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A histone H1 variant is required for erythrocyte maturation in medaka

OSAMU MATSUOKA[1,#], NORIHISA SHINDO[2,#], DAISUKE ARAI[1] and TORU HIGASHINAKAGAWA[1,*]
[1] Center for Advanced Life and Medical Science, Waseda University, Tokyo, Japan and [2] Department of Experimental Pathology, Cancer Institute of the Japanese Foundation for Cancer Research (JFCR), Tokyo, Japan

ABSTRACT Three histone H1 variants were identified in medaka fish and their sequence characteristics were analyzed. This paper reports one of these variants, termed H1-2, because of its possible implication in erythrocyte maturation. The amino acid sequence of H1-2 was phylogenetically similar to that of other replication-dependent histones. The mRNA transcribed from the *h1-2* gene, however, possessed a poly(A) tail without a stem-loop structure, indicating that H1-2 may represent a replication-independent (RI) histone. Transcripts from the *h1-2* gene were largely localized in erythrocytes, and knock-down of the *h1-2* gene with morpholino antisense oligos resulted in failure to develop mature erythrocytes. In the morphants, residual erythrocytes showed severely impaired nuclear compaction. Although not structurally related to chicken RI histone H5, which is required for erythrocyte maturation, H1-2 may constitute its functional counterpart. Our findings may offer evolutionary insights into the function of H1 variants.

KEY WORDS: *replication-dependent histone, replication-independent histone, evolution, medaka*

Introduction

In eukaryotic cells, DNA is packaged with histones and other chromosomal proteins into chromatin, of which the fundamental repeating unit is a nucleosome. Each nucleosome consists of a histone octamer, comprising two copies each of four core histones (H2A, H2B, H3, and H4) around which 146 bp DNA is wrapped. A fifth type of histone, known as linker histone H1, connects adjacent nucleosomes by binding to their DNA (Thomas, 1999).

Unlike core histones, the linker histone H1 exhibits much less evolutionary conservation but diverge into variants or subtypes. Numbers of H1 variants differ among species, and the expression of these variants is characteristically regulated during development and differentiation (Khochbin, 2001). In general, histone H1 plays a direct role in stabilizing nucleosomal and higher order chromatin structure. In addition, it also functions as both general and specific modulator of transcription, by limiting access of regulatory molecules to chromatin (Brown, 2001).

Based on expression patterns and genomic organization, histones can be classified into two different types, namely, replication-dependent (RD) and replication-independent (RI) histones (Doenecke *et al.*, 1997). RD histones, also known as non-replacement histones, are expressed only during S phase of the cell cycle and their genes are organized in clusters. The mRNA of RD histones is not polyadenylated, and possesses an inverted repeat at the 3' region, which potentially forms a stem-loop structure. In contrast, RI histones, or replacement histones, are expressed at low levels but continuously throughout the cell cycle. The genes for RI histones are not found in clusters, but occupy solitary locations within the genome. Unlike RD histones, the mRNA of RI histone is polyadenylated.

Most of our knowledge about the role of histone H1 comes from biochemical studies of chromatin. In order to understand the *in vivo* functions of H1 variants in a developmental context, we have adopted the medaka fish as a model system. In this study, we describe the identification of three medaka histone H1 variants, designated H1-1, H1-2 and H1-3. Of these, H1-2

Abbreviations used in this paper: MO, morpholino oligo; RD, replication dependent; RI, replication independent.

***Address correspondence to:** Prof. Toru Higashinakagawa. Center for Advanced Life and Medical Science, Waseda University, 2-2 Wakamatsu-cho, Shinjuku, Tokyo 162-8480, Japan. Fax: +81-3-5369-7312, e-mail address: toru@waseda.jp

[#] These authors contributed equally to this work

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is shown to be required for erythrocyte maturation through the compaction of the nucleus.

