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Cellular content of ubiquitin and formation of ubiquitin conjugates during chicken spermatogenesis

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Ubiquitin was purified from chicken testis and its content, biosynthesis and formation of conjugates was determined in germinal cells at successive stages of spermatogenesis. Free ubiquitin increased markedly during spermatogenesis, reaching its maximum level in early spermatids. High levels of ubiquitin were still present in late spermatids but were not detectable in mature spermatozoa. Biosynthesis of ubiquitin occurred *in vitro* in a fraction containing meiotic and pre-meiotic cells, and during spermiogenesis, in early and late spermatids. The cellular content of free ubiquitin increased after ATP depletion, especially in early spermatids. Lysates of chicken testis cells, particularly those obtained from spermatids, were able to form nuclear (24 and 27 kDa) and extranuclear (55–90 kDa) ubiquitin conjugates *in vitro*. The presence of increasing levels of ubiquitin and ubiquitin conjugates in chicken spermatids may suggest a possible involvement of this protein in the marked changes of protein turnover, chromatin structure and cell–cell interactions that spermatids undergo during spermiogenesis.

INTRODUCTION

Ubiquitin is a small, highly stable, widely distributed globular protein (Goldstein *et al.*, 1975). Its amino acid sequence has been conserved during evolution suggesting the involvement of ubiquitin in a basic cellular function (Schlesinger & Goldstein, 1975; Gavilanes *et al.*, 1982). Ubiquitin is present in eukaryotic cells either free or covalently bound by an ATP-coupled reaction to target nuclear, cytoplasmic and membrane proteins (Goldknopf & Busch, 1977; Hershko, 1983; Rapoport *et al.*, 1985; Gallatin *et al.*, 1986; Yarden *et al.*, 1986). Nuclear conjugates of ubiquitin (uH2A, uH2B) may be involved in mechanisms of chromatin relaxation (Levinger & Varshavsky, 1982). Ubiquitin may also modulate chromatin structure through stimulation of histone deacetylase activity (Mezquita *et al.*, 1982). Conjugation with ubiquitin may serve as a recognition signal or an unfolding device for degradation of proteins (Hershko, 1983; Rapoport *et al.*, 1985; Bachmair *et al.*, 1986; Parag *et al.*, 1987). Ubiquitin has been identified as an essential component of the stress response system of cells (Bond & Schlesinger, 1985; Finley *et al.*, 1987). Recently another function of ubiquitin has been proposed: ubiquitinated cell surface glycoproteins may be part of a family of cell–cell interaction proteins playing important functions of recognition (Gallatin *et al.*, 1986; Yarden *et al.*, 1986).

Spermatogenesis provides an excellent model for investigating the role of ubiquitin in the marked changes of composition and structure that chromatin undergoes during the differentiation of the germinal cell line (Mezquita, 1985), and the putative mechanisms of protein degradation during spermiogenesis. Ubiquitin may also be involved in the phenomena of cell–cell interaction during the migration of germ cells within the seminiferous tubule, and of free spermatozoa, for specific recognition of oocytes. Previous studies of ubiquitin in spermatogenesis have shown that this protein is abundant in trout testis chromatin and in mammalian testis cells (Watson *et al.*, 1978; Loir *et al.*, 1984). The presence of a putative ubiquitin–histone H2A conjugate that increases during chicken spermiogenesis has also been reported (Agell *et al.*, 1983). In order to study the involvement of ubiquitin in the process of sperm cell differentiation, in this paper, we have determined the levels of free ubiquitin in chicken testis cells at successive stages of spermatogenesis and the formation of ubiquitin conjugates *in vitro* in cells separated by centrifugal elutriation.

EXPERIMENTAL

Purification of ubiquitin

Testes were removed from sexually mature chickens and used either immediately or after storage in liquid N_2. For purification, testicular tissue was homogenized for 5 min at top speed in an Omnimixer with 1.5 vol. of 0.74 M-perchloric acid containing either 5 mM-phenylmethanesulphonyl fluoride (PMSF) or 10 mM-benzamidine. The homogenate was clarified by centrifugation at 15 000 *g* for 15 min. Trichloroacetic acid (100%) was added to the supernatant to a final concentration of 25% (w/v). The precipitate was recovered by centrifugation and washed once with acidified acetone (0.1 ml of conc. HCl/100 ml), twice with acetone, and then dried under vacuum. The proteins thus obtained were dissolved in 0.1 M-HCl. Most of histone H1 and non-histone HMG2, HMG14 and HMG17, and some HMG1, were precipitated from the supernatant with 12.5 vol. of ethanol/HCl (99:1) for 1 h. Ubiquitin and remaining HMG proteins were precipitated from the supernatant with 6 vol. of acetone (−20 °C) overnight

Abbreviation used: PMSF, phenylmethanesulphonyl fluoride.

