# Changes in the Histone H2A Variant H2A.Z and Polyubiquitinated Histone Species in Developing Trout Testis†

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ABSTRACT: The trout histone H2A variant H2A.Z has been identified by its electrophoretic mobility on two-dimensional polyacrylamide gels and its N-terminal amino acid sequence. Similar to bovine H2A.Z and chicken H2A.F (also called H2A.Z and M1), the trout H2A.Z had a two-residue extension when aligned with trout H2A and a 67% sequence homology with the N-terminal portion of trout H2A. The first 29 amino acids of trout H2A.Z were identical with those of chicken H2A.F and differed from those of bovine H2A.Z at only one position. Thus, the N-terminal part of histone H2A.Z appears to be highly conserved. The levels of histone H2A.Z and ubiquitinated species of the histones H2A, H2A.Z, and H2B, which were detected with an anti-ubiquitin antibody, were studied at various stages of trout testis development. At the final stages of spermatogenesis in trout, histones are replaced by protamines. Ubiquitinated and diubiquitinated histone H2A remained at similar levels in early and late stage testis nucleohistone. In the late stage testis chromatin (nucleohistone), ubiquitinated histone H2A.Z was not detected, the level of ubiquitinated histone H2B was reduced, and the amount of diubiquitinated histone H2B increased. There was also a marked reduction in the level of histone H2A.Z. This observation suggests nucleosomes with this histone variant were selectively disassembled during the transition from nucleohistone to nucleoprotamine, indicating that protamine deposition is not a random process in rainbow trout.

The DNA in the nucleus is organized into various hierarchies of chromatin structure (Reeves, 1984). The lowest level of DNA coiling in chromatin is the nucleosome which is constructed by wrapping 146 base pairs of DNA around a histone octamer comprised of two each of the histones H2A, H2B, H3, and H4. The nucleosomes attached by a piece of DNA, called linker, form a chain of repeating subunits. The chromatin fiber may be compacted further into a higher order structure, the 30-nm fiber, whose formation and stabilization are dependent on the presence of histone H1. The 30-nm fiber appears to be folded into loops or domains of chromatin which are believed to be anchored by specific proteins located at the base of the loops to a supporting nuclear structure called the nuclear matrix.

There exists multiple forms of the histones H2A, H2B, and H3 which differ by one of several amino acids in their primary sequence (Wu et al., 1986). Histone variants could generate considerable complexity in the histone octamers of the nucleosomes, and they may be of importance structurally and functionally. One of these variants, H2A.Z, which is a member of the histone H2A family, participates in basal histone synthesis (Wu & Bonner, 1981), appears to be evolutionarily conserved (Wu et al., 1982), and may be enriched in transcriptionally active chromatin (Gabrielli et al., 1981; Ridsdale & Davie, 1987).

The nucleosomal histones are susceptible to a number of postsynthetic modifications including ubiquitination and acetylation. In chromatin, ubiquitin, a 76-residue protein, is found covalently joined via an isopeptide linkage to 5–15% of the nucleosomal histone H2A and also to histones H2B and H2A.Z, but in smaller proportions (Reeves, 1984). The nucleosomal histones H2A, H2B, H3, and H4 can be modified by acetylation at several lysine residues in the amino-terminal portions of the molecules. Several reports indicate that histone ubiquitination and acetylation have roles in the regulation of gene expression as well as chromatin organization [reviewed in Reeves (1984)].

Spermatogenesis is a complex developmental process which sequentially generates several germ cell types: the spermatogonium, the primary and secondary spermatocytes, the spermatid, and the spermatozoan. During spermatogenesis, several morphological and biochemical changes take place, including condensation of chromatin, replacement of the basic nuclear proteins, and cessation of transcription (Gillam et al., 1979). In the final stages of spermatogenesis in rat and rooster, the histones are replaced by low molecular weight proteins. In these systems, several alterations occur in the chromatin composition prior to the replacement process, including the appearance of germ-line-specific histone variants (Trostle-Weige et al., 1982), an increase in the level of the hyperacetylated histone H4 species (Grimes & Henderson, 1983; Oliva & Mezquita, 1982), and an increase in the level of the ubiquitinated histone H2A (uH2A)[1] species (Agnell et al., 1983). It is presently thought that these various components promote the decondensation of the chromatin fiber and facilitate the displacement of the histones.

