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Ubiquitinated Histone H2B Is Preferentially Located in Transcriptionally Active Chromatin†

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ABSTRACT: Using an anti-ubiquitin antibody in Western blotting experiments, we detected polyubiquitinated species of histones H2A, H2A.Z, and H2B in histone preparations of bovine thymus, chicken erythrocyte, and *Tetrahymena* macro- and micronuclei. Histone H2A had the greatest level of polyubiquitinated species, with tetra- to hexaubiquitinated forms of this histone being observed. The fraction of bovine thymus and chicken erythrocyte chromatin enriched in transcriptionally active gene sequences was enriched in mono- and polyubiquitinated species of histones H2A, H2B, and H2A.Z, especially in the ubiquitinated forms of histone H2B. Histones H2A and H2B were ubiquitinated in the transcriptionally active *Tetrahymena* macronucleus, with monoubiquitinated (u) H2B being the predominant ubiquitinated histone species. Ubiquitinated forms of histones H2A and H2B were found in transcriptionally inert micronuclei, but at lower levels than seen in macronuclear histones. Also, the level of micronuclear uH2A was greater than that of uH2B which may be from macronuclei that contaminate the preparation. These results indicate that the mono- and polyubiquitinated species of histone H2B are preferentially located in transcriptionally active chromatin regions. Ubiquitinated histone H2A is located in both expressed and repressed chromatin domains, but expressed chromatin is enriched in mono- and polyubiquitinated forms of this histone. These observations are consistent with the hypothesis that ubiquitinated histones have a role maintaining the structure of transcriptionally active chromatin.

Ubiquitin, a small protein of 76 amino acids, can be covalently attached to histones H2A and H2B and their variants via an isopeptide bond between the ubiquitin C-terminal glycine and the ϵ-amino groups of lysine-119 in H2A (Goldknopf & Busch, 1977) and lysine-120 in H2B (Thorne et al., 1987). In multicellular eukaryotes, ubiquitinated (u) histone H2A replaces approximately 10% of the nucleosomal H2A (Busch & Goldknopf, 1981), and uH2B replaces approximately 1-2% of the nucleosomal H2B (West & Bonner, 1980). In the cytoplasm, ubiquitin is used to "tag" proteins for protease digestion with the rate of proteolysis increasing with the number of attached ubiquitins (Ciechanover et al., 1981). It

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has been suggested that an analogous system may operate in the nucleus with proteolytic removal of nucleosomal proteins from activated chromosomal regions (Levinger & Varshavsky, 1982). There is also evidence that ubiquitinated histones may be involved in preventing the formation of higher order chromosomal structures. Ubiquitinated histones are absent from highly condensed metaphase chromosomes, and decondensation has been correlated with their reappearance (Matsui et al., 1979; Wu et al., 1981; Mueller et al., 1985; Raboy et al., 1986).

The association of ubiquitinated histones with transcriptionally active chromatin is controversial. Varshavsky's group, using the technique of two-dimensional hybridization mapping of nucleosomes, provided evidence that nucleosomal uH2A is preferentially localized in transcribed genes (Levinger & Varshavsky, 1982; Barsoum & Varshavsky, 1985). The two-dimensional hybridization mapping technique resolves nucleosomes of different compositions and then attempts to correlate altered mobility with altered protein composition (Levinger et al., 1981). Results of Garrard's laboratory question the interpretation of Varshavsky's experiments (Huang et al., 1986). Huang et al. (1986) reported that although nucleosomes containing the active immunoglobulin κ chain gene migrated with bulk ubiquitinated particles, the altered mobility of these nucleosomes was not due to the presence of ubiquitinated histones. The altered mobility may be due to other histone modifications or histone variants (Bode et al., 1983; Imai et al., 1986).

In order to gain an insight into the role of histone ubiquitination, we analyzed the distribution of these modified histone species among (1) fractions of bovine thymus and chicken erythrocyte chromatin and (2) *Tetrahymena* macro- and micronuclei. A method to isolate a rare class of chicken erythrocyte chromatin highly enriched (50-fold) in transcriptionally competent β-globin gene sequences was recently reported (Ridsdale & Davie, 1987a). However, as is usually the case with most fractionation procedures, the separation of expressed from repressed chromatin is not complete. Thus, the *Tetrahymena* system is particularly valuable in determining the distribution of modified histone species and histone variants among active and inactive chromatin domains (Allis et al., 1986). In vegetative *Tetrahymena* cells, macronuclei are transcriptionally active and divide amitotically, while micronuclei are transcriptionally inert and divide mitotically (Gorovsky, 1973; Gorovsky et al., 1978). Since these nuclei contain closely related genetic material (Gorovsky, 1973), they serve as a model system for studying how similar genetic material is maintained in different structural and functional states.