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(Walker *et al.*, 1978). The pellet was washed with acidified acetone and acetone as before and fractionated by gel filtration on a Sephadex G-75 column (1.5 cm × 100 cm) eluted with 0.01 M-HCl. The fractions containing ubiquitin were combined and lyophilized.

Amino acid analyses

Amino acid analyses were performed using a Beckman 119C amino acid analyser after hydrolysis of the samples in 6 M-HCl at 110 °C for 24 h. No corrections were made for hydrolytic losses.

Determination of Gly–Gly *C*-terminus

The percentage of intact ubiquitin having the Gly–Gly *C*-terminus was determined by quantifying the amount of Gly–Gly dipeptide liberated by tryptic digestion of the protein. With Gly–Gly dipeptide (Sigma) as standard, a Beckman 119C amino acid analyser was used to perform the analyses.

Iodination of purified ubiquitin

Iodination of purified ubiquitin was carried out by the chloramine T method (Ciechanover *et al.*, 1980). To 30 μg of ubiquitin dissolved in 20 μl of phosphate-buffered saline (pH 7.4), was added 10 μl of a solution containing 0.5 mCi of carrier-free $Na^{125}I$. Chloramine T (10 μl, 2 mg/ml) was added and the sample was mixed vigorously at room temperature for 1 min. The reaction was terminated by adding 10 μl of sodium metabisulphite (2 mg/ml) and the preparation was passed through a column of Sephadex G-25. The specific activity of the ^{125}I-ubiquitin preparation was 6.6×10^6 c.p.m./μg.

Separation of germinal cells at successive stages of spermatogenesis by centrifugal elutriation

Cell suspensions from rooster testes were prepared and separated by centrifugal elutriation essentially as described by Meistrich (1977). Mature testes from Hubbard White Mountain roosters (25–30 weeks old) were decapsulated and minced finely with scissors. The minced tissue was gently suspended in 10 vol. of minimum essential medium (Eagle) containing 0.1% (w/v) trypsin and 2 μg of DNAse I/ml. The suspension was incubated at 31 °C for 30 min with gentle stirring in a water bath. After incubation, the cell suspension was filtered through four layers of surgical gauze and centrifuged for 20 min at 1500 *g* in a JE-7.5 Beckman rotor. The sample was resuspended in 50 ml of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.02% (w/v) soybean trypsin inhibitor, 0.1% bovine serum albumin and 0.1% glucose. The cell suspension was diluted in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to a final concentration of (25–30) $\times 10^6$ ml. A cell suspension of 20 ml was loaded into a JE-6 Beckman elutriator rotor and separations were performed with speeds of 3000 rev./min and flow rates of 3–100 ml/min. Fractions of 125 ml were collected. The following cell types were obtained: (1) testicular spermatozoa and residual bodies (3 ml/min flow rate); (2) elongated spermatids (11 ml/min flow rate); (3) round spermatids (20 ml/min flow rate); (4) meiotic and pre-meiotic cells and multinucleate cells (37 ml/min flow rate).

Labelling of testicular cells with [^{3}H]arginine

Fresh rooster testes (5 g) were finely minced with scissors and suspended in 10 ml of minimum essential medium (Eagle). The suspension was incubated with 1 mCi of [^{3}H]arginine (Amersham; 52 mCi/mmol) at 31 °C for 2 h. Following incubation cells were dissociated for separation by centrifugal elutriation as described in the previous paragraph.

