Expression pattern and localization of β,β-carotene 15,15′-dioxygenase in different tissues

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β,β-Carotene 15,15′-dioxygenase cleaves β,β-carotene into two molecules of retinal, and is the key enzyme in the metabolism of β,β-carotene to vitamin A. The enzyme has been known for more than 40 years, yet all attempts to purify the protein to homogeneity have failed. Recently, the successful cloning and sequencing of an enzyme with β,β-carotene 15,15′-dioxygenase activity from chicken, as well as from *Drosophila*, has been reported. Here, we describe in detail our attempt to enrich the chicken β,β-carotene 15,15′-dioxygenase to such an extent as to allow determination of partial amino acid sequences, which were then used to design degenerate oligonucleotides. Screening of a chicken duodenal expression library yielded a full-length clone containing a coding sequence of 1578 bp. Functional expression in *Escherichia coli* and in eukaryotic cell lines confirmed that we had cloned the first vertebrate dioxygenase that cleaves β,β-carotene at the central 15,15′-double bond. By performing a sequence homology search, the cDNA sequence of the mouse homologue was found as an expressed sequence tag (EST) in the gene bank. At the amino-acid level, the degree of homology between the chicken and mouse sequences is 81 %. Thus β,β-carotene 15,15′-dioxygenase can be considered as being an enzyme that is evolutionarily rather well conserved. We established the expression pattern of β,β-carotene 15,15′-dioxygenase in chicken and mouse tissues with a combination of Northern blots and *in situ* hybridization. The mRNA for β,β-carotene 15,15′-dioxygenase was localized primarily in duodenal villi, as well as in liver and in tubular structures of lung and kidney. These new findings demonstrate that β,β-carotene 15,15′-dioxygenase is also expressed in epithelial structures, where it serves to provide the tissue-specific vitamin A supply.

Key words: central cleavage, epithelial cell expression, retinal, vitamin A.

INTRODUCTION

Carotenoids are a large family of natural pigments [1,2], of which 10 % display provitamin A activity, β,β-carotene being the most important one for animal and human nutrition. Carotenoids serve two essential functions: first, as accessory pigments in the light-harvesting system of photosynthetic organisms [3], and, secondly, in photoprotection from light of short wavelength [4,5]. The characteristic polyene structure of carotenoids is responsible for light absorption, as well as for singlet oxygen quenching or inactivation of 'aggressive' free radicals [6,7]. In the human diet, it has been estimated that up to 80 % of the daily vitamin A intake is derived from the provitamin A carotenoids in fruits and vegetables. Malnutrition in African and Asian countries leads to severe vitamin A deficiency among children [8]. Night blindness and xerophthalmia, as well as a higher susceptibility to infectious diseases, are the result. The benefits of vitamin A and β,β-carotene supplementation to combat vitamin A deficiency have been demonstrated in several studies [9–11,11a].

The β,β-carotene 15,15′-dioxygenase (EC 1.13.11.21) cleaves β,β-carotene into two molecules of retinal, and is the key enzyme in metabolism of carotene to vitamin A. Other carotenoids containing at least one unsubstituted β-ionone ring and a polyene chain attached to it also serve as substrates for the enzyme and, therefore, as precursors of retinoid formation [12]. However, using β,β-carotene as substrate resulted in the highest specific activity when various carotenoids and apo-carotenals were compared [13].

The enzyme was first isolated in the mid-1960s [14,15]. Subsequent experiments suggested a dioxygenase reaction mechanism [16], and the presence of a cofactor containing iron [17,18]. Thus the enzyme was termed carotene dioxygenase, although sound evidence for the detailed reaction mechanism is still lacking. Since then, many attempts have been made to purify and characterize this enzyme by biochemical means [15,17–21]. Experiments by Goodman et al. [16,19] and Fidge et al. [17] showed that β,β-carotene 15,15′-dioxygenase is a soluble cytosolic enzyme. Sklan [22] reported that enzyme activity is associated with a high-molecular-mass lipid–protein aggregate fraction. Highest dioxygenase activity was found in intestinal mucosa and in jejunal enterocytes, with a gradient of decreasing activity from the duodenum/jejunum to the colon [23,24]. In liver, lung, kidney and brain, β,β-carotene 15,15′-dioxygenase activity was also detected [25]. In addition, the mode of cleavage of β,β-carotene has been discussed controversially for many years [26]. Although Glover proposed 'excentric' cleavage as long ago as 1960 [27], the central cleavage pathway was widely accepted as the only metabolic pathway until Wang et al. [28–30] provided evidence for production both *in vivo* and *in vitro* of apocarotenals and retinoic acid as main products of β,β-carotene cleavage. Nagao et al. [31] found stoichiometric formation of retinal with a ratio of 1.9:1, thus presenting evidence for the central cleavage pathway.

Recently, two reports describing the cloning of β,β-carotene 15,15′-dioxygenase have been published [32,33]. von Lintig and Vogt [32] cloned an enzyme displaying β,β-carotene 15,15′-

Abbreviations used: AP, alkaline phosphatase; BHK, baby hamster kidney; BHT, butylated hydroxytoluene; DTT, dithiothreitol; EST, expressed sequence tag; IEC, ion-exchange chromatography; poly(A)$^+$, polyadenylated; RT, reverse transcriptase; TBME, t-butyl methyl ether.

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dioxygenase activity from *Drosophila melanogaster*, while our group [33] published on the cloning of the chicken enzyme. In the present article, we describe in detail our attempts to purify β,β-carotene 15,15′-dioxygenase from extracts of chicken intestinal mucosa, to determine partial amino acid sequences, and to use this information to obtain cDNA sequences for the chicken and mouse β,β-carotene 15,15′-dioxygenase. In addition, we describe both the levels and pattern of expression in various chicken and mouse tissues.