The rainbow trout system is ideal for studying these biochemical changes because large numbers of cells at the same

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; AUT, acetic acid/urea/Triton X-100; uH2A, uH2A.Z, and uH2B, ubiquitin adduct of H2A, H2A.Z, and H2B, respectively; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

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stage of development are present at all times during the process (Drance et al., 1976). As the trout testis matures, the histones are replaced by the protamines. This replacement process occurs in the middle spermatids and is completed in the late spermatids (Louie & Dixon, 1972). An increase in the level of the hyperacetylated histone H4 species has been observed in the late stages of trout testis development (Christensen & Dixon, 1982). In this study, we determined whether there are qualitative and/or quantitative changes in the histone variants and ubiquitinated histone species during the spermatogenesis process in trout. In addition, we have characterized the trout histone H2A variant H2A.Z and demonstrate that the N-terminal amino acid sequence of trout, chicken, and bovine histone H2A.Z is highly conserved.

## Materials and Methods

*Tissues.* Livers and testes at various stages of development were obtained from rainbow trout (*Salmo gairdnerii*, Mount Shasta strain) at the Oregon State University Food Toxicology and Nutrition Laboratory fish hatchery facility, Corvallis, OR. The tissues were stored at −80 °C until use.

The stage of the testis was evaluated by the following: (1) weight and size (Louie & Dixon, 1972; Gillam et al., 1979); (2) DNA to tissue wet weight ratio (early stage, 23 mg/g; intermediate stage, 44 mg/g; late stage, 80 mg/g); and (3) relative content of histones and protamines.

*Preparation of Nuclei.* Trout testes were homogenized in 10 volumes of buffer A [1 M hexylene glycol, 10 mM PIPES, pH 7.5, 2 mM $MgCl_2$, 1% thiodiglycol, 30 mM sodium butyrate, and 10 mM iodoacetamide (Sigma)]. Iodoacetamide is an inhibitor of the enzyme isopeptidase which will remove the ubiquitin moiety from a ubiquitinated protein (Matsui et al., 1982). The scissor-minced tissue was homogenized in a Waring Blendor cup at the lowest speed for 1 min. The homogenate was filtered through four layers of cheesecloth and centrifuged at 750*g* for 10 min. The pellet was resuspended in buffer A and again centrifuged. The resulting pellet was then resuspended in buffer B (1 M hexylene glycol, 10 mM PIPES, pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol, and 30 mM sodium butyrate) and centrifuged as before. This step was repeated once more.

Trout liver nuclei were isolated as described above except that the liver was minced in buffer A and 0.2% (v/v) Nonidet P-40 and homogenized in a Potter–Elvehjem homogenizer. Following centrifugation, the pellet was resuspended in buffer A.

PMSF (1 mM) was included in all the buffers used.

*Histone Isolation.* Liver or testis nuclei were digested with 50 $A_{260}$ enzyme units/mL of micrococcal nuclease (Sigma) at a DNA concentration of 40 $A_{260}$ units/mL at 37 °C for 20 min. The reaction was terminated by the addition of EGTA to a final concentration of 10 mM and the mixture placed on ice. The digested nuclei were collected by centrifugation, yielding the supernatant S0. The digested nuclei were incubated at 0 °C in buffer C (50 mM Tris-HCl, pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol, 25 mM KCl, 10 mM EGTA, 30 mM sodium butyrate, and 0.5 M NaCl) for 30 min. The supernatant S0.5 which contained the majority of the nucleohistone was collected by centrifugation and dialyzed against 1 mM EDTA. A portion of the sample was lyophilized and resuspended in a sample buffer containing protamine (1% protamine sulfate) (grade X from salmon, Sigma), 100 mM Tris–acetate, pH 8.8, 20% glycerol, 8 M urea, 5% 2-mercaptoethanol, 2% thiodiglycol, and 1% cysteamine hydrochloride (Richards & Shaw, 1982). Alternatively, supernatant S0.5 was acid extracted with 0.4 N $H_2SO_4$ (30 min on ice), and the insoluble material was removed by centrifugation. The acid extracts were dialyzed overnight at 4 °C against 0.1 N acetic acid, lyophilized, and redissolved in distilled water.

