

Identification of avian α -melanocyte-stimulating hormone in the eye: temporal and spatial regulation of expression in the developing chicken

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Abstract

The presence and possible physiological roles of α -melanocyte-stimulating hormone (α -MSH) in the peripheral tissues of birds have not been established. By a combination of RT-PCR, immunocytochemistry and *in situ* hybridization, we have examined α -MSH expression in the eye of the chicken during development. In the 1-day-old chick, α -MSH was expressed in the retinal pigment epithelial (RPE) cells, and also at a lower level in the cone cells. The melanocortin receptor subtypes, CMC1, CMC4 and CMC5, were expressed in the layers of the choroid and the neural retina, but not in the RPE cells. It is probable that the RPE cells secrete α -MSH to exert paracrine effects on the choroid and neural retina. During embryonic development, α -MSH immunoreactivity in the RPE cells was initially detected at embryonic day 10, and increased in intensity as develop-

ment proceeded. No cone cells were stained with anti- α -MSH antiserum in any of the embryonic stages tested. The immunoreactivities for two prohormone convertases, PC1 and PC2, were co-localized to the RPE cells with a pattern of staining similar to that of α -MSH. Despite containing α -MSH immunoreactivity, the RPE cells in 1-day-old chicks expressed no immunoreactivity for the endoproteases. Furthermore, in a 3-day-old chick, pro-opiomelanocortin mRNA was detectable by *in situ* hybridization only in the photoreceptor layer and not in the RPE cells. These results suggest that the RPE cells and the cone cells are intraocular sources of α -MSH in the embryonic and postnatal life of the chicken respectively. Embryonic expression of α -MSH in the RPE cells implies a possible role for the peptide in ocular development.

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Introduction

α -Melanocyte-stimulating hormone (α -MSH) is a melanocortin peptide generated from the precursor glycoprotein pro-opiomelanocortin (POMC). The best known source of POMC in most vertebrates is the anterior and intermediate lobes of the pituitary. A series of ordered proteolytic cleavages of POMC with cell type-specific patterns leads to a cell type-specific mixture of melanocortin peptides displaying a wide array of biological effects (Eberle 1988, Smith & Funder 1988, Mains & Eipper 1990). Two kinds of endoprotease involved in this process have been well characterized; prohormone convertase 1 (PC1; also called PC3) generates adrenocorticotrophic hormone (ACTH) from POMC, and prohormone convertase 2 (PC2) cleaves ACTH to produce α -MSH (Benjannet *et al.* 1991, Korner *et al.* 1991, Thomas *et al.* 1991). Restricted expression of PC2 in the intermediate

lobe identifies this part of the pituitary as a secretory gland for α -MSH.

In mammals, α -MSH is now recognized to be widely expressed throughout the body and it has a broad array of physiological actions both in the central nervous system and in the peripheral tissues; this correlates well with its localization and that of its receptors, the melanocortin receptors (MC-Rs) (Eberle 1988, Cone *et al.* 1996). Two recently identified endogenous antagonists of MC-Rs, agouti protein (Bultman *et al.* 1992, Kwon *et al.* 1994, Lu *et al.* 1994, Wilson *et al.* 1995) and agouti-related protein (AGRP) (Ollmann *et al.* 1997, Shutter *et al.* 1997), act in a paracrine manner to regulate MC-R function. However, while the molecular mechanisms of α -MSH actions in mammals are well understood, little is known about the biological activities of α -MSH in birds. The physiological significance of avian α -MSH remains uncertain as the pituitary gland of birds does not possess a

distinct intermediate lobe of the pituitary, the main source of circulating α -MSH in most vertebrates.

We have recently cloned all five receptor genes belonging to the MC-R family in birds, CMC1 (Takeuchi *et al.* 1996a), CMC2 (Takeuchi *et al.* 1998), CMC3 (Takeuchi & Takahashi 1999), CMC4 and CMC5 (Takeuchi & Takahashi 1998); these are chicken homologs of mammalian MC1 (MSH)-R, MC2 (ACTH)-R, MC3-R, MC4-R and MC5-R respectively. CMC1 is implicated in melanogenesis within melanocytes since it has been mapped at the genetic locus which acts to control feather color pigmentation in the chicken (Takeuchi *et al.* 1996b). CMC2 has been suggested to regulate steroidogenesis in the adrenal gland and immune functions in the spleen based on its distribution of expression (Takeuchi *et al.* 1998). Although no function has yet been ascribed to the other subtypes of MC-R in any tissues of the chicken, CMC5 shows a wide distribution of expression as in mammals (Takeuchi & Takahashi 1998), while homologs of the mammalian central MC-Rs, CMC3 and CMC4, display different tissue distributions from mammals. Thus, CMC3 is expressed exclusively in the adrenal gland (Takeuchi & Takahashi 1999) and CMC4 in a wide variety of peripheral tissues as well as in the brain (Takeuchi & Takahashi 1998). Since most of those MC-R-expressing tissues have been found to express the POMC gene, we have hypothesized that avian melanocortin peptides, including α -MSH, serve as local mediators acting in a paracrine and/or autocrine manner to affect a variety of functions in both the brain and peripheral tissues (Takeuchi *et al.* 1999). Furthermore, analyses of the chicken genome as well as of distribution of the expression of AGRP mRNA have suggested the possibility that AGRP could be the sole endogenous antagonist of MC-Rs involved in the regulation of MC-R signalings in numerous peripheral tissues in the chicken (Takeuchi *et al.* 2000). In contrast, nothing is known about the MC-R agonist, α -MSH, in terms of its localization and possible physiological role in peripheral tissues of the chicken.

Many lines of evidence have shown that administration of α -MSH affects numerous functions of the eye in amphibia (frog) and mammals including humans, such as acceleration of the regeneration of visual purple (Hanaoka 1951, 1953), stimulation of the release of dopamine or γ -aminobutyric acid from the retina (Bauer & Ehinger 1980, Bauer *et al.* 1980), increase of the permeability of the blood–aqueous barrier (Dyster–Aas & Krakau 1964, Dyster–Aas *et al.* 1970), and stimulation of the production of eicosanoids in the retinal pigment epithelial (RPE) cells (Ilan *et al.* 1992). The source of the α -MSH that acts in the eye remains unclear, for there is no correlation between circulating α -MSH levels and the eye responses (Dyster–Aas *et al.* 1970). Drager *et al.* (1983) have detected α -MSH immunoreactivity in the eyes of chickens and a variety of mammals. However, this immunoreactivity has been ascribed to the expression of an α -MSH-like epitope by

neurofilaments because the immunostaining was eliminated by preabsorption of the α -MSH antibodies with the neurofilaments. However, it is still possible that α -MSH is expressed within the eye at a level below the immunocytochemical detection limit, and/or that it undergoes temporal changes in expression during development.