EXPERIMENTAL

Enrichment of β,β-carotene 15,15′-dioxygenase from chicken intestinal mucosa [14]

Upon preparation, the duodenum samples were kept on ice or at 4 °C until further use. For isolation of mucosa, chicken (strain LSL Lohmann) intestines were thawed and opened in a Petri dish. The mucosa was scraped off, weighed and suspended in 4 ml of buffer A [100 mM KH_2PO_4/4 mM $MgCl_2$/30 mM nicotinamide (pH 7.8), containing 4 % protease-inhibitor cocktail (5.9 mM benzamidine/HCl, 10 mM 6-aminohexanoic acid and 5 μM soya-bean trypsin inhibitor)] per g of mucosa. The suspension was homogenized with a Potter–Elvehjem homogenizer. After centrifugation at 62000 ***g*** for 1 h, the supernatant was subjected to 20–45 % ammonium sulphate fractionation. The pellet obtained was resuspended in buffer B [10 mM KH_2PO_4/1 mM GSH/0.5 M ammonium sulphate (pH 7.8)] and loaded on a HiLoad 26/10 Phenyl Sepharose High Performance column (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear gradient with buffer C [10 mM KH_2PO_4/1 mM GSH/10 % (v/v) glycerol (pH 7.8)], and activity-containing fractions with a conductivity of < 1 mS/cm were loaded on a 30 ml Blue Sepharose 6 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with buffer C. Dioxygenase activity was recovered in the breakthrough fractions, which were loaded on a 20 ml Poros HQ/M column (PerSeptive Biosystems, Framingham, MA, U.S.A.), also equilibrated with buffer C. β,β-Carotene 15,15′-dioxygenase was eluted with a linear gradient of buffer D [10 mM KH_2PO_4/1 mM GSH/0.5 M NaCl/10 % (v/v) glycerol (pH 7.8)] and recovered in fractions with a conductivity of 10–20 mS/cm. The combined activity-containing fractions were concentrated 40–50-fold in Ultrafree-15 filter units (Millipore, Bedford, MA, U.S.A.), and aliquots of 500 μl were passed over a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech), with buffer E [50 mM KH_2PO_4/1 mM GSH/150 mM NaCl/10 % glycerol (pH 7.8)] as the eluent.

Amino acid sequencing

Multiple aliquots of activity-containing fractions from the final gel-filtration run were separated on a 10 % Tris/glycine gel (Novex, Invitrogen Corp., Carlsbad, CA, U.S.A.). The two protein bands that correlated most closely with β,β-carotene 15,15′-dioxygenase activity were subjected directly to Edman sequencing, or were excised and digested in-gel with trypsin. The tryptic digest was separated by micro-bore reversed-phase HPLC on a 150 mm × 1.0 mm Vydac C18 column (Vydac, Hesperia, CA, U.S.A.). Peptides were eluted with an acetonitrile gradient in 0.1 % (v/v) trifluoroacetic acid, and peptide-containing fractions were collected for further analysis by matrix-assisted laser-desorption ionization–time of flight mass spectrometry ('MALDI–TOF-MS') (Voyager Elite, PerSeptive Biosystems). Two fractions contained one single peptide each, which were sequenced by Edman degradation.

Construction of a chicken duodenal cDNA library

Chickens were killed at 22 weeks of age. The duodenum was removed, washed and cut open. The mucosal layer was scraped off, weighed and homogenized immediately in Trizole reagent (Life Technologies, Rockville, MD, U.S.A.). For total RNA isolation, the standard protocol from Life Technologies was followed. Polyadenylated [poly(A)$^+$] RNA was isolated with the polyATract mRNA isolation kit from Promega (Madison, WI, U.S.A.).

cDNA was prepared from chicken duodenal poly(A)$^+$ RNA with the Copy Kit (Invitrogen) using a polyT/*Not*I primer and a modified Gubler–Hoffman procedure. *Bst*XI linkers were ligated, the cDNA size was selected (0.9–5.5 kb) and subsequently cloned into the *Bst*XI/*Not*I site of the eukaryotic expression vector pcDNA1.1/Amp (Invitrogen). Electroporation into *Escherichia coli* Top10 was accomplished with a Gene Pulser II system (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and this resulted in the generation of a cDNA library of 430000 individual clones.

PCR and reverse transcriptase (RT)-PCR

Two degenerate primers, the degeneracy of which was reduced by using inosine (I), were designed from the peptide sequence Asn-Lys-Glu-Glu-His-Pro-Glu-Pro-Ile-Lys-Ala-Glu-Val-Gln-Gly-Gln-Leu-Pro: 5′-primer, 5′-AAC AAR GAR GAS CAY CCI GA-3′ (20-mer with a degeneracy of × 16); 3′-primer, 5′-SAG CTG ICC CTG IAC YTC SGC-3′ (21-mer with a degeneracy of × 8), where R = A or G, S = C or G, and Y = C or T.

For PCR, 100 ng of chicken duodenal cDNA were taken as the template, and the following PCR steps were performed (with 40 cycles): incubation at 94 °C for 30 s; at 52 °C for 30 s; and at 72 °C for 1 min. The resulting 51 bp fragment was excised from a 10 % TBE/polyacrylamide gel (where 1 × TBE = 45 mM Tris/borate/1 mM EDTA), electroeluted, cloned into pGEM-T Easy (Promega), and then sequenced. From this DNA sequence, a homologous forward primer was derived: 5′-TCTGAATTCC-GGAGCCCATAAAAGC-3′ ('dioxy12'). At the 5′-end an *Eco*RI site (underlined sequence) was included; the subsequent 17 nt are homologous with the previously determined dioxygenase sequence. RT-PCR was performed with a polyT/*Not*I reverse primer (Invitrogen) and with forward primer dioxy12 by using the Titan One Tube RT-PCR kit from Roche Molecular Biochemicals (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions, and by applying the following cycles: 50 °C for 30 s, then 94 °C for 2 min; 10 cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 45 s; then 25 cycles at 94 °C for 30 s, 62 °C for 30 s and 68 °C for 45 s + 3 s/cycle; additional extension at 68 °C for 7 min, then at 4 °C overnight. The resulting 597 bp cDNA fragment was purified on an agarose gel, isolated with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced.

Screening of the chicken duodenal cDNA library

Pools from the original library were plated on agar. Corresponding master and replica filters (nylon membranes; Gene Screen, NEN Life Science Products, Inc., Boston, MA, U.S.A.) were processed and hybridized with the radioactively labelled ([α-^{32}P]dCTP; Amersham Pharmacia Biotech) 597 bp PCR fragment using 500000 c.p.m./ml Church–Gilbert hybridization solution. Filters were washed with 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at room temperature for 20 min,

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and then with 0.1 × SSC/0.1 % (w/v) SDS at room temperature for 20 min, followed by exposure to X-OMAT AR films (Eastman Kodak, Rochester, NY, U.S.A.). Double-positive clones were identified, picked and sequenced on an automated fluorescence-based Vistra DNA sequencer 725 (Amersham Pharmacia Biotech).

