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IMMUNOCHEMICAL STUDIES OF HISTONE H5 FROM *HALOBATRACHUS DIDACTYLUS*

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Abstract—1. Histone H5 from *Halobatrachus didactylus* was isolated by using perchloric acid (PCA) extraction of fish liver nuclei and trichloroacetic acid (TCA) precipitation.

2. A polyclonal antiserum was generated by immunizing rabbits with the antigen purified from SDS–PAGE.

3. By immunofluorescence the serum stains erythrocyte nuclei from *H. didactylus* but it does not react with mammalian cells.

4. By Western blotting, the anti-H5 antibody reacts with the isolated antigen at high titers.

5. Digestion of histone H5 with pepsin and cyanogen bromide suggests that the epitopes are located in the globular and *C*-terminal region of the H5 molecule excluding the *N*-terminal.

INTRODUCTION

Histones are a group of chromosomal proteins maintained in a relatively constant amount with respect to DNA. The synthesis of the major histones, H1, H2a, H2b, H3 and H4, is coupled to DNA replication and is increased in proliferating tissues. In contrast, a minor histone species was identified as being present in non-replicating tissues (Panyim and Chalkey, 1969). This histone was described as the histone H1o on SDS–PAGE and it migrates more rapidly than the major H1 histones. The histone H1o was first detected in several mammalian tissues (Balhorn *et al.*, 1972). The same or a closely related protein was observed in Amphibia (Shimada *et al.*, 1981), reptiles (Rutledge *et al.*, 1981) and fish (Brown and Goodwin, 1983). Analysis of H1o histones from different species and tissues showed that in common with H1 histones, the H1o have a high lysine content and a low content of arginine relative to other histones. Chicken H5 is an erythrocyte-specific histone that accumulates in the nucleus during the maturation of the red blood cells (Appels and Wells, 1972). Similarly, the nucleated erythrocytes of fish contain a basic histone H5 that partially but not completely replaces histone H1 in the mature erythrocyte (Brown and Goodwin, 1983).

Histone H5 shares with H1o a higher degree of sequence homology than with other H1s (Smith *et al.*, 1980). In fact, it has been shown that polyclonal antibodies against H5 cross-react with H1o but not with H1 (Mura and Stollar, 1981). Histone H1 (including H1o and H5), has three distinct conformational domains: the *N*-terminal sequence of approximately 33 residues, the central folded (globular) region of about 80 residues, and the *C*-terminal sequence of 95 residues (Aviles *et al.*, 1978). Structural similarities between H1o and H5 have been examined at the DNA sequence level and by using specific antibodies. The evidence suggests that these proteins have a major homology in the globular region. While this central region appears to have a critical role for the folding ability of the whole molecule, the *C*-terminal domain remains the most likely region for the condensation of the nucleofilament and the very basic and hydrophylic sequence of this domain fits it well for extended ionic interactions with the DNA (Allan *et al.*, 1981).

The evolutionary range between fish, avian and mammals seems sufficiently broad to validate the notion that the amino acid sequence of higher histones H1, H1o and H5, is diagnostic of some functional relatedness. There is a tendency for H1o and H5 histones to accumulate in tissues during development. Further, regenerating tissues (including many cancer cells) have shown a decrease in the level of H1o. In this regard it is interesting to note that H1o and H5 are both thought to restrict, directly or indirectly, the accessibility of the DNA template to transcription or replication. H5 may in fact be a lightly differentiated H1 histone whose sole function is to maintain the highly repressed state in avian and fish erythrocyte chromatin.

In order to study these putative functions of H5 during development of fish erythrocytes, we initially have isolated the protein to homogeneity and generated a polyclonal antibody to this nuclear antigen.

MATERIALS AND METHODS

Histone H5 isolation and cleavages

Whole histone H1 subtypes including H5 were extracted from isolated liver nuclei with 5% perchloric acid and 20% TCA precipitation as described (Smith *et al.*, 1980). Acetone washed pellet was dried and resuspended in the appropriate buffer for further treatments. Histone H5 was purified to

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homogeneity by cutting out several bands from SDS–PAGE and extracted overnight at 4°C in a 10 mM Tris, pH 7.5, 150 mM NaCl buffer including 0.1% SDS. The protein was precipitated by adding three volumes of ethanol for 10 hr at 4°C, and resuspended after centrifugation in Tris–saline buffer.

