Methylation of chick Ubl and UbII polyubiquitin genes and their differential expression during spermatogenesis

Nativitat ROCAMORA*† and Neus AGELL†

*Department of Physiological Sciences and †Department of Cellular Biology, Faculty of Medicine, University of Barcelona, Casanova 143, 08036 Barcelona, Spain

Northern analysis demonstrated that levels of ubiquitin transcript increased during the chicken testis maturation process, in agreement with the previously published increase of ubiquitin during this differentiation process. Specific probes for four different ubiquitin genes (two polyubiquitins, Ubl and UbII, and two ubiquitin-fusion genes, UbCep52 and UbCep80) allowed us to analyse the expression of each individual gene. Ubl polyubiquitin gene was expressed in all the tissues tested, and its transcript was the most abundant ubiquitin RNA in all of them. Unspliced Ubl transcript, already detected in stressed chicken-embryo fibroblast, was also present in immature testis and reticulocytes. UbII, a chicken polyubiquitin gene not previously found expressed and not heat-shock-inducible, was specifically stimulated during the testis maturation process. Two minor ubiquitin fusion transcripts of 0.6 and 0.7 kb, corresponding to UbCep52 and UbCep80 respectively, were also found in chicken testis. Although differentially expressed, it was found that Ubl and UbII chicken polyubiquitin genes had an HTF ('HpaII tiny fragments') island (CpG-rich and constitutively unmethylated region) in their 5' proximal non-coding region. In addition, we demonstrated the coexistence of 3' and/or 5' relatively distal methylated sites together with these 5' proximal HTF islands in both chicken polyubiquitin genes. 3' and 5' distal Ubl CCGG sites were specifically hypermethylated in mature testis, whereas a 3' distal UbII CCGG site was found to be about 50% methylated in all DNAs tested.

INTRODUCTION

Ubiquitin, a highly conserved polypeptide of 76 amino acids present in all eukaryotic cells studied, has been implicated in many fundamental cellular processes, including chromatin structure and intracellular protein turnover. Despite the polypeptide's ubiquity, the free and conjugated cellular pools, as well as specific ubiquitin conjugates, change under different conditions such as stress, developmental stage and cell cycle (for a review, see [1–4]). Ubiquitin levels and those of its conjugates increase during chicken spermatogenesis [5], and this has been related to the elimination of nuclear and cytoplasmic proteins that occurs during this differentiation process. There are two kinds of ubiquitin-encoding genes: one has multiple ubiquitin sequences in the form of contiguous repeats of the coding sequence and the other encode ubiquitin fusion proteins (for a review, see [6]). These two kinds of genes are differentially expressed: whereas ubiquitin fusion transcripts are most abundant in normal dividing cells, high levels of polyubiquitin transcripts are found in cells that undergo sporulation, differentiation or, in general, are under stress [7–10]. Ubiquitin fusion proteins are known to be involved in ribosomal assembly [11]. Bond & Schlesinger [12] described two polyubiquitin genes (PUB) in chicken DNA, Ubl and UbII, containing four and three tandem ubiquitin coding units respectively. Ubl was found to be expressed in normal chicken-embryo fibroblasts and stimulated by heat-shock, consistent with the presence of a heat-shock promoter element in its 5' non-coding region. UbII transcript was not detected in normal or heat-shocked chicken-embryo fibroblasts and it was proposed that its transcription could be regulated in a tissue- or developmentally-specific way [12]. Here we report a specific stimulation of UbII expression during the chicken testis maturation process and we tried to find out whether there is any correlation between

expression of the polyubiquitin genes and their methylation pattern.

DNA methylation has been considered as a putative controlling mechanism in the regulation of gene expression by both its capacity to alter stably the local configuration of a gene and to interfere with the specific protein–DNA binding affinity (for a review, see [13]). Several lines of evidence suggested that CpG methylation may play a role in the regulation of transcriptional activity: methylation of several genes *in vitro* inhibits their expression when inserted into cultured cells [14]; 5-azacytidine, a potent demethylating agent that is thought to act by inhibiting eukaryotic DNA methylases, can selectively activate eukaryotic gene expression and, in some cases, even alter the differentiation state of the cells [15].