Functional expression in eukaryotic cell lines with the Semliki Forest Virus system

The coding sequence of the β,β-carotene 15,15′-dioxygenase cDNA was amplified by PCR, using, as the 5′-primer, 5′-GAGCTAGATCTAGAACGATGGAGACAATATTTAACAGAAAC-3′ (where the *Bgl*II site is underlined) and as the 3′-primer, 5′-CAAGTCTCGAGTTAATGATGATGATGATGATGTTCCGTCTCAGCCCCCAAATCATTC-3′ (where the *Xho*I site is underlined and the His_6 tag is double-underlined). The resulting fragment of 1608 bp was cloned into the *Bam*HI/*Xho*I site of the plasmid $pSFV_2$gen (a gift from Dr K. Lundstrom, F. Hoffmann–La Roche Ltd, Basel, Switzerland). This plasmid was used for *in vitro* synthesis of recombinant RNA by combining 2.5 μg of linearized DNA with 5 μl of 10 × SP6 buffer [400 mM Hepes/KOH (pH 7.4)/60 mM magnesium acetate/20 mM spermidine/HCl], 5 μl of 10 mM CAP [m7G(5′)ppp(5′)G; Amersham Pharmacia Biotech], 5 μl of 50 mM dithiothreitol (DTT), 5 μl of rNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP and 5 mM GTP), 1.5 μl of RNase inhibitor (50 units/μl; Roche Molecular Biochemicals), 3.5 μl of SP6 RNA polymerase (30 units/μl; Amersham Pharmacia Biotech) and 20 μl of water. Incubation was performed at 37 °C for 60 min. Subsequently, a Gene Pulser (Bio-Rad) was used to electroporate the RNA, together with a helper virus RNA, into baby hamster kidney (BHK) cells. The cells were incubated for 24 h at 37 °C/5 % CO_2/95 % humidity, before the medium was filtered through a 0.22 μm membrane (Millex-GP; Millipore). This high-titre virus stock was activated with chymotrypsin (Roche Molecular Biochemicals), and was used to infect the mammalian cell lines HuTu80, CHO (Chinese Hamster Ovary cells) and BHK. After adding protease-inhibitor cocktail, cell extracts were prepared by two cycles of freezing and thawing, and by sonicating the suspension for 5 min.

Dioxygenase activity assay

All work with carotenoids and retinoids was performed in the absence of light and under nitrogen. Measurements of activity were determined according to the method of During et al. [25,34].

Sample preparation

To the assay buffer (0.15 mM Tricine, pH 8.0), 3.0 mg/ml of GSH and 0.21 mg/ml sodium cholate were added before use. The protein pellets [$(NH_4)_2SO_4$ precipitates from intestinal mucosa] were thawed on ice, and then resuspended in cold assay buffer by gentle vortex-mixing at a protein concentration of approx. 10 mg/ml. The suspension was centrifuged at 20000 ***g*** for 10 min at 4 °C, and aliquots of the supernatant were used for the incubation with the substrate. Fractions of recombinant protein were dialysed against assay buffer containing 0.5 mM $FeSO_4$, before activity was determined.

Substrate preparation

Before each assay, β,β-carotene was purified over an Alox (CAMAG Ltd, Muttenz, Switzerland) column: ≈ 1.0 g of Alox (inactivated with 100 μl of water) was poured into a Pasteur pipette and washed with hexane. Of the β,β-carotene stock solution [26.9 mg of all-*trans*-β,β-carotene and 5 μl of α-tocopherol stock solution (10 μmol/ml in hexane) dissolved in 5.0 ml of benzene], 40 μl was poured on to the top of the column, and the hexane wash was initiated. The coloured band was eluted into a 5 ml Pyrex vial containing 50 μl of α-tocopherol stock solution and 200 μl of Tween 40 stock solution (400 μl in 10 ml of acetone). The solvent was evaporated with N_2 at 45 °C. Water (1 ml at 37 °C) was added, and the substrate was solubilized with gentle vortex-mixing. The solution was kept at 37 °C in the dark to avoid crystallization. The concentration of the substrate solution was determined at 452 nm in propan-2-ol (ϵ_{mol} 118300 $M^{-1} \cdot cm^{-1}$).

Each sample was assayed in duplicate. In each assay series, a blank sample and a micellar solution of retinal were included as controls. Assay buffer [0.15 mM Tricine (pH 8.0), including 3.0 mg/ml of GSH and 0.21 mg/ml sodium cholate] (200 μl) and 100 μl of enzyme solution (≈ 1 mg of protein) were placed in an Eppendorf tube and pre-incubated at 37 °C for 5 min. The reaction was started by addition of 75 μl (≈ 18.8 nmol) of the β-carotene substrate solution described above. After incubation for 60 min at 37 °C in the dark with shaking (1 s^{-1}), the reaction was stopped by adding 25 μl of formaldehyde. After an additional incubation period of 10 min, 10 μl of butylated hydroxytoluene (BHT) (0.1 mg/ml in ethanol) was added as protectant to each tube, as well as 10 μl of a Ro11–1430 [$C_{23}H_{31}NO_2$; all-*trans*-*N*-ethyl-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenamide] retinoid stock solution (0.213 mg/ml in ethanol; F. Hoffmann–La Roche Ltd) as an internal standard. Each tube was vortex-mixed, and for detergent removal, transferred quantitatively to a 1 ml Extralut column (Merck, Dietikon, Switzerland). The column was rinsed with 250 μl of a NaCl solution (0.4 g/ml), and was then flushed with 3.0 ml of t-butyl methyl ether (TBME), followed by a second elution with 2.5 ml of TBME. Solvents were evaporated under a gentle stream of N_2 at 37 °C.

Of extraction mixture [ethanol/tetrahydrofurane/TBME (9:1:5, by vol.), including 0.025 % BHT], 200 μl was added and the sample was mixed while slowly turning the tube. A 10 μl aliquot was then analysed on a reversed-phase C_{18} column [Zorbax ODS, Stagroma, Reinach, Switzerland; 250 mm × 4 mm; 1.5 ml/min; isocratic elution with a mixture of methanol and 80 mM ammonium acetate in water (9:1, v/v)]. Retinal was detected at 380 nm, whereas retinol was determined with a fluorescence detector (excitation at 325 nm, emission at 470 nm). In case analysis of β-apocarotenal was required, an aliquot of the sample was additionally injected into a Vydac 218TP54, 250 mm × 4 mm HPLC column [Paul Bucher AG, Basel, Switzerland; 1.5 ml/min; elution with acetonitrile/TBME/(80 mM ammonium acetate in water)/triethylamine, 73:20:7:0.05, by vol.]. A UV–visible light detector was used for detection at 450 nm.