Results

Structural analysis of medaka histone H1 variants

Histone H1 variants share common structural characteristics, although amino acid sequences diverge among organisms. Generally, histone H1 has a central globular domain flanked by less structured amino- and carboxyl-terminal domains. The globular domain is the most conserved among variants and organisms, while the amino- and carboxyl-terminal domains are more diverged. In medaka, we identified two histone H1 variants by molecular cloning and one third variant by database analysis. These variants were named *h1-1*, *h1-2* and *h1-3.* As in other organisms, medaka histone H1 variants shared common structural features (Fig. 1A). Phylogenetic analysis based on amino acid sequence suggests that medaka H1-1 is related to replication-independent (RI) variants, including human, mouse and *Xenopus* H1°, as well as chicken H5. In contrast, H1-2 and H1-3 appear to belong to replication-dependent (RD) variants, such as human and mouse H1.1, and *Xenopus* H1A (Fig. 1B).

Other criteria for differentiating RD from RI histones are via the mode of mRNA processing and genomic organization. The mRNA of the *h1-1* and *h1-2* genes contained poly(A) addition signal in the 3' UTR (Fig. 1C) and their genes are found in solitary location in the medaka genome (GenBank accession nos. BAAF03016248 and BAAF03013006, respectively). In contrast, *h1-3* mRNA possessed a stem-loop structure, which is typical of replication-dependent processing (Fig. 1C) and *h1-3* gene is located in the histone gene cluster on chromosome 1 (GenBank accession no. DF076692) suggesting H1-3 to be of orthodox RD type. To date, the metazoan histone genes that