The *C*-terminal peptide of fish H5 (equivalent by reference to residues 94–189 for chicken histone H5) was prepared by pepsin digestion at 2 μg/ml in 100 mM sodium acetate buffer, pH 4.5, at 37°C for 30 min (Rozalski *et al.*, 1985). The cyanogen bromide peptides of fish H5 were prepared by digestion of whole histones H1 at a 10 mg/ml concentration of the enzyme in 70% formic acid for 30 hr at room temperature (Brown and Goodwin, 1983). Polypeptide products of both cleavages were analysed by SDS–PAGE (Laemmli, 1970).

Antibody preparation

Polyclonal antibody against H5 was elicited in rabbits by immunization with pure H5 protein isolated from SDS–PAGE bands, and emulsified with one volume of complete Freund's adjuvant. Approximately 100 μg of pure H5 was used for the first injection in multiple sites on the back of the animal. Boosted injections were done with 500 μg of the antigen emulsified with incomplete Freund's adjuvant at 4 week intervals. Titers of serum were tested by Western blotting of SDS–PAGE.

Western blotting

For screening and antibody specificity purposes, whole liver fish nuclei proteins and purified H5 were analysed by SDS–PAGE. The separate products were transferred to nitrocellulose filters for 2 hr at 300 mA in Tris–glycine–SDS buffer containing 20% methanol (Towbin *et al.*, 1975). The filters were blocked with 5% non-fat dry milk for 1 hr and reacted with anti-H5 serum and peroxidase-labelled goat anti-rabbit Ig essentially as described by Valdivia and Brinkley (1986).

Immunofluorescence microscopy

For indirect immunofluorescence analysis, mature fish erythrocytes were fixed onto coverslips with 3% formaldehyde in PBS for 30 min at room temperature, and lysed afterwards with 0.1% Triton in PBS for 90 sec. After washing with phosphate–saline buffer (PBS) for 10 min, they were covered with 3% albumin in PBS for 15 min and washed in PBS for 10 min. Cells were incubated with anti-H5 serum at 1:100–1:200 dilutions at 37°C for 45 min. After removing the excess of antibody, the cells were reacted with fluorescein-conjugated goat anti-rabbit Ig at 1:40 dilution for 45 min at 37°C. After a final washing with PBS, the coverslips were mounted with glycerol–PBS containing 0.5 μg/ml of Hoechst 33528 and photographed with a Nikon Labophot microscope.

RESULTS

Extraction of *Halobatrachus didactylus* liver nuclei with perchloric acid gave three bands upon electrophoresis in SDS gels (Fig. 1A, lane 1). An identical pattern was obtained when erythrocyte nuclei from the same species were used as a source of whole histone H1 (data not shown). These polypeptides migrated close together with an apparent mol. wt of 28,000–31,000. This pattern corresponds quite well with that described for chicken histone H1 and H5 as compared with whole human H1 as an example (Fig. 1A, lane 2). While major bands for mammalian cells are those migrating slowly on the SDS–PAGE, in fish the major band is the one moving faster on the gel (Fig. 1A, lane 1). This fish histone corresponds to H5 with a mol. wt very close to that of H1o from human (see minor faint band in lane 2 of Fig. 1A).

