The S.//.A.IG amino acid motif is present in a replication dependent *late* H3 histone variant of *P. lividus* sea urchin

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Abstract A novel gene encoding a new H3 histone variant (H3L) has been identified in *P. lividus* sea urchin embryo. It encodes a H3 histone protein showing the S.//.A.IG amino acid motif typical of the replication independent H3.3 variants but in a mRNA showing the 3′ terminal stem-loop nucleotide sequence that is typical of the replication dependent variants. The gene is intronless, the corresponding short transcript is non-polyadenylated and its expression is replication dependent with a timing of a *late* variant. The new H3 variant is expressed as a minor component with respect to a major replication dependent *late* H3 histone here identified by partial cDNA sequence. These results show that classification of histones in replication dependent and independent variants only on the basis of their amino acid sequences should be reconsidered.

Key words: H3 histone; Replication dependent variant; *Late* histone; Sea urchin embryo; *P. lividus*

1. Introduction

In higher eukaryotes histones are classified into two major groups, replication dependent type and replication independent replacement variants [1]. The two groups show various differences. Genes of the replication dependent histone group are intronless and encode short transcripts (about 500 bp) not undergoing polyadenylation [2]. The corresponding mRNAs have short 5′ and 3′ UTRs and a very well conserved stem-loop structure that defines the 3′ end of the transcript [3]. Genes for the replacement replication independent variant group contain at least one intron and are transcribed into longer polyadenylated mRNAs [2]. Almost invariably, the histones of the two groups show differences in particular amino acid positions. Some authors have suggested that interaction of replacement histone variants with DNA may be the mechanism that causes structural alterations of particular chromatin domains [4]. It has also been suggested that the non-dividing cells may require synthesis of the histone variants during interphase [5]. Replacement variants have been reported for almost all histone types [6–9]. However, the largest number of variants have been found for histone H3 in several animal species, in plants and protozoa [9–16]. Comparison of amino acid sequences of the replacement variants has shown that all those classified as H3.3 share the amino acid motif S.//.A.IG, whose functional importance is still unknown [9].

We have already reported on the presence of a H3.3 histone variant in *P. lividus* sea urchin embryo [16]. Northern blot analysis of the cDNA for this variant reveals, in addition to its long polyadenylated mRNA, the presence of a short non-polyadenylated mRNA having the pattern of accumulation, during development, typical of a *late* replication dependent H3 histone [16]. This finding provided the first evidence about the postulated occurrence of a *late* histone type in *P. lividus* embryo and indicated that both replication dependent and independent histone mRNAs are present at the same time in the sea urchin developing embryo.

Here we extend that analysis and show the occurrence of a novel type of H3 histone gene in *P. lividus* sea urchin. This gene encodes a protein with the amino acid sequence typical of a replication independent variant in a mRNA molecule with a structure and stability typical of a replication dependent variant. We show also, in the same embryonic preparations, the presence of a replication dependent mRNA with the size and structure of a replication dependent variant encoding a major *late* H3 histone. The findings reported here, together with the previous report about the presence of a polyadenylated H3.3 mRNA histone variant [16], demonstrate that in the *late* sea urchin embryo, in addition to a major *late* histone type, there are at least two other different H3 histone variants (H3L and H3.3) encoded on mRNAs with different nucleotide structures.

2. Materials and methods

2.1. Screening of the genomic library

A genomic library was prepared from the sperm cells of a single adult *P. lividus* sea urchin. DNA was digested with *Eco*RI and cloned into λ-EMBL4 vector (Stratagene) using standard procedures [17]. Three probes were used to screen the library: the coding region of the *P. lividus* H3 *early* histone gene (*Pvu*II fragment of pPH70 clone [18]), the complete cDNA of the histone H3.3 cDNA of *P. lividus* and its 3′ UTR (fragment *Bam*HI and fragment *Xba*I-*Hin*dIII, respectively, of the clone 11C11 [16]). Filters were hybridized at 65°C, washed at high stringency and then exposed to X-ray film (Fuji) as described [16]. Studies were performed on subclone H3L of phage 6, which hybridized only with the coding region of the H3.3 histone (see Section 3).

