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Structural analysis of the chicken hydroxyindole-*O*-methyltransferase gene

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Hydroxyindole-*O*-methyltransferase (HIOMT) catalyzes the final step of melatonin synthesis, a neurohormone involved in photoperiodism and produced specifically in the pineal gland and in the retina. In the chicken, HIOMT gene transcription appears to be controlled by a circadian oscillator located in the pineal gland. We have characterized the chicken HIOMT gene over 17 kb, including 2.9 kb of 5'-flanking sequence. The major transcript (1.6 kb) is composed of eight exons distributed over 7.5 kb of genomic DNA. A ninth alternative exon was identified 6 kb downstream of exon 8. It was found in minor transcripts in the pineal gland and in the retina. Sequence similarity between exons 8 and 9 suggests their origin by exon duplication. Due to early stop codons, inclusion of exon 9 truncates the open reading frame by up to 33%. A restriction fragment length polymorphism was detected for a *Bgl*III site in intron 8. Fluorescence hybridization localized the HIOMT gene on chicken chromosome 1q22. The 5'-flanking region contains GATTAA and TAATCC sequences that may be related to tissue-specific expression. An ATTTAAAT sequence at position -29 would play the role of a TATA box, as evidenced by electrophoretic mobility shift assay. Information obtained in this study opens the way to further studies aimed at analyzing the circadian rhythm of transcription at promoter level.

Keywords: chicken; pineal; retina; melatonin; HIOMT gene.

Many vertebrate species respond to seasonal changes in daylength (photoperiod) with altered physiology and behavior. A circadian rhythm of melatonin production plays a major role in conveying this photoperiodic information to the organism [1, 2]. The major source of circulating melatonin is the pineal gland. However, melatonin is also produced in the retina, where it acts as a local modulator [3, 4]. The metabolic pathway to melatonin is tryptophan → 5-hydroxytryptophan → serotonin → N-acetylserotonin → melatonin. The last-acting enzyme in this pathway is hydroxyindole-*O*-methyltransferase (HIOMT) [5]. Several aspects of HIOMT expression motivate a structural analysis of the gene encoding this enzyme. First, its tissue distribution. Consistent with the limited localization of melatonin synthesis, HIOMT is found to be expressed predominantly in retinal photoreceptors and in photoreceptor-derived pineal cells [6–9]. Highest levels of HIOMT expression are regularly found in the pineal gland, whereas retinal expression of the enzyme varies greatly among species [10]. In the chicken retina, HIOMT immunoreactivity is unequally distributed among the different photoreceptor subtypes [9]. In human, very low levels of HIOMT expression have been observed in the retina [11]; while higher HIOMT levels in Y79 retinoblastoma have suggested this retinal cell line may have acquired a more pinealocyte-like phenotype [12]. Elucidating the molecular mechanisms that control tissue-specific ex-

pression of the HIOMT gene would provide an insight into the melatoninergic differentiation of photoreceptors. Another interesting aspect of HIOMT gene expression is its regulation by a circadian oscillator. In chicken and other non-mammalian vertebrates, the day/night rhythm of melatonin production is driven by a circadian oscillator located in the pineal gland itself [13, 14]. The molecular basis of this oscillator remains undefined. Early studies using inhibitors of transcription had suggested the day/night rhythm of melatonin synthesis might be generated at the transcriptional level [15]. More direct evidence of the circadian transcription of melatonin-related genes was recently obtained in different laboratories. Our contribution to this line of research was to demonstrate the existence of a day/night rhythm of HIOMT mRNA levels in the chicken pineal gland and retina. In the retina, peak levels of HIOMT mRNA can be detected at the end of the night. This rhythm does not show a photorefractory period and, therefore, may reflect a passive response to the light/dark cycle [9]. In the pineal gland, HIOMT mRNA levels are threefold higher at midday than at midnight [16]. Unlike in the retina, the pineal HIOMT mRNA rhythm appears to depend on an endogenous mechanism; it persists in organ culture and displays a circadian component in constant darkness [17]. Circadian changes in mRNA levels have also been reported, in the chicken pineal gland, for two other enzymes of the melatonin pathway: tryptophan hydroxylase [18] and arylalkylamine-*N*-acetyltransferase [19]. All three genes represent good candidates for studying the mechanisms of transcriptional regulation exerted by the circadian oscillator of the chicken pineal gland. Sequence information on the 5'-flanking regions of chicken tryptophan hydroxylase, arylalkylamine-*N*-acetyltransferase and HIOMT genes is therefore needed to initiate studies aimed at identifying *cis*-regulatory regions involved in circadian control

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Abbreviations. HIOMT, hydroxyindole-*O*-methyltransferase; TBP, TATA-binding protein; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; wt, wild type; mut, mutated.

Enzymes. HIOMT (EC 2.1.1.4); tryptophan hydroxylase (EC 1.14.16.4); arylalkylamine-*N*-acetyltransferase (EC 2.3.1.87).

Table 1. Oligonucleotides used in this study and their location in the chicken HIOMT gene. The arrows indicate their 5'→ or 3'← orientation. S = sense; AS = antisense. This list does not include all oligonucleotides used for sequencing.

Oligo-nucleotide	Sequence 5'→3'	Location
S47	CCTCAAATCATCTTTCAATAC	→ exon 1
S104	GCCTGTGAGCTGGGCGTGTTTGA	→ exon 2
AS128	GATCAAACACTCCCTAACTCAC	← exon 2
S309	AGTCAAGTCCCAAGTCTCA	→ exon 2
AS376	GTGCCAGCACAAAGTAGACGG	← exon 3
S409	TCAATATGAAAGAGCTTTTGG	→ exon 4
AS457	GGCTCCAAAGAGGTCTTTA	← exon 4
AS540	GTAAGTACATCTCTGCCACA	← exon 5
S542	GCATTTGACCTTTCCCTT	→ exon 5
S677	AAAGAGCGATTGGTCCCCCG	→ exon 6
AS685	TCGCTCTTTGGCCACTTGTAC	← exon 6
S743	TCAATCCCTGAAGCTGACCT	→ exon 7
AS797	CATCCAGTCATGCAGTATCT	← exon 7
S849	GTGGTGGAGTGTCTGGT	→ exon 8
AS944	CTCGGTCTGCACCAAGCATGTTTCA	← exon 8
S1248	GGCTTGCCCTTTGGATTGAATAGCAG	→ exon 8
AS1333	GCAACTCTGCACGTTTCTT	← exon 8
EA733	TTTGCTGGCATTGTGTACCTCGCTG	← 5' of exon 9
EA1541	CCACAAGAAAGGAAACAGCAG	← exon 9

of transcription. In the present work, we report the cloning of the chicken HIOMT gene and the sequence analysis of its putative promoter region. Structural analysis demonstrates the existence of splice variants and allelic polymorphism, while *in situ* hybridization assigns the HIOMT gene to chicken chromosome 1q22.