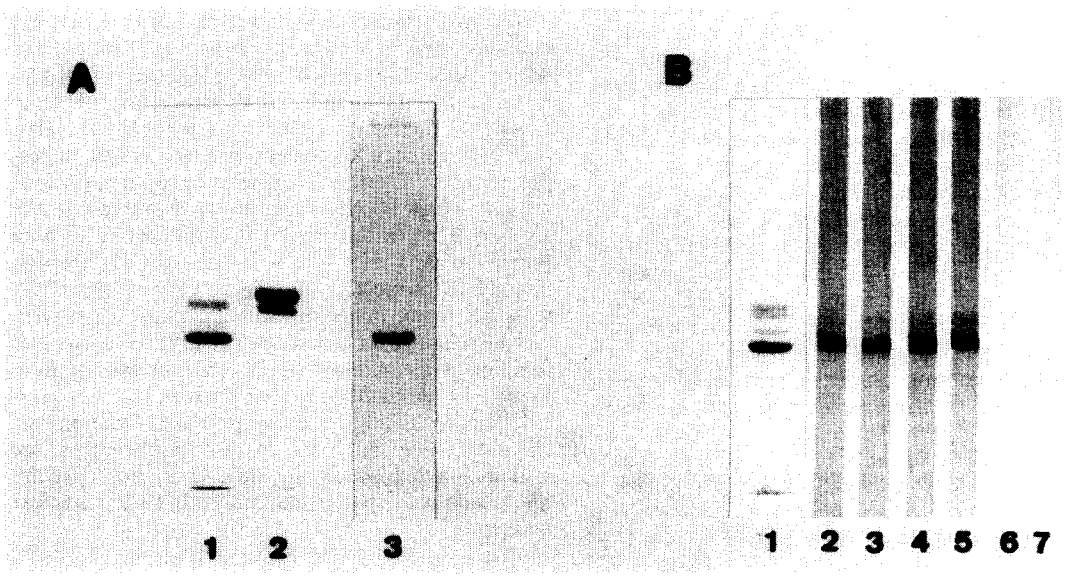

Fig. 1. Isolation of *Halobatrachus didactylus* histone H1 and immunoblot with anti-H5. (A) Coomassie Blue staining of 5% perchloric acid extracted proteins from fish liver nuclei (lane 1) and HeLa cells nuclei (lane 2). Fish histone H5 purified protein is shown in lane 3. (B) Whole fish histone H1 as shown by Coomassie Blue staining in lane 1 was assayed with polyclonal sera to purified fish histone H5 by immunoblot. Increasing amounts of immunoglobulins against histone H5 were used in lanes 2–5 (from 1:2000 to 1:100). At the lowest dilution 1:100 (lane 5) cross-reactivity is observed with other polypeptides. Negative controls using preimmune serum (lane 6) or in the absence of second antibody (lane 7) demonstrated the specificity of the assay.

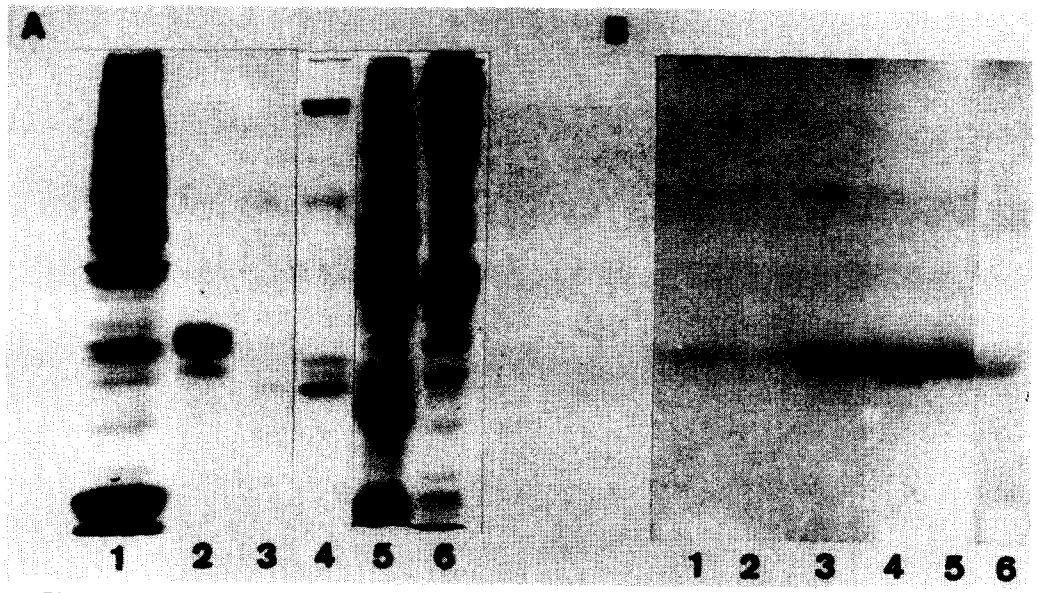

Fig. 2. Immunoblot of different cells extracts with anti-histone H5. (A) Coomassie Blue staining of human placenta nuclei cell extract (lane 1), purified human placenta histone H1 (lane 2) purified fish histone H5 (lane 3), purified fish histone H1 (lane 4), fish liver cell extract (lane 5) and fish kidney cell extract (lane 6). (B) Immunoblot of a similar gel shown in (A). It is clear the specificity of the polyclonal serum to fish histone H5. No cross-reactivity is observed with human histone H1A and H1B. In lane 1 a very faint band shows a putative reactivity with the human related histone H1o.