Although CpG dinucleotide is highly suppressed in the bulk vertebrate DNA, genes coding for housekeeping functions contain CpG-rich and constitutively unmethylated 'islands' in their 5' end regions. These islands, also called 'HpaII tiny fragments' (HTF), are DNA regions in which CpG frequency has not been suppressed, probably because they had never been methylated. It has been proposed that the lack of methylation would render HTF islands constitutively available to the transcriptional machinery and so distinguish them from bulk DNA (for a review, see [16,17]). A nuclear protein that specifically binds to methylated CpGs has been recently isolated from mammalian nuclei, and it has been suggested that it could be involved in the stabilization of inactive chromatin structures [18,19]. On the other hand, upstream regions of tissue-specific genes have a decreased CpG frequency and are specifically hypomethylated in expressing tissues, but are hypermethylated in non-expressing ones (for reviews, see [13,20,21]).

Tissue-specific genes are hypermethylated in vertebrate sperm DNA, correlating with its transcriptional inactivity, but

Abbreviations used: HTF (island), HpaII tiny fragments (CpG-rich and constitutively unmethylated gene regions).

† To whom correspondence and reprint requests should be addressed.

'housekeeping' genes maintain their CpG-rich islands unmethylated. Global DNA methylation data, by contrast, show a quantitatively important (30–50%) and almost widespread male-germ-line-specific undermethylation with, at present, unknown physiological significance [22–25]. Although there are considerable data on DNA methylation of tissue-specific and housekeeping genes in sperm, and also on repetitive sequences, very few DNA methylation analyses of 'spermatogenic genes' has been done.

Our results show that, despite the fact that UbI and UbII chicken polyubiquitin genes are differentially expressed during spermatogenesis, both contain a CpG-rich and constitutively unmethylated island (HTF) in their 5' proximal non-coding region, whereas 5' and 3' relatively distal sites are partially and/or differentially methylated.

MATERIALS AND METHODS

Tissues, cells and probes

Liver, testis and blood of Hubbard White Mountain roosters (25–30 weeks old) and sexually immature chickens (8 weeks old) were used in the present study. Testes and liver when isolated were immediately kept in liquid N₂. Reticulocytes were obtained from immature chickens after 1 week of phenylhydrazine treatment: 0.4 ml of 2.5% phenylhydrazine/kg of animal weight was injected daily. Spermatogenic nuclei were purified in citric acid and separated by sedimentation at unit gravity [26]. The probes used, (see Fig. 1) were: 1, 2, 3, 5, 6 and PCR from UbI and UbII chicken polyubiquitin genes, and Cep52 and Cep80 (specific probes for the non-ubiquitin region of the two human ubiquitin fusion genes UbCep52 and UbCep80). UbI and UbII probes (described in [12]) were a gift from Dr. M. J. Schlesinger. UbCep52 and UbCep80 cDNAs were provided by Dr. R. T. Baker and Dr. P. K. Lund respectively. UbI and UbII probes were isolated from their plasmids by digestion with the corresponding restriction enzymes and purified by the DEAE-agarose method [28]. C-Terminal extensions, Cep52 and Cep80 (non-ubiquitin region of ubiquitin fusion probes), were isolated from the ubiquitin region of UbCep52 and UbCep80 by digestion with restriction enzymes *HhaI* and *DdeI* respectively (Fig. 1) and purified in the same way as the other probes.

RNA analysis

Frozen material was pulverized in liquid N₂, directly homogenized in guanidium isothiocyanate, and ultracentrifuged throughout a CsCl cushion as described by Chirgwin *et al.* [29]. Total RNA was electrophoresed on denaturing 1.2%-agarose/2.2 M-formaldehyde gels. RNA was transferred to Hybond-N membranes (Amersham International) and fixed by u.v. irradiation according to the manufacturer's protocols. All probes were ³²P-labelled to specific radioactivities (between 10⁸ and 10⁹ c.p.m./μg of DNA) by the oligo-labelling procedure [30], by using [α -³²P]-CTP (3000 Ci/mmol; New England Nuclear) and the random primed DNA labelling kit from Boehringer Mannheim. Homologous probes (chicken polyubiquitin UbI and UbII probes) were hybridized under high-stringency conditions. Prehybridization was done in 5 × SSPE[180 mM-NaCl/10 mM-NaH₂PO₄(pH 7.4)/1 mM-EDTA]/5 × Denhardt's (0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% BSA)/0.5% SDS/50% (v/v) formamide/10% (w/v) dextran sulphate/denatured sonicated salmon sperm DNA (500 μg/ml) overnight at 42 °C. Hybridization was done for 16–20 h under the same conditions, denatured labelled probes (10⁶–10⁷ c.p.m./ml) being added. Filters were washed sequentially for 20 min each in 2 × SSPE/0.1% SDS, 1 × SSPE/0.1% SDS and 0.1 × SSPE/0.1% SDS, all at 65 °C. Human Cep52 and Cep80 probes were hybridized under the