EXPERIMENTAL PROCEDURES

Screening of chicken genomic library. A λEMBL-3 chicken genomic library (Clontech) was plated on Luria-Bertani/agarose plates, at a density of 30 000 phage particles/plate (total 700 000 phage particles). Nitrocellulose replicas of the plates

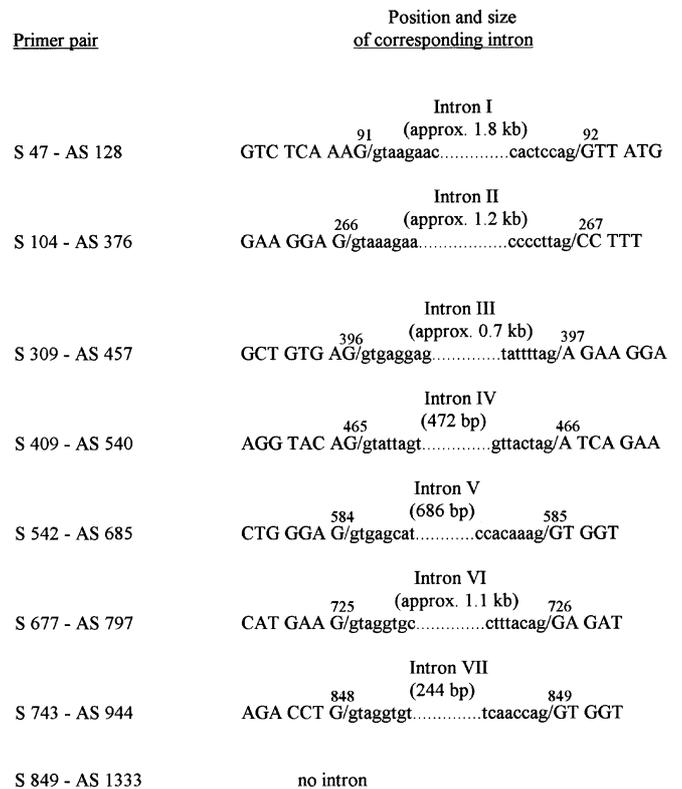


Fig. 2. Intron-exon junction sequences of the chicken HIOMT gene. The positions and sizes of the introns were determined by PCR amplification of cloned and genomic DNA. The primer pairs used for amplification are shown. S = sense, AS = antisense, numbers = position of the 5' extremity of the oligonucleotide in the cDNA, starting from the cap site (i.e. add 17 bp to position in the published cDNA sequence, EMBL X62309). Intron-containing PCR products were sequenced with the primers used for amplification. The sequences of the intron-exon junctions are shown: upper-case letters = exon, lower-case letters = intron, numbers = positions in the cDNA (as above).

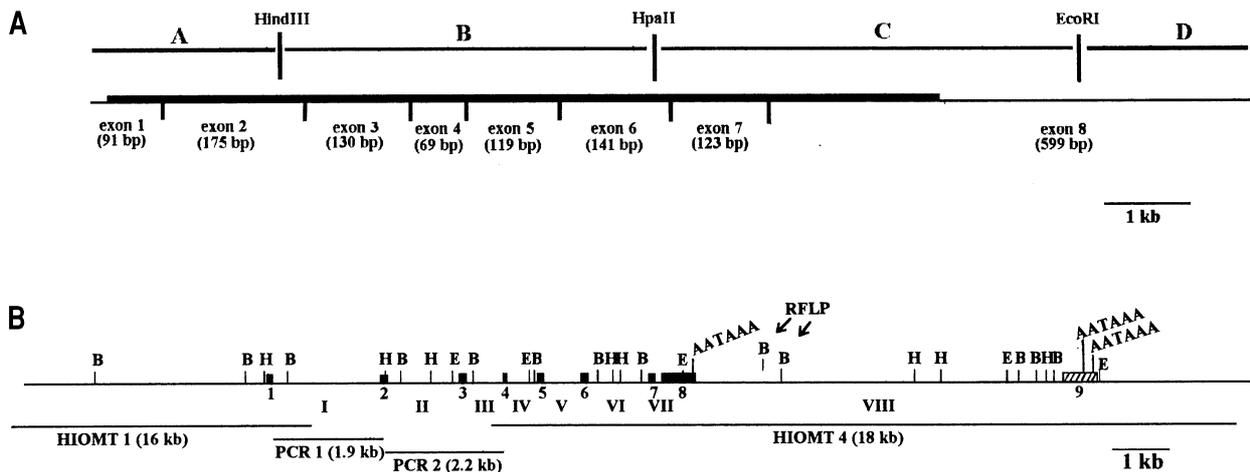


Fig. 1. Map of the chicken HIOMT gene. (A) Outline of the chicken HIOMT cDNA showing the exon composition, and the short probes A, B, C and D used in hybridization experiments. The coding region is shown as a filled bar. (B) Organization of the chicken HIOMT gene deduced from the analysis of clones HIOMT1 and HIOMT4, and of the PCR products 1 and 2, obtained by amplification of chicken genomic DNA. The map shows the positions and relative sizes of the eight exons composing the major HIOMT transcript (filled boxes), as well as the alternative exon 9 (hatched box) which has sequence identity with exon 8 (see also Fig. 3). The restriction sites for *Bgl*II (B), *Eco*RI (E) and *Hind*III (H) are indicated. The position of an RFLP for *Bgl*II in intron VIII is shown, the site retained is that found in clone HIOMT4. The positions and sizes of the introns were determined by PCR amplification of genomic and cloned DNA, followed by partial sequencing (see also Fig. 2). The restriction fragments were arranged in 5' to 3' order, by hybridization with the short probes described in (A).