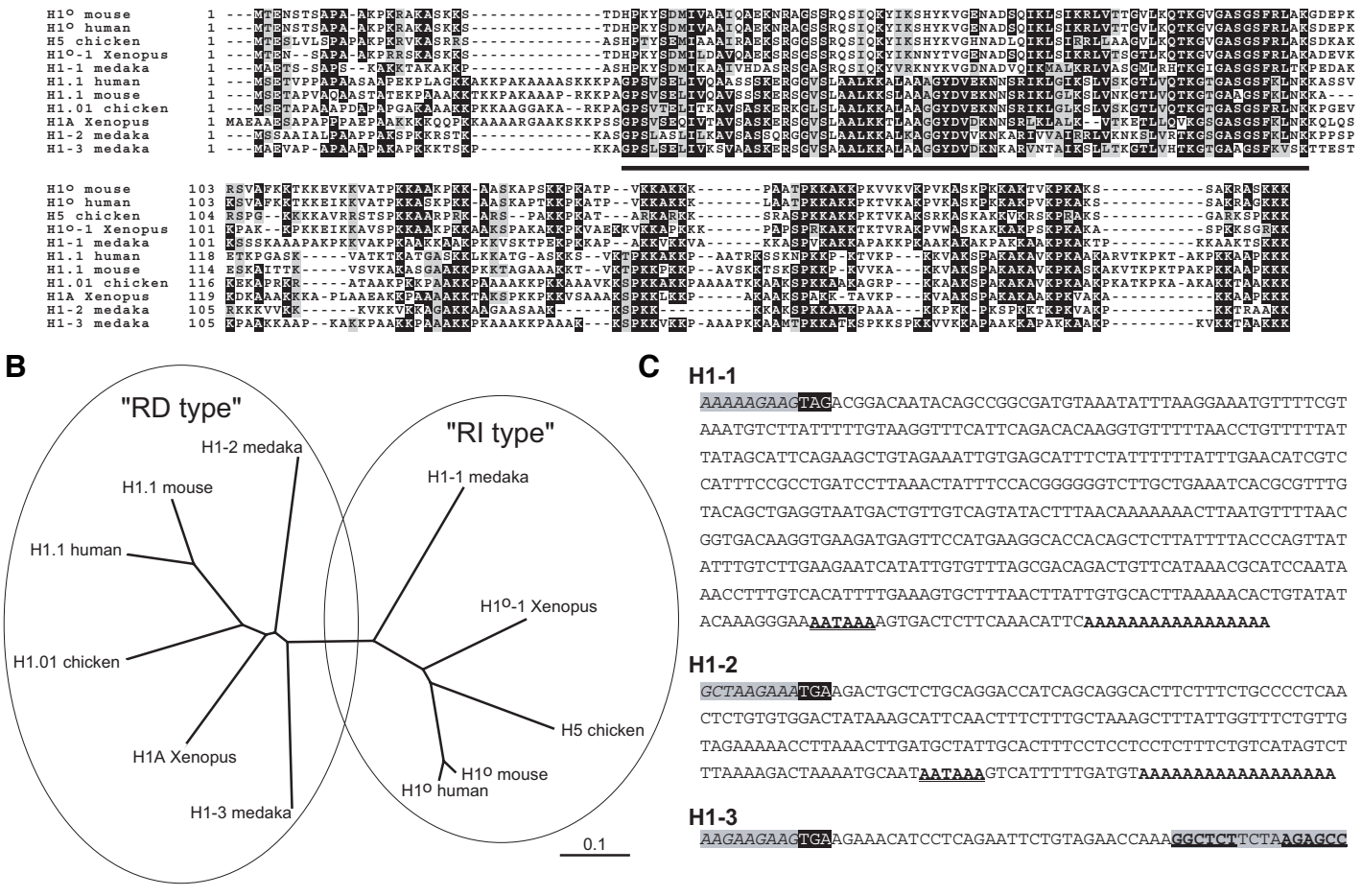

Fig. 1. Structural analysis of medaka histone H1 variants. (A) *Medaka H1 variants possess structural features that were observed among other histone H1 proteins. Multiple sequence alignment was carried out using the CLUSTAL X program (Thomson* et al.*, 1997). Identical residues appear on a black background and similar residues are on a gray background. Numbers to the left of the sequences indicate position of the leftmost amino acid. Medaka H1-1 and H1-2 and H1-3 share structural features that are common among H1 variants. The globular domain is underscored with a thick line.* **(B)** *Phylogram of histone H1 sequences. "RD type" and "RI type" stand for "replication-dependent type histone" and "replication-independent histone", respectively.* **(C)** *3' UTR of medaka histone H1 transcripts. Portions of sequences in italics highlighted in light gray are the ends of the coding regions, terminating with the stop codon highlighted in black. The polyadenylation signal AATAAA is in bold and double underlined and the poly(A) tail indicated in bold. The stem-loop structure is highlighted in gray with inverted repeat nucleotides in bold and underlined. GenBank accession nos.: medaka H1-1, BR000413; medaka H1-2, BR000412; medaka H1-3, BR000411; human H1.1, X57130; mouse H1.1, Y12290; chicken H1.01, X01752;* Xenopus *H1A, S69089; human H1°, Z97630; mouse H1°, X13171; chicken H5, J00870;* Xenopus *H1°-1, Z71502.*

encode polyadenylated mRNA are not cell cycle regulated and of RI type (Marzluff and Duronio, 2002). Our data suggest that H1-2 may represent an RI type histone although its protein structure can be classified as an RD histone.

Expression of medaka h1-2 *during embryonic development*

Intrigued by the structural complication of H1-2, further analysis was focused on the behavior of this molecule. Developmental expression profiles of *h1-2* were examined by RT-PCR and whole-mount *in situ* hybridization. The *h1-2* transcripts showed a sharp increase at 30 somite stage (i.e. 2 days and 16 hours) (Fig. 2A) and it was largely restricted to erythrocytes in blood vessels, such as the duct of Cuvier (the main vessel on the yolk sac, arrows in Fig. 2B). Note that the dark anterior staining at somite completion stage (i.e. 4 days and 5 hours) indicates the pigmentation of the eyes (arrowheads in Fig. 2B). No appreciable *h1-2* mRNA was detected at 18-19 somite stage (i.e. 2 days and 2 hours), which is consistent with the RT-PCR results. These observations suggest that H1-2 may play a role in erythrocyte formation during medaka development.

Knock-down of h1-2 *gene resulted in reduced production of erythrocytes*

To explore a possible function of H1-2 in erythrocyte production, we performed knock-down experiments using morpholino antisense oligos (MO) targeted to *h1-2* mRNA. We injected *h1-2* MO into both blastomeres of 2-cell embryo. An initial injection of 5 ng MO resulted in total lethality following gastrulation (data not shown). However, injection of the same dose of 5mis *h1-2* MO, which contained 5 base mismatches, resulted in approximately 10% of lethality, suggesting that H1-2 variant possesses some developmental significance. Consequently, the amount of MO was gradually reduced to a point where levels of lethality were insignificant, but still hypomorphic conditions were produced. With 0.25 ~ 1 ng of *h1-2* MO, overall lethality was finally reduced to a level of approximately 5~6%.

