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Expression and Regulation of the Gene for Arginase I in Mouse Salivary Glands: Requirement of CCAAT/Enhancer-Binding Protein α for the Expression in the Parotid Gland[1]

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Arginase in salivary glands is potentially involved in the synthesis of proline, glutamate, and polyamines that play specific physiological roles in the glands, and also in depletion of arginine in the oral cavity to protect teeth from microorganisms. We detected protein and mRNA for the type I isoform of arginase in mouse salivary glands. Enzymes of the arginine-biosynthetic pathway were also detected. Immunohistochemical analysis revealed that arginase I was enriched in the striated duct, and was also present in the acinus, demilune and granulated duct. Mice with targeted disruption of the gene for C/EBPα, which is a transcription factor essential for expression of the arginase I gene in the liver, showed dramatically reduced immunoreactivity for arginase I in the parotid gland but not in the submandibular and sublingual glands. Therefore, C/EBPα is specifically required for expression of the arginase I gene in the parotid gland.

Key words: gene targeting, immunohistochemistry, ornithine cycle, transcription factor, urea cycle.

Arginase is an enzyme that catalyzes the hydrolysis of arginine to urea and ornithine [as a recent review see Ref. *1*]. Genes for two arginase isoforms have been identified in mammals. The type I isoform, arginase I, is highly expressed in the liver and involved in detoxification of ammonia as a member of the urea cycle. Arginase I is also present in red blood cells (*2*) and salivary glands (*3, 4*). The type II isoform, arginase II, is expressed mainly in the kidney, small intestine and prostate, and at lower levels in a number of other organs (*5–7*). The role of arginase II in these organs is thought to be the provision of ornithine for the synthesis of polyamines, proline and glutamate (*1*). Both arginase isoforms are induced by treatment with lipopolysaccharide in macrophages, and are possibly involved in the regulation of nitric oxide production (*8–10*).

In salivary glands, arginase has been implicated in the production of proline and glutamate, which are enriched in salivary proteins (*11*), and polyamines that stimulate mucin secretion (*12*), as well as in the reduction of arginine in saliva to repress microorganisms causing dental diseases (*13*). Arginase activity in the submandibular (submaxillary) gland is among the highest in extrahepatic organs (*3*), and has been attributed to the type I isoform (*3*) or both isoforms (*4*), based on electric charge and immunological properties. The detection of protein and mRNA species for the arginase isoforms, and determination of their histological localization in salivary glands remain to be done.

Transcription factors involved in regulation of the gene for arginase I have been characterized, and members of the CCAAT/enhancer-binding protein (C/EBP) family were shown to play critical roles (*14–17*). Studies on mice with disruption of the genes for C/EBP family members indicated that C/EBPα is crucial for expression of the arginase I gene in the liver during the neonatal period (*18*), while C/EBPβ is required for induction of the gene in response to glucocorticoids and/or glucagon in primary-cultured hepatocytes (*19*).

Here, we examined the expression of arginase isoform genes in mouse salivary glands, and detected protein and mRNA for arginase I. Immunostaining revealed that arginase I protein was enriched in the striated duct. C/EBPα-deficient mice exhibited dramatically reduced expression of arginase I in the parotid gland, indicating the essential role of this factor in this gland.

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Abbreviations: AGPC, acid guanidinium thiocyanate-phenol-chloroform; AL, argininosuccinate lyase; AS, argininosuccinate synthetase; C/EBP, CCAAT/enhancer-binding protein; CPS, carbamylphosphate synthetase; OTC, ornithine transcarbamylase; PCNA, proliferating cell nuclear antigen.

EXPERIMENTAL PROCEDURES

Animals—Mice heterozygous for disruption of the gene for C/EBPα (*20, 21*) or C/EBPβ (*22*) were mated, and the genotypes of their offspring were determined by Southern blot analysis as described (*20*).