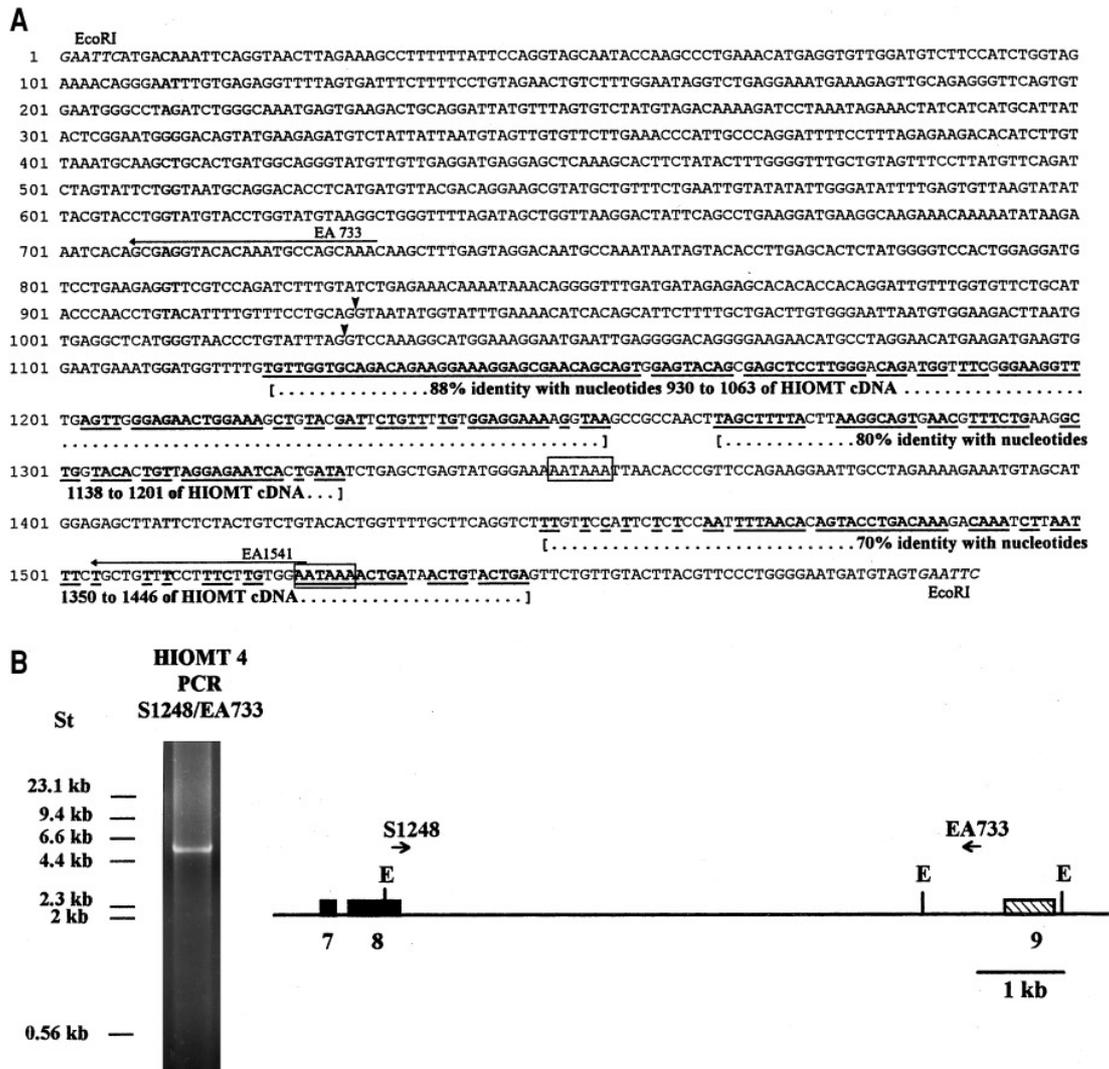


Fig. 3. Characterization and localization of the repeated sequence containing exon 9, in the chicken HIOMT gene. (A) The 1.6-kb *EcoRI* fragment was subcloned in M13mp19 and sequenced. Regions of identity with exon 8 are indicated by underlined bold letters. Two polyadenylation signals are framed. The positions of two antisense primers are shown (EA733 and EA1541). Primer EA733 was used in PCR experiments, to localize the 1.6-kb *EcoRI* fragment in the chicken HIOMT gene (see B). Primer EA1541 was used in RT-PCR, to show that the repeated sequence is expressed as an alternative exon 9 (see Fig. 4). The positions of the acceptor splice sites of exon 9 are labelled with an arrowhead. (B) PCR amplification (30 cycles) of clone HIOMT4 (20 ng) was performed with primers S1248 (in exon 8) and EA733 (see above). A 6-kb PCR product can be observed by ethidium bromide fluorescence (left panel). Its position at the 3' extremity of the HIOMT gene is outlined (right panel): exons 7 and 8 are shown as filled bars, the repeated sequence (exon 9) is shown as a hatched bar and the PCR primers as arrows. *EcoRI* restriction sites (E) are indicated.

were hybridized with a ^{32}P -labelled full-length HIOMT cDNA probe [21] under high stringency conditions: hybridization step at 42°C in 40% formamide, 10% dextran sulfate, 4×NaCl/Cit (10×NaCl/Cit, 1.5 M NaCl, 0.15 M sodium citrate, pH 7), 1×Denhardt [poly(vinylpyrrolidone), bovine serum albumin fraction V, Ficoll 400; 0.02% each], 20 mM Tris/HCl, pH 7.4, and 0.3 mg/ml salmon sperm DNA; washing step at 52°C in 0.1×NaCl/Cit, 0.1% SDS for 2 hours [20]. After three rounds of screening, the DNA from HIOMT-positive clones was purified [22] and analyzed on dot-blots by hybridization with the short HIOMT cDNA probes described (Fig. 1A).

Restriction mapping of the chicken HIOMT gene. Chicken genomic DNA prepared according to [20] and DNA from HIOMT-positive clones was digested with *EcoRI*, *BglII* and *HindIII*. The restriction fragments were separated on 0.8% agarose gels, transferred onto nitrocellulose membranes and hybridized with the short HIOMT cDNA probes (Fig. 1A).

PCR in exon/intron mapping. Chicken genomic DNA (0.5 µg) and DNA from HIOMT-positive clones (50 ng) was used as template for PCR amplification of introns I–VII. Primers were designed to encompass sequences of the cDNA that might have resulted from a splicing event (A/C AGG or AGG T/G; Breathnach and Chambon, 1981). Reactions were performed in 50 µl containing 50 pmol each primer, 10 mM Tris/HCl, pH 9, 50 mM KCl, 1.5 mM MgSO₄, 0.1% Triton X-100, 0.2 mg/ml bovine serum albumin, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase (Appligene). The temperature cycle was: 95°C for 1 min (95°C for 1 min, 5°C below primers' t_m for 45 s, 70°C for 1–2 min) 30 cycles, 70°C for 5 min. Intron VIII (6 kb) was amplified from cloned DNA (50 ng) using the *Taq* Extender™ system (Stratagene), according to the manufacturer's instructions. The reaction products were analyzed on 0.8% agarose gels. The HIOMT-specific primers (Eurogentec) are listed in Table 1.