2.2. DNA sequence

DNA sequences were determined on both strands with the Sanger method [19], using Sequenase 2.0 kit (United States Biochemical), following the manufacturer's protocol. BLAST and PCgene programs were used to compare the nucleotide sequences and the encoded amino acid sequences with those in the databases.

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The nucleotide sequence data reported in this paper will appear in the Genome Sequence DataBase with the following accession numbers: clone 10, GSDB:S:1216219; PCR clone 9, GSDB:S:1216220; clone H3L, GSDB:S:1216221.

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2.3. Northern blot analyses

Total RNA was isolated from unfertilized *P. lividus* sea urchin eggs and from embryos at different hours of development, following the described procedures [20]. 7 μg of each RNA sample was fractionated on 1% agarose-formaldehyde gel and transferred to a Hybond nylon membrane (Amersham). Hybridization was performed in 60% formamide at 42°C, using as a probe either the PCR product obtained with the primer pair H3L1/H3L3 (clone PCR9, see Section 2.4) or the insert of the genomic subclone H3L (see Fig. 1). 30 ng of each probe was labelled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), using the random primer labelling kit of the manufacturer.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from two cultures of *P. lividus* embryos, at 30 h of development, each deriving from the fertilization of the eggs of one female with the sperms of one male [20]. 1 μg of RNA, treated with RNase-free DNase (Boehringer), was reverse transcribed with AMV-RT (Promega), following the manufacturer's instructions, in the presence of direct primer SPE2 (5′-TAAGGCCCCGATTGCAGCAC-3′) corresponding to the nucleotide position 407–426 of the H3L subclone. Polymerase chain reactions were performed on the resulting cDNAs using the SPE1 reverse primer (5′-ACAGGCCTGGAACAGTGT-3′) corresponding to the nucleotide position 289–272 of the H3L subclone. Another H3 *late* histone cDNA (PCR9 clone) was identified by the RT-PCR method already described using the pair of primers H3L1 (5′-GGAGTCAAGAAGCCTCA-3′) and H3L3 (5′-TTGGCGTGGATGGCGCA-3′). The amplified cDNAs were cloned in a pGEM 3 vector (Promega).

2.5. Blocking DNA duplication

DNA synthesis was blocked for a period of 2 h in embryos at 19 h of development by adding 5 mM hydroxyurea to the sea water [16]. At 21 h of development embryos were collected and RNA isolated and analyzed on Northern blots using as a probe the insert of the genomic subclone H3L.

3. Results and discussion

3.1. Isolation of the H3L genomic clone

A primary screening of the genomic library prepared in λ-EMBL4 vector was performed to identify *late* H3 histones using as a probe the coding fragment of the H3.3 histone cDNA that reveals a short non-polyadenylated H3 *late* histone transcript in addition to its full-length mRNA [16]. Forty phages, positive to this hybridization, were screened again using as probes the sea urchin *early* H3 histone gene and the 3′ UTR of the H3.3 histone cDNA to eliminate clones encoding those two variants. Genomic phage 6 was positive only to the initial hybridization and was then chosen for further studies. Digestion of the DNA of this phage with *Sal*I restriction enzyme generated several bands, among which a 2 kb fragment was positive to the hybridization using the coding fragment of the H3.3 histone cDNA. This fragment was subcloned and amplified in pBluescript SK$^-$ vector. Digestion of this genomic clone with *Hin*dII restriction enzyme generated one fragment of 599 bp, which was subcloned into pGEM3 vector, and a fragment containing the remaining 1400 bp of the insert still bound to the pBluescript vector, which was ligated again. The complete sequence of the 599 bp *Hin*dII fragment (clone H3L) and part of the sequence of the 1400 bp fragment still bound to the vector (clone 10) were determined, as shown in Fig. 1a. The sequence shows that the H3L clone contains an ORF for an H3 histone starting at position 148 having 81% and 87.5% nucleotide identity, respectively, with the coding region of *P. lividus* H3.3 histone and with *late* H3 histones of other sea urchins. Comparison with the known genes for H3 histones indicates that position 93 of the insert might represent the start of transcription. The

