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Characterization of a chicken polyubiquitin gene preferentially expressed during spermatogenesis

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We have previously reported that a chicken polyubiquitin gene (Ub II) not expressed under normal or heat shock conditions in chick fibroblasts is transcribed during spermatogenesis [(1987) Nucleic Acids Res. 15, 9604]. The level of Ub II mRNA is several-fold higher in testis cells than in somatic tissues. The gene Ub II possesses characteristic features not seen in the polyubiquitin gene expressed in heat shock conditions (Ub I). The 5' noncoding region of Ub II shows the consensus cAMP regulatory element (CRE) followed immediately downstream by a CA dinucleotide. It has been proposed that this extended CRE may be involved in the coordinate expression of various genes during spermatogenesis.

Ubiquitin; Polyubiquitin gene; cAMP regulatory element; Spermatogenesis

1. INTRODUCTION

Ubiquitin is a small, highly conserved protein found both free and covalently attached to various nuclear, cytoplasmic, and cell membrane proteins [1]. Ubiquitin is also conjugated with viral structural proteins [2]. A major function of ubiquitin is to mark certain proteins for proteolytic elimination [3]. In addition ubiquitin probably modulates the function of other proteins without triggering proteolysis [1].

The advanced genetic methods available for yeast have shown that conjugation of ubiquitin is essential for cell cycle control, DNA repair, resistance to stress and sporulation [4,5]. Sporulation is accompanied by a compact packing of the chromosomal DNA and may require drastic changes in protein composition of chromatin. These changes are probably analogous to those occurring during spermiogenesis in higher eukaryotes. We have proposed that ubiquitination of chromosomal proteins may be involved in the process of chromatin remodelling during spermiogenesis [6].

Polyubiquitin genes are expressed constitutively in certain systems [7,8] and in a developmentally specific manner in others [9,10]. Bond and Schlesinger [11] observed no expression of the polyubiquitin gene Ub II in normal or heat-shocked chick fibroblasts and they postulated that the expression of this gene could be developmentally regulated in certain tissues. In accordance with their proposal, we showed that the Ub II gene was expressed during chicken spermiogenesis [12]. The same observation has been recently reported [13]. The goals of the present study were to know whether or not the expression of the chicken polyubiquitin gene Ub II was testis-specific and how the putative specificity or preferential expression of the gene during spermatogenesis was dependent on special characteristics of its promoter region.

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2. MATERIALS AND METHODS

2.1. *Separation of chicken testis cells by centrifugal elutriation*

Hubbard White Mountain roosters (6–12 months old) were used in all the experiments. Chicken testis cells were prepared and separated by centrifugal elutriation as previously described [14].

2.1. *Isolation of DNA and RNA*

RNA was isolated by the guanidine isothiocyanate method of Chirgwin et al. [15]. The RNA was pelleted in a CsCl gradient step. The DNA was also obtained from the gradient and further purified by standard methods [16].

2.2. *Hybridization analysis*

Two subclones, one containing the *Pst-Bgl*II 5'-region and the other the *Xmn*I-*Sac*I 3' region of the Ub II gene were constructed to prepare specific probes. Additional probes were obtained from the coding region. The probes were labeled using the multiprime system from Amersham and [α-^{32}P]dCTP. Agarose gels (1%) were used for Southern blots. Total RNA was electrophoresed in 1.2% agarose/2 M formaldehyde gels. DNA and RNA were transferred to nylon filters (Hybond N from Amersham). Blots were baked, prehybridized and hybridized as recommended by manufacturers (Amersham and NEN) using stringent conditions. With 5' probes, of high GC content, washing temperatures of 85°C were necessary to obtain specific signals.

2.3. *Cloning of polyubiquitin gene Ub II*

A library constructed by partial *Sau*3AI digestion of chicken genomic DNA and ligation into *Bam*HI-digested phage EMBL3, provided by Dr Rafael Oliva, was screened by plaque hybridization essentially as described [17] using as a probe ubiquitin coding sequences

from a cDNA clone. *E. coli* NM 539 was used as the host. Hybridizing clones were isolated by two further rounds of plaque purification. The phage was purified on CsCl gradients and the DNA prepared by the formamide method [18].

A *Sall-Sac*I fragment of 1.5 kb containing the whole Ub II gene was subcloned in Bluescript KS. Additional subclones were constructed in Bluescript to obtain specific probes and for sequencing.

2.4. *DNA sequencing*

Sequencing was carried out by the dideoxy chain termination method [19] using T7 polymerase from Pharmacia and the conditions recommended by this company.

2.5. *Analysis of methylation*

Methylation was studied using the isoschizomers *Hpa*II and *Msp*I. 10 μg of each DNA sample were digested with 60 units of *Msp*I or *Hpa*II and incubated at 37°C overnight. Twenty more units of enzyme were then added and incubation was extended for two additional hours to ensure complete digestions. *Hin*dIII digestion was performed after *Msp*I incubation with 70 units for 4 h. Samples were electrophoresed at 30 V for 16 h and blotted as described.