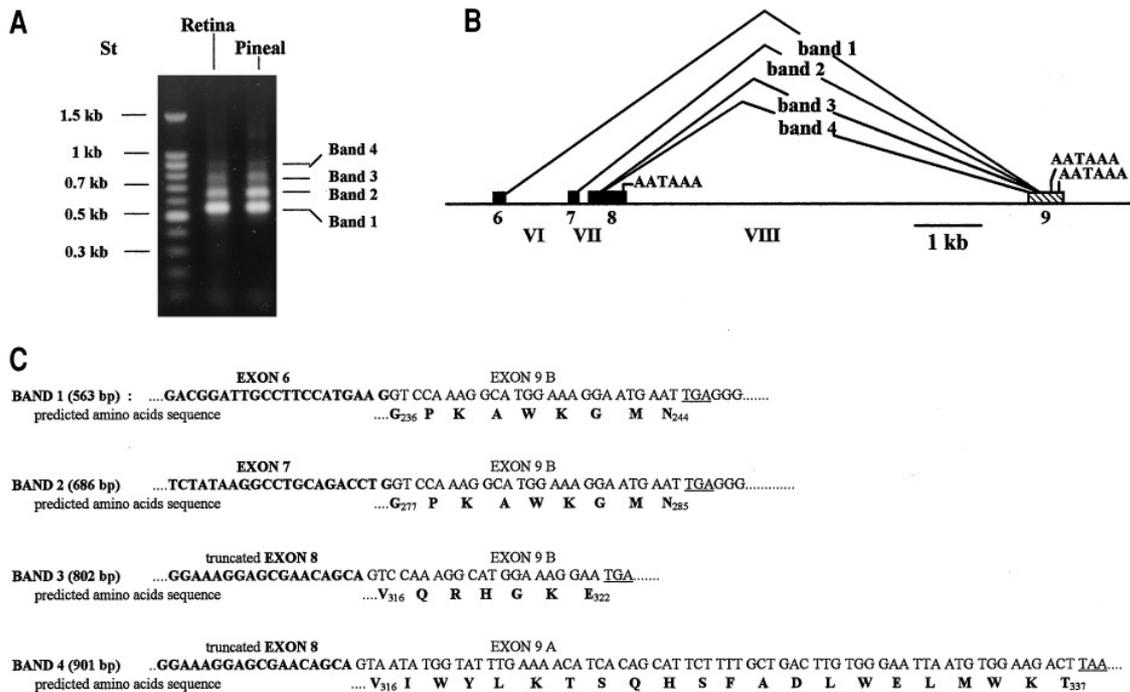


Fig. 4. Detection and structure of four HIOMT splice variants containing exon 9. (A) Chicken pineal and retinal cDNA immobilized on Dynabeads™ (1 μ l suspension), was amplified by PCR (35 cycles), with primers S677 (exon 6) and EA1541 (exon 9). The reaction products (20% of the reaction) were analyzed on a 1.5% agarose gel and visualized by ethidium bromide fluorescence. (B) Outline of the alternative splicing giving rise to bands 1–4. The four PCR products obtained from retinal cDNA were gel purified and sequenced (see C for details). Exons composing the major transcript are shown as filled bars, exon 9 is shown as a hatched bar with its polyadenylation signals. (C) The sequences of bands 1–4 at the junction of exon 9 are shown. The portions of sequence that are common with the major transcript are in bold letters. The in-frame stop codons are underlined. The predicted amino acid sequences of the variant carboxy terminals are shown in one-letter code, with numbers indicating the positions of the amino acids.

5'-rapid amplification of cDNA ends. The 5'-RACE was performed using a modified method based on the 5'-AmplifINDER RACE kit from Clontech. First, chicken pineal and retinal cDNA was synthesized on Dynabeads™ oligo (dT)₂₅ (Dynal), according to a previously described procedure [11]. Briefly, total RNA (50 μ g) extracted from chicken pineal or retina using the LiCl/urea procedure [23] was reverse transcribed on 0.5 mg Dynabeads oligo (dT)₂₅ using a mixture of Superscript™ (Life Science Technologies) and Retrotherm™ (Epicentre Technologies). The anchor primer (5'-AGCGGCCGC-CAGCCTACCGAC-3') was ligated to the 3' end of the single-stranded cDNA immobilized on Dynabeads, according to a previously described procedure [11], using T4 RNA ligase (New England Biolabs). The amplimer primer (complementary to the anchor primer) was used in combination with HIOMT-specific primer AS457 to PCR amplify the 5' end of HIOMT cDNA. The amplification was performed as described above, with 1 μ l pineal-sensitized or retina-sensitized beads and the following temperature cycle: 95°C for 1 min (95°C for 30 s, 55°C for 30 s, 72°C for 2 min), 30 cycles, 72°C for 10 min.

PCR in splice variants detection. Pineal or retinal cDNA immobilized on Dynabeads™ was used as template (1 μ l sensitized beads) for PCR amplification with a sense primer localized in exon 6 (S677) and an antisense primer localized in exon 9 (EA1541). The reaction was performed as described above, with the following temperature cycle: 95°C for 2 min (95°C for 40 s, 60°C for 40 s, 72°C for 1 min) 35 cycles, 72°C for 5 min.

Subcloning and sequencing. PCR products (including the 5'-RACE products) were T/A cloned using the pGEM-T vector system (Promega) according to the manufacturer's instructions. Restriction fragments from λ EMBL-3 HIOMT-positive clones were subcloned in M13mp19. The templates used for sequenc-

ing were either single-stranded DNA obtained after amplification of the M13 clones in *Escherichia coli*, or double-stranded DNA obtained by PCR amplification of the inserts of pGEM-T clones. Some PCR products were sequenced without pGEM-T cloning. In this case, the template was fractionated on an agarose gel, then purified by Gene-Clean™ (BIO 101). Sequencing of single-stranded or double-stranded DNA was performed using either the Sequenase 2.0 kit (USB) or the ABI Prism kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were analyzed on 6% acrylamide/8 M urea denaturing gels.