The aim of the present study was to test our hypothesis that α -MSH exerts paracrine/autocrine effects in peripheral tissues by determining whether α -MSH is expressed within the chicken eye. By a combination of immunocytochemistry, RT-PCR and *in situ* hybridization, we demonstrate here for the first time that α -MSH is expressed within the chicken eye, and that its expression during embryonic development is temporally and spatially regulated. The results suggest a novel role for α -MSH in the embryonic development of the chicken eye and support our hypothesis that avian α -MSH is a paracrine/autocrine hormone (Takeuchi *et al.* 1999, 2000).

Materials and Methods

Animals

Tissues for RNA extraction, immunocytochemistry and cell culture were obtained from 1-day-old Rock Cornish chicks and/or embryos (embryonic day 7, 10, 13 and 17) purchased from a commercial grower (Fukuda Poultry Breeding Farm, Okayama, Japan). For *in situ* hybridization, tissue samples were taken from a 3-day-old female chick of the J-line strain of Brown Leghorns (Roslin Institute flock).

Isolation of eye layers

One-day-old Rock Cornish chicks were killed by cervical dislocation. The eyes were then rapidly dissected and placed in Ca²⁺- and Mg²⁺-free Hanks' solution containing 20 mM Hepes and 0.3% bovine serum albumin (BSA; fraction V; Sigma, St Louis, MO, USA) (CMF–HSH–BSA). An incision was made slightly posterior to the corneal limbus, and the anterior sections, lens and vitreous humor were then removed. The remaining posterior cup was divided into three to four pieces and placed in 0.5 mM EDTA in CMF–HSH–BSA at 4 °C for 30 min, and subsequently at 37 °C for 40 min. The tissue was washed twice with CMF–HSH–BSA. The layers of the RPE, the neural retina and the choroid were membranously separated from the tapetum using forceps. These tissues were immediately frozen in liquid nitrogen and stored at –70 °C before being used for RNA preparation. To assess the purity of the isolated tissues, some tissue fragments were subjected to standard histochemical analysis.

Isolation and cultivation of RPE cells

RPE cells were isolated from 1-day-old Rock Cornish chicks by the method of Hayashi *et al.* (1978) with some

modifications. The membranous RPE layers were isolated as described above and processed by sequential incubation at 37 °C in the following: 0.1% trypsin (Type III; Sigma) in CMF-HSH-BSA (5 min); 0.1% soybean trypsin inhibitor (Sigma) in CMF-HSH-BSA (10 min); 0.005% DNase I (Sigma) in CMF-HSH-BSA (10 min). After these treatments, a single cell suspension was obtained by gentle pipetting. Cell yield was calculated by counting the cells with a hemocytometer, and cell viability was checked using a trypan blue exclusion test. The single cell suspension was collected by centrifugation, and resuspended in a culture medium of Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). The resulting cells were inoculated into 9 cm well plates (Becton Dickinson, Lincoln Park, NJ, USA) at a cell density of 6×10^5 cells/ml (6×10^6 cells/well), and cultured for 3 days at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Reverse transcription (RT)-PCR analysis

Total RNA was prepared from tissues and culture cells using the method of Chomczynski & Sacchi (1987). One microgram of each total RNA was reverse transcribed using a SuperScript II reverse transcriptase (Gibco BRL) according to the manufacturer's directions. A one-tenth aliquot of the reactions was used in each PCR using specific primers for POMC, AGRP or MC-R subtypes (CMC1-CMC5). The primers for CMC1 were CAGGA TGGCGTTGTTGCGGTAAGT and GAATCTGCAC TCGCCACGTA. The sequence of other primers have been described elsewhere (for POMC, Takeuchi *et al.* 1999; for AGRP, Takeuchi *et al.* 2000; for CMC2, Takeuchi *et al.* 1998; for CMC3, Takeuchi & Takahashi 1999; for CMC4 and CMC5, Takeuchi & Takahashi 1998). The PCRs were carried out using an AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA) and a thermal cycler (Gene Amp PCR System 9600; Applied Biosystems). The conditions for the PCRs were as follows: after activation of the DNA polymerase by incubating for 9 min at 95 °C, 42 cycles of reactions including denaturation for 30 s at 95 °C and extension for 1 min at 60 °C were performed, followed by additional extension for 10 min at 60 °C. For AGRP, the incubation temperature for extension and annealing was altered to 66 °C. A one-tenth aliquot of each reaction was electrophoresed on a 2.0% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination. The gel was subsequently transferred onto a Hybond-N+ (Amersham Pharmacia Biotech, Uppsala, Sweden) and subjected to Southern blot analysis using corresponding cDNA probes as described elsewhere (Takeuchi *et al.* 1996b, 1998, 1999, 2000, Takeuchi & Takahashi 1998, 1999). Labeling and detection of the probes were carried out using an ECL Random Prime Labeling and Detection

system (Amersham Pharmacia Biotech) according to the manufacturer's directions, except for the AGRP probe which was radiolabeled using α -[³²P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) and a Random Primer DNA Labeling Kit Version 2 (Takara, Shiga, Japan). The primers for POMC should produce 1239 and 426 bp products from genomic and complementary DNA of the chicken POMC respectively. Similarly, primers for AGRP should produce 1521 and 426 bp products respectively. Each primer set for MC-R subtypes produces PCR products with the same size, when cDNA or genomic DNA is the template. To rule out the possibility that PCR products resulted from the amplification of genomic DNA contamination in the RNA samples, 1 μ g total RNA was also subjected to PCR using primers for the MC-R subtypes. The size of amplicon was 369, 567, 435, 649 and 448 bp for CMC1, CMC2, CMC3, CMC4 and CMC5 respectively.