Histone H5 was purified to homogeneity by eluting the specific band from several SDS gels and the result is shown in Fig. 1A, lane 3. Pure fish histone H5 induced a high titer specific polyclonal serum when it was used as an immunogen in rabbits (Fig. 1B). By Western blotting the anti-H5 antibody reacted predominantly with H5 when total fish H1 proteins were used in the assay (Fig. 1B, lanes 2–5). Some putative cross-reactivity was detected with another H1 polypeptide when the serum was used at a very high concentration (Fig. 1B, lane 5). Appropriate controls demonstrate the specificity of the immunoblot (Fig. 1B, lanes 6, 7).

To show the wide presence of histone H5 in different tissues in fish, we checked by immunoblotting the polyclonal anti-H5 serum with several cell extracts. As shown in Fig. 2, Histone H5 is expressed in several fish tissues indicating that its presence and role in chromatin organization and function is something ubiquitous. Identical results were obtained with other *Halobatrachus didactylus* tissues than those shown in Fig. 2 (data not shown). Figure 2 also indicates the absence of reactivity of our antibody with mammalian H1. A very small reaction occurs with histone H1o (Fig. 2B, lane 1). The high titer and specificity of the serum was evident when we compared the staining by Coomassie Blue and the results of the immunoblotting (Fig. 2A and B, lanes 2 and 3). Even in the presence of an undetectable amount of H5 using Coomassie Blue, the antiserum still reacts very strongly. In contrast, large amounts of human H1 did not react with anti-H5.

To localize the antigen recognized by the polyclonal serum in fish cells, we used indirect immunofluorescence in fixed fish erythrocytes. These types of cells have been shown to be nucleated and contain large amounts of histone H5 (Brown and Goodwin, 1983). As shown in Fig. 3B, anti-H5 antibody reacts very strongly with erythrocyte nuclei producing no reaction at all with the cytoplasm. Some putative proliferating non-erythrocyte cells showed minor staining (see legend in Fig. 3 and comments in the Discussion). Preimmune serum as control demonstrated the specificity of the staining (Fig. 3C). As a correlation with the immunoblotting results we tested the staining in mammalian cells with anti-H5 serum. Negligible cross-reactivity was detected by immunofluorescence in nuclei from different mammalian sources, indicating the absence of a major antigen reactive with anti-H5 serum (Fig. 4).

In order to identify the epitopes on the H5 molecule reactive with our polyclonal serum, we subjected histone H5 to digestion with pepsin. The electrophoretically resolved peptides resulting from this procedure (Fig. 5B, lanes 1, 2) were transferred to nitrocellulose paper and incubated with anti-H5 antibody. This revealed that some H5 peptides do not contain epitopes for the antiserum produced. From this result and the peptide pattern originated by pepsin digestion on the H5 molecule (Rozalski *et al.*, 1985), we conclude that our antibody recognizes epitopes localized mainly on the *C*-terminal region of H5 molecule excluding the *N*-terminal region.

Cyanogen bromide has been shown to cleave H1o at a single methionine (residue 30 for chicken H5). Therefore we decided to use this reagent to reveal the presence of that amino acid on fish histone H5 and to help to localize the epitope(s) reactivated with our anti-H5 antibody. Total fish histone H1 in 70% formic acid was incubated at room temperature in the dark with a 100-fold excess of cyanogen bromide (CNBr) for 30–40 hr. Figure 5A shows the Western