Electrophoretic mobility shift assay (EMSA). EMSA was performed following a previously described method [24]. Protein extracts were prepared from pineal glands and retinas of one-week-old chicks. The DNA probe used for EMSA was a double-stranded oligonucleotide containing the putative TATA box of the chicken HIOMT promoter ('HIOMT-TATA': 5'-GAGCTTATTTAAATTAAGAGGAG-3'). This probe was labelled with [γ -³²P]ATP using T4 polynucleotide kinase (Pharmacia). The DNA-protein binding assay was done with 1 fmol ³²P-labelled probe and 20 μ g protein, in a reaction buffer containing 16 mM Hepes, pH 7.9, 2.3 mM MgCl₂, 84 mM NaCl, 20 mM KCl, 0.16 mM EDTA, 0.8 mM dithiothreitol, 0.4 μ g/ml leupeptin, 0.4 μ g/ml pepstatin, 0.7 μ g/ml aprotinin, 17% glycerol and 0.05 μ g/ml poly[d(I-C)] · poly[d(I-C)]. Competition experiments were performed by incubating the protein extracts with 200–1000-fold molar excesses of unlabelled double-stranded oligonucleotides, before adding the ³²P-labelled probe. Three types of oligonucleotides were used for competition: 'HIOMT-TATA'; 'wt-TATA' (5'-GCAGAGCATATAAATGAGG-TAGGA-3'), the consensus binding site for the TATA-box-binding protein (TBP) and 'mut-TATA' (5'-GCAGAGCAGCTAAA-

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-2921 GGATCTATCC TTATTGCTCA TAAAGAAAGT TGCATGCATG AAAATGCTGT CAGTGTCAT TTCTCACCAG CAGGGACATT AATTCGCTT GAAATCTGCT
-2821 TTATTCATTA ATATTCTTTT TAAGTGTAC ACAAAAATGT TGCTTTGTTT TTGTCATATT TGTCTGTAGT ACAAAGATAA ATTTATCATT AGAACAGAAG
-2721 CTTGGAGATA AAGTATTAC TTTAGCCTGT GTGTGATCTA AGGCCTATGA CAAAAACCTT GATGGGGTTT TCAGAGGCGAG AAAGCCACTG CAAGTTCATC
-2621 TTCATGAATG CATTTTTAGC TTAAGAGGAG CTTAGGCTTT AAGCCCCAGT AACAAATTAC TTAGAACAG CAACACAGTG TGTGGCTCTG CATCATTCTG
-2521 ATGTGAGTTA TCCGTAACCC TCCTCCCCTG TATCTCGCTG CTGGTAGTAA TCGCATTAGA TGATTACATC TGGTGTCTCT AACAGATGGA TTTAGTCAAT
-2421 TGAAAAAGTA CTCCAGGTGG TTTAATCGAC TGTGTGGTTG TTTTATAACC AGAGCCCTCCT GTACCTACAA CGTTGCCTTA AGTGGAGTGG ATCTATGGCC
-2321 CATTACCAGG CTCTATCAGT TTTGCATGTA ACCATCTTGT TATTAACCTAC TACAGGAATT GAGATGTCTT GAGAGTTTTT TTTTCTGTA GAACTGTCTT
-2221 TAGAATCGGT CTGAGGAACT GGGGAGAATG GTTTTAGATT TGGTCAAGTG AATGAATTTT GCAGAATCAC AATTACAATA GCATTACCT AAATGATAGC
-2121 TGTGATTAGT CAGTACCCTT TGGGGTGTGG GGATAGCTGG GGAAGATGCT CATTTTTTTT CATGTGGCTG TGTACTGAA ATCTGTGTC TAGTTTTCTT
-2021 TCAGAGAGAC CACCTCTTGT TAAATGCAAG CTGGTGGATG CAGCGGTCAC TGGCAGGGAG GTATGGTTTG ATCTGCAGCA TTCACAATAC AAAACCAACA
-1921 AAATCTAACG TGACAGACA CATCAGGCAA GGCCATAGCA CTGTACTTGC AGGCTGTGTT GTCCAGGATG GTGAGCTCTA AGTGCTTGTG CAGTTGGGGT
-1821 GTGCCATAGC TTTTITATGT TTGGCTGAAG TGTTTTCTA ATGCAGTACA CCTTGTGTTG ATAGAATATG GAAACATAG ATGGTTTCTG TGTGTGCAC
-1721 AGAAGGAAAA AAGGATGACA ACCTACTTCA AGAGTTAGTT ACGTTTAGCT AGTCATATTT CACTGTTTTA TTACTGGCTA AAACAAATAC AGTATTTTCT
-1621 GGCTATAATG AAAGAACAT AATAGAAAAT ATGGATTGTC AAAAGGATTA CATAGTGGAA ACTTTGAGTT ATTAAGATTT CACTGCATCC TTGGAGGGGC
-1521 TAATAGGAAA GCATAGGAAA CTCTGAACCT GTCTATTCTT TCTAAAAATG AGGTAGGATC AAAGGAAAAA CATTGAACCT ACCTGCAGTA GCAGATAAAT
-1421 ACAACCCATA TTCTTAGGAA AAGGAGAAGA TCAAAATTTT GTGTTGGGAC AGTGCTGTGA GTGCCCATTT TTAAGATGC ACTAAGGTAA GGTACACATA
-1321 TTGTTGATCT GCATCATATT GCTCAGGTTT TGCTGTAA CCTTACTGCT GAAGCATGGA CAGAACTAC AACTGGAAAA GCATTATTTT GTAACGTGTT
-1221 GCCACTGTCA TACCACCTAA GTCATGGTCT GATTTAATCT TACTGCCTGA AGGGTCACTA AATGTGTAAT ATCAAAACTG TGATTCTTTC CTTTCTGGAA
-1121 AGGGCCTGCT TTTTACAAAA TGCTGAGGGC ATGATGATCA GCATTTACAA GCTGGTGTAA TGAACAGTGG GGCCAATGAT GAAGATGACA TCCATTACAC
-1021 ATCTGTCATT TGGAGGTCAT TTCATTGTCA TCTGCAAAAT AAAGGAAAGC ATAAAGAGTA AGTTTAGTTC CTCATAGGCT TTGCTTGAGT GACAACTGCC
-921 AGAACAAACT GATCACTGTG ACAAAGCAGG ACACCAGACA GGCTCTTGTG GCCCTCCTTG GCCTTGCTGT TTCTGTACCT CTGGATAGAG TGTGCCCTCAT
-821 GGCAGTCTGT GGACCTGTGC CCATCATAGC ACTTCTCAGC TCCCCTCCTG CCTGCTTTCG AGTAGTTATG CCAGTCTTTA ACTTGATGTC TAAGCTTGAT
-721 GCCCCAGGCA TGCTATTCTG ATCTGAACAA GTCAGTGTCT CAGTGGGATC GAGGTAGAGG CTGGTGCCTA TGCTCCTAGC TCATGATGAT CAACAGATGA
-621 GTGAAACTGG GGAGATCAGT GATGCTGGGG CTCGACCAGG AGGATGTCCA GACCACTCTG AACTGAGTGG ATGCTTCTCT ACTCATAATC GGTGAGAATG
-521 TCCAGTTTC ACATCTAAAG AAGCATGTCA CCAAGTTGTC TTCTGTCTC TGGTCCATTT CATTGGGCTT CTGCTCTAGG GGATGCTCTC CAGCATCAAG
-421 TGTGATGGGG TAGAGCTGCT TTTGAAGAAG CCTAGCATAA GACTCCAGTT TGGTTTATGA ACTGGTGCAT TATACTGAGT TCAGTCTCTC CATATTTGCT
-321 CTATAGTGAG ATCTTCAGAG CTTGTTTTTC CCTCAGATGT CTTCAACTTT CAGTTGTTTT TGAAGTACCA TCATTTAAT TGAACCGTGA ACCGATGGTA
-221 CTGCTTTAAT CTGCACTGCT TCTTAGCCTG GTGCTTGATG ATGAAAGATG CAAAGAAATA GTTGAGGCTG CCTAAAAAAA CCCTGTTGAA GGTAAATAAC
-121 TTAGCTTATG TGTGTTTAA CCAAAGTATT TCTCAGAAGC AATGATGATG GTGGATCAG CTGATGTGCT GTGCCATAGT GGAAAGAAGC TTATTTAAT
-21 TAAGAGGAGC AGAGAATCTG TGcctcaata ctaaaggaag atggactcca cagaagacct tgactatcct caaatcatct