*Isolation of Histone H2A.Z.* Total trout testis histones were fractionated by gel exclusion chromatography on a Bio-Gel P30 column (110 × 2.5 cm, Bio-Rad) eluted at room temperature with 50 mM NaCl/10 mM HCl (flow rate 40 mL/h). Typically, 75 mg of histones was dissolved in 10 mM Tris–acetate, pH 8.8, and 10% 2-mercaptoethanol and loaded onto the column. The elution of the histones was monitored at 230 nm. The pooled fractions were dialyzed against 0.1 N acetic acid, followed by water, and lyophilized. Trout histone H2A.Z was isolated from fraction 3 (see Figure 2) by preparative AUT–polyacrylamide gel electrophoresis (Francis et al., 1984).

*Peptide Mapping.* Histones were electrophoretically resolved on a 0.5 mm thick AUT gel. Following electrophoresis, the bands were visualized by staining with 8-anilino-1-naphthalenesulfonic acid (Francis et al., 1984). The gel slices containing the protein of interest were placed perpendicular to the surface of the stacking gel. The peptide mapping was performed as described by Davie (1985) except AUT gels were used. Cleavage of the proteins with cyanogen bromide was done as described by Tung et al. (1984).

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis was performed as described by Davie (1982) and Francis et al. (1984). The two gel systems typically used in this study were AUT gels [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide gels] and SDS gels (sodium dodecyl sulfate/15% polyacrylamide gels).

Silver staining was performed according to the method of Morrissey (1981) with the following modifications: (i) gels were fixed in 50% methanol overnight with one change; (ii) prior to being dried, the gel was soaked twice for 30 min in a solution containing 0.1 g of citric acid, 146 mL of water, 50 mL of ethanol, 4 mL of glycerol, and 90 μL of aqueous methylamine (40% v/v) according to Eschenbruch and Burk (1982).

*Electrophoretic Transfer of Proteins from SDS–Polyacrylamide Gels to Nitrocellulose.* Two-dimensional polyacrylamide gel electrophoresis (AUT → SDS) was used to resolve histone proteins and their variants. The proteins (50 μg) were analyzed on gels measuring 16 cm × 16 cm × 0.75 mm. Second-dimension SDS–polyacrylamide gels were equilibrated 30 min to transfer buffer (25 mM Tris, 192 mM glycine, and 0.01% w/v SDS) before electroblotting. Nitrocellulose filters (BA85, Schleicher & Schuell) were hydrated overnight. Transfers were performed with a Bio-Rad Transblot apparatus (4 V/cm, 16 h with cooling). The filter was removed, blotted dry with filter paper, and air-dried before heat fixing at 65 °C for 30 min. Anti-ubiquitin IgG was prepared as described by Haas and Bright (1985). Immunochemical staining for ubiquitin using an anti-ubiquitin IgG and $^{125}I$-labeled protein A was performed as described by Haas and Bright (1985). India ink staining of the proteins that had been electrophoretically transferred onto nitrocellulose paper was done as described by Hancock and Tsang (1983).

*Amino Acid Sequence Determination of Trout Histone H2A.Z.* Trout testis H2A.Z was electroeluted from AUT–polyacrylamide gels, and the intact protein was partially sequenced as described in Allis et al. (1986).

## Results and Discussion

*Identification of the Trout Histone H2A.Z.* Histones isolated from early stage trout testis (a germ line tissue) and from trout liver (a somatic tissue) were electrophoretically resolved