same conditions, except that prehybridization and hybridization temperatures were decreased from 42 to 37 °C, and the final wash was in 1 × SSPE/0.1% SDS at 60 °C. Quantitative densitometric analyses were performed on films exposed in the linear region.

Genomic Southern-blot analysis

High-molecular-mass DNA was isolated as described in [31] and completely digested with the appropriate restriction enzymes. Restriction digestions (4 μg of enzyme/μg of DNA) were carried out for 4 h (three times) under the conditions recommended by the suppliers (Boehringer Mannheim and New England Biolabs). Complete digestion was monitored by adding λ-phage DNA and by the analysis of the hybridization pattern obtained. The digested DNA was precipitated with ethanol, washed and re-suspended in the electrophoresis buffer. DNA was electrophoresed through 0.8- or 2%-agarose/TBE (89 mM-Tris/borate/89 mM-boric acid/2 mM-EDTA) gels and transferred as indicated for the RNA. Where indicated, DNA was electrophoresed on a 5% polyacrylamide/7 M-urea gradient DNA-sequencing gel and electrotransferred to nylon membranes as described in [32]. DNA filters were prehybridized, hybridized and washed in the same way as RNA filters.

RESULTS

Differential expression of ubiquitin genes in chicken mature testis versus immature testis and some somatic tissues

Northern blots were performed with equal amounts of total RNA from chicken mature testis, immature testis, liver, reticulocytes and from human lymphocytes. RNAs were hybridized with: (a) a probe of ubiquitin coding region (PCR), (b) specific probes for the two chicken polyubiquitin genes UbI and UbII [12] and (c) the non-ubiquitin region of two human ubiquitin-fusion cDNAs: Cep52 [33] and Cep80 [27] (Fig. 1). The ubiquitin-coding region is common to all the ubiquitin transcripts so PCR hybridization revealed a very complex pattern of bands (Fig. 2). Probes 2 and 5 discriminated between the UbI and UbII gene transcripts respectively. Probe 1 corresponds to the intronic region of UbI only, and its hybridization revealed the presence of unspliced RNA.

Consistent with previous data, probes 2 (UbI 5'-specific probe) and PCR revealed a transcript of 1.2 kb in all the tested chicken tissues. In addition, these two probes and the probe 1 hybridized with a larger RNA of approx. 1.9 kb in immature testis and reticulocytes, which demonstrated the presence of unspliced RNA in these tissues, but not in mature testis nor in liver. Probe 5 (UbII 5'-specific probe) hybridized with a transcript of approx. 0.9 kb that was found in large amounts only in mature testis (longer exposures showed weak hybridization of this probe with immature testis and reticulocyte RNA, but not with liver RNA) (Fig. 2).

Both human Cep52 and Cep80 hybridized with 0.6 kb RNAs, in human lymphocytes, as described by Baker & Board [34]. Probes Cep52 and Cep80 hybridized with two chicken RNAs of 0.6 and 0.7 kb respectively (Fig. 2), which were also detected as minor bands in the PCR hybridization, suggesting that they would correspond to UbCep52 and UbCep80 transcripts respectively. These two ubiquitin-fusion transcripts were found in mature and immature testis, the UbCep52 transcript level being specifically increased in the testis maturation process.