The MO injected embryos, called morphants, were left to develop up to the somite completion stage and subjected to microscopic inspection (Fig. 3 A-H). Compared with control uninjected embryos or 5mis *h1-2* morphants, *h1-2* morphants exhibited a remarkable reduction in the number of blood cells in the duct of Cuvier (Fig. 3A). Staining of hemoglobin by *o*-dianisidine further confirmed the reduction in the levels of blood cells in *h1-2* morphants in a dose dependent manner (Compare Fig. 3 F,G). *In situ* hybridization at the 18-19 somite stage using embryonic α-globin probe (Sakamoto *et al.*, 2004) confirmed that the *h1-2* morphants had intact intermediate cell mass (ICM), a region where primitive hematopoiesis occurs (Fig. 3 I,J). This finding and the observation that *h1-2* expression is not yet prominent at the 18-19 somite stage (Fig.2) suggest that the H1-2 function is specific to the later stage of blood cell maturation. Essentially the same results were obtained using another MO (*h1-2* MO2) that binds to a different site of the *h1-2* transcript (data not shown). In addition, *h1-2* morphants exhibited various other phenotypes, such as deformed heart, small eyes, small head and shortened tail. These observations suggest that H1-2 may also participate in other developmental processes that are not directly associated with blood cell maturation, although the detailed mechanisms involved await further investigation.

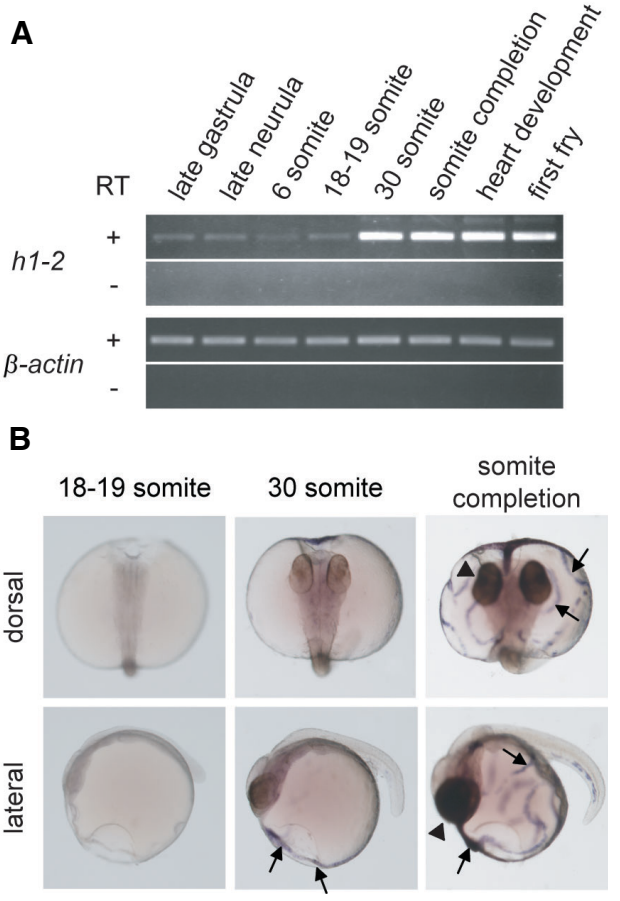

Fig. 2. Temporal and spatial expression of medaka *h1-2* during development. (A) *RT-PCR expression profiles of medaka histone* h1-2. *β*-actin *expression is shown as a control.* **(B)** *Whole-mount* in situ *hybridization profiles of medaka histone* h1-2. *Arrowheads indicate pigmented eyes and arrows indicate the duct of Cuvier expressing* h1-2. *The stage first fry designates the hatched fries until fin rays appear in the caudal and pectoral fins.*