The distribution of ubiquitinated histones in chromatin was examined by resolving proteins by two-dimensional polyacrylamide gel electrophoresis and immunochemical detection of ubiquitinated histone species with a polyclonal anti-ubiquitin IgG. We found that chromosomal proteins from the transcriptionally active macronuclei of *Tetrahymena* and chromatin fractions enriched in transcriptionally active/competent gene sequences were enriched in polyubiquitinated and monoubiquitinated species of histones H2A and H2B, especially those of H2B. The results of this study suggest that the mono- and polyubiquitinated species of histone H2B are preferentially located in transcriptionally active chromatin domains. Ubiquitinated histone H2A is found in both expressed and repressed chromatin regions, but expressed chromatin is enriched in mono- and polyubiquitinated forms of this histone. These observations suggest that histone ubiquitination has a role in maintaining the structure of transcriptionally competent chromatin.

Materials and Methods

Fractionation of Bovine Thymus Chromatin. Bovine thymus nuclei were isolated, digested, and chromatin fractionated as described previously (Davie & Nickel, 1987). Briefly, micrococcal nuclease digested nuclei were resuspended in 10 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF), and the supernatant, T (total chromatin), was collected by centrifugation at 12000*g* for 20 min. The chromatin fraction T was made 0.15 M NaCl by the dropwise addition of 2 M NaCl while stirring. Centrifugation at 12000*g* produced the supernatant (S) and pellet (P). The pellet was resuspended in 10 mM EDTA and 1 mM PMSF. The distribution of A_{260}-absorbing material was 77.0% in the EDTA-solubilized fraction (total), 26.7% in the soluble fraction (S), and 50.4% in the salt-insoluble fraction (P).

Fractionation of Mature Chicken Erythrocyte Chromatin. Mature chicken erythrocyte nuclei were prepared and digested with micrococcal nuclease as described by Ridsdale and Davie (1987a). Digested nuclei were sequentially incubated with buffer C [50 mM Tris-HCl, pH 7.0, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol, 25 mM KCl, 10 mM EGTA, and 30 mM sodium butyrate] containing increasing concentrations of NaCl (0.1 M NaCl and 0.6 M NaCl) as described by Sanders (1978), yielding chromatin fractions SS0.1, SS0.6, and insoluble pellet(P). The distribution of A_{260}-absorbing material was 5.0% in SS0.1, 71.2% in SS0.6, and 18.9% in P.

Mature chicken erythrocyte chromatin was also fractionated by the method of Ridsdale and Davie (1987a). Briefly, micrococcal nuclease digested nuclei were resuspended in 10 mM EDTA and 1 mM PMSF and incubated on ice for 2 h to release chromatin fragments into solution. Insoluble nuclear material was removed by centrifugation at 12000*g* for 20 min. The EDTA-soluble material (SE) was made 0.15 M NaCl by the addition of 4 M NaCl while mixing. Centrifugation at 12000*g* for 20 min yielded a supernatant (S0.15) and a pellet. The S0.15 fraction was concentrated against poly(ethylene glycol), and the chromatin fragments were resolved by gel exclusion chromatography on a Bio-Gel A-5m column (110 × 2.5 cm) equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.15 M NaCl.

Proteins were prepared as previously described (Davie & Nickel, 1987).

Fractionation of Tetrahymena Nuclei. *Tetrahymena thermophila* were cultured axenically in enriched protease peptone as previously described (Gorovsky et al., 1975). Macronuclei and micronuclei were isolated according to published procedures (Gorovsky et al., 1975) and stored frozen (−80 °C) as pellets under medium A (Gorovsky et al., 1975) until needed. Histones were prepared as previously described (Allis et al., 1979).