Although ubiquitin transcripts were present in the four tissues tested, the total amount and distribution of the different RNAs changed from one tissue to another. Quantification of ubiquitin transcripts on the basis of densitometric analyses of exposed films is shown in Table 1. These data demonstrated that

Fig. 1. Probes used in the Northern and Southern blots

UbI and UbII correspond to the chicken polyubiquitin genomic sequences [12] and UbCep52 and UbCep80 correspond to the human ubiquitin cDNA sequences [28,33]. The third repeat of the UbI coding region (probe *) was used to screen ubiquitin DNA sequences and transcripts. Probes 1, 2, 3, 5, 6, Cep52 and Cep80 are used to discriminate between the different ubiquitin genes and transcripts and their regions. The protein coding region (PCR) is indicated. The mRNA-coding sequence of UbI is also indicated.

reticulocytes and mature testis were the tissues with highest levels of ubiquitin transcripts, the UbI transcript always being the most abundant one. The most striking result was the substantial and specific increase during the testis maturation process of the UbII gene transcript, a gene which had not previously been found to be expressed.

Southern-blot analysis of different chicken ubiquitin genes: polymorphism

Hybridization of *PstI* + *HindIII*-restricted chicken DNA with PCR and specific probes for the different ubiquitin genes resulted

in a DNA fragment pattern identical in all tissues studied, allowing us to analyse different ubiquitin genes independently (Fig. 3). PCR probe hybridization revealed two major bands of 1.3 and 1.9 kb, corresponding to the UbI and UbII fragments respectively, as expected from the published restriction map of the two genes ([12] and Fig. 1) and confirmed by using probes 1 and 5 (Fig. 3a). Probe 2 hybridization revealed a fragment of 430 bp and probe 3 one of 350 bp. The extra bands revealed with probes 1 and 5 [Figs. 3a and 5c (below)] could be due to the presence of repetitive sequences in these probes (probe 1 is totally intronic and probe 5 could also contain an intron, because of the

Fig. 2. Northern hybridization analysis

Total RNA (15 µg) samples of chicken mature testis, immature testis, liver, reticulocytes and human lymphocytes were electrophoresed through a 1.2%-agarose gel containing 2.2 M-formaldehyde as described in the Materials and methods section. It was stained, transferred to a nylon membrane (Hybond-N) and covalently fixed to the membrane by u.v. treatment. The fixed RNA was hybridized with different probes under different stringency conditions depending on their sequence similarity, as indicated in the Materials and methods section. Lanes a, b, c, d and e are reticulocyte, mature testis, liver, immature testis and human lymphocyte RNAs respectively. 5* corresponds to a longer exposure of probe 5 hybridization (15 versus 3 days). The 1.2 kb band present in the Cep80 hybridization could be due to the incomplete purification of this probe from the PCR region.

Table 1. Relative levels of the different ubiquitin transcripts in the tissues analysed

Abbreviations: IT, immature testis; MT, mature testis; R, reticulo-cyte; and L, liver. 'A' columns represent the relative increases of ubiquitin transcripts between tissues with the value for immature testis standardised to 1. Ub total transcript was measured from PCR hybridization (probe *; see Fig. 1). Ubl, UblI, UblII, UbCep52 and UbCep80 transcripts were measured with probes 2, 5, Cep52 and Cep80 respectively. '(B)' columns represent the percentage of each transcript with respect to the total amount of ubiquitin RNA for each tissue. The percentages in immature testis were measured from the PCR hybridization (probe *), and all the other percentages were estimated from these immature-testis values together with the increases described in columns A.

Transcripts (ubiquitin)	IT		MT		R		L	
	A	(B)	A	(B)	A	(B)	A	(B)
Total	1		2.8		2.0		0.3	
Ubl	1 (88)		1.8 (74)		2.6 (97)		0.4 (98)	
UblII	1 (8)		6.0 (22)		0.8 (3)		0	
UbCep52	1 (2)		2.3 (2)		0.1 (0.1)		0.05	
UbCep80	1 (2)		1.3 (1)		0		0.2 (1)	

widespread presence of introns in the 5' end of the characterized polyubiquitin genes). Although the two principal PCR bands (1.3 and 1.9 kb) correspond to the two chicken polyubiquitin genes (Ubl and UblII), at least three other weaker bands, one of more than 4 kb in length and two others of 1.0 and 0.6 kb, also appeared. The 0.6 kb PCR fragment also hybridized with the