Northern blot analysis

Total RNA from chicken duodenum was separated on a 1 % formaldehyde/agarose gel in 1 × Mops buffer. The RNA gel was vacuum-blotted with 20 × SSC on a Zeta-Probe membrane (Bio-Rad) for 4 h. The membrane was cross-linked with a UV-Stratalinker (Stratagene, La Jolla, CA, U.S.A.) and pre-hybridized for 1 h in 50 % (v/v) formamide, 5 × SSC, 5 × Denhardt's solution (where 1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA), 1 % SDS and 100 μg/ml denatured herring sperm DNA.

As a probe, a part of the coding sequence of β,β-carotene 15,15′-dioxygenase was amplified by PCR (5′-primer, 5′-GGT-

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Table 1 Summary of the various steps in the purification of β,β-carotene 15,15′-dioxygenase from three chicken intestines

Shown are the means for 3–4 measurements.

Purification step	Total protein (mg)	Total activity (nmol/h)	Yield (%)	Specific activity (pmol · h^{-1} · mg^{-1})	Purification factor
$(NH_4)_2SO_4$ pellet	779	8.61	100	11.0	–
Phenyl-Sepharose	80.8	8.27	96.1	102	9.27
Blue Sepharose	16.0	8.86	103	554	50.1
Poros HQ	1.56	3.90	45.3	2500	226

ACTTCAATTGTTGATAAAGG-3′; 3′-primer, 5′-TTCTGTT-GCATAGACATACTTG-3′) and purified over a S-400 spin column (Amersham Pharmacia Biotech). Denatured DNA (25 ng) was labelled with 50 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; Amersham Pharmacia Biotech) using the High Prime random labelling kit from Roche Molecular Biochemicals. The labelled DNA probe was purified over a S-200 spin column (Amersham Pharmacia Biotech). Hybridization was performed overnight at 42 °C with 7×10^5 c.p.m./ml. Filters were washed with 2 × SSC for 15 min at room temperature, with 0.1 × SSC/0.1 % SDS for 15 min at room temperature, and, when required, with 0.1 × SSC/0.1 % SDS for 15 min at 65 °C, and were then exposed overnight on X-OMAT AR films (Eastman Kodak) at −80 °C with double intensifying screens.

The mouse multi-tissue Northern blot was purchased from Clontech. The probe was labelled as described above. Hybridization was performed using 1×10^6 c.p.m./ml in ExpressHyb hybridization solution (Clontech), according to the protocol supplied.

In situ hybridization

Chickens at an age of 18 weeks were fed with a low vitamin A diet for 4 weeks. Before killing (4 days), the animals were given daily 20 μmol of the RARα antagonist Ro41–5253 ($C_{28}H_{36}O_5S$; p-{(*E*)-2-[3′,4′-dihydro-4′,4′-dimethyl-7′-(heptyloxy)-2′H-1-benzothiopyran-6′-yl]propenyl}benzoic acid 1′,1′-dioxide; F. Hoffmann–La Roche Ltd) in order to up-regulate the β,β-carotene 15,15′-dioxygenase mRNA. All *in situ* hybridization experiments were performed at frimorfo Ltd (Fribourg, Switzerland) according to the method of Fleming et al. [35]. Tissues were fixed immediately in 4 % paraformaldehyde, and stored in a 5 % sucrose/PBS solution for cryosections. The tissues were then frozen at −20 °C, and sectioned at 10 μm using a Microm HN 505 M Cryostat.

For preparation of the hybridization probe, a 1120 bp *Pst*I fragment from the coding region of the cDNA was subcloned into pBSKII (Stratagene), and both the antisense and the sense RNA probes were synthesized using the *in vitro* transcription kit from Roche Molecular Biochemicals. Hybridization of sections was performed overnight at 50 °C with 400 ng of digoxygenin-labelled RNA probe per ml of hybridization buffer [300 mM NaCl/10 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 × Denhardt's solution/10 % dextran sulphate/30 mM DTT/50 % formamide/200 μg/ml tRNA/200 μg/ml single-stranded DNA]. After a first wash step with 4 × SSC (2 × 30 min), the sections were treated with RNase in 0.5 M NaCl/10 mM Tris/HCl, pH 7.5/1 mM EDTA. Washing steps were performed consecutively with 2 × SSC, 0.1 × SSC/1 mM DTT and 0.1 % Tween 20 in PBS. Incubation with an α-digoxygenin/alkaline phosphatase (AP) antibody (1:500 dilution) was performed for 2 h in blocking buffer (10 % BSA/0.1 % Tween 20 in PBS). The slides were rinsed with 0.1 % Tween 20/PBS and then incubated with 5 % levamisole in AP buffer (100 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) for 5 min. Incubation with the AP substrate Nitro-Blue Tetrazolium chloride/5-bromo-4-chloro-3′-indoly-phosphate p-toluidine salt ('NBT-BCIP') was performed overnight in AP buffer containing 5 mM levamisole. Finally, the slides were washed with 96 % ethanol until the background staining disappeared.

RESULTS

Enrichment of β,β-carotene 15,15′-dioxygenase, identification of peptide sequences, and cloning and sequencing of its cDNA

Starting with a cytosolic fraction from chicken intestine, we have used a fast purification protocol encompassing ammonium sulphate fractionation, hydrophobic interaction chromatography, Blue Sepharose chromatography, ion-exchange chromatography (IEC) and gel filtration. Hydrophobic interaction chromatography, Blue Sepharose chromatography and IEC allowed a 226-fold enrichment of enzyme, with 45 % recovery, yielding a preparation with a specific activity of 2.5 nmol · h^{-1} · mg $protein^{-1}$ (Table 1). However, gel filtration and SDS/PAGE of the concentrated IEC eluate demonstrated that the final preparation was far from being pure.