Polyacrylamide Gel Electrophoresis and Immunochemical Detection of Ubiquitinated Histone Species. One-dimensional AUT [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide gel] and two-dimensional [AUT into SDS (sodium dodecyl sulfate)/15% polyacrylamide gel] gel electrophoreses were performed as described by Davie (1982), except that AUT gels were not preelectrophoresed. Proteins were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG and ^{125}I-protein A as described previously (Nickel et al., 1987). To facilitate comparison of the levels of ubiquitinated histones associated with different fractions, several precautions were taken. AUT gel electrophoresis was used to determine

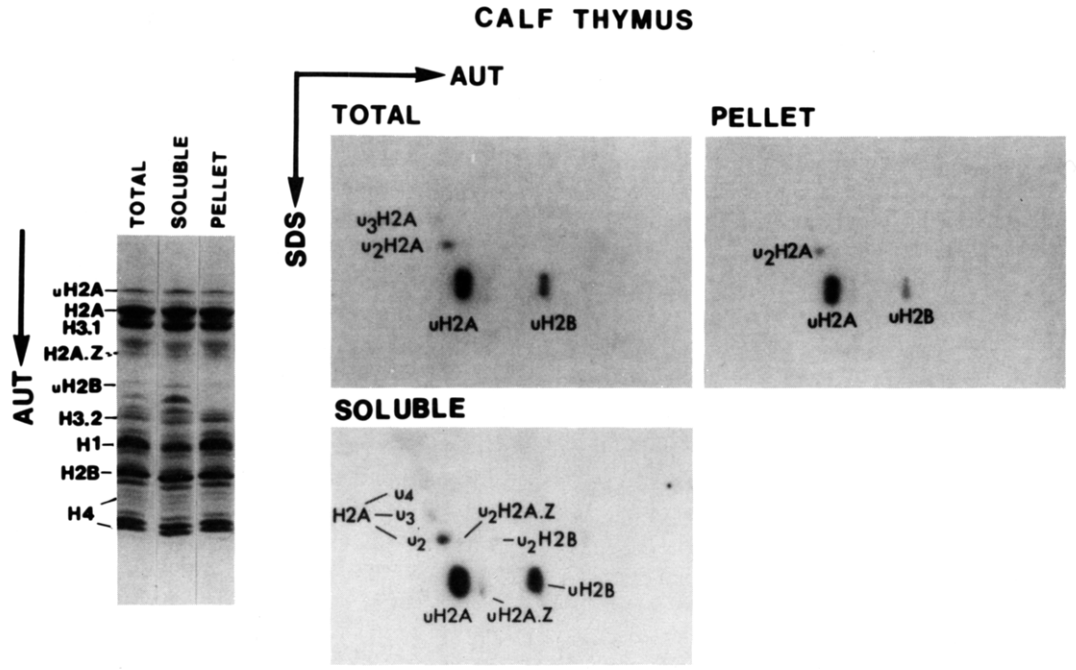

FIGURE 1: Ubiquitinated histone species are enriched in the salt-soluble fraction of calf thymus chromatin. (Left panel) Chromosomal proteins from EDTA-solubilized chromatin (total), 150 mM NaCl soluble (soluble), and 150 mM NaCl insoluble (pellet) fractions were analyzed by AUT gel electrophoresis. The gel was stained with Coomassie blue. (Right panel) Histones from the above fractions were resolved by two-dimensional gel electrophoresis (AUT into SDS), electrophoretically transferred to nitrocellulose, 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, H2B, and H2A.Z are denoted as uH2A, uH2B, and uH2A.Z, respectively. The polyubiquitinated histone species are labeled as u_2, u_3, and u_4 representing the attachment of two, three, and four ubiquitins, respectively. Note that on SDS gels the monoubiquitinated histone species migrate as doublets.

the amount of sample required for equivalent histone levels. Typically, 9 μg of protein was used. Following Coomassie blue staining and destaining, the lanes were scanned with a densitometer. The protein load was then calculated to give equal densities of histones H2B and H3. Protein blots from the same fractionation experiment were analyzed together, or as close in time to each other as possible. Following immunochemical detection, blots from the same experiment were exposed to film for autoradiography together.

Results

The Low Salt Soluble Fraction of Bovine Thymus Chromatin Is Enriched in Ubiquitinated Histone Species. The low salt soluble chromatin fraction of micrococcal nuclease digested bovine thymus nuclei is enriched in transcriptionally active gene sequences (Davie & Saunders, 1981). This fraction is depleted in the H1 histones and enriched in acetylated and ubiquitinated histone species, with uH2B being enriched to the greater extent (Davie & Nickel, 1987; Davie & Saunders, 1981; Figure 1).