Cep52 probe indicating the presence of a UbCep52-like sequence in chicken DNA (Fig. 3a). We were unable to detect hybridization signals with the Cep80 probe, probably because of a lower sequence similarity or the presence of introns in the chicken gene. Densitometric analysis of PCR hybridization confirmed that Ubl (1.3 kb band) contains four ubiquitin coding units and UblII (1.9 kb) contains three. The intensity of the PCR weaker bands (1.0 and 0.6 kb) is less than that expected for a single ubiquitin coding unit, suggesting that this sequence would be cut by *Pst*I or *Hind*III enzymes. The presence of *Pst*I or *Hind*III sites in the ubiquitin coding region is not compatible with the conserved ubiquitin amino acid sequence, but these sites could be present in intronic regions which have been described in the middle of ubiquitin coding sequences in human and yeast fusion genes [9,34].

Seven among eight individual DNAs analysed showed the described PCR hybridization pattern, whereas the eighth individual showed an additional major band of approx. 1.5 kb with PCR hybridization (Fig. 3b). The relative intensity of the 1.5 kb with respect to the 1.3 kb band, which contains four ubiquitin repeats, suggests that the former has five ubiquitin coding units. This band hybridized with the same probes as the 1.3 kb one (Ubl); it was positive with probe 1 and negative with the others. In addition, the approx. 200 bp length increase of this fragment with respect to the Ubl 1.3 kb band is consistent with its identification as a Ubl-like fragment with five contiguous ubiquitin coding units. PAGE demonstrated the presence of other polymorphisms undetectable in agarose gels; hybridization with probe 2 of a *Pst*I+*Hind*III-restricted DNA demonstrate the presence of a heterozygotic individual when analysed on polyacrylamide gels, but not on agarose gels (Fig. 3c). Poly-

Fig. 3. Southern-blot analysis of chicken ubiquitin genes

Chicken mature testis DNAs (10 µg) from eight different individuals were digested with *Pst*I+*Hind*III, fractionated on a 2%-agarose gel and transferred to a nylon membrane (Hybond-N). Filters were sequentially hybridized with the indicated probes, and seven individuals gave the pattern indicated in (a), whereas only one individual gave the pattern indicated in (b). (c) Shows the hybridization pattern of probe 2 with the digested DNAs electrophoresed on a polyacrylamide gel. Lanes from (c) are: size standards and liver and testis of four different individuals from left to right.

morphisms in the coding unit number ([34,35] and M. J. Schlesinger, personal communication) and restriction-fragment-length polymorphism [36] have already been described in polyubiquitin genes.

UbI and UbII chicken polyubiquitin genes have CpG-rich and unmethylated islands in their 5' 'proximal' non-coding regions

UbI C+G content in the UbI published sequence is 53.7%. The observed GpC dinucleotide frequency (7.0%) is close to the calculated one from the C and G percentages (6.99%) and is randomly distributed across the sequence. CpG dinucleotide frequency is lower (3.33%) than the calculated one, as can be expected from the known CpG suppression in vertebrates (CpG/GpC normal values in vertebrate DNA are about 0.2–0.25). CpG density, however, increases in the 5' non-coding region, and the highest CpG/GpC level coincides with the probe 2 region (Fig. 4 and Table 2). The extent of CpG deficiency in the PCR region (CpG/GpC ratio is 0.24) is inversely correlated with an excess of TpG and CpA dinucleotides, as can be expected from the increased mutation rate of methylated CpG [37]. There are 22 nucleotide changes between the different ubiquitin-coding units, in the UbI published sequence. Eleven of these changes are C/T and four of them have a G in the 3' position. Only two CpG sequences are conserved in all of the four repeats, and preliminary *HhaI* restriction results showed that they are hypomethylated (results not shown).