Immunocytochemistry

One-day-old Rock Cornish chicks or embryos were killed by cervical dislocation and the eye layers dissected as described above. Pieces of the posterior cup were fixed with Bouin's solution (24 h at 4 °C) and processed by sequential immersion: twice in 70% ethanol (8 h at 4 °C); in 80%, 90%, 95%, 100%, 100% ethanol (20 min each at 4 °C); twice in 100% xylene (5 min at room temperature); in a 1:1 mixture of paraffin (Paraplast; Sherwood Medical, St Louis, MO, USA)-xylene (10 min at 50 °C); in Paraplast (10 min at 50 °C); and embedded in Paraplast. Sections (5 μ m) were cut and mounted onto microscope slides. Slides bearing eye sections were deparaffinized and rehydrated by sequential immersion at room temperature: twice in xylene (15 min); in 100%, 100%, 90%, 70% ethanol (5 min each); and in distilled water (DW, 5 min); and treated with 0.3% hydrogen peroxide in methanol for 10 min at room temperature to inhibit endogenous peroxidase activity. After being rinsed three times in DW and once in 0.01 M phosphate-buffered saline (PBS; 0.14 M sodium chloride in 0.01 M sodium phosphate, pH 7.6), the slides were subjected to immunostaining using specific antisera and a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's directions. The antisera used as first antisera were anti- α -MSH (1:2000; Biogenesis Ltd, Poole, Dorset, UK), anti-rat-ACTH (1:4000; Yanaihara Institute Inc., Shizuoka, Japan), anti-mouse-PC1/PC3 (ST-28; 1:1000) and anti-mouse-PC2 (ST-29; 1:1000). The production and characterization of the antisera against PC1/PC3 (ST-28) and PC2 (ST-29) used in this study have been described elsewhere (Tanaka *et al.* 1996). The antibodies were generated in rabbits by using, as immunogens, two synthetic peptides corresponding to amino acids 442-459 (ST-28) of mouse PC1/PC3 and amino acids 613-629 (ST-29) of mouse PC2. To enhance the

sensitivity of immunostaining for PC1 and PC2 (Tanaka *et al.* 1996, 1997, Kurabuchi & Tanaka 1997), and to decolorize melanin pigments in the RPE cells, some slides were oxidized for 10 s at room temperature with Gomori's oxidization mixture consisting of 0.5% potassium permanganate and 0.25% sulfuric acid, bleached in 3% sodium bisulfite for 20 s, and then rinsed by sequential immersion at room temperature: three times in DW (5 min); in 0.1 M Tris buffer, pH 8.0 (5 min); twice in DW (5 min); and in 0.01 M PBS, pH 7.6 (5 min) prior to being subjected to immunostaining. Non-specific immunoreactivity was blocked by incubating the slides in 2% low-fat milk (w/v) in 0.01 M PBS, pH 7.6 for 1 h at room temperature. They were then incubated with each first antiserum in 0.01 M PBS for 24 h at 4 °C in a humidified box, and washed three times with 0.01 M PBS, pH 7.6. The slides were incubated with biotinylated anti-rabbit IgG in 0.01 M PBS, pH 7.6 (1:100) for 1 h, and then with avidin-biotinylated peroxidase complex for 30 min at room temperature. They were then washed three times with 0.01 M PBS, pH 7.6. Sections were immunostained in the dark for 90 s at room temperature in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (w/v) solution containing 0.005% hydrogen peroxide and 0.05 M Tris, pH 7.6, or two to three drops/50 ml intensifier included in the Vectastain ABC kit and 0.05 M sodium phosphate, pH 7.6. The resulting slides were washed three times with DW for 5 min, stained with hematoxylin for 5 min, and then rinsed under running water for 5 min. They were dehydrated by passage through a graded ethanol series (70, 90, 100 and 100%, 5 min each) and xylene (5 min twice), mounted in paramount and cover-slipped. As negative controls, some sections were processed as above with the exception that 2% low-fat milk (w/v) was used in place of each first antiserum. For the detection of α -MSH immunoreactivity, the primary antibody was absorbed with 10 μ g/100 μ l antiserum of purified α -MSH (Sigma) or BSA (Sigma) for 16 h at 4 °C before being used for immunostaining.

In situ hybridization

A 3-day-old female chick of the J-line strain of Brown Leghorns was killed by cervical dislocation. The eyes were then rapidly dissected, placed in embedding compound (Bright Cryo-M-Bed; Bright Instruments, Huntingdon, Cambs, UK) and immediately frozen in powdered dry ice. The tissue was stored at -70 °C before being sectioned on a cryostat (Model OT; Shandon, Runcorn, Cheshire, UK) at 15 μ m thickness. Sections were thaw-mounted onto microscope slides (Superfrost Plus; Cellpath, Hemel Hempstead, Herts, UK) and stored at -70 °C. Slides bearing eye sections were processed by sequential immersion at room temperature in: 4% paraformaldehyde (5 min); twice in 0.1 M sodium phosphate (PB; 5 min each); 20 μ g/ml proteinase K in 5 mM EDTA, 10 mM