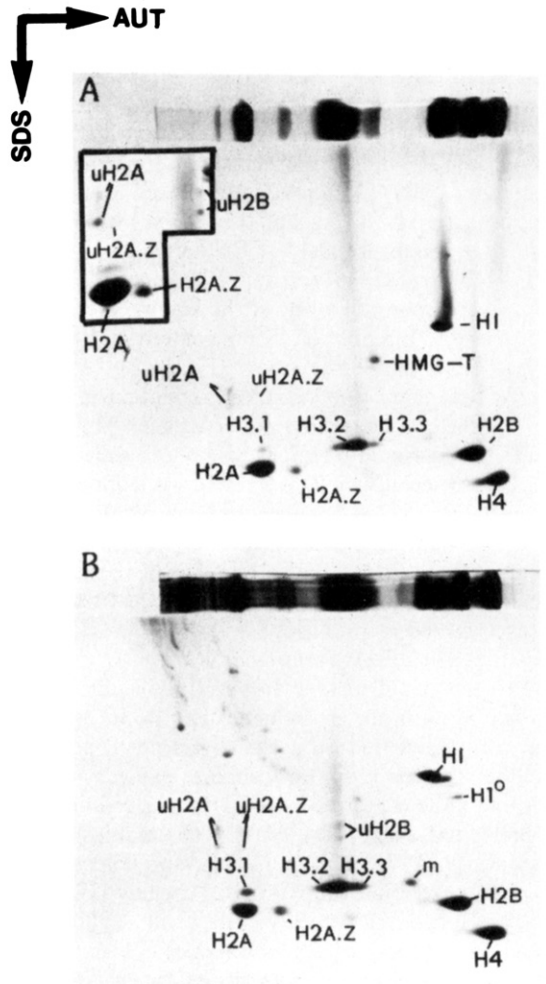


FIGURE 1: Two-dimensional electrophoretic patterns of protamine-released proteins from early stage trout testis nuclei and trout liver nuclei. Histones (10 μg) from fraction S0.50 from either early stage trout testis (A) or trout liver nuclei (B) were obtained by the modified protamine release technique of Richards and Shaw (1982) (see Materials and Methods). The proteins were electrophoretically resolved by two-dimensional gel electrophoresis with the first (horizontal) dimension consisting of a 15% polyacrylamide gel composed of 0.375% Triton X-100 (w/v) and 6.7 M urea and the second (vertical) dimension consisting of a 15% polyacrylamide–SDS gel. m is micrococcal nuclease. (A) *Inset*: A portion of a two-dimensional gel pattern containing 40 μg of early stage trout testis histones. The ubiquitin adducts of H2A, H2A.Z, and H2B are denoted as uH2A, uH2A.Z, and uH2B, respectively.

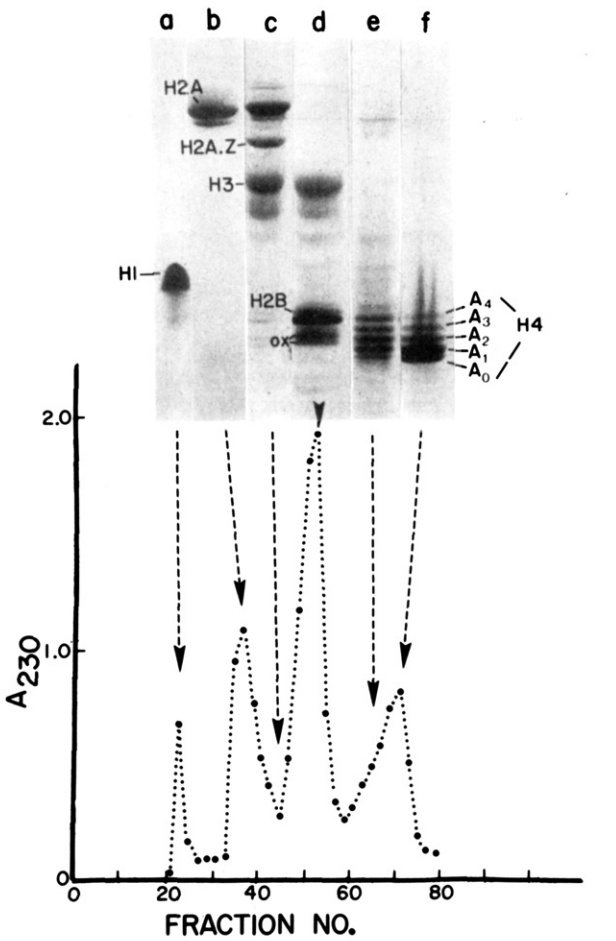


FIGURE 2: Gel exclusion chromatography of the trout testis histones. Trout testis histones were fractionated on a Bio-Gel P30 column. The histones were pooled into six fractions (a–f). The histones in each fraction were electrophoretically resolved on an acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide minislab gel. ox denotes oxidized H2B. $A_0$, $A_1$, $A_2$, $A_3$, and $A_4$ represent the un-, mono-, di-, tri-, and tetraacetylated species of H4, respectively.