H1-2 is required for erythrocyte maturation through compaction of nucleus

It has been reported that nuclear compaction accompanied by chromatin condensation occur during terminal differentiation of erythrocytes (Yamamoto and Iuchi, 1975; Rowley and Ratcliffe, 1987; Morioka and Minamikawa-Tachino, 1993). In mammals, a multi-functional chromatin-associated protein called Heterochromatin Protein 1 (HP1), is implicated in heterochromatin-mediated silencing and terminal differentiation process. In frogs and chicken, histone H1 also is involved in this process (Thomas *et al.*, 1985; Bergman *et al.*, 1988; Wangh *et al.*, 1995; Dimitrov and Wolffe, 1996; Gilbert *et al.*, 2003). In fish, the involvement of histone H1 in erythrocyte terminal differentiation has been suggested but thus far not confirmed. Our observation of H1-2 function in medaka may suggest a role for histone H1 in nuclear compaction of red blood cells. If medaka H1-2 is indeed involved in the nuclear

TABLE 1

PRIMER PAIRS AND OTHER CONDITIONS USED FOR RT-PCR ANALYSIS

Gene	Primer	Sequence	Annealing temperature (°C)	Product size (bp)	Cycles	Accession no.
h1-2	H1-2-F	CTT GTC TGT GAC CTT ACT GAC CTG	57	746	30	BR000412
	H1-2-R	ACA GAA AGA GGA GGA GGA AAG TGC				
β-*actin*	β-actin-F	AAC TCA TTG GCA TGG CTT C	60	501	20	S74868
	β -actin-R	TAG TCA GTG TAC AGG TTT GGC				

F means forward and R means reverse

compaction of erythrocytes, knock-down of *h1-2* would result in the formation of immature erythrocyte with loosened nucleus and decondensed chromatin.

To assess this possibility, we collected blood from both control embryos and morphants produced by injection of 0.25 ng *h1-2* MO. Blood cells were collected by cutting the tails of embryos at the somite completion stage. Since this stage represents the onset of blood circulation in the tail, collecting cells at this time avoids the possibility of picking up morphants with delayed development. Collected blood cells were stained by May-Grünwald-Giemsa (MGG) method and with DAPI. In both control and morphant embryos, MGG staining revealed spherical erythrocytes with non-compacted nucleus at the 24 somite stage (i.e. 2 days and 10 hours) (Figs. 4A and B). At the somite completion stage control erythrocyte had a compacted nucleus in its center (Fig. 4C). In contrast, however, at the same developmental stage, morphant erythrocyte displayed spherical morphology with a non-compacted nucleus (Fig.4D), essentially similar to that seen at the 24 somite stage for these embryos (Fig. 4A and 4B). Visualization by DAPI staining further showed that morphant erythrocytes had non-compacted nucleus compared with control erythrocytes at the somite completion stage (Figs. 4G and H). Finally, digital image analysis of DAPI stained erythrocyte nuclei confirmed this observation (Fig. 4I). Between stages compared, control erythrocytes showed statistically significant compaction ($p<0.05$, Student's t-test) whereas, in morphants, no statistically significant differences in erythrocyte compaction were detected ($p = 0.16$, Student's t-test).