The differential methylation sensitivity of *MspI/HpaII* isoschizomers (*MspI* cuts CCGG regardless of its CpG methylation status, whereas *HpaII* is unable to cut the CCGG site when methylated) allowed us to analyse the methylation pattern of chicken polyubiquitin genes from mature and immature testis, liver, erythrocytes, reticulocytes and cells at successive stages of spermatogenesis (spermatogonia, spermatocytes, round and elon-

gated spermatides and spermatozoa). CCGG sites in the proximal upstream and downstream regions and in the structural sequences of these genes were deduced from the published UbI sequence and/or from the *MspI* restriction patterns of the probes. The 5' region of UbI corresponding to probe 2 contains several CCGG sequences, but none of the other UbI regions contains this sequence. Also CCGG sites in the UbII gene are only found in the 5' region (probe 5). The methylation pattern of 5' proximal non-coding regions of the two polyubiquitins were studied by *MspI/HpaII* redigestion of *PstI*+*HindIII*-digested DNAs. Hybridization with PCR, 5 and 6 probes demonstrated that UbII DNA, when digested with *MspI* or *HpaII*, changed mobility from 1.9 kb to approx. 1.3 kb in all tissues studied. Hybridization with probe 5 of DNA analysed on agarose (Fig. 5a) or polyacrylamide gels (results not shown) revealed an identical pattern of small bands when the DNA was redigested with *MspI* or *HpaII* enzymes in all the DNAs studied, indicating that the CCGG sites of this UbII 5' DNA fragment were always hypomethylated. The *PstI*+*HindIII* initial 1.3 kb fragment (UbI) from the PCR and probe 1 hybridization is maintained in all the redigested DNAs, as expected from the lack of CCGG sites in the published sequence. Hybridization of the same filters with probe 2 also showed an identical restriction pattern for the two enzymes, demonstrating that CCGG sites were also hypomethylated in the 5' UbI region. The hybridization patterns of probes 3 and 6 confirmed the absence of CCGG sites in the corresponding genomic regions (Fig. 5a). Cep52 hybridization of *MspI/HpaII*-redigested DNAs showed two bands of 246 and 130 bp length, the smaller one also being revealed with the PCR probe (Fig. 5b; and results not shown). These results demonstrated the presence and hypomethylation of a CCGG site in the ubiquitin coding region of the UbCep52-like gene.

In summary, the richness in CpG dinucleotide in the 5' end of

Fig. 4. Distribution of CpG and GpC dinucleotides across the UbI polyubiquitin gene

Each CpG (upper diagram) or GpC (lower diagram) dinucleotide is indicated as a vertical bar.

Table 2. C+G content and dinucleotide ratios (GC/CG, TG/GT and CA/AC) in the UbI gene

The 5' region corresponds to the complete UbI 5' region (probes 1, 2, and 3).

DNA region	Length (bp)	C+G (%)	Dinucleotide ratio		
			CG/GC	TG/GT	CA/AC
UbI	2199	54	0.47	1.75	1.56
5'	1105	56	0.77	1.09	0.94
Probe 3	335	53	0.72	1.28	1.40
Probe 2	430	64	1.0	0.90	0.82
Probe 1	340	48	0.50	1.21	0.63
PCR	904	55	0.24	5.39	2.11
Probe 4 (3')	190	34	0	1.09	2.55

Fig. 5. DNA methylation of UBI and UBII chicken polyubiquitin genes

(a) Southern-blot analysis of *MspI/HpaII + HindIII + PstI*-digested DNA. Chicken DNA was digested with *PstI + HindIII* (lanes a), with *PstI + HindIII + MspI* (lanes b) or with *PstI + HindIII* (lanes c). Portions of digest [5 μ g (lanes a) or 10 μ g (lanes b and c)] were electrophoresed on a 2%-agarose gel, transferred to a Hybond-N membrane and sequentially hybridized with the indicated probes. (b) Cep52 hybridization of mature testis, immature testis and liver DNAs. Mature testis, immature testis and liver DNA (from left to right in each panel: a, b and c) and digested with (a) *PstI + HindIII*, (b) *PstI + HindIII + MspI* and (c) *PstI + HindIII + HpaII*, electrophoresed on a 5%-C polyacrylamide gel, transferred to a Hybond-N membrane, and hybridized with the Cep52 probe. (c) Southern-blot analysis of *MspI/HpaII*-digested DNA. Chicken DNAs (8 μ g) from mature testis (lanes a), liver (lanes b) and immature testis (lanes c) were digested with *MspI* (M) or *HpaII* (H) and electrophoresed on a 0.8%-agarose gel. The gel was transferred to a Hybond-N membrane and sequentially hybridized with the probes indicated.