on two-dimensional gels (AUT → SDS) (Figure 1). The putative trout H2A.Z was designated as such because it co-migrated with calf thymus H2A.Z on the two-dimensional gels (not shown). Three variants of histone H3 (H3.1, H3.2, and H3.3) were observed. It should be noted that the relative levels of the H3 variants did not change when iodoacetamide was not included in the nuclei isolation buffers. Also, the histones were isolated by the protamine release method of Richards and Shaw (1982) which was modified in order to minimize protein oxidation, and thiodiglycol, which is an efficient scavenger of oxidizing agents, was included in the isolation of the nuclei to prevent partial oxidation of methionine residues (Urban et al., 1979). Thus, it is unlikely that the histone H3 variants are artifacts of oxidation.

An inspection of the protein patterns demonstrated that there were no qualitative differences in the histone variants associated with liver and testis chromatin. However, there were several quantitative differences in the relative levels of the histone variants H3.1 and H3.3 in the liver and testis chromatin, with these variants being more abundant in liver than in testis (Figure 1). Thus, trout testis cells do not display germ cell specific histone variants like those observed during mammalian spermatogenesis (Trostle-Weige et al., 1982).

The putative trout H2A.Z was enriched by gel-exclusion chromatography on a Bio-Gel P30 column. Fraction c contained the putative trout H2A.Z (Figure 2), and this fraction was used to provide H2A.Z for peptide mapping and amino acid sequence determination.

On SDS gels, the peptide pattern of H2A.Z was similar to H2A but not to H2B (Figure 3A). Under these conditions, the *Staphylococcus aureus* protease would likely cleave at glutamoyl and aspartoyl bonds, so the results suggested that these residues were placed at similar intervals in H2A and H2A.Z but not in H2B. The peptide patterns of histones H2A.Z, H2A, and H2B were also compared on AUT gels under which conditions the *S. aureus* protease has a preference for glutamoyl bonds (Davie, 1985). On these gels, the peptide pattern of H2A.Z differed from that of H2A (Figure 3B).

Neither bovine H2A.Z nor chicken H2A.Z (also called H2A.F and M1) contains methionine residues (Ball et al., 1983; Harvey et al., 1983; Urban et al., 1979). To determine whether the putative trout H2A.Z had methionine residues, trout testis histones were treated with cyanogen bromide which will cleave the peptide at methionine residues. Putative trout H2A.Z and H2A, which does not have methionine residues (Connor et al., 1984), were not cleaved while histones that do

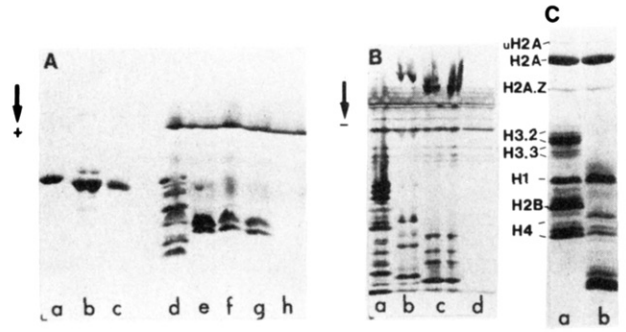


FIGURE 3: Peptide mapping of the histones H2A, H2A.Z, and H2B. (A) Trout testis histones H2A, H2A.Z, and H2B with and without *S. aureus* (V8) protease were electrophoretically separated on a 15% polyacrylamide–SDS minislab gel. The gel was stained with Coomassie blue. Lanes a, b, and c, H2B, H2A, and H2A.Z, respectively. Lanes d, e, f, and g, the histone digested with V8 protease was H2B, H2A, H2A.Z, and H2A.Z, respectively. Lane h was V8 protease. (B) Trout testis histones H2A, H2A.Z, and H2B with V8 protease were electrophoretically resolved on an acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide minislab gel. Lanes a, b, c, and d correspond to H2B, H2A, H2A.Z, and V8 protease, respectively. (C) Trout testis histones were treated with cyanogen bromide as described under Materials and Methods. The proteins were electrophoretically resolved on an AUT–15% polyacrylamide gel which was stained with Coomassie blue. The lanes correspond to untreated histones (a) and cyanogen bromide treated histones (b).

contain methionine residues (e.g., histones H3, H2B, and H4) were processed (Figure 3C). These results suggest the putative trout H2A.Z does not contain internal methionine residues as would be expected if this protein were similar to other H2A.Z proteins.