Since we failed to achieve a better standard of purification at that time, an indirect approach had to be chosen in order to obtain amino acid sequence information concerning β,β-carotene 15,15′-dioxygenase. When the concentrated IEC eluate was subjected to gel filtration, β,β-carotene 15,15′-dioxygenase activity was eluted at a molecular mass of approx. 50 kDa. SDS/PAGE analysis of the fractions revealed that out of ≈ 15 discrete protein bands, only two correlated reasonably well with β,β-carotene 15,15′-dioxygenase activity (see [33]). Edman degradation of the lower band yielded a sequence of 19 amino acids demonstrating homology with a thioesterase ([36]; GenBank/EMBL accession no. L05493) that was considered to be unlikely to display β,β-carotene 15,15′-dioxygenase activity. Since the second band was N-terminally blocked, the protein was excised from a gel and subjected to in-gel trypsin digestion. Upon separation of the tryptic peptides by micro-bore reversed-phase HPLC, the following two overlapping sequences were obtained by Edman degradation: (1) Ala-Glu-Val-Gln-Gly-Gln-Leu-Pro; and (2) Asn-Lys-Glu-Glu-His-Pro-Glu-Pro-Ile-Lys-Ala-Glu-Val-Gln-Gly-Gln-Leu-Pro.

Degenerate oligonucleotides were derived from these sequences and were used for PCR amplification of a 51 bp DNA fragment within the longer peptide. This new sequence served as a template for the synthesis of a homologous primer. The RT-PCR fragment of 597 bp obtained with this primer together with a polyT primer was radioactively labelled and used for the screening of a chicken duodenal expression library. The isolated full-length cDNA for β,β-carotene 15,15′-dioxygenase (EMBL accession no. AJ271386) has a length of 3.1 kb. The coding sequence spans 1578 bp and encodes a protein of 526 amino acids (Figure 1).

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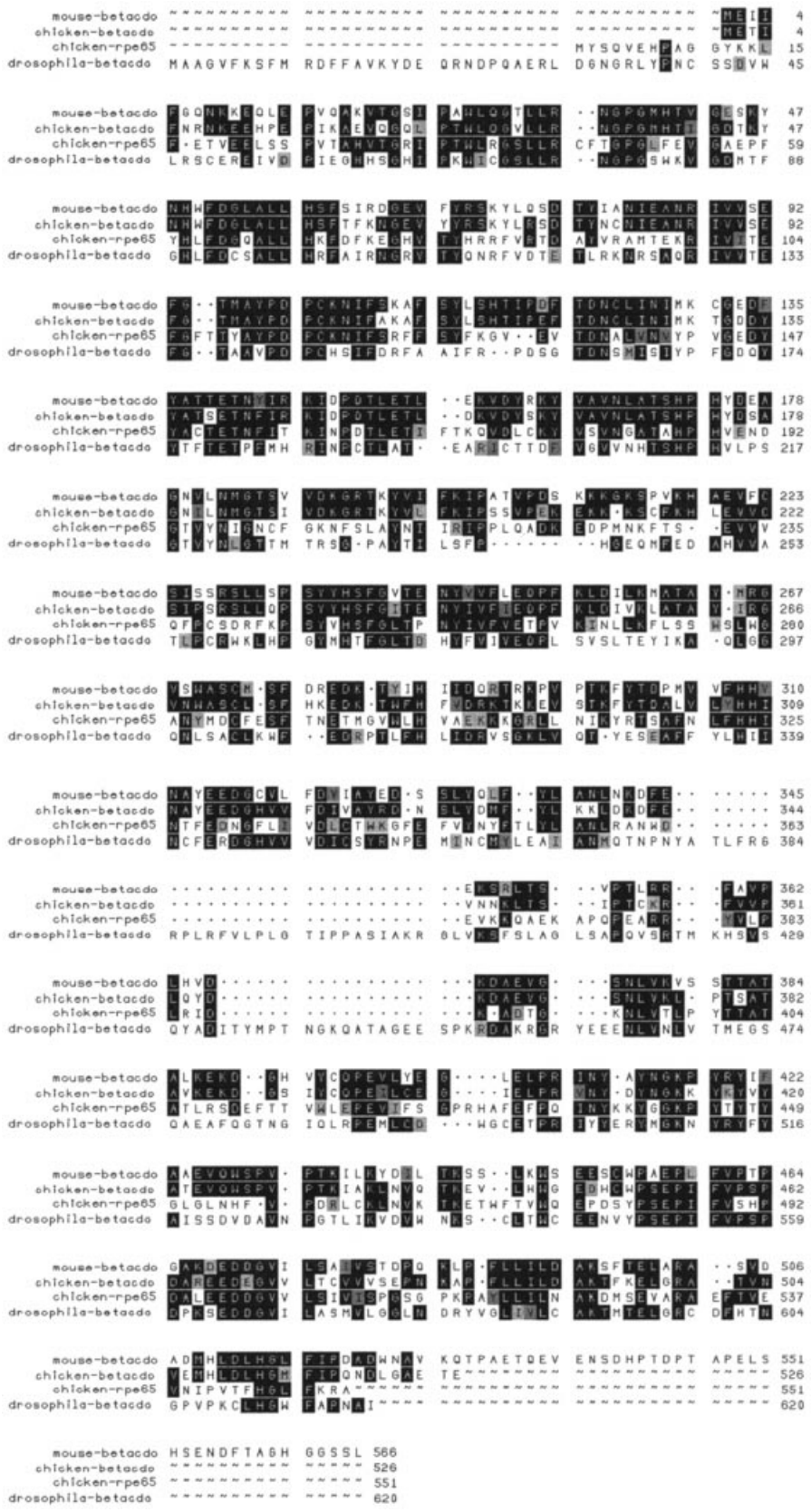

Figure 1 PRETTYBOX alignment of the β,β-carotene 15,15′-dioxygenase amino acid sequences of mouse, chicken and *D. melanogaster* with chicken RPE65

Identical amino acids are shown in black; similar amino acids are in grey. The alignment was performed with the GCG software package using the default matrix. The homology between the chicken and the mouse sequence is 81%; the *Drosophila* β,β-carotene 15,15′-dioxygenase shows 50% homology with the chicken, and 48% with the mouse, sequences. betacdo, β,β-carotene 15,15′-dioxygenase.