Western Blot Analysis—Mouse tissues were homogenized in nine volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM antipain, 50 μM leupeptin, 50 μM pepstatin A, and 50 μM *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal. The homogenates were centrifuged at 15,000 ×*g* for 20 min at 4°C, and the supernatants were used as tissue extracts. The extracts (10 μg of protein) were subjected to SDS-PAGE, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham Pharmacia Biotech, Tokyo), and antibodies against amylase (Biogenesis, Poole, UK), carbamylphosphate synthetase (CPS) (*23*), ornithine transcarbamylase (OTC) (*24*), argininosuccinate synthetase (AS) (*25*), argininosuccinate lyase (AL) (*25*), and arginase I (*26*).

RNA Preparation—Total RNA was isolated from mouse tissues using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction procedure (*27*). Since the resultant RNA samples from the submandibular/sublingual glands were not pure enough to be competent for Northern analysis, they were further purified by proteinase K treatment. The reaction was carried out in 100 μl of a mixture containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.1% SDS, 30 μg of the crude RNA, and 20 μg of proteinase K. The mixture was incubated at 37°C for 20 min. After serial extraction with phenol, phenol/chloroform, and chloroform, RNA was precipitated with two volumes of ethanol, washed with 70% ethanol, and dissolved in water.

Northern Blot Analysis—RNA (2 μg per lane) was electrophoresed in denaturing formaldehyde-agarose (1%) gels. After visualizing 28S and 18S rRNAs by ethidium bromide staining to check the integrity of RNA samples and equal loading, the RNA was blotted onto nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized using a transcription kit (Roche Diagnostics, Tokyo), from cDNAs for the following proteins: CPS at nucleotide positions 2799–3237 (*28*), OTC (*29*), AS at nucleotide positions 326–775 (*30*), AL (*31*), arginase I (*32*), arginase II (*5*), C/EBPα (*33*), and C/EBPβ (*33*). Hybridization, washing and chemiluminescent detection on X-ray films were performed as recommended by Roche Diagnostics.

Tissue Preparation and Immunohistochemistry—Mice were anesthetized with ether, and then perfused through the heart with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 (pH 7.4), and 1.5 mM KH_2PO_4. Salivary glands were removed, fixed at room temperature overnight in the same paraformaldehyde solution, dehydrated in serially increasing concentrations of ethanol and then in xylene, and finally embedded in paraffin. Tissue sections, with a thickness of 3.5 μm, were cut. De-waxing was performed by heating the slides in an oven at 60°C for 1 h, followed by immersion in xylene three times (30, 15, and 15 min). The slides were then rehydrated in serially diluted ethanol (95, 70, and 30%) and then in PBS. The sections were subjected to antigen retrieval by microwave heating for 5 min in 10 mM sodium citrate (pH 6.0) (*34*). After inhibition of endogenous peroxidase activity by treatment with periodate, the sections were subjected to incubation with a primary antibody diluted with PBS for 1 h at room temperature. The primary antibodies used were rabbit anti-human arginase I antisera (*26*) (1:500 dilution) and a rabbit anti–human proliferating cell nuclear antigen (PCNA) antibody (1.3 μg/ml IgG; sc-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h, the sections were further incubated with a mixture of avidin and horseradish peroxidase-conjugated biotin for 1 h. Peroxidase activity was visualized using 3,3′-diaminobenzidine as a substrate. Sections were slightly counterstained with hematoxylin and methyl green to immunostain arginase I and PCNA, respectively, and then the slides were mounted in Aquatex (E. Merck, Darmstadt, Germany).

RESULTS AND DISCUSSION

Detection of Arginase I Protein in Mouse Salivary Glands—Salivary glands and other organs were dissected from adult wild-type mice, and subjected to Western analysis (Fig. 1). Since mouse submandibular and sublingual glands are joined and difficult to separate, the two glands were dissected en masse. Correct dissection of the submandibular/sublingual and parotid glands was confirmed by detecting amylase protein as a marker of salivary glands at prominently high levels compared to in other organs.

Arginase I protein was detected in the salivary glands as two immunoreactive bands whose migration distances were coincident with those for the liver. The two protein species have been believed to result from translational initiation from two different methionine codons (*35*). Arginase I protein was under the detectable level in the brain and kidney, coinciding with the view that the distribution of the enzyme is rather tissue-specific (*36*).