Allis et al. (1986) have partially sequenced a *Tetrahymena* macronuclear-specific histone variant, hv1. Although hv1 resembled chick H2A.F in several ways, antibodies against hv1 failed to cross-react with bovine H2A.Z. We investigated whether anti-hv1 IgG would cross-react with the putative trout H2A.Z. Cross-reactivity was not observed (not shown).

Further support that we have identified the trout histone H2A variant H2A.Z comes from an analysis of the sequence of the 32 amino-terminal residues. Similar to bovine H2A.Z, trout H2A.Z had an unblocked amino terminus. Comparisons of the first 30 amino acid residues of trout H2A.Z with those of trout H2A, bovine H2A.Z, and chicken H2A.F shown in Figure 4 demonstrated the following points. First, trout H2A.Z had the conserved H2A sequence AGLQFPV (Wu et al., 1986). Second, 18 of the first 27 residues in trout H2A are identical with those in trout H2A.Z. Third, the first 29 amino acids of trout H2A.Z were identical with those of chicken H2A.F and differed from those of bovine H2A.Z at only one position. Thus, the amino-terminal part of histone H2A.Z appears to be highly conserved.

*Histone H2A.Z Is Selectively Removed from the DNA in the Late Stages of Testis Development.* Histones were isolated from testes which were at different stages of development. On the basis of the work of Louie and Dixon (1972), we expect the different testis stages to contain a preponderance of the following cell types: early stage, primary and secondary spermatocytes; intermediate stage, early and middle spermatids; and late stage, late spermatids and spermatozoa. The percentage of DNA which was complexed as nucleohistone was approximately 70, 30, and 7 in early, intermediate, and late stage testis, respectively. Christensen and Dixon (1981, 1982) demonstrated that testis maturation was accompanied with an increase in the levels of the hyperacetylated histone H4 species and a reduction in the content of HMG-T. We have confirmed their observations (Figure 5). Moreover, we observed that there was a marked reduction in the level of H2A.Z in the late stage testis nucleohistone. This observation suggests that chromatin regions containing nucleosomes with H2A.Z were selectively converted to nucleoprotamine. Alternatively, since H2A.Z can be ubiquitinated, it is conceivable that in late stage testis chromatin H2A.Z was converted to its ubiquitinated form. Thus, the levels of the ubiquitinated histone species at various stages of trout testis development were ascertained.

*Identification of the Trout Ubiquitinated Histone Species.* Ubiquitinated trout histone species were identified with an anti-ubiquitin antibody. Trout testis histones were electrophoretically separated on a two-dimensional gel (AUT → SDS) and transferred to nitrocellulose paper, and ubiquitin-conjugated proteins were detected with anti-ubiquitin IgG and $^{125}$I-labeled protein A (Figure 6). The ubiquitinated species of histones H2A, H2A.Z, and H2B were detected.

The existence of polyubiquitinated species of histone H2A has recently been reported (Kanda et al., 1986). The assignment of the polyubiquitinated species of histone H2A was based on the following observations: (1) several ubiquitinated species coeluted with histone H2A in the Bio-Gel P30 column fraction b (Figure 6C); (2) when the protein pattern visualized by India ink staining of the nitrocellulose was superimposed on the autoradiogram shown in Figure 6C, it was evident that some of the ubiquitinated species and histone H2A were on a diagonal line on the two-dimensional gel pattern; (3) when the relative mobilities on SDS gels of histone H2A and these ubiquitinated forms were plotted vs. the log polypeptide molecular weight, the ubiquitinated species labeled uH2A, $u_2$-H2A, and $u_3$H2A increased by increments of 8.5 kDa, corresponding to the addition of one ubiquitin molecule (Watson et al., 1978); and (4) treatment of the protein sample (Bio-Gel column fraction b) with cyanogen bromide did not alter the pattern shown in Figure 6C. (Note that neither histone H2A, histone H2A.Z, nor ubiquitin contains internal methionine residues.) Histone H2B, uH2B, and the ubiquitinated species identified as diubiquitinated H2B also were on a diagonal line on the two-dimensional gel pattern, and they were cleaved by cyanogen bromide treatment.