Ubiquitin–protein conjugation assay

In order to determine the ability of testicular cell lysates to catalyse the formation of cytoplasmic and nuclear conjugates of ubiquitin, chicken testis cells (2×10^7) separated by centrifugal elutriation were incubated for 2 h at 37 °C in 300 μl of 10 mM-Tris/HCl (pH 7.8), 5 mM-$MgCl_2$, 1 mM-$CaCl_2$, 0.1 mM-leupeptin, 1 mM-PMSF, 5 mM-dithiothreitol, 100 units of aprotinin/ml, 10 mM-creatine phosphate, 1 mM-ATP, 1.8 units of creatine phosphokinase and 10 μg of ^{125}I-ubiquitin (6.6×10^7 c.p.m.). After centrifugation at 3000 *g* for 15 min, the supernatant was precipitated by addition of trichloroacetic acid (25% final concn.) and the pellet was suspended in a solution containing 0.25 M-sucrose, 10 mM-Tris/HCl (pH 7.8), 5 mM-$MgCl_2$, 5 mM-PMSF, 100 units of aprotinin/ml, 5 mM-*N*-ethylmaleimide and 0.1% Triton X-100, homogenized, and centrifuged through 0.88 M-sucrose for purification of cell nuclei.

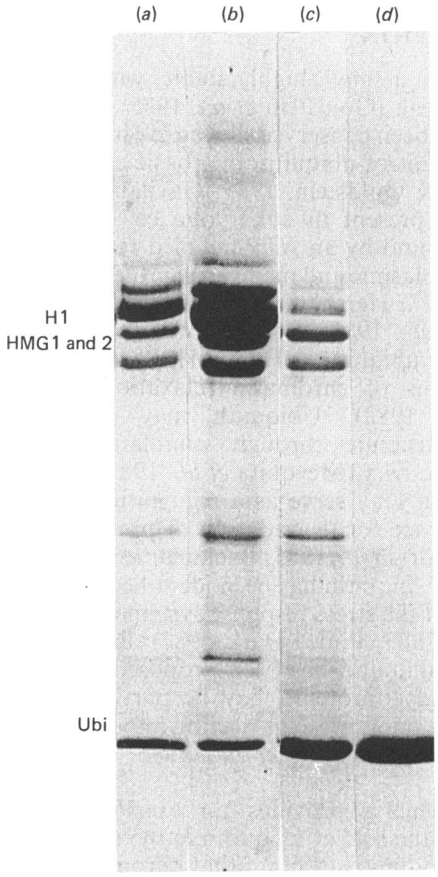

Fig. 1. Purification of ubiquitin (Ubi)

SDS/18% polyacrylamide-gel electrophoresis of different fractions obtained during the purification process. (*a*) Proteins extracted with 0.74 M-perchloric acid and precipitated with 25% (w/v) trichloroacetic acid. (*b*) Proteins precipitated with 12.5 vol. of ethanol/HCl (99:1). (*c*) Proteins precipitated with 6 vol. of acetone. (*d*) Fraction containing ubiquitin eluted from a Sephadex G-75 column.

Table 1. Amino acid composition of ubiquitin purified from chicken testis

The amino acid composition of ubiquitin isolated from chicken testis is compared with the amino acid composition deduced from the sequence of the chicken ubiquitin gene (Bond & Schlesinger, 1985).

Amino acid	Chicken testis	Ubiquitin gene
Lys	7.42	7
His	1.22	1
Arg	3.85	4
Asp	7.32	7
Thr	6.06	6
Ser	2.75	3
Glu	12.48	12
Pro	2.78	3
Gly	6.44	6
Ala	2.13	2
Val	3.80	4
Met	0.72	1
Iso	6.29	6
Leu	8.48	8
Tyr	1.17	1
Phe	2.98	3

Purified nuclei were extracted with 0.74 M-perchloric acid and subsequently with 0.2 M-H_2SO_4.

Analytical methods

Electrophoreses were performed either in SDS/polyacrylamide gels (Thomas & Kornberg, 1978) or in acetic acid/urea/polyacrylamide gels (Orrick *et al.*, 1973). The methods of Bonner & Laskey (1974) and Laskey & Mills (1975) were used to prepare gels for fluorography. ATP was quantified by the procedure of Beutler (1975). DNA was determined by the diphenylamine reaction (Burton, 1968) and proteins were quantified by the procedure of Lowry *et al.* (1951).