Tris buffer, pH 7.5 (7.5 min); PB (5 min); 4% paraformaldehyde (5 min); PB twice (5 min each); water (<5 s); 0.1 M triethanolamine, pH 8.0 (TEA; <5 s); 0.15 M acetic anhydride in 0.1 M TEA (10 min); 2 \times SSC; 70%, 95% and 100% ethanol (3 min each); and blow-dried. Slides were then stored at room temperature until application of hybridization solution. The riboprobe used for *in situ* hybridization was generated by RT-PCR amplification of a plasmid containing chicken POMC cDNA (Takeuchi *et al.* 1999). Oligonucleotide primers were designed to amplify a 299 base chicken POMC cDNA fragment. Primers used were ATGCTGGGAGAACAGCAAGT GCC and GTTCCAACCCTTCTCCATCTTC, corresponding to positions 2315-2337 and 3409-3430 respectively of the chicken POMC gene (accession number AB019555). The fragment was inserted into the EcoRV site of pBluescript II SK⁺ (Stratagene, Cambridge, Cambs, UK) and transformed into *E. coli* XL1-Blue (Stratagene). The orientation and identity of positive clones were established by sequencing and restriction mapping. After linearization with EcoRI, a 378 base antisense chicken POMC riboprobe was transcribed from the T7 promoter of the plasmid using T7 RNA polymerase (Roche Diagnostics, Lewes, E Sussex, UK). The riboprobe was labeled with [³⁵S]UTP α S (Amersham, Little Chalfont, Bucks, UK). Hybridization solution contained chicken POMC riboprobe at approximately 2 \times 10⁶ d.p.m./100 μ l, 5 mg/ml yeast total RNA and 0.01 M dithiothreitol (DTT) in a buffer consisting of 50% formamide, 10% dextran sulfate and 1 \times Denhardt's solution in 300 mM NaCl, 10 mM Tris and 1 mM EDTA, and 100 μ l was applied to each slide. Slides were then cover-slipped with parafilm and incubated in a humidified chamber at 45 °C overnight. All steps prior to and including hybridization were conducted in RNase-free conditions. Following hybridization, cover-slips were removed and slides were washed twice (15 min each) in 4 \times SSC and 2 mM DTT. Slides were then placed in a pre-warmed RNase buffer consisting of 0.01 M Tris, pH 8, 0.5 M NaCl and 1 mM EDTA and containing 0.03 mg/ml RNase A. Sections were incubated for 30 min at 37 °C before washing in 2 \times SSC and 2 mM DTT for 30 min at room temperature. Slides were then placed in a hot wash of 0.1 \times SSC and 2 mM DTT at 55 °C for 30 min and washed in 0.1 \times SSC for 3 min at room temperature. Sections were dehydrated by immersion in 50% and 85% ethanol containing 300 mM ammonium acetate (3 min each) and a final wash in 100% ethanol (3 min) before air-drying. Processed slides were dipped in Ilford K2 emulsion diluted 1:1 with DW, dried overnight, and exposed in the dark for 2 weeks at 4 °C. Slides were then developed in D19 (Kodak) for 2.5 min, washed for 30 s in DW, fixed in Ilford Hypam diluted 1:4 in DW for 3 min, and rinsed under running water for 15 min. Developed slides were dried, mounted in DPX and cover-slipped.

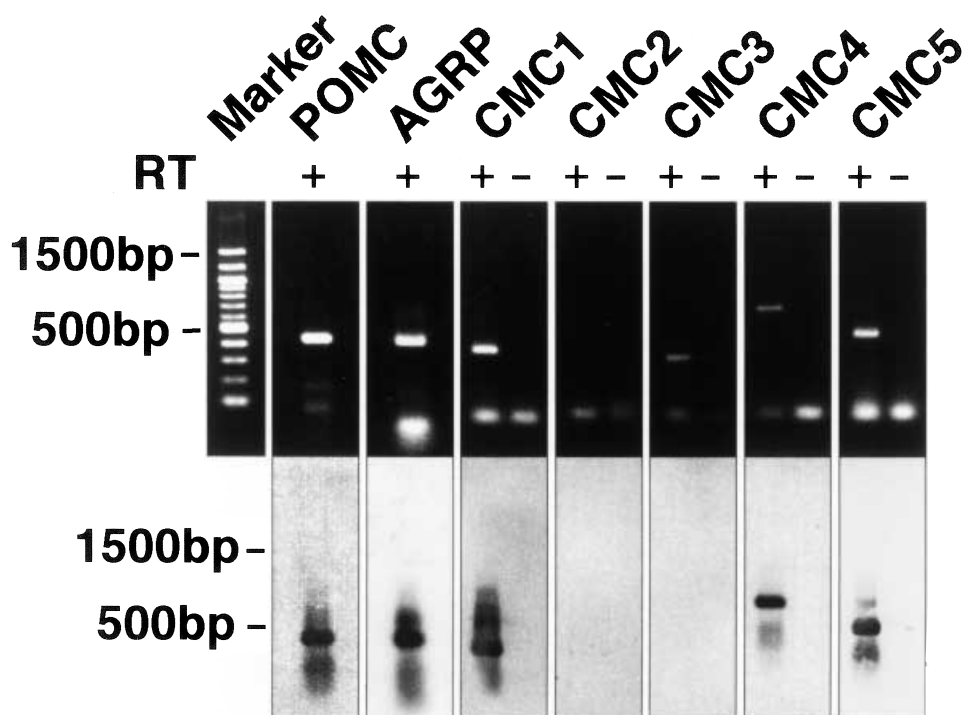


Figure 1 RT-PCR analysis of the expression of POMC, AGRP and MC-Rs mRNAs in the eye of 1-day-old Rock Cornish chicks. The upper panel shows the electrophoretic pattern of PCR products and the lower panel a Southern blot hybridization of the same gel to the chicken cDNA probes for POMC, AGRP and MC-R subtypes. A 100 bp ladder used as a molecular marker is indicated on the left. Negative controls are indicated by RT (-). A band observed in the CMC3 lane was revealed to be an artifact of PCR by sequence analysis.

Results

RT-PCR analysis was used to determine whether or not the melanocortin system exists in the chicken eye. As shown in Fig. 1, the eyes of 1-day-old chicks expressed mRNAs for POMC and AGRP, a common precursor of melanocortin peptides and an endogenous antagonist of MC-Rs respectively. Moreover, specific primers for MC-Rs produced cDNAs for CMC1, CMC4 and CMC5. These results suggest the existence of an intraocular melanocortin system in the chicken. Although ligand specificity of those MC-R subtypes remains to be determined, their mammalian orthologs respond to α -MSH either preferentially or as well as ACTH (Cone *et al.* 1996, Takeuchi 1997), which led us to examine whether α -MSH is produced by the processing of POMC in the eyes.

Immunostaining was performed on eye sections of 1-day-old chicks using antiserum directed against α -MSH. As shown in Fig. 2A, the RPE cells were stained intensely. Less intense α -MSH immunoreactivity was observed in the region between the outer limiting membrane and the RPE, particularly in some inner segments of photoreceptor cells. The immunoreactive

inner segments were large in size, and their cell bodies containing nuclei were arranged in a single row immediately beneath the outer limiting membrane, the morphological characteristics of the cone cells. The α -MSH immunoreactive cells in the photoreceptor layer are therefore presumed to be cone cells. The immunoreactivity of the RPE cells disappeared completely after preabsorption of the antiserum with α -MSH, whereas the cone cells remained stained, although staining intensity was apparently reduced (Fig. 2B). In the immunostaining with anti-ACTH antiserum or in the control, no immunoreactivity was observed (Fig. 2C and D). These results indicate that α -MSH-like immunoreactive material exists in the RPE cells, and possibly also in the cone cells of 1-day-old chicks.