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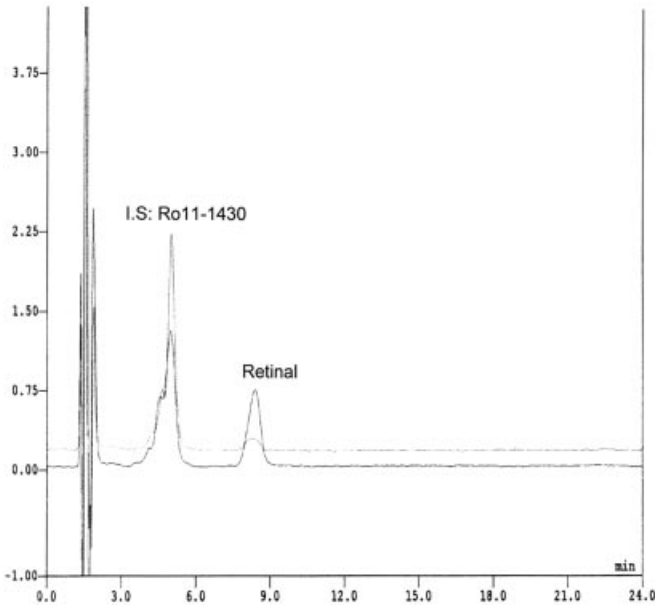

Figure 2 HPLC chromatogram overlay confirming the product from the β,β-carotene 15,15′-dioxygenase activity assay

The upper chromatogram represents a sample from an assay using recombinant β,β-carotene 15,15′-dioxygenase, whereas the lower chromatogram represents the same sample spiked with a retinal standard. As an internal standard, the retinoid Ro11–1430 [$C_{23}H_{31}NO_2$; all-*trans*-*N*-ethyl-9-(4-methoxy-2,3,6-trimethyl-phenyl)-3,7-dimethyl-2,4,6,8-nonatetraenamide] was used. The only peak detected with this sample was thereby confirmed as retinal.

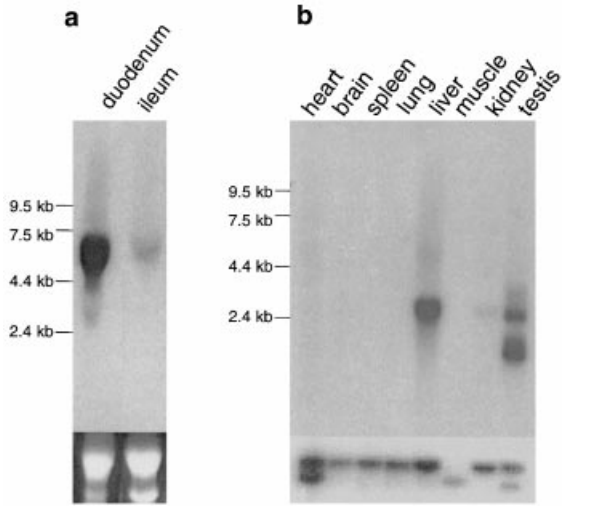

Figure 3 Northern blot of total RNA from chicken duodenal mucosa and ileum (a), and a mouse multi-tissue Northern blot using poly(A)$^+$ RNA from eight different tissues (b)

(**a**) Below the Northern blot, the RNA gel shows that similar amounts of RNA were loaded. The size of the message was estimated to be about 5.5 kb. (**b**) In three of the tissues, namely liver, kidney and testis, positive signals were obtained. The blot was also hybridized with a mouse β-actin probe (lower picture) to show the amounts of mRNA loaded.

The cDNA reveals a high similarity of β,β-carotene 15,15′-dioxygenase to RPE65 [37,38], a retinal pigment epithelium-specific protein of, at present, unknown function. By searching the EMBL gene bank, we identified a mouse expressed sequence tag (EST) (EMBL accession no. AW044715) containing the putative mouse homologue of β,β-carotene 15,15′-dioxygenase (resubmitted; EMBL accession no. AW278064). The regions of amino acids 97–108 and 132–155 are almost identical and, therefore, have been highly conserved during evolution. In addition, von Lintig and Vogt [32] have recently cloned and expressed the cDNA for the *Drosophila* enzyme displaying β,β-carotene 15,15′-dioxygenase activity. A PRETTYBOX alignment is shown in Figure 1. The chicken and mouse β,β-carotene 15,15′-dioxygenases show highest homology (81 % on the amino acid level), whereas the *Drosophila* β,β-carotene 15,15′-dioxygenase sequence shows 50 % homology with the chicken, and 48 % with the mouse, amino acid sequences (Figure 1). Although the *Drosophila* protein has two additional stretches (amino acids 1–30 and 378–406), the rest of the sequence is very similar to the vertebrate dioxygenase sequences.

Functional expression in eukaryotic cells

Chicken β,β-carotene 15,15′-dioxygenase was overexpressed in CHO cells. For this purpose, the coding sequence of β,β-carotene 15,15′-dioxygenase was amplified by PCR, and the resulting fragment was cloned into the plasmid pSFV$_2$gen. This vector is part of the Semliki Forest Virus expression system [39], which can be used successfully with most mammalian cell types. The plasmid was used for production of a high-titre virus stock, and subsequently CHO cells were infected. The recombinant protein was purified using a Co^{2+}-chelate affinity column, and the protein-containing fractions were dialysed against assay buffer and tested for β,β-carotene 15,15′-dioxygenase activity (Figure 2). When β,β-carotene was used as substrate, retinal was detected as the only reaction product. The resultant peak was identified by co-chromatography with a retinal standard solution (Figure 2), as well as by liquid chromatography–MS (results not shown). On the other hand, HPLC analysis revealed no β-apo-carotenals or other metabolites (results not shown).

Northern blotting

β,β-Carotene 15,15′-dioxygenase is expressed primarily in the digestive tract of chicken (Figure 3a). Highest expression was found in the duodenum, whereas in the ileum, the mRNA level was markedly lower. From the chicken Northern blot, the size of the message was estimated to be approx. 5.5 kb. On a mouse multi-tissue Northern blot with eight poly(A)$^+$ RNAs (Figure 3b), expression of β,β-carotene 15,15′-dioxygenase in three additional tissues was confirmed. In liver, quite a strong signal appeared, whereas expression in kidney was only marginal. No expression was observed in lung. Most interestingly, three distinct bands were clearly identified in mouse testis: a message of $\approx$ 2.4 kb, a fainter band at $\approx$ 2 kb and the most prominent band, at $\approx$ 1.7 kb. Whether these three bands represent alternative splicing products of a single gene, or three distinct β,β-carotene 15,15′-dioxygenase genes, remains to be established. If the occurrence of particular isoenzymes of β,β-carotene 15,15′-dioxygenase in testis can be confirmed, this might point to an important function of this enzyme during maturation of germ cells.