RESULTS

Purification of ubiquitin from mature chicken testes

Ubiquitin was extracted from mature chicken testes with 0.74 M-perchloric acid and partially purified by differential precipitation and gel filtration on Sephadex G-75. Ubiquitin was identified by electrophoretic mobility (Fig. 1) and amino acid analyses (Table 1). Using PMSF as a protease inhibitor, it was found that 34.5% of ubiquitin molecules possessed Gly–Gly at their *C*-terminals and using benzamidine as inhibitor, 49% of ubiquitin was obtained in the intact form.

Cellular content of free ubiquitin in mature testes of chicken in comparison with immature testes, liver, spermatozoa and erythrocytes

A comparison of the cellular content of free ubiquitin measured in different chicken tissues is shown in Fig. 2 and Table 2. The protein is apparently more abundant in cells of mature chicken testes than in cells of immature testes, liver, spermatozoa and chicken erythrocytes.

Biosynthesis of ubiquitin at successive stages of spermatogenesis

Small fragments of sexually mature chicken testis were incubated with [^{3}H]arginine as described in the Experimental section. After incubation, testicular cells were dissociated and separated by centrifugal elutriation. Proteins soluble in 0.74 M-perchloric acid were electrophoresed in acetic acid/urea/polyacrylamide gels and

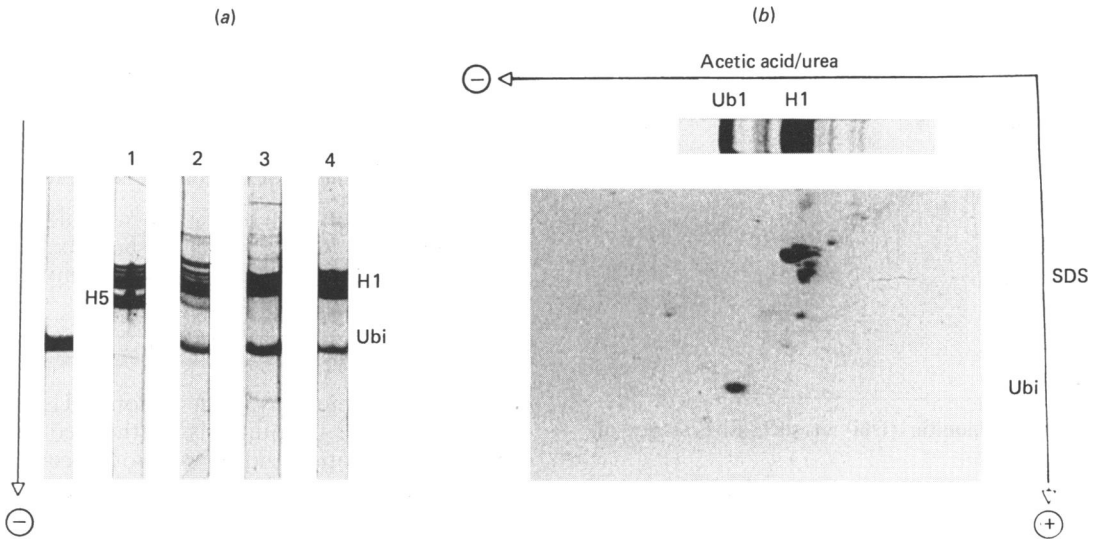

Fig. 2. Comparison of the cellular content of ubiquitin (Ubi) in different chicken tissues

(*a*) Acetic acid/4.5 M-urea/10% polyacrylamide-gel electrophoresis of purified ubiquitin and 0.74 M-perchloric acid-soluble proteins obtained from chicken erythrocytes (1), chicken liver (2), mature chicken testes (3), and immature chicken testes (4). (*b*) Two-dimensional polyacrylamide-gel electrophoresis of 0.74 M-perchloric acid-soluble proteins extracted from mature chicken testes. First dimension: acetic acid/4.5 M-urea/10% polyacrylamide-gel electrophoresis. Second dimension: SDS/18% polyacrylamide-gel electrophoresis. The band corresponding to ubiquitin obtained in the first dimension cannot be further resolved in the second dimension in any of the tissues studied. For this reason, the cellular content of ubiquitin was determined from densitometer scans of acetic acid/urea-gel electrophoresis.

Table 2. Cellular content of free ubiquitin in different chicken tissues

	Content	
	(μg of ubiquitin/ mg of DNA)	(μg of ubiquitin/ mg of protein)
Mature testes	66±13	2.37±0.86
Immature testes	47±8	1.53±0.15
Liver	44±12	0.55±0.07
Erythrocytes	N.d.	N.d.
Spermatozoa	N.d.	N.d.