It has been reported that α -MSH-like immunoreactive material is detectable in the cone cells and other neuronal cells in adult retinas from a variety of vertebrates including chickens (Drager *et al.* 1983). However, this has been assumed to result from cross-reactivity to an epitope expressed by neurofilaments because no immunoreactivity was observed when anti- α -MSH antibodies were preabsorbed with the neurofilaments (Drager *et al.* 1983).

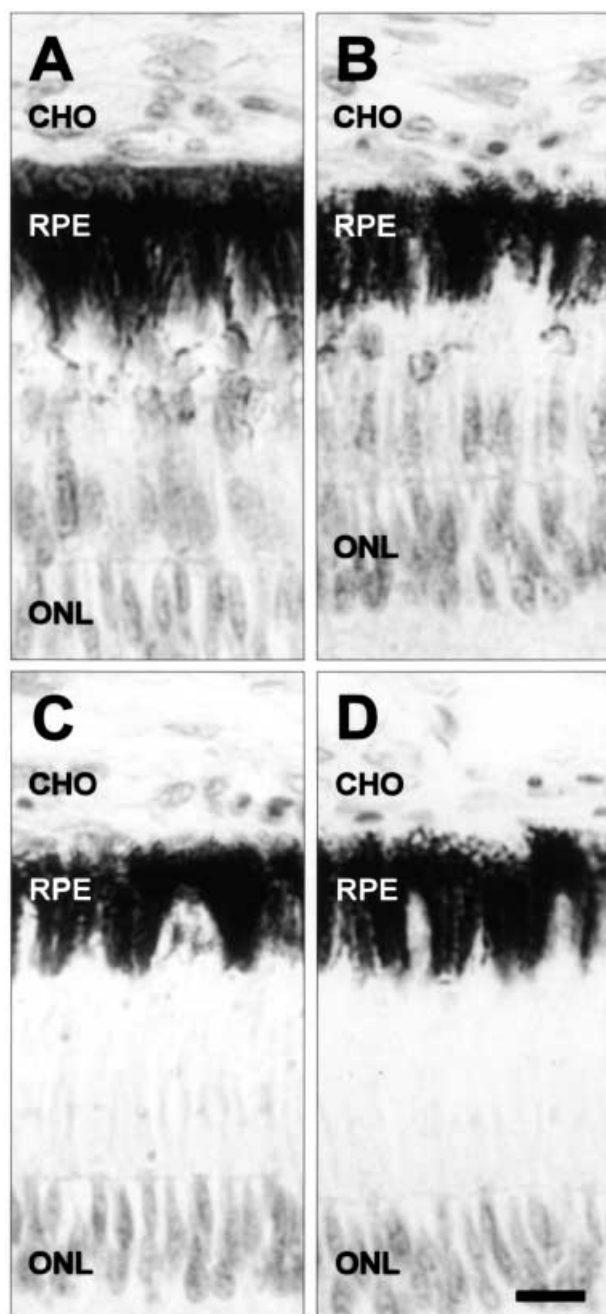


Figure 2 Immunocytochemical localization of α -MSH in the eye of 1-day-old Rock Cornish chicks. Light micrographs are representative examples of immunostaining using anti- α -MSH antiserum preabsorbed with BSA (A) or α -MSH (B) and anti-ACTH antiserum (C) as first antisera, and using low-fat milk instead of first antiserum (D; control). CHO, RPE and ONL indicate layers of the choroid, retinal pigment epithelium and outer nuclear of photoreceptors respectively. Immunoreactivity is represented by half-tone staining. Scale bar = 10 μ m.

Since the neurofilaments are widely distributed in the central nervous system (Drager *et al.* 1983, Trojanowski *et al.* 1985), it is possible that the immunoreactivity detected here represents this cross-reactivity. Furthermore, even if the immunoreactive material in the RPE cells was α -MSH, its source remains unclear since RPE cells actively take up materials from the stroma of the choroid or from the retina proper, such as vitamin A and the rod outer segments (Dowling & Gibbon 1962). Thus, there are limitations in immunocytochemical studies for the precise localization of the sites of α -MSH biosynthesis. To show that RPE cells express POMC at the mRNA level, RPE cells were isolated from 1-day-old chick eyes and cultured for 3 days. During the cultivation period, cells from neural retina and/or choroid disappeared so that only melanin-containing cells with an epithelial cell-like shape, the RPE cells, increased in number (data not shown). Total RNA was prepared from the RPE cells and subjected to RT-PCR analysis. As expected, the cultured RPE cells did express POMC mRNA (Fig. 3A), indicating that α -MSH is produced in the RPE cells of 1-day-old chicks.

Notably, while MC-R mRNAs were detected by RT-PCR in whole eyes, the RPE cells in primary culture did not express mRNA for any MC-R subtype. Bovine RPE cells have been suggested to express MC-Rs since α -MSH stimulated the production of prostaglandin E_2 and prostacyclin in the cells in organ culture (Ilan *et al.* 1992). It is possible that the chicken RPE cells do express the MC-Rs, and their expression was repressed due to the cells being isolated and cultured for 3 days. To address this possibility, an attempt was made to isolate individual cell layers of the RPE, choroid and neural retina from 1-day-old chicks. The RT-PCR analysis of RNAs from those layers revealed that all MC-R subtypes detected in whole eyes, CMC1, CMC4 and CMC5, were expressed in the layers of the choroid and the neural retina, but not in the RPE (Fig. 3B–D). These results not only indicate that the chicken RPE cells do not express the MC-Rs *in vivo* and *in vitro*, but also suggest that α -MSH produced in the RPE cells affects the adjacent layers, the choroid and the neural retina in a paracrine fashion.

The POMC mRNA expression was observed in the layer of the neural retina as well as in the RPE cells. It is possible that the α -MSH immunoreactivity detected in the cone cells could be α -MSH produced in the cells themselves. A faint band of POMC cDNA was also detectable in the choroid (Fig. 3D), which might result from contamination with RPE cells since histochemical observation of the isolated choroid tissues revealed that small amounts of RPE cells remained attached to the choroid layers (data not shown). Similar analyses were performed to localize AGRP expression, and showed that all eye layers and the cultured RPE cells express AGRP mRNA (Fig. 4).