Fig. 6. UBI and UBII methylation maps

Maps are derived from the data shown in Fig. 3 and other data. Vertical bars indicate CCGG sites derived from the published sequence and confirmed in the present work. CCGG sites outside the sequenced area deduced from the present work are indicated by an asterisk. The asterisk in parentheses in the downstream UBI region indicates the presence of at least one more CCGG site, between the other two, deduced from *MspI* partial digestion. Half solid circles (◐) indicate variable methylated sites; the two sites in UBI are hypermethylated in mature testis and the one in UBII is about 50% methylated in all tissues studied. Boxes indicate a CpG-rich hypomethylated region present in both genes. The four small solid circles under the UBI map (●, in the HTF box) indicate the presence of four SpI-binding sequences.

the UBI gene, together with the hypomethylation of this region in all tissues tested (Fig. 5a), demonstrated the presence of an HTF island in the non-translated 5' region of the gene. The core of this HTF island is approx. 500 bp long and is located between nucleotides -75 and +430. Although the UBII sequence has not been published, digestions of probe 5 revealed the presence of many *MspI* and *HhaI* restriction sites (results not shown), indicating a high frequency of CpG. That, and the hypomethylation of the non-translated 5' end of UBII found in all DNAs studied (Fig. 5a), demonstrated that UBII also has a 5' HTF island.

Distal methylation sites in UBI and UBII chicken polyubiquitin genes

Single *MspI*/*HpaII* digestion of DNA and its sequential hybridization with the different specific probes allowed us to analyse the methylation state in a more extended genomic region (Fig. 5c). Complete *MspI* digestion of DNA showed, after PCR hybridization, two major bands of 2.7 and 1.8 kb, which were also found with specific UBI and UBII probes, the 2.7 kb band with probe 1, and the 1.8 kb band with probes 5 and 6. Complete *HpaII* versus *MspI* digestion showed, after PCR hybridization, two additional bands of 3.5 and 2.1 kb. The 2.1 kb band also appeared with probes 5 and 6 (UBII). The amounts of the 1.8 and 2.1 kb bands (specific UBII bands) were approximately equal in all DNAs studied. The 3.5 kb band was also detected with probe 1. It accounted for 70–80% of the total UBI-PCR hybridization in mature testis, but was almost undetectable in liver and immature testis. Probe 3 (the more 5' distal UBI probe) revealed a unique *MspI* band of 1.0 kb. *HpaII* digestions revealed the same unique 1.0 kb band in liver DNA, but a different and also unique band of 5.0 kb in DNA from mature testis and separated spermatogenic cells (results not shown). In immature testis both bands were found, the ratio between them being approx. 0.7.

MspI/*HpaII* single digestions, together with the demonstrated presence of a 5' constitutively hypomethylated region in both UBI and UBII genes, allowed us to locate several 'distal' CCGG sites and determine their methylation in these two genes (Fig. 6). Hybridization patterns with probes 1, 5 and 6 made us conclude that there is at least one partially methylated site downstream of the two genes: UBII has two CCGG downstream sites (approx. 1 and 1.3 kb from PCR), the first one being around 50%

methylated in all tested tissues; UBI has a 3' distal region, located between 1.2 and 2 kb from the protein-coding region, with several CCGG sites (*MspI*-partially-digested DNA showed the presence of many methylatable sites), the first of them being 80% methylated in mature testis and nearly unmethylated in all other tissues. Probe 3 hybridization revealed the presence of at least one site in the UBI distal upstream region (500 bp from the 'Pelham box') that is specifically methylated in germ-line cells (40% methylated in immature testis and totally methylated in mature testis).

DISCUSSION

Two chicken polyubiquitin genes, UBI and UBII, have been described, but only UBI was found expressed, and it was proposed that UBII gene might encode a tissue- or developmentally-specific transcript [12]. The major objectives of the present project were to analyse the expression and methylation of these two chicken polyubiquitin genes during spermatogenesis.

Northern and Southern blots hybridized with the PCR probe showed a very complex pattern of ubiquitin bands that cannot be completely accounted for by the two polyubiquitins. Hybridization analysis with specific non-ubiquitin probes of two chicken polyubiquitin (UBI and UBII) and two human ubiquitin-fusion (UbCep52 and UbCep80) genes, demonstrated the presence of these four genes in the chicken genome and also their differential expression in the chicken tissues analysed. In addition, hybridization of Southern blots with 5' and 3' non-coding probes allowed us to determine the methylation pattern of each polyubiquitin gene.