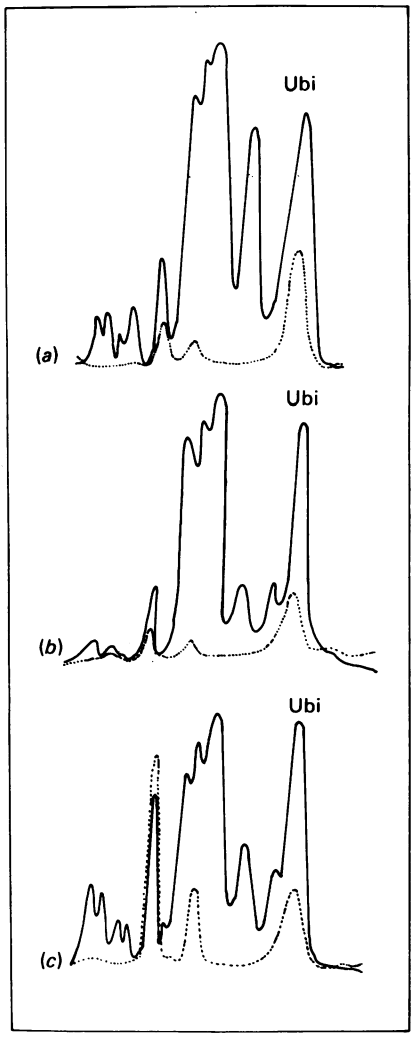

Fig. 3. Biosynthesis of ubiquitin (Ubi) at successive stages of spermatogenesis

Densitometric scans of gels (——) and fluorograms (----) of 0.74 M-perchloric acid-soluble proteins, labelled with [^{3}H]arginine, extracted from testis cells separated by centrifugal elutriation. (*a*) Meiotic and pre-meiotic cells. (*b*) Round spermatids. (*c*) Elongated spermatids.

the incorporated radioactivity was determined by fluorography. Three main bands were detected in the fluorograms (Fig. 3): one band with the mobility of ubiquitin, a second band with the mobility of HMG2 and a third band of lower mobility than histone H1, HMG1 and HMG2 proteins. Ubiquitin is synthesized in a fraction containing meiotic and pre-meiotic cells in round spermatids and in elongated spermatids.

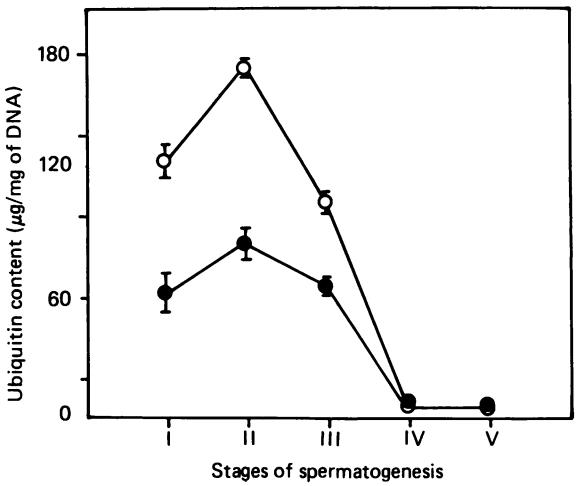

Fig. 4. Changes in free ubiquitin content at successive stages of chicken spermatogenesis

Determinations of ubiquitin were performed without depletion of ATP (●) and after depletion of ATP (○). I, Meiotic and pre-meiotic cells; II, round spermatids; III, elongated spermatids; IV, testicular spermatozoa; V, spermatozoa from the vas deferens. For ATP-depletion testicular cells were incubated as described in the legend of Fig. 5.