In adult chickens, as mentioned above, immunoreactive α -MSH has been localized to the cone cells and other

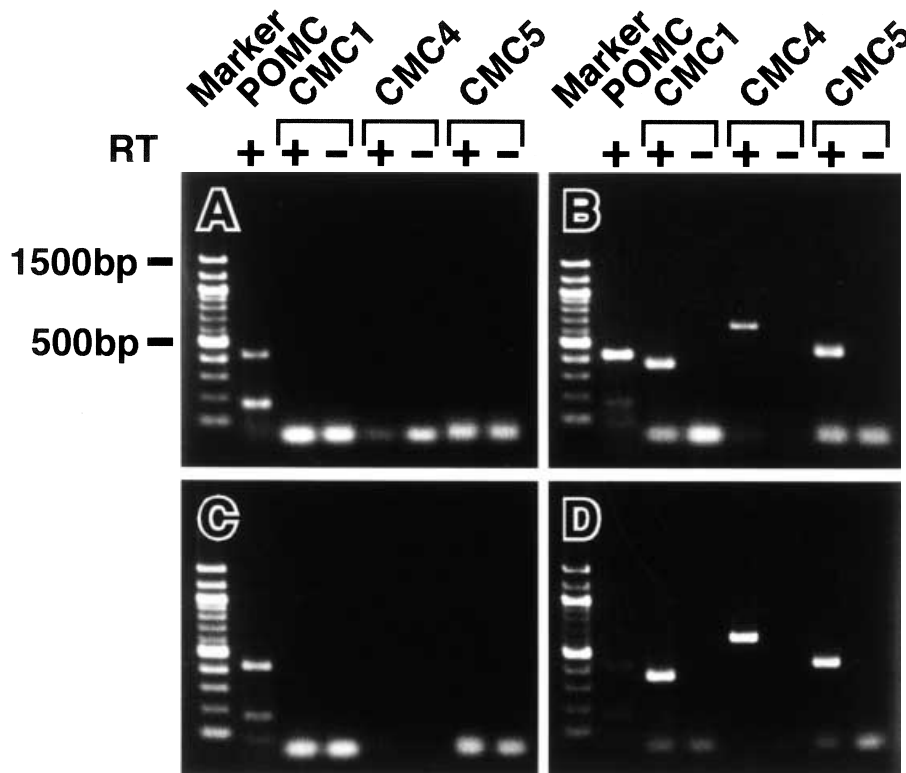


Figure 3 RT-PCR analysis of the expression of mRNAs for POMC and some MC-R subtypes in RPE cells in culture and in eye layers of 1-day-old Rock Cornish chicks. The electrophoretic pattern of PCR reactions amplifying cDNAs from the RPE cells in primary culture (A) and from individual cell layers of the neural retina (B), RPE (C) and choroid (D) is shown. In each case, a 100 bp ladder used as a molecular marker is indicated on the left. Negative controls are indicated by RT (-).

neuronal cells in the retina, but not in the RPE cells (Drager *et al.* 1983). On the other hand, in 1-day-old chicks, α -MSH was predominantly produced in the RPE cells, and the cone cells expressed relatively small amounts of α -MSH immunoreactivity. The difference between chicks and adult chickens in localization of α -MSH (or immunoreactive α -MSH) led us to speculate that the expression pattern of α -MSH in the eye changes during development or growth in the chicken.

To address this possibility, we compared the expression of α -MSH in the eyes between chicken embryos at different developmental stages and in 1-day-old chicks by immunocytochemistry (Fig. 5). In the embryonic stages tested (embryonic days 7, 10, 13 and 17), α -MSH immunoreactivity was initially detected in the RPE cells at embryonic day 10, and the staining intensity increased as embryonic development proceeded. The RPE cells of 1-day-old chicks expressed relatively low levels of immunoreactivity compared with those of embryos at 17 days of incubation, and no photoreceptor cells were stained with anti- α -MSH antiserum in any of the embryonic stages tested. The occurrence of α -MSH expression

in the RPE cells was further confirmed by demonstrating that the prohormone convertases PC1 and PC2 immunoreactivities were co-localized to the RPE cells, and their expression levels correlated well with that of α -MSH as assessed by the intensity of the immunostaining. No immunoreactivity was detected with anti-ACTH antiserum. The RPE cells of 1-day-old chicks contained immunoreactivity for α -MSH, but not for the endoproteases, suggesting that the production of α -MSH in the RPE cells ceases after hatching.

Further evidence for the change in localization of α -MSH expression after hatching is provided by the observation that POMC mRNA was detectable only in some cells in the photoreceptor layer, and no positive signal was observed in the RPE cells (Fig. 6). This result, together with our observations in the 1-day-old chick that the level of α -MSH immunoreactivity was lower in the neural retina than in the RPE cells, suggest that it is probable that the production of α -MSH in the RPE cells is extensive during middle to late embryonic development and ceases after hatching and that, in contrast, the cone cells express α -MSH postnatally.

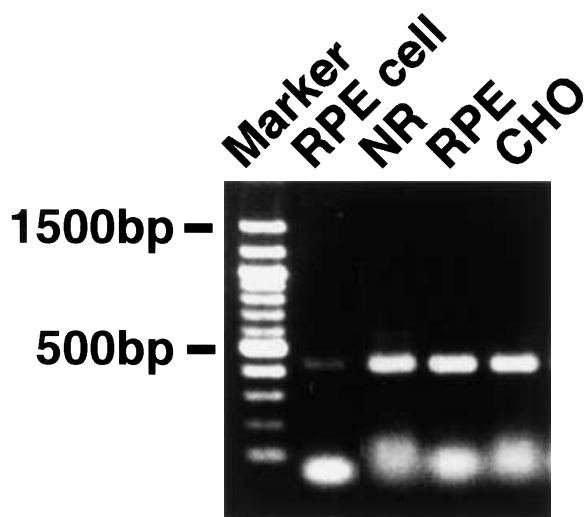


Figure 4 RT-PCR analysis of the expression of AGRP mRNA in RPE cells in culture and in eye layers of 1-day-old Rock Cornish chicks. The electrophoretic pattern of each PCR reaction using cDNA from the RPE cells in primary culture (RPE cell) and from individual cell layers of the neural retina (NR), RPE and choroid (CHO) is shown. A 100 bp ladder used as a molecular marker is indicated on the left.