Analysis of the proteins by two-dimensional polyacrylamide gel electrophoresis and immunochemical staining for ubiquitin allow better resolution and increased sensitivity in the detection of ubiquitinated histone species. Analysis of total EDTA-solubilized chromatin (total), 150 mM NaCl solule (soluble), and 150 mM salt insoluble (pellet) fractions of calf thymus chromatin shows that more than one ubiquitin can be attached to histones H2A, H2A.Z, and H2B (Figure 1). Note that histone H2A is polyubiquitinated to the greatest extent. In comparison to total or salt-insoluble (pellet) histones, the histones of salt-soluble chromatin are enriched in uH2A, uH2A.Z, polyubiquitinated species of H2A, H2B, and H2A.Z, and, most strikingly, uH2B.

Poly- and Monoubiquitinated Histone Species Are Enriched in Chicken Erythrocyte Chromatin Fractions Which Are Enriched in Competent Gene Sequences. Immunochemical analysis of chicken erythrocyte histones demonstrates that this chromatin is associated with polyubiquitinated histone species of H2A and H2B, with histone H2A having the greater level of polyubiquitinated species (Figures 2 and 3). Note that in the low salt soluble fraction (SS0.10 in Figure 2) where the level of uH2B is equal to or slightly greater than that of uH2A, histone H2A is still polyubiquitinated to a greater magnitude.

Fractionation of mature chicken erythrocyte chromatin using the Sanders' (1978) technique enriches for the transcriptionally competent β-globin gene in the low salt eluted fraction (SS0.1) (Ridsdale & Davie, 1987b). The low salt eluted chromatin fraction (SS0.1) is enriched in acetylated and ubiquitinated histone species and depleted in linker histones H1 and H5 (Figure 2). Analysis of ubiquitinated proteins associated with the different fractions shows an increase in the levels of ubiquitinated histone species (uH2A, uH2B, and polyubiquitinated H2A) in the SS0.1 fraction (Figure 2).

Fractionation of chicken erythrocyte chromatin using the procedure described by Ridsdale and Davie (1987a) results in the isolation of a rare chromatin fraction (less than 1% of total genomic DNA) which is salt soluble as polynucleosomes and highly enriched in β-globin gene sequences. This atypical chromatin fraction is enriched in ubiquitinated and acetylated histone species and depleted in linker histones H1 and H5. Comparison of ubiquitinated histones from total EDTA-solubilized chromatin, salt-soluble polynucleosomes, and salt-soluble mononucleosomes shows that polynucleosomes are enriched in uH2A, uH2B, uH2A.Z, and polyubiquitinated histone species of H2A and H2B, with the increase in levels of uH2B and u_2H2B being the most striking (Figure 3).

Ubiquitinated Histone Species Are Preferentially Located in the Transcriptionally Active Macronuclei of Tetrahymena. Tetrahymena macronuclear histones are highly acetylated while the acetylated forms of the nucleosomal histones are reduced or absent in micronuclei (Figure 4; Gorovsky et al.,