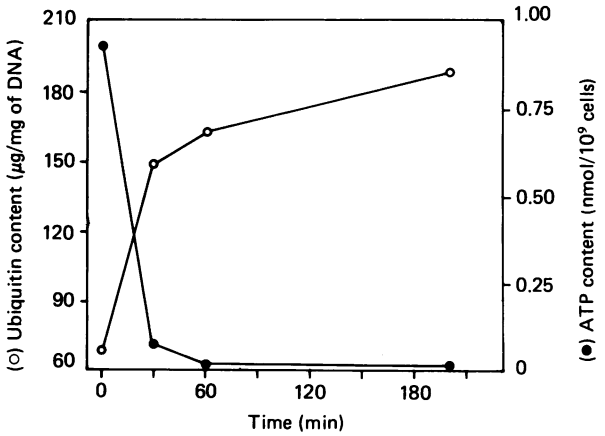

Fig. 5. Depletion and ubiquitin content in chicken testis cells

As a control of ATP depletion, testicular cells were incubated at 37 °C in a phosphate-buffer saline medium containing 25 mM-2-deoxyglucose/0.25 mM-2,4-dinitrophenol for the times indicated.

Cellular content of free ubiquitin at successive stages of chicken spermatogenesis in the presence of ATP and after depletion of ATP

The cellular content of ubiquitin at successive stages of spermatogenesis was determined after separation of testicular cells by centrifugal elutriation. Ubiquitin increased during spermatogenesis, reaching its maximal level in early spermatids, was still present in late spermatids, and finally disappeared in spermatozoa (Fig.

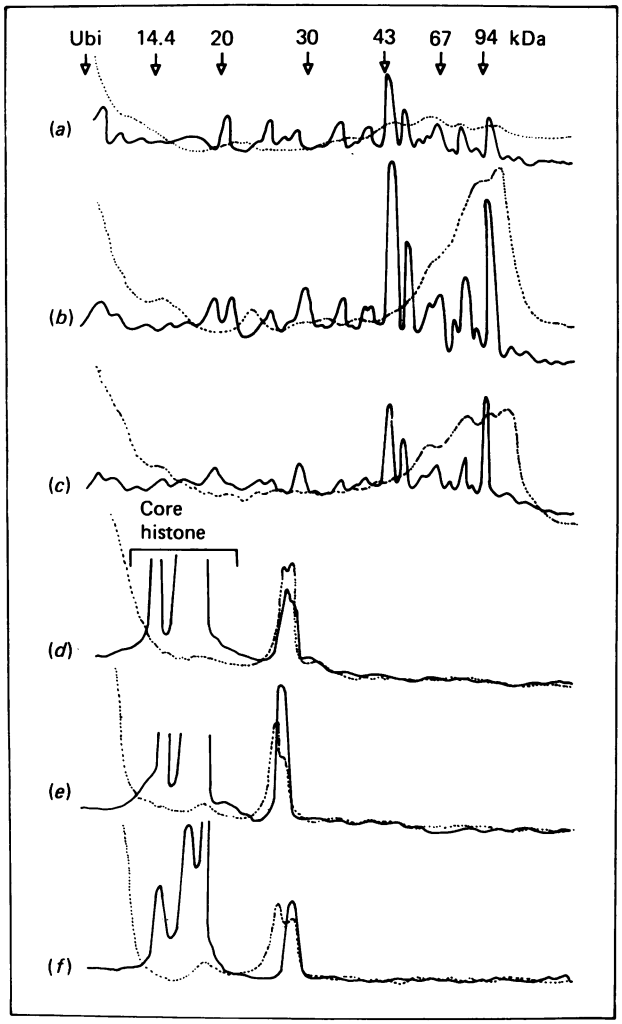

Fig. 6. Formation of ubiquitin–protein conjugates by chicken testis cell lysates

Densitometric scans of gels (——) and autoradiograms (----) of ^{125}I-ubiquitin–protein conjugates formed by chicken cell lysates. Lysates obtained from testicular cells separated by centrifugal elutriation were incubated in the presence of ^{125}I-ubiquitin as described in the Experimental section. Proteins obtained from the supernatants and purified nuclei were analysed by SDS/18% polyacrylamide-gel electrophoresis and ubiquitin (Ubi) conjugates visualized after autoradiography. (*a*) Supernatants of meiotic and pre-meiotic cells. (*b*) Supernatants of round spermatids. (*c*) Supernatants of elongated spermatids. (*d*) Nuclear proteins from meiotic and pre-meiotic cells. (*e*) Nuclear proteins from round spermatids. (*f*) Nuclear proteins from elongated spermatids. Nuclear proteins were extracted with 0.2 M-H_2SO_4 from nuclei previously washed with 0.74 M-perchloric acid. No conjugates were formed in the absence of ATP.