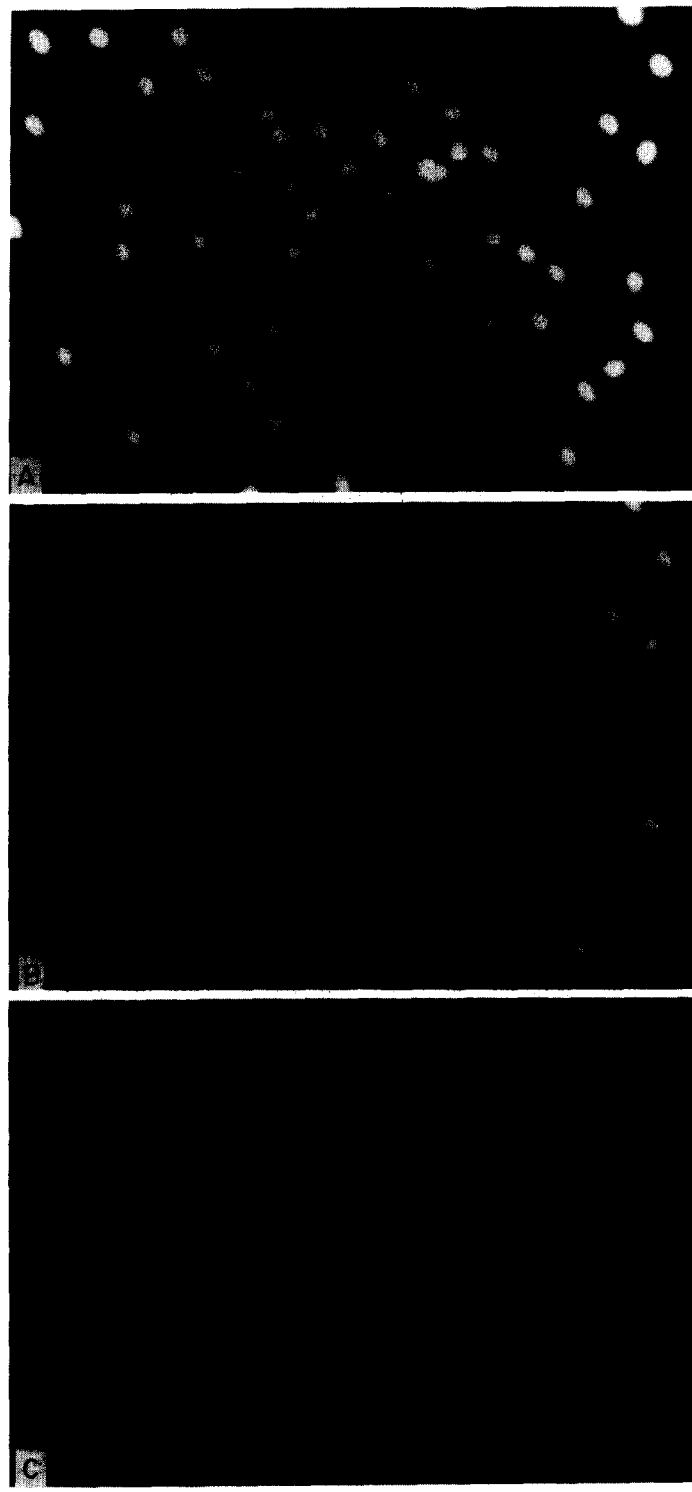

Fig. 3. Immunofluorescence microscopy of fish erythrocytes with anti histone H5 antibody. Note the bright staining pattern observed in the nuclei by the serum used (B). In (A) the same preparation as in (B) was stained with Hoechst 33528 to the DNA. Note that some nuclei do not stain well with anti-H5. They probably represent non-erythroid cells present on the preparation. Control with preimmune serum does not show any reactivity (C).