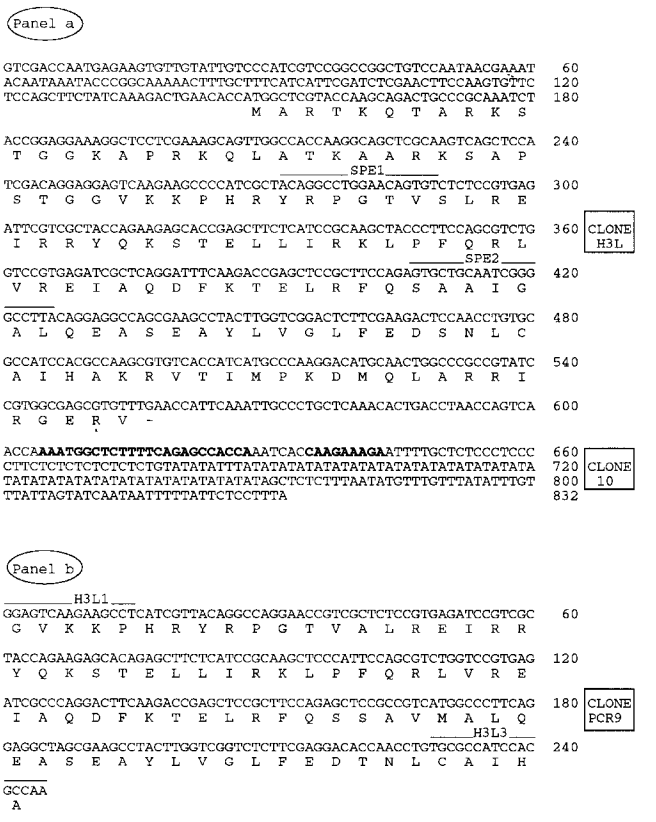

Fig. 1. Nucleotide sequences and putative encoded amino acids of the H3L clone (a) and the PCR9 clone (b). The nucleotide sequence of clone 10 containing part of the 3′ UTR is shown. The high conserved motifs in the 3′ terminal region of histone genes are in bold.

putative 5′ promoter region contains sequence motifs common to almost all the eukaryotic genes: a non-canonical TATA box at −30 and two CAAT boxes at positions −45 and −87 from the putative start of transcription. The 3′ UTR is contained in part in the H3L clone and in part in clone 10 and shows the typical stem-loop sequence observed in all replication dependent variants. The encoded protein has both S replacing A at position 31 and the A.IG amino acid motif at positions 87–89, as peculiar of the replication independent H3.3 histone variants. In addition, it shows E replacing D at position 81, as typical of all the echinoid H3 histones and four additional substitutions at positions 47, 107, 124 and 135, when compared with the consensus H3 histone sequence [21]. The two latter substitutions occur at positions where differences are observed also in protozoa and in yeasts. The result is that the gene for this H3 histone variant is intronless, with the stem-loop structure at the 3′ region of the transcript typical of the replication **dependent** variants, while the encoded protein presents the amino acid motif typical of the replication **independent** variants.