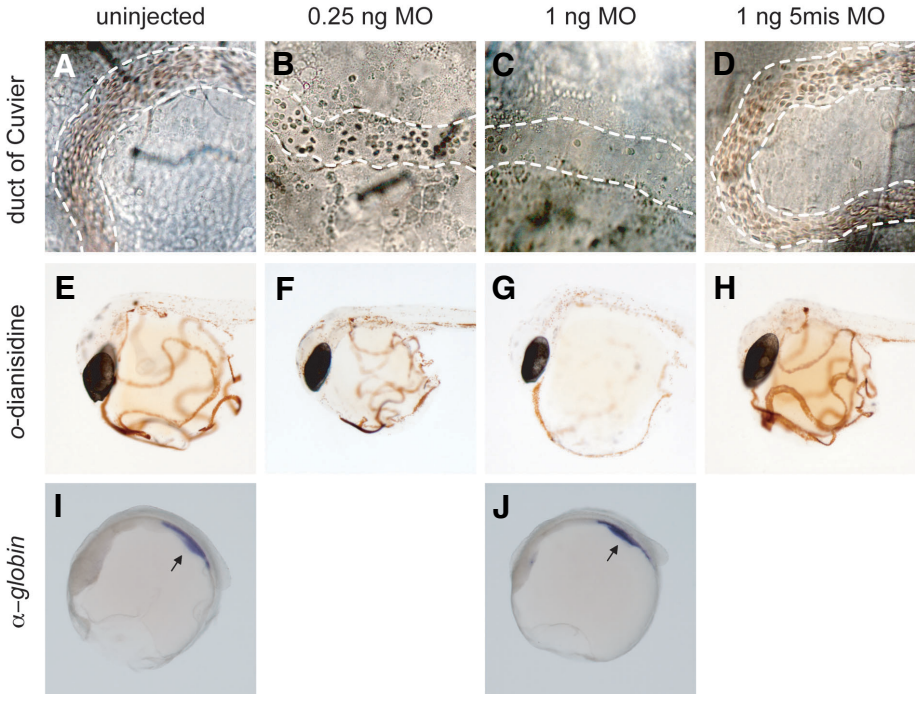

Fig. 3. Injection of *h1-2* MO resulted in reduced numbers of blood cells. *Somite completion stage (4 days and 5 hours) embryos were inspected under the light microscope.* **(A-D)** *Circulating blood cells in the duct of Cuvier, the main vessel on the yolk sac.* **(E-H)** *Hemoglobin staining by* o-*dianisidine. 5mis MO was used for a control (see Materials and Methods). White broken lines demarcate the duct of Cuvier.* **(I,J)** *Whole-mount* in situ *hybridization using embryonic α-globin probe on control embryos and 1 ng* h1-2 *morphants at 18-19 somite stage (2 days and 2 hours). Arrows indicate the intermediate cell mass (ICM).*

Discussion

We have identified three histone H1 variants, termed H1-1, H1-2 and H1-3, in medaka *Oryzias latipes,* either through EST-based cloning (H1-1 and H1-2) or by *in silico* survey (H1-3). As in other metazoan, medaka histone H1 molecules possessed a typical tripartite structure. Based upon the transcript structure and their location in the genome, we predict H1-1 and H1-2 to be of RI type and H1-3 to be of RD type. Interestingly, amino acid sequence of H1-2 suggests that this variant is of RD type (Figs. 1A and 1B). In all cases to date, however, histone genes that encode a polyadenylated message are not cell cycle regulated and of RI type (Marzluff and Duronio, 2002). In addition, given that in mouse RI histone H3.3 differs from the primary RD histone H3.1 by just four amino acid residues (Hraba-Renevey and Kress, 1989), it appears that mRNA structure provides a much stronger basis for histone classification as opposed to polypeptide sequence. Our observation of a close phylogenetic relationship of H1-2 with other RD histones may instead have an evolutionary origin (see below).

Prompted by a growing body of evidence for the crucial roles of RI histones in development (Khochbin and Wolffe, 1994; Doenecke *et al.*, 1997; Khochbin, 2001) and the structural complication as described above, we examined the expression profiles of *h1-2* during development. The *h1-2* mRNA increased intensely at 30 somite stage and was mostly restricted to erythrocytes (Fig. 2). This erythrocyte restricted expression is reminiscent of erythrocyte specific expression of chicken H5, which is also of RI type. Indeed, knock-down analysis using *h1-2* MO resulted in reduced