3. RESULTS

3.1. *Sequence of the chicken polyubiquitin gene Ub II*

The polyubiquitin gene Ub II encodes a translation product consisting in a polyprotein composed of three identical repeats of ubiquitin. The last of the three ubiquitin repeats is followed by a single Asn residue (Fig. 1).

A cAMP responsive element (CRE) that shows a perfect match with the 8-bp palindromic mammalian consensus sequence 5′-TGACGTCA [20] is present at position −87 of the ATG start codon (doubly underlined in Fig. 1). The dinucleotide CA is present immediately downstream to the CRE sequence. This feature has been observed in other genes expressed during spermatogenesis [21]. In addition to the CRE, the polyubiquitin gene Ub II contains several sequences in the 5′-flanking region of the gene related to the AP-2 sequence (CGCGGGC, CCGGCGGC, CCCCGCCC, TCCCTTCC at positions −268, −250, −122, and −16 respectively) [20]. Six GGGCGG hexanucleotides (CG boxes), the recognition sequence for the transcription factor Spl [22], are also present in the 5′-noncoding region of the gene (underlined in Fig. 1). The whole 5′ region of the gene is very CG-rich (74%).

Two typical polyadenylation signals AATAAA are found in the 3′ noncoding region of Ub II (underlined in Fig. 1). In a polyubiquitin Ub II cDNA clone prepared from poly(A)$^+$ mRNA isolated from chicken spermatids the first polyadenylation signal is used [12]. The poly (A)$^+$ tail starts at position +757. Downstream the second polyadenylation signal is found a motif, ATTTA, which may contribute to mRNA instability [23].

3.2. *Expression of the polyubiquitin Ub II gene in different chicken tissues and at successive stages of spermatogenesis*

The polyubiquitin Ub II mRNA is present in all chicken tissues examined: testis, brain, kidney, heart,

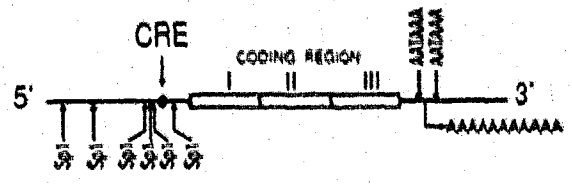

Fig. 1. Nucleotide sequence of chicken polyubiquitin gene Ub II and predicted amino acid sequence of the encoded protein. Numbers on the right of the sequence indicate nucleotide position beginning with the first nucleotide of the coding region. Doubly underlined nucleotides in the 5′-region match the consensus sequence for the cAMP responsive element (CRE). Underlined nucleotides in the 5′-region indicate the Spl binding sequences (CG boxes). Underlined nucleotides in the 3′-region indicate polyadenylation signals and a potential mRNA destabilizing sequence.

liver, lymphocytes and ovary. The level of Ub II mRNA in testis is 5-fold higher than in brain, 7-fold higher than in kidney and more than 10-fold higher than in heart or liver (Fig. 2). Most of the polyubiquitin Ub II mRNA expressed during chicken spermatogenesis is present in spermatids (50% in round spermatids and 21% in elongated spermatids). A mixed fraction of meiotic and premeiotic cells contains the remaining 29% of the Ub II mRNA (Fig. 3).

3.3. *DNA methylation at the polyubiquitin Ub II gene*

The relative proportion of DNA methylation at CCGG sequences around the polyubiquitin Ub II gene has been determined by Southern blot analysis using isoschizomeric restriction enzymes *Hpa*II and *Msp*I (Fig. 4). Digestion with *Msp*I yielded a single band of 1.9 kb. *Hpa*II digestion of DNA obtained from various chicken cells revealed different proportions of one additional band of 2.1 kb. Double digestion with *Msp*I and *Hin*dIII resulted in a fragment of 1.4 kb, indicating that the methylated site is at the 3′-region of the gene. The

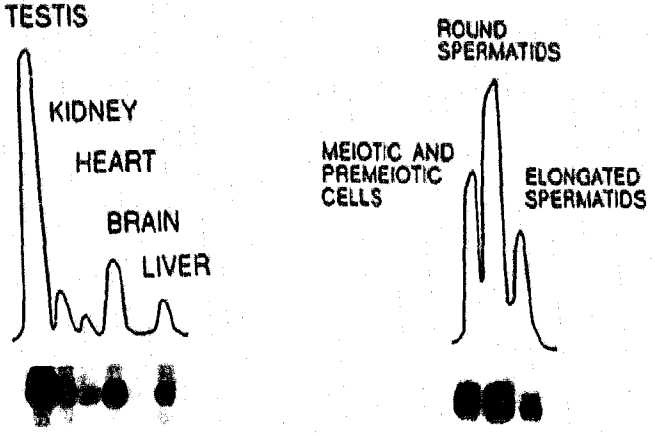

Fig. 2. Polyubiquitin Ub II mRNA levels in different chicken tissues. Total RNA was isolated and 20 μg were applied to each lane. Densitometric measurements on appropriately exposed autoradiograms (within the linear range of film density) were performed with a Hoefer Scanning densitometer.