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Fig. 5. 5'-flanking sequence of the chicken HIOMT gene. The nucleotide sequence is numbered relative to the transcription initiation site (indicated by an arrowhead). Exon 1 is shown in lower-case letters and 5'-flanking sequence in upper-case letters. The initiation codon ATG and the potential TATA box are underlined. Six potential Crx-binding sites that may confer tissue-specific expression [35–38] are boxed. Direct and inverted CCAAT boxes (positions –1049 and –460) and an AP2 element (position –721) that may confer regulation by protein kinase C and/or protein kinase A [40, 41] are double underlined.

ATGAGGTAGGA-3') an oligonucleotide containing a mutated TBP-binding site. The DNA-protein complexes were analyzed on non-denaturing gels (4.5% acrylamide in 8 mM Tris, 54 mM glycine and 0.4 mM EDTA).

EMSA with the purified TBP was performed as previously described [25]. Briefly, 20 ng purified human TBP (Promega) was incubated with 5 fmol ³²P-labelled oligonucleotide for 1 h 45 min at room temperature in a binding buffer containing 20 mM Hepes, pH 7.9, 25 mM KCl, 0.1 mM EDTA, 2 mM MgCl₂, 100 µg/ml bovine serum albumin, 0.025% Nonidet P-40, 0.1 mM dithiothreitol and 10% glycerol. DNA-protein complexes were analyzed on a non-denaturing 6% acrylamide gel in 50 mM Tris, 40 mM borate, 0.5 mM EDTA, 4 mM MgCl₂ and 0.02% Nonidet P-40.

Chromosome preparation. The primary fibroblast cell lines were isolated by trypsinization from 9-day chick embryos. Meta-phase chromosome spreads were prepared as previously described [26]. Cell cultures were synchronized with a double thymidine block during S phase in order to increase the yield of metaphase. To induce R-banded chromosome spreads, cell cultures were treated with 5-bromodeoxyuridine (10 mg/ml) during the second half of the DNA synthesis phase.

In situ hybridization. The HIOMT probe was generated by PCR amplification of clone HIOMT 4 (primers S542 and AS1333) and labeled by nick translation according to Boehringer's protocol in the presence of biotin-16-dUTP. The labelled probe was precipitated in the presence of a 500-fold excess of sonicated chicken genomic DNA to reduce background. The *in situ* hybridization was performed as described [27]. The probe was used at about 150 ng/slide. The biotinylated probe was detected first by goat anti-biotin antibody (Vector Biosis) followed by a fluorescein-labelled anti-goat IgG (Tebu Nordic). The slides were stained with propidium iodide (1 mg/ml). To reveal directly the R-banding pattern, the slides were mounted with P-phenylenediamine. The chromosomes were classified according to ICSAK recommendation [28].

RESULTS

Isolation and characterization of genomic clones. Four clones were isolated after screening the chicken EMBL-3 genomic library with the full-length HIOMT cDNA probe. Hybridization with short cDNA probes (Fig. 1A) indicated that clone HIOMT1

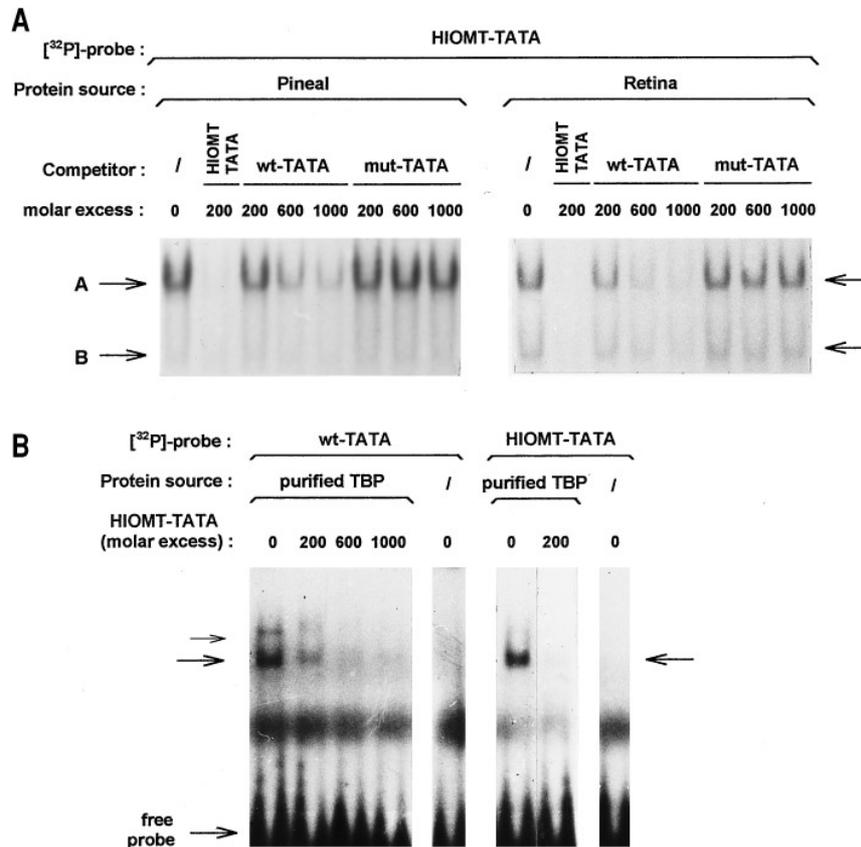


Fig. 6. Protein-binding characteristics of the putative HIOMT TATA box. The protein-binding characteristics of the putative HIOMT TATA box were analyzed by EMSA. (A) Proteins (20 μ g) from chicken pineal gland or retina were incubated with 1 fmol putative HIOMT TATA-box (³²P-labelled) with or without the indicated amounts of unlabelled oligonucleotide competitors ('HIOMT-TATA' or 'wt-TATA' or 'mut-TATA'). DNA-protein complexes were separated on non-denaturing 4.5% acrylamide gels. Two specific complexes (A and B) are indicated by arrows. (B) Purified TBP (20 ng) was incubated with 5 fmol ³²P-labelled 'wt-TATA' or 'HIOMT-TATA', with or without the indicated amounts of unlabelled 'HIOMT-TATA' competitor. DNA-protein complexes were separated on non-denaturing 6% acrylamide gels. Specific DNA/protein complexes are indicated by arrows.