In situ hybridization

To investigate the expression pattern of β,β-carotene 15,15′-dioxygenase, we performed *in situ* hybridization on 10 μm

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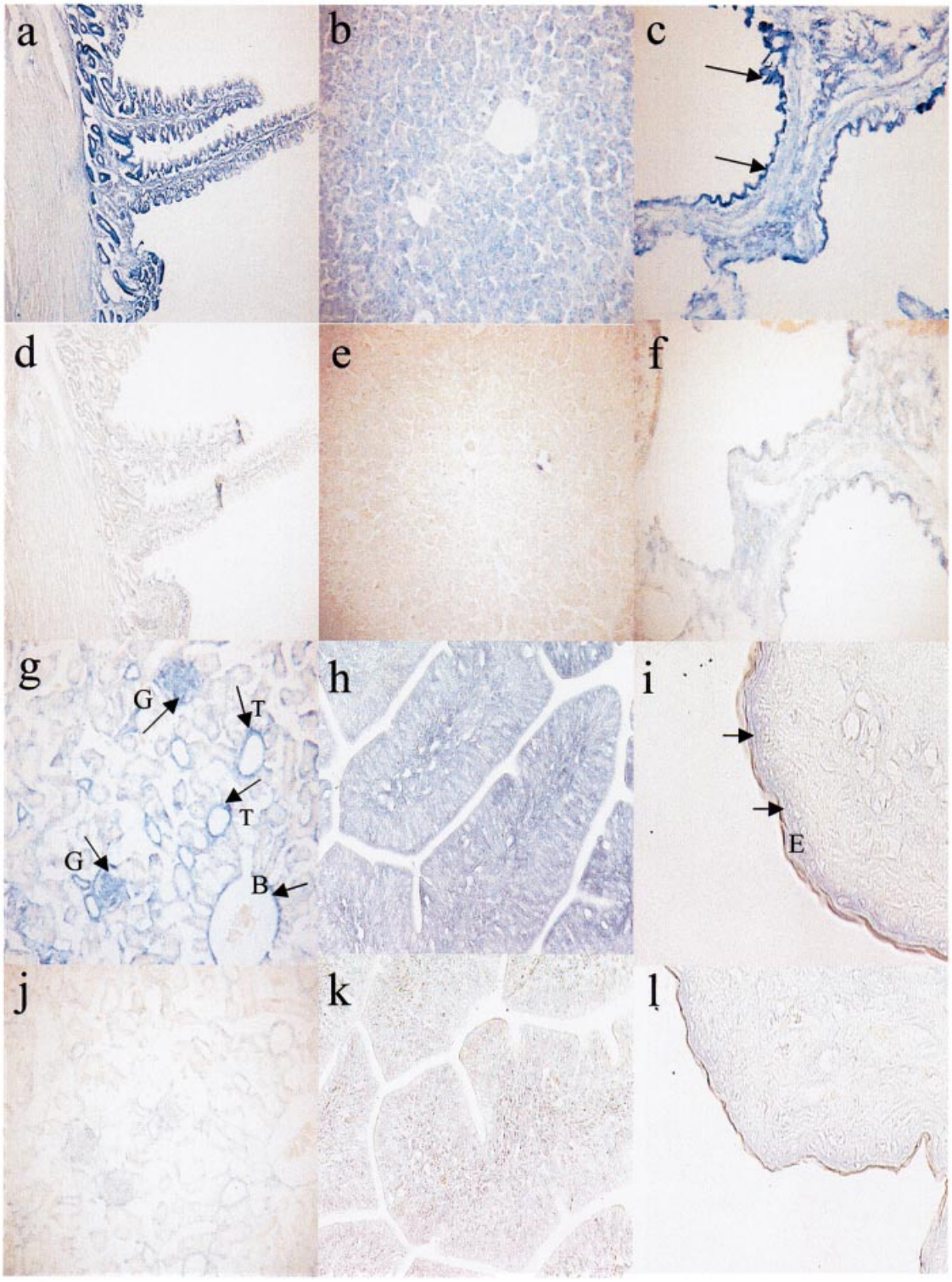

Figure 4 ***In situ*** **hybridization on chicken cryosections**

Shown are six *in situ* hybridizations, with the antisense probe (**a**–**c**, **g**–**i**), and, below, the respective control experiments with the sense RNA probe [**d**–**f** (corresponding to **a**–**c**) and **j**–**l** (corresponding to **g**–**i**)]. All sections are magnified $\times 100$, except for skin (**i** and **l**), which is shown at a magnification of $\times 400$. Strong signals were obtained in epithelial cells of duodenal crypts and villi (**a**). A diffuse positive signal was detected in liver hepatocytes (**b**). In lung (**c**), the epithelial cells of bronchioles were strongly stained. In the control sections (**f**), a slight background staining, as well as some substrate precipitates, were observed. The epithelial layers of glomeruli (labelled 'G') were strongly stained in the kidney cryosection (**g**). Most of the tubuli (T) were only weakly positive; some, however, gave rise to a stronger signal. The endothelium of blood vessels (B) was also stained in kidney. In ileum (**h**), a homogeneous expression was detected, whereas in skin (**i**) only the epidermis (E) gave a positive signal when compared with the control (**l**).

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cryosections from chicken tissues. In duodenum (Figure 4a), the epithelial cells of the crypts and villi gave rise to a strong signal. Stromal cells in the villi were also positively stained. These signals might arise, at least in part, from cells involved in the immune response (lymphocytes and plasma cells). Further identification as to whether these cells are immune cells has yet to be performed.

In liver (Figure 4b), a diffuse signal was detected in hepatocytes. Compared with the control staining with the sense probe (Figure 4e), these signals were considered to be clearly positive. The possibility that other cell types are also stained cannot be excluded. The 'foaminess' of the hepatocyte signal might have been caused by the presence of glycogen in the cytoplasm, or by the preparation of the sections. In lung sections (Figure 4c), a strong signal was seen in epithelial cells of bronchioles. Weaker signals were seen in alveolar walls. In kidney, the endothelial cells of glomeruli and tubuli were positively stained (Figure 4g). In this tissue, the endothelial cells of blood vessels also appeared to be stained. Ileum was positively stained (Figure 4h). In skin, dioxygenase staining was also increased compared with the control section. For all sections, counterstaining with haematoxylin and eosin was performed (results not shown), as were control incubations with a sense RNA probe (Figures 4d–f and 4j–l).