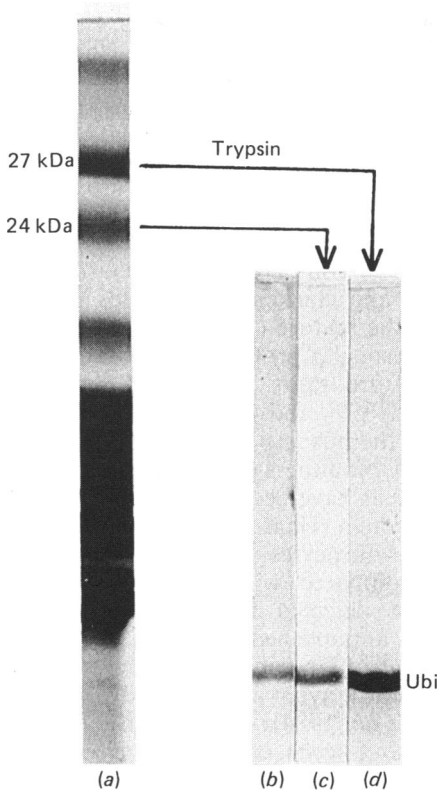

Fig. 7. Nuclear conjugates of ubiquitin (Ubi)

(*a*) Proteins of 24 kDa and 27 kDa, identified as putative nuclear conjugates of ubiquitin, were separated by preparative SDS/18% polyacrylamide-gel electrophoresis (3 mm gels). (*b*) Bands were stained and eluted as described by Hager & Burgess (1980). The proteins were redissolved in 100 μl of 0.1 M-*N*-ethylmorpholine (pH 7.8) and digested with 2 μg of trypsin at 37 °C for 48 h. Purified ubiquitin (15 μg) was incubated with trypsin under the same conditions. Ubiquitin is resistant to trypsin digestion. (*c*) and (*d*) The 24 kDa and the 27 kDa proteins yield bands with the same electrophoretic mobility as ubiquitin.

4). When cellular ATP is depleted in chicken testis cells (Fig. 5), the cellular content of free ubiquitin during spermatogenesis increases, reaching its maximum level in early spermatids (Fig. 4).

Formation of ubiquitin conjugates in chicken testis cell lysates

Ubiquitin labelled with ^{125}I was incubated in the presence of ATP with cell lysates obtained from chicken testis cells. The densitometer traces of the radioautograms of the conjugates formed between ^{125}I-ubiquitin and cellular target proteins are shown in Fig. 6. Most of the extranuclear labelled proteins migrate with electrophoretic mobilities in the range 70–100 kDa. The nuclear acid-soluble labelled proteins, extracted with 0.2 M-H_2SO_4 after perchloric acid extraction of the nuclei, were two main radioactive bands of 24 and 27 kDa. The nuclear protein of 27 kDa greatly increases during chicken spermiogenesis (Agell *et al.*, 1983). Both the 24 and 27 kDa acid-soluble nuclear proteins yield ubiquitin when digested with trypsin (Fig.7).

DISCUSSION

We have shown that ubiquitin purified from chicken testis in the presence of the protease inhibitors PMSF or benzamidine showed 34.5% and 49% intact Gly–Gly *C*-terminals respectively. These results are in accordance with the percentage of intact ubiquitin obtained from trout testis (Watson *et al.*, 1978), and are at variance with the content of intact free ubiquitin determined in mam-

malian testis, where all ubiquitin was devoid of the Gly–Gly *C*-terminals and presented an Arg *C*-terminal (Loir *et al.*, 1984). Both avian and mammalian ubiquitin sequences obtained at the genomic level possessed Gly–Gly *C*-terminals, and only the intact molecule is able to form protein conjugates. The absence of Gly–Gly *C*-terminals can be attributed to their artifactual removal by proteases.

Ubiquitin is an abundant protein in mature chicken testis cells when compared with immature testes, liver, spermatozoa and chicken erythrocytes. The amount of ubiquitin in the mature chicken testis represents 0.23% of the total testicular protein; the amount is only 0.15% in the immature testis and 0.05% in chicken liver. Amounts of 0.05% and 0.07% were detected in rabbit liver and calf thymus respectively (Low *et al.*, 1979; Haas *et al.*, 1985). Ubiquitin is present in mammalian erythrocytes; these cells have been used for large-scale purification of the protein (Haas & Wilkinson, 1985). However, in chicken erythrocytes (cells with marked metabolic differences compared with mammalian erythrocytes), we have not detected free ubiquitin (N. Agell and C. Mezquita, unpublished work).