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                                10                  20                  30
Trout H2A.Z      (A) G G K A G K D S G K A K A K A V S R S Q R A G L Q F P V (V) (R) I
Trout H2A             S G R * * T G * * * R * * * K T * * S * * * * * * * * G   •   V
Chicken H2A.F    *  * * * * * * * * * * * * * * * * * * * * * * * * * * * * G   *   *
Bovine  H2A.Z    •  * * * * * * * * * * T * * * * * * * * * * * * * * * * * G
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FIGURE 4: Amino acid sequences of the amino-terminal residues of trout testis histones H2A.Z and H2A and other H2A.Z proteins. The N-terminal amino acid sequences of trout histone H2A.Z along with trout H2A (Connor et al., 1984) are shown. For comparison, the sequences of chicken H2A.F (Harvey et al., 1983) and bovine H2A.Z (Ball et al., 1983) are also shown. Parentheses in the trout histone H2A.Z sequence indicate that only a tentative identification of these amino acids has been made. Amino acids which are identical with those in trout histone H2A.Z have been given an *asterisk*, and those amino acids which are different are indicated.

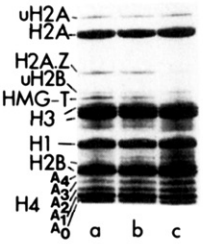


FIGURE 5: Histone composition in developing trout testis. Acid-extracted histones isolated from early (a), intermediate (b), and late (c) stage trout testis were separated on an AUT–15% polyacrylamide slab gel. The gel was stained with Coomassie blue.

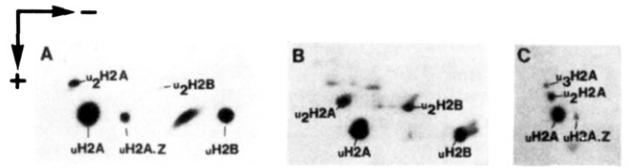


FIGURE 6: Identification of trout testis ubiquitinated histone species. Histones isolated from early stage trout testis (A), late stage trout testis (B), and Bio-Gel P30 column fraction b (shown in Figure 2) (C) were electrophoretically separated on two-dimensional gels (AUT → SDS). The proteins were electrophoretically transferred to nitrocellulose paper and immunochemically stained for ubiquitin with anti-ubiquitin IgG and $^{125}$I-labeled protein A. The autoradiograms are shown. The ubiquitin adducts of histones H2A, H2A.Z, and H2B are denoted as uH2A, uH2A.Z, and uH2B, respectively. The polyubiquitinated histone species are labeled as $u_2$ and $u_3$, representing the attachment of two and three ubiquitins, respectively.

The level of the ubiquitinated histone species in early and late stage trout testis chromatin (nucleohistone) was compared (Figure 6). For both sources, uH2A was the predominating ubiquitinated histone species, and the levels of the ubiquitinated and diubiquitinated H2 species were similar. Qualitative and quantitative differences in the levels of the other ubiquitinated histone species were noted. Ubiquitinated H2A.Z was not detected in the late stage trout testis. The content of uH2B was reduced while the amount of diubiquitinated histone H2B increased in the late stage testis chromatin.

These results demonstrate that the reduced levels of H2A.Z in the late stage trout testis chromatin were not due to conversion of H2A.Z to uH2A.Z and suggest that the protamine deposition is not a random process because nucleosomes with histone H2A.Z appear to have been selectively disassembled in the late stage testis. We do not observe an increase in the levels of the ubiquitinated histone species in the late stages of trout spermatogenesis like that seen in rooster. However, it is conceivable that the presence of the ubiquitinated histone species in the late stage testis nucleohistone may aid in the replacement process, perhaps synergistically with histone hyperacetylation. Moreover, polyubiquitination of the histones may prepare or tag the histone for degradation.

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