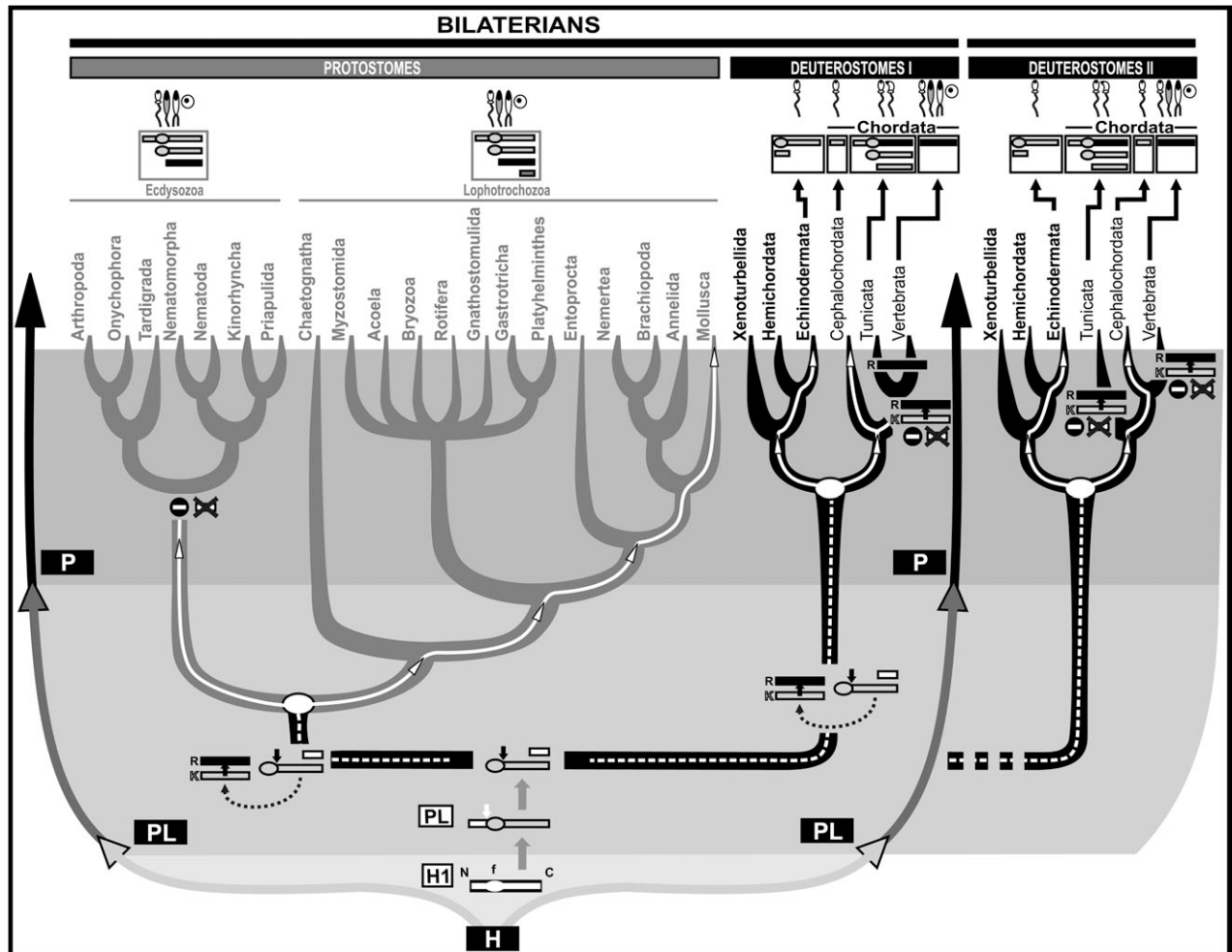


FIG. 6.—Schematic representation of the evolutionary process followed by SNBPs across bilaterians since their origin from a precursor histone H1 molecule (Eirín-López et al. 2006), indicating the major events leading to the differentiation of actual protamine-like and protamine lineages. The evolutionary relationships among the major groups of animals have been represented following recent reports based on a phylogenomic sampling (Dunn et al. 2008; Telford 2008). The different stages in the parallel vertical evolution of SNBPs (H ↔ PL ↔ P) are highlighted with light grey background (H stage), grey background (PL stage) and dark grey background (P stage). The differentiation of the three H1 domains was followed by a first segregation event (indicated by a white arrow) leading to the independization of the N-terminal region of H1 (indicated in white). Shortly after, a second segregation event (indicated by a black arrow) resulted in the segregation of the lysine-rich C-terminal domain (indicated in grey), whose preferred codon usage for lysine residues (mostly AGA) allowed a lysine (K) to arginine (R) transition (being AAG the preferred codon for this later amino acid) based on a frameshift mutation, involving a shift from negative selection to a rapid adaptive selection process (the resulting arginine-rich SNBP component is represented in black). Such specialized protamines acquired priority as the preferential choice for DNA packaging in the sperm nucleus, leading to the extinction of arginine-poor SNBPs, including the lineage corresponding to the N-terminal region of H1. Nevertheless, this later SNBP component was able to subsist in concrete groups of both protostomes (mollusc PL-III) and deuterostomes (amphioxus and sea urchin SNBPs) as a ‘fossil’ lineage (whose evolutionary pathway is indicated by white lines across the diagram). The different SNBP lineages and types of spermatozoa found in actual bilaterians are represented in the top of the figure. Given that SNBPs provide different efficiencies in DNA packaging in the sperm, they can probably influence the shape of the sperm nucleus, bearing critical implications for fertility and sperm competition (Ausió, Eirín-López, and Frehlick 2007). The different hypothesized relationships among chordates are represented in the right hand margin of the figure as follows: while in one hypothesis (DEUTEROSTOMES I) the cephalochordates are viewed as the most ‘basal’ chordates, in the other one (DEUTEROSTOMES II) tunicates are considered as the earliest offshot of the chordate lineage, and amphioxus as the closest group to vertebrates. In this later case, the