(17 kb) contained the 5' extremity of the coding region, plus about 16 kb of the 5'-flanking region of the gene, while clone HIOMT4 (18 kb) covered the last 80% of the coding region as well as the 3'-flanking region. Clones HIOMT2 and HIOMT3 turned out to be short versions of clone HIOMT4 (data not shown). Since clones HIOMT1 and HIOMT4 were not overlapping, the missing portion of the gene was generated by PCR amplification of chicken genomic DNA (Fig. 1B). Together, the HIOMT1 and HIOMT4 clones and the PCR-generated fragments (PCR1 and PCR2) cover approximately 38 kb of contiguous DNA in the HIOMT locus (Fig. 1B).

Structural organization of the chicken HIOMT gene. The sizes and positions of the introns were determined by PCR with cDNA-specific primers (Fig. 2). The sequence of the intron/exon boundaries was determined by direct sequencing of the PCR products (Fig. 2). This analysis clearly indicated that the previously cloned chicken HIOMT cDNA is composed of eight exons distributed over 7.5 kb of genomic DNA (Figs 1B and 2). These exons are separated by seven introns ranging from 244 bp to 1.80 kb (Figs 1B and 2). Complete sequences have been obtained for introns IV, V, VII and the partial sequences (at least 50%) have been obtained for introns I, II, III, VI (data available upon request). A restriction map of the gene was established for *Bgl*III, *Eco*RI and *Hind*III (Fig. 1B). Restriction fragments analysis on chicken genomic DNA gave no indication of

multiple copies of the HIOMT gene. However, *Bgl*III digestion of genomic DNA from 10 individual chickens revealed the presence of a restriction fragment length polymorphism (RFLP) immediately downstream of exon 8 (Fig. 1B).

Alternative splicing. A 1.6-kb *Eco*RI fragment located 6 kb downstream of exon 8 (Fig. 1B) was found to carry an alternative exon (exon 9) showing sequence similarity with exon 8. The sequence of the 1.6-kb *Eco*RI fragment and its localization by PCR on the HIOMT gene are illustrated in Fig. 3. Sequence similarity with exon 8 was found in three regions of 134 bp, 64 bp and 99 bp, respectively (Fig. 3A). The presence of two polyadenylation signals in this sequence suggested it might contain an alternative exon. This was confirmed by reverse transcription (RT)-PCR on pineal and retinal RNA, using a sense primer located in exon 6 (S677, see Table 1) and an antisense primer located immediately upstream of the second polyadenylation signal (EA1541, see Table 1). Four PCR products of respectively 563 bp, 686 bp, 802 bp and 901 bp were generated from both pineal and retinal cDNA (Fig. 4A). All four PCR products displayed day/night variations in intensity that matched the rhythm of the major HIOMT transcript, i.e. with a daytime increase in the pineal gland and a night time increase in the retina (data not shown). Partial sequences and splicing patterns of these four PCR products are illustrated (Fig. 4B and C). All four PCR products contain in-frame stop codons within the first

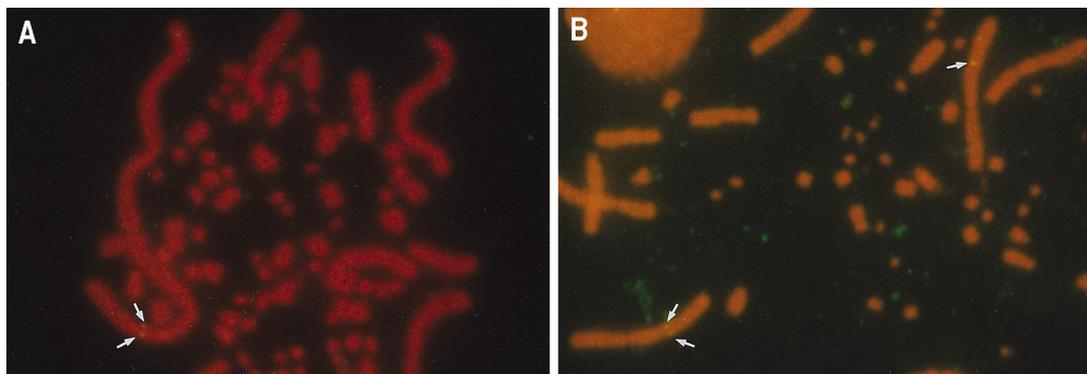


Fig. 7. Localization of the HIOMT gene by fluorescence hybridization to the chicken chromosome 1. (A) Partial metaphase spreads following *in situ* hybridization with the HIOMT gene probe on chicken chromosome 1q22; arrows indicate a specific signal on both chromatids of the same chromosome. (B) Partial metaphase spreads following *in situ* hybridization with the HIOMT gene probe on chicken chromosome 1q22; arrows indicate a specific signal on both chromatids of the same chromosome (left) and a single signal (right).

24–69 bp of exon 9, i.e. before reaching the region of homology with exon 8. The variant C-terminal amino acid sequences encoded by the mRNA species containing exon 9 are illustrated in Fig. 4C. RT-PCR analysis of the major HIOMT transcript (exons 1–8) produced no evidence of alternative splicing either in the pineal gland or in the retina (data not shown).

Nucleotide sequence of the 5'-flanking region. Clone HIOMT1 contains approximately 16 kb of the 5'-flanking region of the chicken HIOMT gene. This region was sequenced over 2.9 kb upstream of the ATG start codon (Fig. 5). The transcription start site was determined by 5'-RACE amplification of pineal and retinal cDNA, using an HIOMT-specific primer located in exon 4 (AS457, see Table 1). The 5'-RACE products were cloned in pGEM-T, sequenced and compared with the sequence of clone HIOMT1. Out of 24 clones analyzed for the 5'-RACE of pineal mRNA, 21 had the same transcription initiation site located 19 nucleotides upstream from the ATG start codon (Fig. 5). Three clones contained shorter products, probably due to staggering of the reverse transcriptase. Analysis of the 5' end of retinal HIOMT mRNA (four clones analyzed) revealed the same transcription initiation site as in the pineal gland. The 5'-flanking region was analyzed for consensus regulatory elements using the TESS software (URL: <http://agave.humen.upenn.edu/teess/index.html>). Some of the sequences identified as potential *cis*-regulatory elements are shown (Fig. 5). The chicken HIOMT proximal promoter lacks a consensus TATA box. However, a TA-rich region (TTATTTAAATTAA) was found at position –29. The protein-binding characteristics of this sequence were investigated.