3.2. Expression of H3L genomic clone and identification of the cDNA for a major late H3 histone variant at 30 h of development

The possible expression of the gene encoded in clone H3L was studied by RT-PCR on total RNA isolated from sea urchin embryos at 30 h of development. Various pairs of primers corresponding to the H3L clone sequence were used, to take into consideration the possible presence of indi-

vidual polymorphisms and the preferential codon usage of the sea urchin. The products of amplification were sequenced. Among those tested, the pair of primers SpeI-Spe2 amplified products that corresponded to the cDNA of the sequenced gene (Fig. 2, lanes 2, 3, 5 and 6). These primers have made it possible to distinguish the different cDNAs because they correspond to short sequences of the coding region in which there are various differences in the nucleotide composition of the transcripts for *early*, *late* and replacement histones of the sea urchin. Another couple of primers, H3L1-H3L3 (Fig. 2, lanes 1 and 4), amplified a partial cDNA that encoded another sea urchin H3 variant. This was cloned and sequenced and proved to lack the S.//.A.IG motif (PCR9 clone; Fig. 1b). The sequence of the partial cDNA clone has a high level of identity (92–93%) in nucleotide sequence with the coding regions for the *late* H3 histones of other sea urchins [21]. The putative protein is exactly what was expected on the basis of the consensus sequence for sea urchin replication dependent major *late* H3 histones.

As shown in Fig. 2 the two *late* H3 histone variants are expressed at 30 h of development in two embryo cultures deriving from the eggs of two females each fertilized with the sperms of a different male. In fact, H3L cDNA (Fig. 2, lanes 2, 3, 5 and 6) and PCR9 cDNA (Fig. 2, lanes 1 and 4) are amplified in each of the two samples. The same level of amplification of the two mRNAs was obtained using four times more H3L cDNA than PCR9 cDNA, indicating that the two transcripts occur at different concentrations in the embryo. The different levels of expression of the two *late* histone variants are confirmed also by the different times of exposure required to obtain similar spot intensity in Northern blot experiments. The simultaneous expression of two H3 variant histones, H3L and H3.3, together with a major *late* H3 molecule in the progeny of the same animal suggests the presence of a high multiplicity of histones in the sea urchin embryo.

3.3. *Analysis by Northern blot of the expression of H3L and PCR9 clones during development*

The pattern of expression of the genes for the two H3 variants during embryonic development of *P. lividus* sea urchin is shown in Fig. 3a. The insert of the H3L clone reveals a single band of mRNA (about 550 nt) in embryos starting at 10 h of development (Fig. 3a). The intensity of the band increases between 10 and 30 h of development and slightly decreases at later stages. This pattern corresponds to those of the major *late* histone genes already identified in other sea urchins. If DNA replication is blocked between 19 and 21 h with 5 mM hydroxyurea, the transcript decreases to an almost undetectable amount (Fig. 3b). This result confirms that this H3 histone is replication dependent, as suggested by other features. In addition, Northern blot experiments on polyadenylated and non-polyadenylated fractions of RNA demonstrate that the transcript is polyA(−) (data not shown). The insert of the PCR9 cDNA clone reveals a single band of mRNA (about 550 nt) in embryos starting at 10 h of development (Fig. 3a). The pattern of accumulation of this transcript is consistent with the PCR9 clone being a major *late* H3 histone as expected on the basis of the nucleotide sequence.

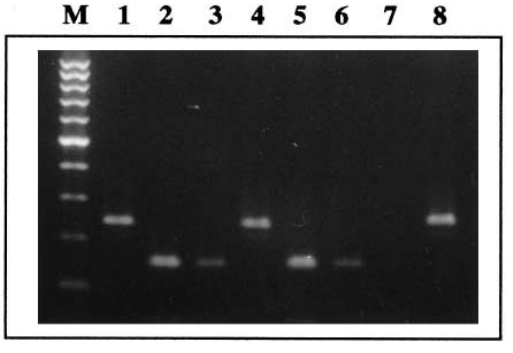

Fig. 2. Agarose gel electrophoresis of products amplified by RT-PCR performed on total RNA isolated from embryo cultures at 30 h of development. DNA bands produced on RNAs of culture I (lanes 1–3) and culture II (lanes 4–6). Lane 7: reaction mix with no addition of cDNA. Lane 8: PCR positive control experiment using the DNA of H3L clone as a template. M: 100 bp ladder molecular weight marker (Promega). Two different pairs of primers were used: H3L1/H3L3, (lanes 1, 4, 7, 8) and SpeI/Spe2 (lanes 2, 3, 5, 6).