Consistent with the previously reported high levels of ubiquitin during chicken spermatogenesis [5], our Northern-blot results demonstrated an increased level of ubiquitin transcripts during this differentiation process.

All eukaryotic organisms studied contain one or more constitutively expressed polyubiquitin genes that can be stimulated under specific conditions. We showed a widespread distribution of UBI transcripts in all the tissues analysed and a specific increase of UBII transcripts in mature testis. These data, together with those of Bond & Schlesinger [12], suggest that the two chicken polyubiquitin genes are stimulated under different condi-

tions: Ubl by heat-shock and chemical stress, and UbII during spermatogenesis. Other genes, including 'housekeeping' genes, that express a particular form during spermatogenesis have been described [38,39].

Accumulation of unspliced Ubl transcripts in stressed chicken-embryo fibroblasts has been reported [12] and, in general, heat-shock and other stresses have been shown to inhibit RNA splicing in several systems [40]. The presence of unspliced Ubl RNA in immature testis of unstressed animals could be due to a cell programmed preparation to the dramatic changes that these cells undergo in their differentiation process. Other authors have suggested a role for heat-shock proteins in the development of the mammalian male germ-cell lineage, because they found members of the hsp70-gene family highly expressed in isolated mouse spermatogenic cells [41].

We have demonstrated the presence of an HTF island in the proximal upstream non-coding region of the Ubl and UbII genes. Methylation studies have not previously been carried out on the ubiquitin genes, but a 5' HTF island has been found in a human X-chromosome-located housekeeping gene which has a ubiquitin-related sequence and, perhaps, function [42]. The constitutive expression of chicken Ubl agrees with the presence of an HTF island in its 5' non-coding region; UbII also contains an HTF island in its 5' region, which indicates that its specific stimulation during the testis maturation process does not correlate with a change in the methylation pattern of its proximal promoter region.

Despite the presence of HTF islands in the 5' proximal non-coding regions of both polyubiquitin genes, distal methylation sites have also been found. The spermatogenic specific hypermethylation of 5' and 3' distal Ubl sites parallels the general hypermethylation of non-HTF gene sequences in sperm DNA [43]. In contrast, we found the 3' distal UbII region equally methylated in spermatogenic and somatic cells. The lack of more upstream UbII probes prevented us from detecting any putative 5' distal methylation. This may be the general reason why 5' distal methylation sites have not been usually found in HTF-containing genes (most of the analyses have been done using PCR, 3' or proximal 5' non-coding probe hybridization).

Although the presence of an HTF island is associated with a potentially active gene structure, some HTF-containing genes are differentially stimulated [44], indicating that other additional factors can modulate their expression. The inability to detect UbII transcripts in liver and embryo fibroblasts may be due to the presence of bound repressors as has been postulated for other HTF-containing tissue-specific genes [17]. Specific stimulation of UbII in spermatogenic cells could be due to the presence of other 5' and/or 3' relatively distal regulatory sequences, as is the case of the 'Pelham box' in the 5' non-coding Ubl region.

Finally, partially methylated sites found in haploid-cell populations (round and elongated spermatids), suggest the presence of different methylation patterns between individual cells which may represent differentially imprinted genetic information in each individual gamete. DNA methylation has been postulated as a putative mechanism responsible for the differential 'genomic imprinting' [45].

In summary, the presence of the heat-shock promoter element and the four specific Sp1 binding sequences around the Ubl transcriptional origin, together with its constitutive expression and the transcription and methylation results presented, are consistent with the classification of Ubl as a 'housekeeping' and 'stress-inducible' gene. Although UbII also has an HTF island, its specific stimulation in spermatogenic cells could be related to a special genomic context of this gene. It may be linked to other genes specifically stimulated in spermatogenesis, probably because of a related chromosomal situation and/or a possession of

'*cis-acting*' germ-line-specific sequences. Although the possibility still remains that the observed transcript levels are the result not only of the transcriptional activity but also the differential stabilization of the transcripts, sequence analysis of the 5' region of UbII may allow us to clarify the control of UbII expression during spermatogenesis.

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