EMSA analysis of the DNA/protein interactions on the putative HIOMT TATA box. We first tested the ability of the TA-rich region ('HIOMT-TATA': TTATTTAAATTAA) to interact with pineal and retinal proteins. Two retarded complexes were detected by EMSA analysis with proteins from both pineal gland and retina; a major complex, designated as 'A' and a minor one, designated as 'B' (Fig. 6A). The formation of complexes 'A' and 'B' was inhibited by the homologous unlabelled probe as well as by an unlabelled consensus wild-type TATA probe (wt-TATA), but not by a mutated TATA probe (mut-TATA; Fig. 6A). Conversely, an excess of unlabeled 'HIOMT-TATA' oligonucleotide prevented purified TBP from binding to the ³²P-labelled consensus TATA probe (Fig. 6B). Finally, we showed that the ³²P-labelled 'HIOMT-TATA' oligonucleotide can bind purified TBP (Fig. 6B) and, therefore, may play the role of a TATA-box.

Chromosomal localization of the chicken HIOMT gene. The biotinylated HIOMT probe was hybridized to metaphase spreads of chicken chromosomes. About 66 metaphase spreads were analyzed in each of three experiments, giving 80–90% signals on one or both chromatids of each homologous chromosome. Very little non-specific background was observed. The HIOMT gene was mapped precisely on band 1q22 (Fig. 7A and B).

DISCUSSION

The chicken HIOMT gene has been characterized over 17 kb including 2.9 kb 5'-flanking region. The major HIOMT transcript is composed of eight exons that are distributed over 7.5 kb genomic DNA. All the exon/intron junctions follow the GT-AG rule [29]. Exons 1–8 of the chicken HIOMT gene correspond to exons 1B, 2, 3, 4, 5, 7, 8, 9 of the human HIOMT gene [11], with exact conservation of the positions of the introns. The LINE1 fragment which corresponds to exon 6 in the human gene is not present in the chicken HIOMT transcript. The chicken gene also differs from the human gene by the presence of an alternative exon 9 (more precisely, exons 9A and 9B), located 6 kb downstream from exon 8 and showing sequence similarity with the latter. This sequence similarity suggests exons 8 and 9 originated from exon duplication, as previously described for other genes ([30] and references therein). Analyzing this exon duplication in different species would provide an insight into the evolution of the HIOMT gene. The functional significance of exon 9 remains to be investigated. Due to early stop codons in exons 9A and 9B, the minor splice variants described herein would encode HIOMT proteins lacking up to 33% of their carboxy end. Scanning the HIOMT sequence for protein domains that might be added or deleted by the alternative splicing (PROSITE search on EMBL data bank) revealed only few changes that concerned mostly its potential phosphorylation sites. Since the active site of HIOMT has not been identified, expression studies are needed to determine whether the enzyme activity is conserved, or maybe increased, in the truncated forms of the protein resulting from the incorporation of exon 9.

In this study, we report the localization of the HIOMT gene by fluorescence *in situ* hybridization to the chicken chromosome 1q22. The human HIOMT gene has been mapped to the pseudo-autosomal region X(Xp22.3) [31]. The chicken karyotype is complex, with 38 pairs of autosomes and a pair of sex chromosomes, Z and W. Our results confirm previous observations indicating that some genes that map to sex chromosomes in mammals are autosomal in birds and vice versa [32, 33]. In fact, up

to now, there has been no evidence of homology between the mammalian X chromosome and the avian Z chromosome. However, relatively few loci have been investigated to evaluate this degree of homology.

Several features of the 5'-flanking region of the chicken HIOMT gene are worth noticing because they are shared with other genes expressed specifically in photoreceptors. First, the ATTTAAAT sequence at position -29 from the transcription initiation site resembles a motif previously noted as a potential TATA-box in the 5'-flanking region of the phosphodiesterase-3 gene [34]. To our knowledge, the present study provides the first evidence that this element binds purified TBP as well as proteins extracted from pineal and retina, indicating it may act as a TATA-box.

Using the ALIGN software (URL: http://www.infobiogen.fr/services/analyse/cgi-bin/alignn_in.pl), no significant similarity was detected between the 5'-flanking region of the chicken HIOMT gene and the putative promoters A and B of the human HIOMT gene [11]. However, a GATTAA sequence at position -76 in the chicken HIOMT gene is also present at position -71 in promoter B of the human HIOMT gene [11]. This element has been reported to direct tissue-specific expression of a reporter gene in the pineal gland and retina of transgenic mice [35]. Recent studies have identified a paired, homeodomain protein (Crx) that binds to this element and plays a major role in photoreceptor differentiation [36, 37]. Based on an improved definition of the Crx-binding site [38], it appears that six such sequences can be found at positions -76, -246, -1577, -1784, -2119 and -2474 of the chicken HIOMT gene. Promoters A and B of the human HIOMT gene each contain at least one copy of the Crx-binding site [38]. These *cis*-acting elements are also found in the promoters of a number of genes of the phototransduction cascade [35-38] and, therefore, might be responsible for the coordinated differentiation of the light-sensing and melatoninergic functions of photoreceptors.

A previous study had indicated that transcription of the human HIOMT gene could be initiated from two different promoters, one of them being specifically active in the retina [11]. This suggested to us that the tissue-differences of the HIOMT mRNA rhythm observed in the chicken (day-time peak in the pineal and night-time peak in the retina) [9, 16] might be due to the presence of two different promoters. This hypothesis should be rejected because our 5'-RACE experiments indicate that HIOMT is transcribed from the same promoter in the chicken pineal gland and retina.

One unusual aspect of HIOMT gene regulation is its circadian rhythm of transcription in the chicken pineal gland [16, 17]. The biochemical components of the circadian oscillator of chicken pineal cells have not been identified. Therefore, the chicken HIOMT promoter should prove useful to analyze day/night changes in DNA/protein interactions that may be relevant to circadian rhythms of transcription in the pineal gland. This approach may help characterize the mode of action of putative clock-genes such as *per*, *tau* and *clock* ([39] for review) or it may lead to the identification of new candidates for this function.

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