Discussion

The present study was undertaken to determine whether α -MSH is produced in the chicken eye. A combination of immunocytochemistry and RT-PCR provided evidence for α -MSH expression in the RPE cells, and also at a lower level in the cone cells of 1-day-old chicks. Since its receptors, CMC1, CMC4 and CMC5, were expressed in the layers of the choroid and the neural retina, adjacent to the RPE cells, it is probable that the RPE cells secrete α -MSH to exert paracrine effects on those layers. α -MSH expressed in the cone cells, on the other hand, might be acting as a neurotransmitter or neuromodulator in the neural retina. The expression of AGRP was also detected in the eyes. It remains unclear at present, however, whether AGRP is involved in this intraocular α -MSH system since its expression did not show a cell type specificity. As we suggested previously (Takeuchi *et al.* 2000), the AGRP gene might be expressed ubiquitously at low levels in the chicken. Alternatively, the AGRP expression pattern observed might result from an unusual mutation analogous to the A^y agouti allele in mice (Bultman *et al.* 1992), and the expression pattern may vary among different chicken breeds. From this point of view, it would be of great interest to examine the cellular distribution of AGRP expression in the chicken brain, as AGRP in mammals is expressed mainly in the arcuate nucleus where it plays an important role in the hypothalamic control of feeding and energy homeostasis by

antagonism of central melanocortin 4 receptors (Ollmann *et al.* 1997, Shutter *et al.* 1997).

In the present study, immunocytochemistry and *in situ* hybridization revealed temporal and spatial patterns of α -MSH expression in the eye during development of the chicken. The expression of α -MSH was initially detected in the RPE cells at embryonic day 10, and its level increased as embryonic development proceeded. After hatching, the production of α -MSH probably ceased since the RPE cells in 1-day-old chicks did not contain either of the two endoproteases involved in α -MSH generation from POMC, and POMC mRNA could not be detected in the RPE cells of 3-day-old chicks by *in situ* hybridization. On the other hand, expression of α -MSH or POMC was observed in the cone cells only after hatching (1- and 3-day-old chicks in the present study) and possibly in adults in an earlier report (Drager *et al.* 1983). Although detailed temporal and spatial localization of α -MSH expression is required, it is probable that the RPE and cone cells produce embryonic and postnatal α -MSH respectively. Many lines of evidence have shown that proliferation, differentiation and apoptosis of retinal cells during ocular development are humorally regulated by growth factors generated within the eye, such as fibroblast growth factors (Guillemot & Cepko 1992, Consigli *et al.* 1993, Tcheng *et al.* 1994, Pittack *et al.* 1997, Yokoyama *et al.* 1997, Desire *et al.* 1998) and insulin-like growth factors (de La Rosa *et al.* 1994, Hernandez-Sanchez *et al.* 1995, Calvaruso *et al.* 1996), by an autocrine and/or paracrine mechanism. The expression pattern in the RPE cells suggests a possible involvement of α -MSH in regulating the embryonic development of the chicken eye in a similar fashion. However, the exact physiological role of α -MSH during ocular development remains to be elucidated. It is unclear at present whether the intraocular α -MSH system exists in other vertebrates. Similar analyses on mouse eyes are now in progress. Further analysis of the ocular α -MSH system will contribute to our understanding of how eye development is regulated by α -MSH, and provide a foundation for future attempts to develop methods of treatment for eye diseases in which melanocortin peptides may be the causative agents, such as the central serous retinopathy linked to the therapeutic use of ACTH or to endogenous ACTH hypersecretion (Zamir 1997).

α -MSH was discovered 90 years ago as a potent stimulator of pigment cells, modulating color change in amphibia (Eberle 1988). Surgical ablation of the intermediate lobe of the pituitary results in frogs turning pale, giving rise to the idea that the gland plays a key role in α -MSH function. The absence of this region in the avian pituitary has led to the general thought that α -MSH is a hormone of negligible interest with no physiological roles in birds. Up to now, neither the occurrence nor a physiological role for α -MSH has been established in birds. In mammals, α -MSH exerts a multiplicity of effects