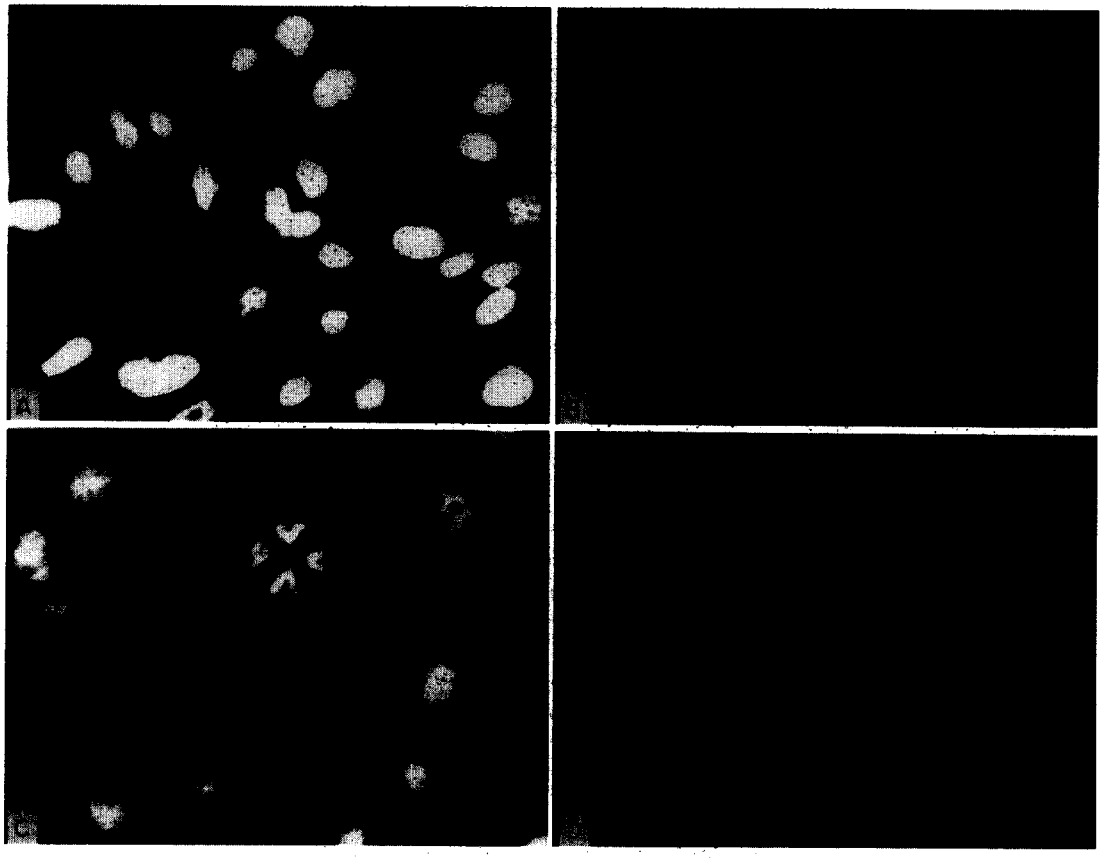

Fig. 4. Immunofluorescence of mammalian cells with anti-fish histone H5 antibody. Nuclei from CHO cells (B) and HeLa cells (D) were not recognized by the H5 antiserum. Hoechst staining of DNA of both CHO and HeLa cells is shown in (A) and (C).

blotting analysis of the H5-CNBr cleavage experiment. It seems that in contrast with chicken H1o, fish H5 probably contains more than one methionine residue. This result is interesting because it is the first evidence of the presence of several methionines in H5 for any species so far studied. Antiserum to H5 reacts strongly with one of the CNBr-peptides. According to the CNBr–chicken H5 digestion pattern described by Rutledge *et al.* (1981), the epitopes are located in the globular and *C*-terminal region of the H5 molecule. However, owing to the absence of data for localization of the methionine residues in the fish H5 molecule, it has to be determined more accurately where the epitopes reactives are localized within the antiserum anti-H5.

DISCUSSION

Histone H5 is an early marker of the avian and fish erythroid lineage. Expression of H5 is regulated during erythrocyte differentiation so that its content increases with cell maturity. The rate of H5 gene transcription increases at the erythroblast stage and is maintained thereafter (Affolter *et al.*, 1987). Accumulation of H5 results in inhibition of DNA synthesis and the arrest of the cells in G1 (Lennox and Cohen, 1983). So proliferating cells may need to keep the H5 gene under negative control to maintain the content of H5 at a level that does not interfere with growth. In fact H5-containing nucleated erythrocytes are virtually inactive in transcription, and mammalian H1o is mostly found in terminally differentiated cells. The fish erythroid lineage, therefore, provides a natural system to examine the regulation of the histone genes during terminal differentiation.

The availability of antibodies that are specific for different portions of the H5 molecule provides reagents that will be useful for the study of where this protein occurs and of its structural orientation within chromatin. The cross-reactive anti-H5 antibodies for the globular or charged carboxyl-terminal half of the molecule, can serve this purpose in part. Monoclonal examples of chicken anti-H5 antibodies have been prepared, and their targets have been restricted to various portions of the globular region (Mendelson and Bustin, 1984), as are those induced by the H1o globular region itself (Yasuda *et al.*, 1984). The polyclonal serum reported in this paper raised against the fish H5 appears to be highly specific to this protein, judging from the lack of cross-reactivity with other H1-type-histones from the same species. The species specificity of anti-H5 was also demonstrated in this study, since a related protein present in human cells (H1o) was only weakly immunoreactive. Moreover our anti-H5 antibody