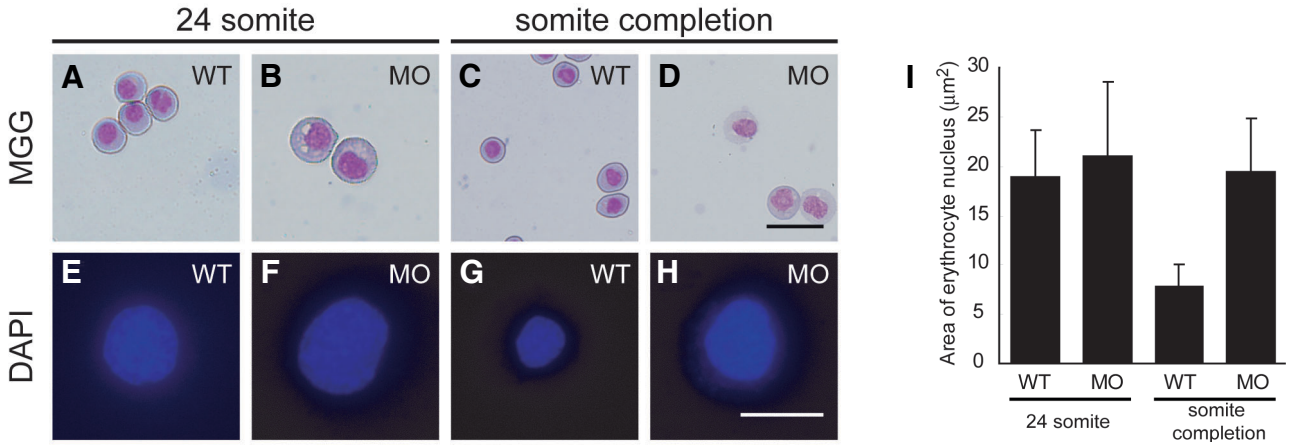

Fig. 4. H1-2 is required for erythrocyte maturation through the compaction of the nucleus. *Blood cells were collected from both control wild type embryos (WT) and 0.25 ng* h1-2 *morphants (MO) at 24 somite stage (2 days and 10 hours) and somite completion stage. May-Grünwald-Giemsa (MGG) staining* **(A-D)** *and DAPI staining* **(E-H)**. *Scale bars, 20 μm (A-D), 10 μm (E-H).* **(I)** *Area of erythrocyte nucleus measured by Scion image program. Mean values ± SD are given. 24 somite stage WT (18.84 ± 4.83 μm², n = 73), 24 somite stage MO (21.03 ± 7.47 μm², n = 65), somite completion stage WT (7.78 ± 2.30 μm², n = 80), somite completion stage MO (19.40 ± 5.44 μm², n = 64).*

production of erythrocytes (Fig. 3).

Nuclear compaction observed in terminally differentiated erythrocytes represents a model system in which to study the developmentally regulated chromatin condensation (Weintraub, 1984; Rowley and Ratcliffe, 1987). Several studies have shown a close relationship between nuclear size and chromatin condensation, which involved H1 histones (Yamamoto and Iuchi, 1975; Morioka and Minamikawa-Tachino, 1993; Gilbert *et al.*, 2003). In chicken erythrocytes, histone H5 caused the compaction of the nucleus (Bergman *et al.*, 1988) and the condensation of chromatin fiber (Thomas *et al.,* 1985). In nucleated erythrocytes in *Xenopus*, accumulation of histone H1°-2 led to the cessation of proliferation and chromatin condensation (Koutzamani *et al.*, 2002) whereas its removal results in nuclear swelling and presumably chromatin loosening (Wangh *et al.*, 1995; Dimitrov and Wolffe, 1996). A recent study showed the involvement of HP1 in this process in mammals, but not the case in chicken, frog, and fish erythrocytes (Gilbert *et al.*, 2003).

We provide the first evidence suggesting that fish erythrocytes contain RI histone variant that functions in nuclear compaction. Erythrocytes collected from *h1-2* morphants displayed immature characteristics (Figs. 4A-D) and non-compacted nuclei (Figs 4E-I). These results suggest that H1-2 is required for erythrocyte maturation through the compaction of the nucleus, presumably associated with chromatin condensation. Highly probable is that, through chromatin condensation, H1-2 silences certain group of genes necessary for terminal differentiation of the erythrocytes.

Erythrocyte specific expression and the *h1-2* morphant phenotype together suggest that H1-2 represents a medaka counterpart of chicken H5 and *Xenopus* H1°-2, both RI histone. Our phylogenetic analysis at the amino acid sequence level, however, revealed that H1-2 is more closely related to other RD histones (see Fig. 1B). Therefore, we predict that the resemblance of H1-2 to a RD histone may have an evolutionary significance. For example, Brocard *et al.* (Brocard *et al.*, 1997) proposed that, based on comparative amino acid sequence analysis, the chicken erythrocyte specific histone H5 and the *Xenopus* $H1^0$ genes share a common ancestral $H1^0$ -like gene. They suggest an interesting possibility that, over evolutionary time, the ancestral H1°-like gene of RD type was incorporated into an erythroid-specific genomic region and became erythroid specific and of RI type. We speculate an analogous situation during the evolution of fish. An ancestral form of H1-2, which was originally a RD histone and located in a histone cluster, may have been translocated into an erythroid-specific genomic region and acquired the function required for erythrocyte development. This evolutionary scenario is in line with the hypothesis that RI histones were originated from clustered RD histones and became permanently localized to a solitary region of the genome (del Gaudio *et al.*, 1998; Eirin-Lopez *et al.*, 2005). Further analysis of the genomic structure of the *h1-2* gene in medaka will help to elucidate a more complete picture of the evolutionary origin of this histone variant.