The hybridizations with the sense probe occasionally produced weak unspecific signals, which could be clearly distinguished from the positive signals obtained with the respective antisense probe. In conclusion, in all tissues examined, mainly the epithelial cells of tubular structures expressed β,β-carotene 15,15′-dioxygenase.

DISCUSSION

Since 1965, a number of attempts have been made to purify β,β-carotene 15,15′-dioxygenase. In these studies, purification factors of up to $\times 240$, and specific activities of up to 3.3 nmol of retinal formed/h per mg of protein have been reported. The failure to obtain a more complete purification was hypothesized to be due to an amphiphilic character of β,β-carotene 15,15′-dioxygenase, which might render the protein unstable in aqueous solution [21].

In spite of using a fast purification protocol, encompassing ammonium sulphate fractionation, hydrophobic interaction chromatography, Blue Sepharose chromatography, IEC and gel filtration, our final preparation, with a specific activity of approx. 2.5 nmol $\cdot$ h^{-1} $\cdot$ mg protein^{-1}, was still far from being pure. Several lines of evidence suggest that, in our preparation too, amphiphilic characteristics of β,β-carotene 15,15′-dioxygenase might have prevented further enrichment of the protein: (a) major losses in enzyme activity were observed both during IEC (60 %) and upon 50-fold concentration of the IEC eluate before gel filtration (75 %) (both an increased ionic strength and a higher protein concentration are likely to promote aggregation and, presumably, precipitation of an amphiphilic protein); (b) inclusion of 10 % glycerol in most of the elution buffers considerably stabilized the protein; and (c) incubation of the Blue Sepharose eluate with 1 M ammonium sulphate resulted in 80 % inactivation of β,β-carotene 15,15′-dioxygenase.

Despite the difficulties encountered in protein purification, we obtained sufficient amino acid sequence information to clone the chicken cDNA for this elusive enzyme. The isolated chicken cDNA clone had a length of 3.1 kb, of which 1578 bp are coding for the 60.4 kDa protein. The mouse cDNA had a size of only 2.1 kb, of which 1698 bp are coding for a protein of 63.9 kDa. Since gel-permeation chromatography suggested a molecular mass for the chicken enzyme of $\approx$ 50 kDa, β,β-carotene 15,15′-dioxygenase most likely is a monomeric protein, at least in solution.

Northern blots showed that the mRNA is indeed longer in chicken ($\approx$ 5.5 kb; Figure 3a) than in mouse ($\approx$ 2.4 kb; Figure 3b). The alignment of the protein sequences of β,β-carotene 15,15′-dioxygenase from three different species (chicken, mouse and *D. melanogaster*) with chicken RPE65 (Figure 1) reveals high homology between all these proteins, which form a new family of carotenoid and/or retinoid binding/cleaving enzymes, as also suggested by von Lintig and Vogt [32]. In this family, the membrane receptor for the plasma retinol-binding protein [40] can also be included. Most of the highly conserved regions are located in the N-terminal part of the protein, e.g. a purported amphipathic helix (between amino acids 100 and 120 of the chicken sequence), which is highly conserved in all species examined. These conserved regions might well be important for substrate binding and/or catalysis. Another possibility is that this helix links the protein to the membrane, where the hydrophobic substrates, i.e. β,β-carotene, are normally located after uptake into the cell.

β,β-Carotene 15,15′-dioxygenase was found to be a cytosolic enzyme [17,19]. We also found cleavage activity exclusively in cytosolic fractions, and no β,β-carotene 15,15′-dioxygenase activity was associated with membrane fractions of the eukaryotic expression systems used. In addition, there is no evidence from the amino acid sequence for transmembrane segments of the protein.

Owing to the formation of inclusion bodies and to a high frequency of internal initiation products in *E. coli*, we have chosen the Semliki Forest Virus system and eukaryotic cell lines (BHK, CHO and HuTu80) for overexpression of β,β-carotene 15,15′-dioxygenase. In CHO cells, an expression level of 1–2 mg of soluble and active protein per litre of medium was obtained.

In all of our experiments, retinal was identified as the only reaction product (Figure 2), thereby lending conclusive support for central cleavage of β,β-carotene at the 15,15′-bond. In contrast, neither β-apo-carotenals nor other metabolites were detected by HPLC. In the future, other provitamin A carotenoids will be tested, and the respective reaction products analysed.

Investigation of the expression pattern in different chicken and mouse tissues revealed, as expected, highest expression levels in the duodenum (Figure 3a), the tissue from which the cDNA was cloned. Ileum gave rise to a much weaker signal on Northern blots. Among eight mouse tissues analysed, liver, kidney and testis were positive.

In situ hybridization was used to analyse the expression pattern of β,β-carotene 15,15′-dioxygenase in selected chicken tissues in more detail (Figure 4). In duodenum, the epithelial cells of crypts and villi were positively stained. Some stromal cells were also stained, hypothetically representing T- or B-cells; however, this needs to be elucidated. There is growing evidence that vitamin A plays an important role in immunological processes. Thus the requisite vitamin A demand might be met by the dioxygenase in the corresponding cells and tissues.

In chicken-lung tissue, a clear signal was obtained with *in situ* hybridization on cryosections (Figure 4c); however, on the mouse multi-tissue Northern blot, no band was observed in lung. This could be explained by an interspecies difference of expression, or by the tissue-specific regulation and induction of the β,β-carotene 15,15′-dioxygenase by the RARα antagonist (see the Experimental section). In general, the results from *in situ* hybridization cannot be quantified, but show the localization within a tissue. All differences in expression levels have to be investigated further and confirmed by quantitative RT-PCR,

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which is the most accurate method available for measuring mRNA levels in tissues.

The cloning of the first vertebrate β,β-carotene 15,15′-dioxygenase is an important step forward in terms of acquiring a more detailed knowledge of β,β-carotene and vitamin A metabolism. We have cloned the chicken and mouse β,β-carotene 15,15′-dioxygenases, two new members of the family of carotene-cleaving enzymes, which appear to be highly conserved throughout evolution. Our studies have also demonstrated that many tissues express relatively high levels of this enzyme, leading to the conclusion that additional vitamin A supply has to be met by tissue-specific cleavage of β,β-carotene.

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