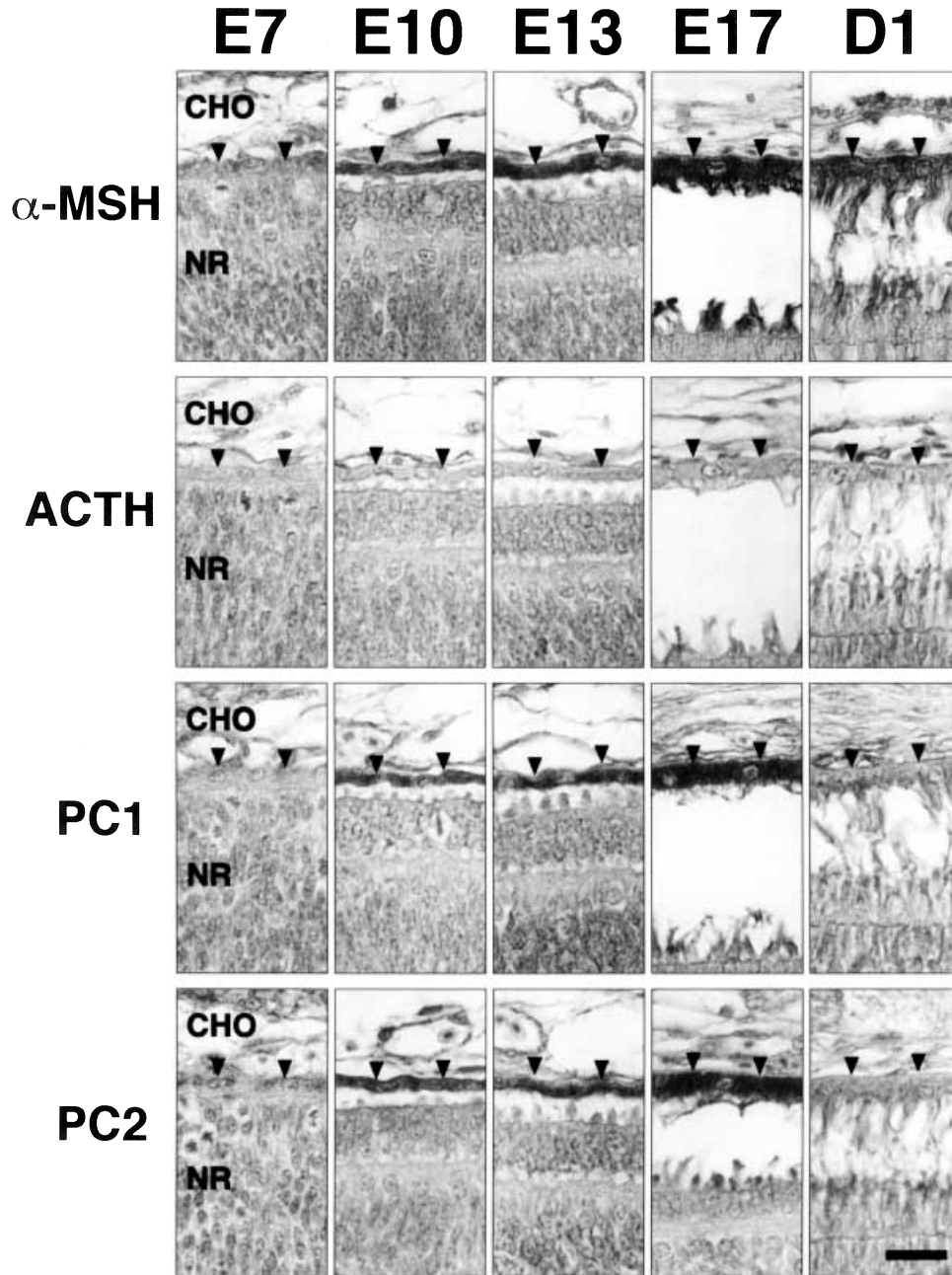


Figure 5 Immunostaining for α -MSH, ACTH and PC1 and PC2 in the eye of Rock Cornish chicken at different developmental stages. Light micrographs are representative examples of immunostaining using anti- α -MSH antiserum preabsorbed with BSA, anti-ACTH antiserum, and antisera directed against the two prohormone convertases, PC1 and PC2, as first antisera. Developmental stages are indicated on the top of each set of panels; E7–E17 and D1 indicate embryonic days 7–17 and day 1 after hatching respectively. Sections were oxidized with Gomori’s oxidization mixture prior to immunostaining to enhance the sensitivity of immunostaining for PC1 and PC2, and to decolorize melanin pigments in the RPE cells. In most sections, an artifactual separation is found between the photoreceptor layer and the RPE, which is a fixation artifact due to lack of actual anatomical connection between the two layers. The retinal pigment epithelium is indicated by arrowheads. CHO and NR indicate layers of the choroid and neural retina respectively. Scale bar=20 μ m.

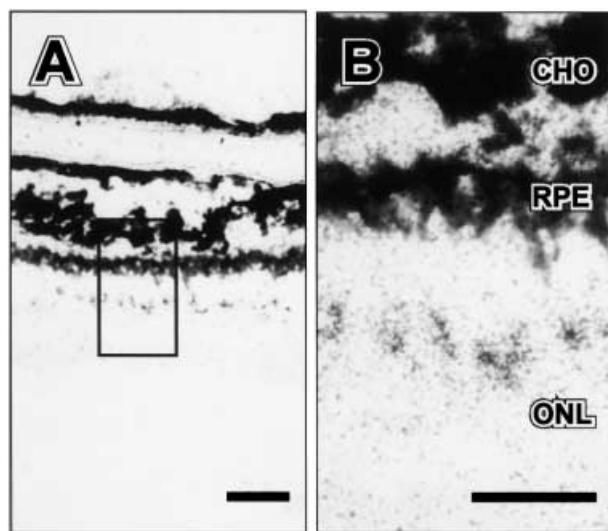


Figure 6 Localization of POMC mRNA by *in situ* hybridization in the eye of a 3-day-old Brown Leghorn chick. Light micrographs at lower ($\times 20$) (A) and high ($\times 80$) (B) magnification are presented. Positive signals (densely clustered silver grains) are arranged in a single row in the photoreceptor layer. The choroid (CHO), RPE and outer nuclear layer (ONL) are labeled. Scale bars are 20 μ m (A) and 40 μ m (B).

throughout the body where it acts as a neuroendocrine, paracrine and autocrine local hormone. To obtain insight into the evolution of the α -MSH endocrine system, we have cloned the chicken POMC and AGRP genes, and those of all five MC-R subtypes. Our analyses of the tissue distribution of expression of these genes have revealed their widespread expression throughout the body of the chicken (Takeuchi *et al.* 1996a, 1998, 1999, 2000, Takeuchi & Takahashi 1998, 1999), raising the possibility that the physiological role of α -MSH has been conserved during evolution so that it acts as a paracrine and/or autocrine hormone in birds as it does in mammals. Besides birds, some mammalian species, including humans, also lack the intermediate lobe of the pituitary (Eberle 1988). Based on these observations, we have proposed the hypothesis that most of the functions assigned to α -MSH in higher vertebrates may be modulated by locally produced α -MSH via paracrine and/or autocrine mechanisms *in vivo*. The intermediate lobe of the pituitary may have developed as the primary secretory gland of circulating α -MSH involved in physiological color change in lower vertebrates, and the main physiological roles of the gland might have altered during evolution towards animals bearing feather or hair. α -MSH may be essentially a paracrine and/or autocrine hormone (Takeuchi *et al.* 1999, 2000). The present study provides the first evidence for α -MSH expression in peripheral tissues in birds, and sets the scene for investigation into paracrine and/or autocrine effects of the hormone.

In conclusion, the present study demonstrates for the first time that α -MSH is expressed within the chicken eye, and that its intraocular expression is temporally and spatially regulated during development. Embryonic expression of α -MSH in the RPE cells suggests its role in the development of the eye. This is the first report demonstrating the expression of α -MSH in peripheral tissues in birds, and supports our hypothesis that avian α -MSH is a paracrine/autocrine hormone.

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