Materials and Methods

Fish

Wild type HO4C inbred strain of Japanese medaka, *Oryzias latipes*, was used. Adult fish were maintained in aquaria under an artificial photoperiod of 14/10-h light/dark cycle at 26.5°C. Developmental stages of the embryo were determined according to the criteria described by Iwamatsu (Iwamatsu, 2004).

Cloning of medaka histone H1 *genes*

Sequences coding for medaka histone H1 were identified as medaka expressed sequence tags (EST) using alignment analysis with known H1 sequence. One EST clone, MF01SSA117H10 (GenBank accession nos. BJ022051 and BJ008276), which corresponds to full length histone H1 cDNA, was kindly provided by Dr. H. Takeda (University of Tokyo), and termed medaka histone *h1-1*. Another histone variant, termed medaka histone *h1-2,* was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed based on the sequences of EST fragment (GenBank accession no. AM339419). The sequence of *h1-3* gene was based on EST clone MF01ASD039j21 (GenBank accession no. BJ885804).

RT-PCR analysis of medaka histone H1 *mRNA*

Total RNA was isolated from embryos at successive developmental stages using the RNeasy Mini kit (Qiagen). Complete removal of DNA was carried out using the RNase-free DNase set (Qiagen). Complementary

DNAs were generated using the Omniscript RT kit (Qiagen) and Oligo dT primer (Ambion). Primer pairs used are shown in Table 1. PCR conditions were as follows; initial denaturation for 1 min at 94°C, denaturation for 30 sec at 94°C, annealing for 30 sec at the temperature optimized for each primer pair and extension for 1 min at 72°C.

Plasmid constructs and whole-mount* in situ *hybridization

Digoxigenin-labeled antisense RNA probes were synthesized using a DIG-RNA labeling mix (Roche Diagnostics). Whole-mount *in situ* hybridization was carried out as described previously (Shindo *et al.*, 2004).

Microinjection of morpholino antisense oligos

Two morpholino antisense oligos (MO) were obtained from Gene Tools, LLC. The *h1-2*MO (5'-GCAGAAGACATCGTGGATCTCTGAG-3') was targeted to the start codon of histone *h1-2* mRNA. Another MO, termed *h1*-2 MO2 (5'-GAGTCAGGTCAGTAAGGTCACAGAC-3') was targeted to the upstream of the *h1-2*MO binding site. A 5mis *h1-2*MO (5'-GCAcAAcACATCcTGGATgTCTcAG-3') containing 5 base mismatches was prepared as a negative control. Morpholinos were dissolved in nuclease-free water, and microinjected as described previously (Shindo *et al.*, 2005).

o–dianisidine staining

Staining of hemoglobin by *o*-dianisidine was performed as described (Paffett-Lugassy and Zon, 2005). To remove chorion, embryos were incubated at 26°C in a solution containing pronase (10 mg/ml, Sigma) and hatching enzyme (a crude extract of embryos just before hatching) until the chorion became soft. Enzyme-treated embryos were transferred to balanced salt solution (111.2 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.4 mM $CaCl_2$, 0.01 % $NaHCO_3$), and the chorion was peeled with fine forceps.

Isolation and staining of embryonic blood cells

Blood cells were collected by cutting the tail of dechorionated embryos in phosphate-buffered saline containing 1% bovine serum albumin. The blood cells were centrifuged at 1300 rpm for 2 minutes onto a glass slide using CytoFuge 2 (Statspin). Cells were stained by the May-Grünwald-Giemsa (MGG) method and with DAPI. Nuclei of DAPI stained cells were photographed with a digital camera interfaced with a fluorescence microscope. The area of each nucleus was calculated using the Scion Image Program, Beta 4.0.3 (Scion Corporation).

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