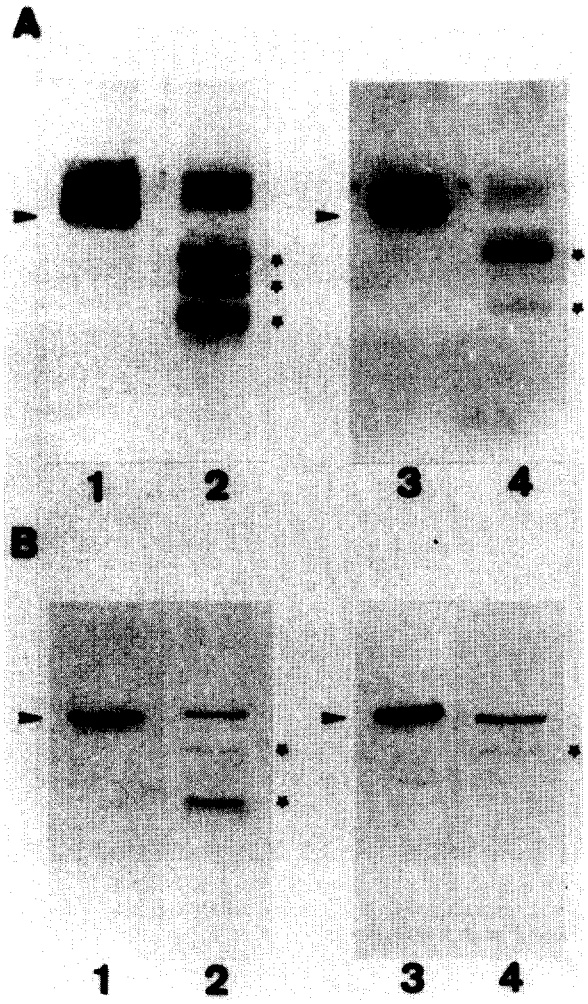

Fig. 5. Immunoblot of histone H5 peptides after cleavage with cyanogen bromide (A) and pepsin (B). Peptides were prepared as described in Materials and Methods separated on 12.5% SDS–PAGE and transferred to nitrocellulose. One set was stained with Coomassie Blue (lanes 1 and 2) and two identical sets were incubated with polyclonal anti-H5 (lanes 3 and 4). Cyanogen bromide digestion of fish histone H5 originates three peptides indicated by stars (lane 2 in A). A major reactivity is observed with the highest mol. wt peptide, a minor one is observed with the lowest peptide. According to Rutledge *et al.* (1981) the epitopes are located in the globular and *C*-terminal region of the H5 molecule. Digestion with pepsin originates two peptides (*), (lane 2 in B). Only one peptide reacts with the antibody. Again this result confirmed, in accordance with Rutledge *et al.* (1981), some epitopes are present on the globular region of fish histone H5.

does not react with chicken histone H5 (data not shown), which suggests that epitopes occur exclusively on the fish antigen. The high specificity of the antibody will allow us to define the distribution of H5 in various tissues of fishes.

In fish, methionine-containing H1 subfractions in the liver and kidney of rainbow trout have been described by Brown and Goodwin (1983). Our results of the cyanogen bromide cleavage of H5 show that it contains at least a residue of methionine at about the same distance from the *N*-terminus as mammalian H1o and avian H5, as shown by SDS–PAGE and immunoblotting. Polyclonal and monoclonal antibodies raised against avian H5 have shown binding to the central globular segment of the molecule. However, monoclonal antibodies obtained from mice immunized with a complex of H5–RNA react with the *N*- or *C*-terminal of H5 (Mendelson and Bustin, 1984). This globular region of the H5 molecule extends from residues 22–100 (Mura and Stollar, 1981). Our polyclonal serum contains epitopes included, but not restricted to, that part of the molecule as demonstrated by CNBr and pepsin cleavages.

The content of H5 relative to H1 remains modest in proliferating precursor cells. However, H5 accumulates in the nucleus once the cells enter the final stages of maturation. This pattern of behaviour is emphasized in immature erythrocytes. Although the chromatin structure of erythroid-specific genes of immature and mature chicken erythrocytes has been studied, nothing is known in fish erythrocytes.

Erythropoiesis is an appropriate model for analysing cell cycle kinetic events and the maturation process in morphological changes during evolution from primitive precursor cell to the mature erythrocyte. The structural changes occurring during differentiation are associated with striking biochemical alterations. In this context, we are interested in studying the structure, regulation and function of histone H5 of fish in order to understand the chromatin behaviour during fish erythrocyte maturation.

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