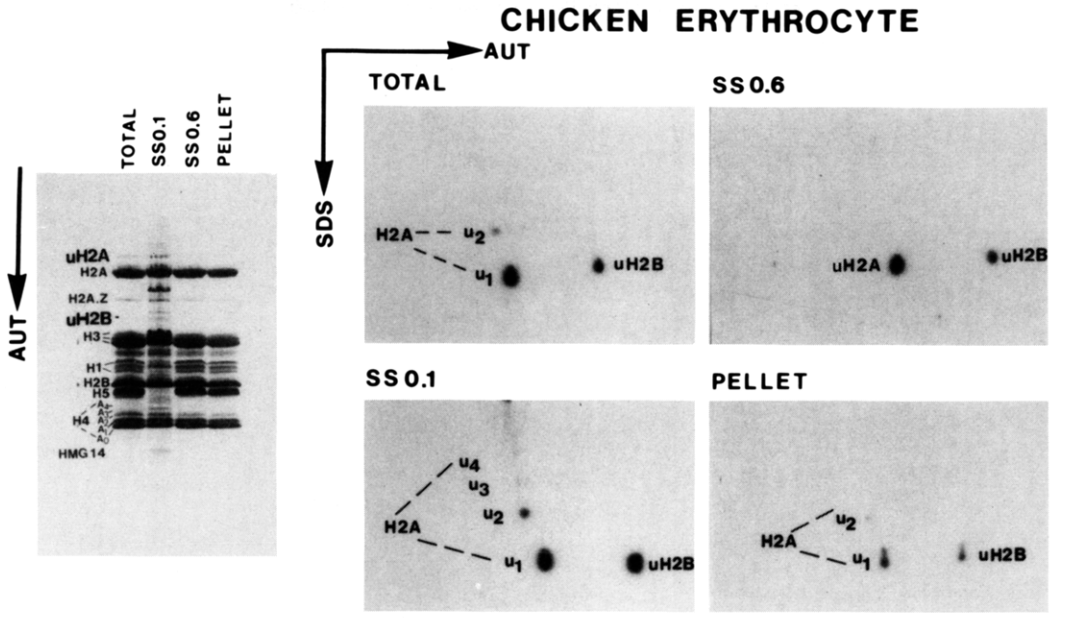

FIGURE 2: Ubiquitinated histone species are enriched in the low salt soluble fraction of chicken erythrocytes. Chromosomal proteins from total, low salt soluble (SS0.1), high salt soluble (SS0.6), and insoluble (P) fractions were analyzed by AUT gel electrophoresis and immunochemical staining for ubiquitin conjugates as described in Figure 1.

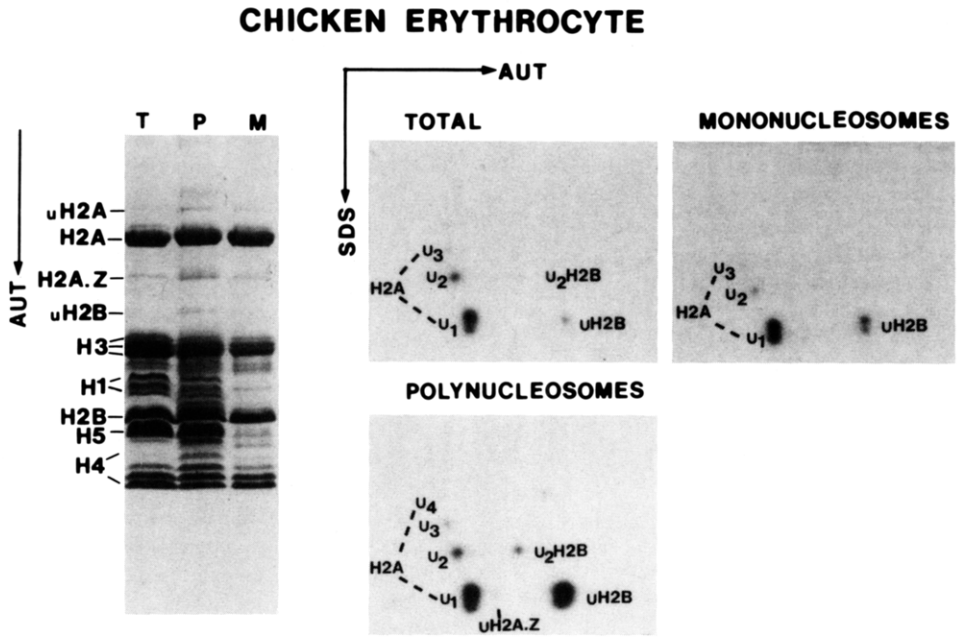

FIGURE 3: Ubiquitinated histone species are enriched in salt-soluble polynucleosomes. Chromosomal proteins from mature chicken erythrocyte chromatin (T or total), salt-soluble polynucleosomes (P or polynucleosomes), and salt-soluble mononucleosomes (M or mononucleosomes) were analyzed by AUT gel electrophoresis and immunochemical staining for ubiquitin conjugates as described in Figure 1.

1978; Vavra et al., 1982; Chicoine & Allis, 1986). The position of the monoubiquitinated forms of H2A and H2B on the AUT gel pattern shown in Figure 4 is indicated. Two-dimensional gel patterns (AUT into SDS) show that several other proteins comigrate with the ubiquitinated histone species on the AUT gel (not shown). Analysis of chromosomal proteins from the transcriptionally active macronuclei and transcriptionally inactive micronuclei of *Tetrahymena* demonstrates that polyubiquitinated H2A is present in both types of nuclei, with the level of these modified species being greater in macronuclei (Figure 4). The level of ubiquitinated H2B species associated with macronuclei is much greater than that associated with micronuclei. Moreover, uH2B is the major ubiquitinated histone species in macronuclei.