Fig. 3. Polyubiquitin Ub II mRNA levels at successive stages of spermatogenesis. Total RNA was isolated from each fraction of chicken testis cells and 20 μg were applied to each lane. Densitometric measurements were performed as indicated in legend to Fig. 2.

5'-region remains free of detectable methylation in all tissues explored.

4. DISCUSSION

It has been previously reported that the chicken polyubiquitin gene Ub II is not expressed in certain cells or tissues [11,13]. We have detected expression of this gene in all the cells and chicken tissues explored: testis, brain, kidney, liver, heart, ovary and lymphocytes. Constitutive expression of polyubiquitin genes has been observed in different systems [7,8]. The presence of a methylation-free island and Sp1 CG boxes, such as those present in the 5'-flanking region of the chicken polyubiquitin Ub II gene is often associated with housekeeping genes [26].

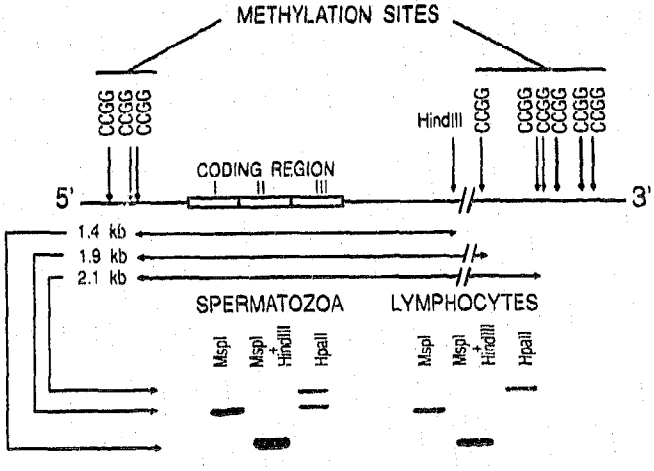

Fig. 4. Location of methylation CCGG sites around the polyubiquitin Ub II gene. The CCGG sites were determined by sequencing the 5'- and 3'-regions of the gene. Digestion with *Msp*I, *Hpa*II and *Msp*I + *Hin*dIII showed that while the 5'-region remains free of detectable methylation in all chicken tissues explored, the first methylation CCGG site downstream the coding region is differently methylated in different chicken cells and tissues studied (testis, ovary, erythrocytes, lymphocytes and spermatozoa). The figure shows the methylation pattern of spermatozoa and lymphocytes.

Although the Ub II mRNA is present in different chicken tissues, the levels of Ub II mRNA are several-fold higher in testis than in other chicken tissues. The presence of unmethylated 5'-CpG island in the Ub II gene of both testis and somatic tissues makes it unlikely that DNA methylation plays a role in the physiological transcriptional regulation of this gene. Preferential expression of the polyubiquitin gene Ub II during spermatogenesis may be related to the presence of the mammalian consensus cAMP regulatory sequence in the 5'-flanking region of the gene. The cyclic AMP responsive element supports a dual role in gene expression, as a basal transcription element and as an inducible enhancer [20]. The CRE has been identified in the 5'-flanking regions of cell type-specific genes subjected to hormone regulation. In testis, cAMP mediates the cellular response to the pituitary gonadotropins [24]. The cyclic AMP responsive element is present in several genes expressed during spermatogenesis such as the protamine genes and the transition protein genes [21]. A common characteristic of the cAMP responsive elements of these genes and the CRE of the Ub II gene is the existence of a CA dinucleotide immediately following the CRE sequence [21]. It has been proposed that the CRE consensus core extended by the dinucleotide CA might confer the necessary specificity for the coordinated expression of genes during spermatogenesis [21]. In addition to CRE other sequences related to the AP-2 *cis*-acting element have been identified in the 5'-region of the Ub II gene. The AP-2 sequence is involved in the activation of transcription by cAMP and phorbol esters [20]. Both cAMP regulatory elements, CRE and AP-2, have been reported in the proenkephalin gene, a gene expressed during spermatogenesis [25].

The 3' region of the Ub II gene contains two polyadenylation signals. The start of the poly(A)$^+$ tail, determined by sequencing of a Ub II cDNA clone isolated from chicken spermatids, shows that the first polyadenylation signal is used during spermatogenesis. We do not know if the second polyadenylation signal is used in any chicken tissue. The second signal is followed by a potential mRNA destabilizing ATTTA sequence [23].

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