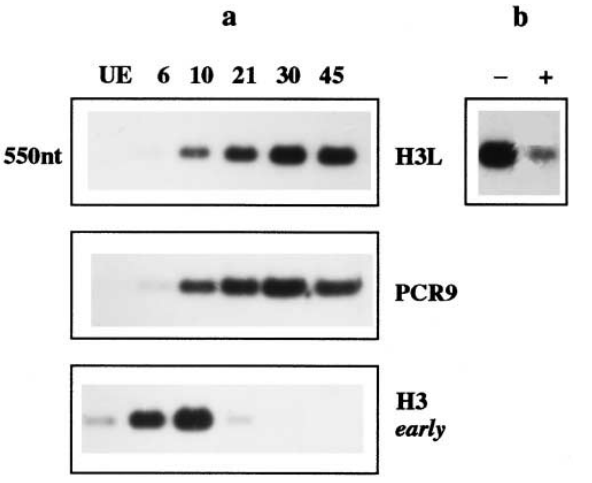

Fig. 3. Northern blot analyses. a: Total RNAs isolated from sea urchin unfertilized eggs (UE) and embryos at the indicated hours of development. b: RNAs isolated from control embryos at the 21 hour of development without (−) and with (+) previous block of DNA replication for 2 h. Probes as indicated at the side.

4. Conclusions

The critical role of histones in structuring eukaryotic DNA in chromatin is widely accepted, but only recently it appeared clear that the packaging of DNA in chromatin is not homogeneous probably in correlation with different local functional requirements [4]. Nucleosomal core histones with specific amino acid sequences might contribute to the production of the local structural alterations at the base of the functional differentiation of chromatin domains known to occur by enzymatic modification of chromosomal core histones [22]. Consistent with this possibility, it has recently been demonstrated that, in yeast, mutations that cause amino acid substitutions in the structured domain of the H3 histone partially relieved mutations in the SW1-SNF regulatory complex [23].

It is interesting that of the H3 and H4 histone pair, which is critical in the binding of the nucleosomal histone core to DNA and in chromatin assembly (for review see [24]), mainly histone H3 appears to tolerate mutations [9]. In the H3 replacement variants the characteristic amino acid motif S.//.A.IG has been identified, well conserved, in both inverte-

brates and vertebrates [9]. Are these amino acid substitutions simply the effect of neutral polymorphism or indicative of conserved functions requiring a turnover independent of DNA replication? The high conservation of the S.//.A.IG motif in H3.3 variants in invertebrates and vertebrates is not consistent with the hypothesis of their maintenance by neutral polymorphism. The occurrence in the sea urchin embryo of a histone protein that has both the amino acid motif typical of the replication independent replacement variants and the mRNA features of the replication dependent variants argues against the hypothesis that cell cycle independent mRNA regulation and a particular protein composition are necessarily correlated characteristics. The synthesis of a H3.3 protein molecule might indeed be the expression of a particular cell requirement but that variant does not appear to be necessarily encoded on a mRNA with a specific structure. Signals of polyadenylation and stem-loop structure have already been shown to be both present in the same gene for some histone replacement variants [6,25]. In our case two different genes, one for the H3.3 replacement variant [16] and the other for the H3L variant reported here, appear to exist in the sea urchin genome, in addition to the major *late* component, and both are expressed as revealed by the RNA analyses.

Moreover the identification of this novel gene lends support to the hypothesis that the H3.3 gene is a common ancestor of animal and fungal H3 histones [9] and that, after the divergence of the fungi, there was a transition from the unique split gene encoding the proteins with the mentioned amino acid motif, encoded on polyadenylated transcripts, to non-split genes with a modified amino acid composition and a consensus sequence typical of non-polyadenylated transcripts.

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