It is possible that the low amount of uH2B in the micronuclei is due to macronuclear contamination. To estimate the level of this contamination within the micronuclear preparation used in Figure 4, acid-soluble proteins from the same micro- and macronuclei were fractionated on a one-dimensional SDS gel, transferred to nitrocellulose, and probed with antibodies specific to the macronuclear-specific histone variant, hv1 [Figure 5B; see Allis et al. (1980, 1982) and Wenkert and Allis (1984) for details]. The corresponding immunoblot reveals significant levels of hv1 in the micronuclear preparation (arrow), suggesting that the low but detectable levels of uH2B in the micronuclear preparation may be due to contamination by macronuclei.

Discussion

Our results are consistent with the hypothesis that ubiquitinated histones are preferentially located in transcriptionally active/competent regions of chromatin. The low salt

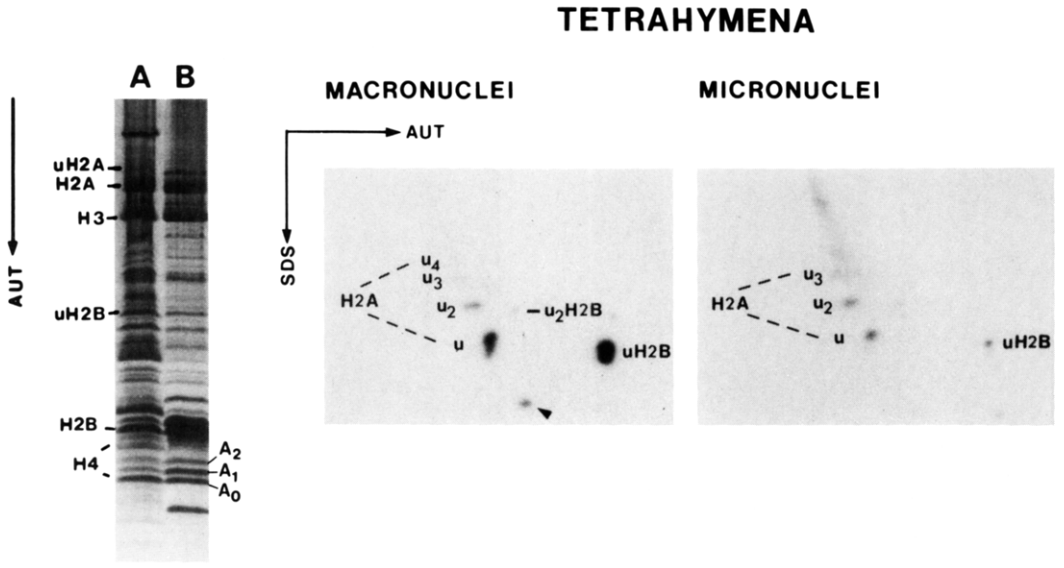

FIGURE 4: Ubiquitinated histone species are enriched in the transcriptionally active macronuclei of *Tetrahymena*. Chromosomal proteins from *Tetrahymena* macronuclei and micronuclei were analyzed by AUT gel electrophoresis (lane A, micronuclei; lane B, macronuclei) and immunochemical staining for ubiquitinated proteins as described in Figure 1. A_0, A_1, and A_2 correspond to the un-, mono-, and diacetylated species of histone H4. The arrow shows a staining artifact.

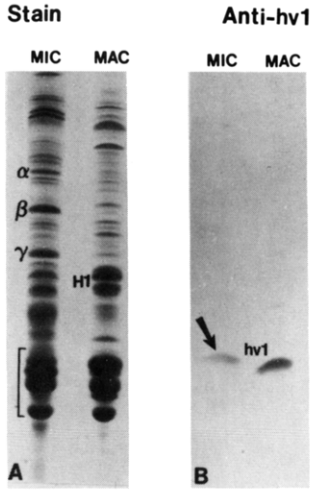

FIGURE 5: Immunochemical detection of the macronuclear-specific histone variant hv1. Acid-soluble proteins from the same micro- and macronuclear preparation shown in Figure 4 were electrophoretically resolved on a SDS gel and either stained directly (A) or transferred to nitrocellulose and probed with antibodies which are specific to the macronuclear-specific histone variant hv1 (B). Labeled in the stained parallel gel are the micronuclear-specific linker peptides α, β, and γ and macronuclear-specific H1. The bracket includes all of the major core histones, H2A, H2B, H3, and H4. The corresponding immunoblot reveals the levels of hv1 in the micronuclear preparation (arrow).