appearance of already highly differentiated arginine-rich protamines as early as in tunicates seems incompatible with the prevalence of the PL lineage later on during the differentiation between cephalochordates and vertebrates. Oppositely, a ‘basal’ position of cephalochordates would agree with the early differentiation between the SNBP lineage from amphioxus (N-terminal region of H1) and the highly differentiated arginine-rich protamines typical from tunicates and vertebrates (C-terminal region of H1).

of tunicates. In this new scenario, the lysine to arginine transition observed in tunicates could no longer be considered as an early event in bilaterians or stand for the origin of protamines in vertebrates. The existence of tunicate PLs which are already highly specialized and reminiscent of P-type SNBPs, together with the sharp contrast they present with respect to the primitive amphioxus PL-type SNBP lin-

age, make tempting to hypothesize that such observations support the basal condition of amphioxus among chordates (fig. 5B). Furthermore, the Phi-0 protein from the sea cucumber *Holothuria tubulosa* (Prats and Cornudella 1995) displays certain sequence similarities, including the presence of introns, with the *B. floridae* SNBP as well as with PL-III (figs. 3 and 4). This further supports the origin of this

SNBP lineage as early as before the differentiation between protostomes and deuterostomes. However, and even by taking into account that protamines have been successfully used as molecular markers in the past (Eirín-López, Frehlick, and Ausió 2006; Frehlick et al. 2006), these results must be interpreted with caution given that tunicates have very derived genomes and body plans.

The SNBP component corresponding to the H1 N-terminal region appears to be the initial choice for sperm DNA packaging in early metazoans. The extinction of this PL lineage in the genome was probably due to its inability to undergo a lysine to arginine transition based on the observed patterns of codon usage bias for lysine residues. In this regard, the PL-III protein from molluscs and the SNBP component identified in amphioxus would represent end points of the SNBP evolutionary process in deuterostome and protostome genomes that were promptly discarded by the fast specialization experienced by arginine-rich PL- and P-type SNBPs which arose through a process of positive selection.

Supplementary Material

Supplementary table and figure are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>). The sequence described in the present work has been deposited in the GenBank database with accession number EU271675.

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