The amount of free ubiquitin compared with that of DNA is similar in avian and mammalian spermatogenic cells (Bucci *et al.*, 1984). Ubiquitin is also an abundant protein in trout testis cells (Watson *et al.*, 1978). In chicken immature testis enriched in spermatogonia, the amount of free ubiquitin is 1.53 μg/mg of protein. This amount increases in fractions containing pre-meiotic and meiotic cells obtained by centrifugal elutriation (2.71 μg of ubiquitin/mg of protein) and further increases, reaching a maximum, in fractions containing round spermatids (3.24 μg of ubiquitin/mg of protein). A high content of free ubiquitin still persists in elongated spermatids in spite of the drastic removal of proteins that takes place at the end of spermiogenesis. Free ubiquitin is no longer detectable in mature spermatozoa.

We have detected biosynthesis of ubiquitin in different fractions of chicken testis cells including late spermatids. Biosynthesis of ubiquitin has also been detected in pre-meiotic, meiotic and round spermatids during rat spermatogenesis (Bucci *et al.*, 1984).

After ATP depletion the content of free ubiquitin increases in all the stages of chicken spermatogenesis, with the exception of mature spermatozoa, where ubiquitin is still undetectable. Formation of ubiquitin conjugates with cellular target proteins is dependent on ATP-coupled reactions, and the release of ubiquitin from conjugates by the isopeptidase enzymatic activity is independent of ATP (Hershko, 1983). For these reasons, when cellular ATP is depleted, the cellular content of free ubiquitin should increase.

In the presence of ATP, lysates of testicular cells were able to form nuclear and extranuclear conjugates of ubiquitin. One of these conjugates, a nuclear acid-soluble protein of 27 kDa, increases markedly at the end of chicken spermiogenesis (Agell *et al.*, 1983). This conjugate and the 24 kDa conjugate show solubilities, electrophoretic mobilities and amino acid analysis characteristics that are similar to the uH2B and uH2A conjugates, and both yield ubiquitin upon trypsin digestion. The observed electrophoretic heterogeneity of the ubiquitin conjugates in SDS gels also could be a consequence of post-translational modifications or conjugation of more than one molecule of ubiquitin.

The presence of high levels of free ubiquitin in chicken spermatids and its ability to form conjugates with target nuclear and extranuclear proteins suggest that ubiquitin could be involved in the marked changes of protein turnover, chromatin structure and cell–cell interactions occurring during spermiogenesis. Conjugation of ubiquitin with nuclear and extranuclear proteins during spermatogenesis might be part of an ATP–ubiquitin-dependent proteolytic process. All histones and most of the non-histone chromosomal proteins are lost at the end of chicken spermiogenesis (Mezquita & Teng, 1977). Ubiquitin conjugation may participate in this process inducing conformational changes in the proteins undergoing proteolysis.

In addition, nuclear conjugates of ubiquitin in spermatids could play a role in the structural transition of chromatin from nucleohistone to nucleoprotamine during chicken spermiogenesis (Agell *et al.*, 1983). Relaxation of chromatin and exposition of binding sites for protamine in spermatids could be induced by modification of nucleosomes by the presence of ubiquitin–histone conjugates. On the other hand, free ubiquitin stimulates histone deacetylase *in vitro* (Mezquita *et al.*, 1982) and the presence of high levels of ubiquitin in chicken spermatids might be related to the high turnover of acetyl groups of histones detected in these cells (Oliva & Mezquita, 1982).

Finally, ubiquitin through formation of conjugates with membrane proteins, could be involved in recognition phenomena between spermatogenic cells in a similar way as that demonstrated for lymphocyte homing receptors (Gallatin *et al.*, 1986). Migration of meiotic and post-meiotic cells through the seminiferous tubule interacting with Sertoli Cells, and the final recognition of oocytes by spermatozoa, might be mediated by membrane glycoproteins conjugated with ubiquitin. The identification of nuclear and extranuclear conjugates of ubiquitin will shed light on the possible functions of ubiquitin during spermatogenesis.

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