soluble chromatin fractions of bovine thymus and mature chicken erythrocytes, which are enriched in transcriptionally active DNA sequences (Davie & Saunders, 1981; Ridsdale & Davie, 1987a,b), are enriched in mono- and polyubiquitinated species of histones H2A and H2B. Among the monoubiquitinated histone species, uH2B is enriched to the greatest extent in the competent enriched chromatin. Compared to unfractionated chromatin, the relative enrichment of uH2B is greater than that of uH2A in the low salt soluble fraction of bovine thymus chromatin (1.69 for uH2B vs 1.36 for uH2A; Davie & Nickel, 1987) and in the salt soluble polynucleosomes of chicken erythrocytes (3.7 for uH2B vs 1.3 for uH2A; Ridsdale & Davie, 1987a).

Since the partitioning of active/competent chromatin from repressed chromatin is not complete, i.e., the salt-insoluble chromatin fraction also contains active/competent gene sequences [see Ridsdale and Davie (1987a)], the presence of ubiquitinated histones in all of the chromatin fractions may reflect the amount of active/competent gene sequences in these fractions. Conversely, ubiquitinated histone species or a subset of these modified histones (e.g., uH2A but not uH2B) may also be located in transcriptionally inactive chromatin regions. Thus, in order to find whether the ubiquitinated histone species are located in repressed chromatin, we examined the level of ubiquitinated histone forms in the transcriptionally active *Tetrahymena* macronucleus and transcriptionally inert micronucleus. Our results show that macronuclear histones are ubiquitinated while the ubiquitinated forms of H2A and H2B are greatly reduced in micronuclei. When the macronuclear contamination of the micronuclear preparation is considered, it is possible that the ubiquitinated species of H2B are totally absent in micronuclei. However, micronuclear histone H2A is likely ubiquitinated. If the presence of ubiquitinated histones in the micronuclear preparation was due solely to macronuclear contamination, then the level of micronuclear uH2B would be greater than that of uH2A. Since the level of uH2A is greater than that of uH2B in the micronuclear preparation (Figure 4), this indicates that micronuclei have low levels of poly- and monoubiquitinated H2A. These observations suggest that the mono- and polyubiquitinated forms of H2B are preferentially located in transcriptionally active/competent regions of chromatin. Mono- and polyubiquitinated species of histone H2A are present in both competent and repressed chromatin domains, but expressed chromatin is enriched in these modified histones.

Polyubiquitinated species of histones H2A, H2B, and H2A.Z have been detected in preparations of bovine thymus, chicken erythrocytes, *Tetrahymena* macronuclei and micronuclei, trout testis, trout liver, and trout hepatocellular carcinoma (this study; Nickel et al., 1987; Davie et al., 1987). Histone H2A had the greatest level of polyubiquitinated forms, with tetra- to hexaubiquitinated forms being observed (Figure 2; Davie et al., 1987). Thus, in general, histone H2A is the preferred substrate or most accessible substrate for the enzyme(s) which catalyze the addition of multiple ubiquitin molecules onto histone. In nucleosome cores, the C-terminal

sequence of histone H2A is accessible to proteolytic enzymes while the C-terminal sequence of histone H2B is not (Hatch et al., 1983; Rosenberg et al., 1986). The same accessibility is expected with respect to the enzymes catalyzing the addition and removal of ubiquitin. The increased accessibility of the C-terminal tail of histone H2A compared to the relative inaccessibility of the C-terminal portion of histone H2B may explain why H2A is polyubiquitinated to a greater extent than H2B, and in unfractionated chromatin, uH2A is usually severalfold greater than uH2B, with exceptions being (1) trout liver and *Physarum polycephalum*, where the levels of uH2A and uH2B are similar (Nickel et al., 1987; Mueller et al., 1985), and (2) *Tetrahymena* macronuclei, where the amount of uH2B is much greater than that of uH2A.

The preferential location of uH2B in transcriptionally active/competent chromatin may be a result of an alteration or disruption of nucleosome structure during transcription such that the C-terminus of histone H2B becomes accessible to the modifying enzymes. It has been proposed that the H2A–H2B dimer is displaced from the nucleosome during transcription (Jackson & Chalkley, 1981; Baer & Rhodes, 1983; Loidl, 1988). It is possible that histone H2B is a substrate for the enzymes catalyzing the addition of ubiquitin only when it is released from the nucleosome.

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