Other ornithine cycle enzymes were also examined. As for the first enzyme, CPS, a faint protein band comigrating with liver CPS was visible for the submandibular/sublingual glands, and a very faint band for the parotid gland. Examination of the second enzyme, OTC, revealed complicated profiles: while a band comigrating with authentic liver OTC was detected for salivary glands, another band comigrating with a cross-reacting material in the brain was also observed. The nature of this material is unknown. The third and fourth enzymes, AS and AL, were obviously detected in salivary glands.

Detection of Arginase I mRNA in Mouse Salivary Glands—Total RNA prepared from submandibular/sublingual glands by the usual AGPC extraction procedure exhibited 28S and 18S rRNA profiles that were different from those of liver RNA (Fig. 2A, compare lanes 1 and 2). One possible cause for these abnormal profiles was that specific features of salivary proteins (*37*) disturb elimination of the proteins through AGPC extraction, and that the contaminating proteins interfere with correct migration of RNAs on electrophoresis. Therefore we examined if proteinase K treatment of the RNA samples followed by phenol extraction could improve the electrophoretic profile, and showed

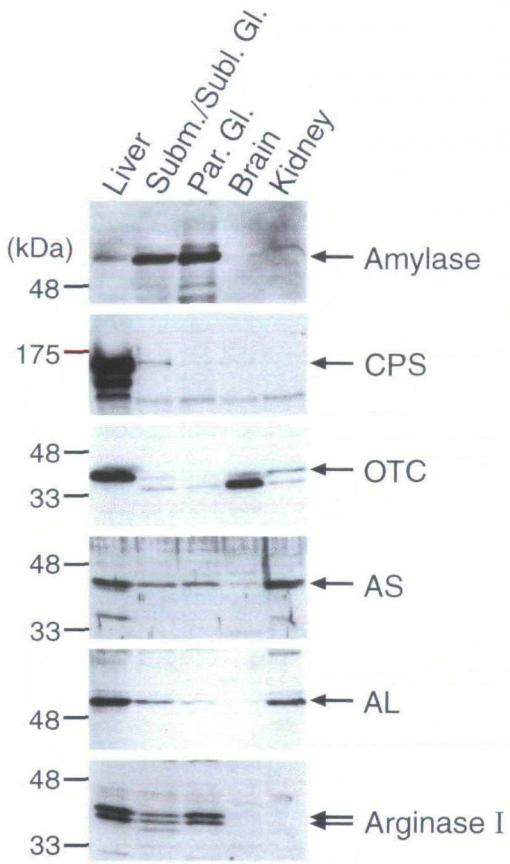

Fig. 1. **Western blot analysis of mouse salivary glands.** Protein extracts of submandibular/sublingual glands, parotid glands, and other organs of wild-type mice were subjected to electrophoresis in SDS gels consisting of 10% polyacrylamide, 6% for CPS, transferred to nitrocellulose membranes, and then immunodetected with specific antibodies against the indicated enzymes. The molecular weight markers (Prestained Protein Marker; New England Biolabs, Beverly, MA, USA) were MBP-β-galactosidase (175 kDa), aldolase (48 kDa), and triosephosphate isomerase (33 kDa).

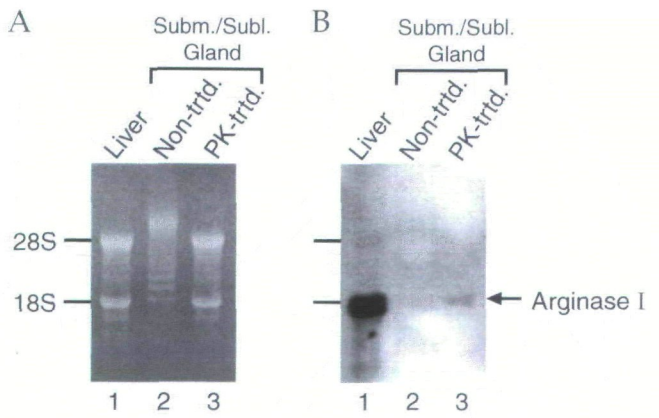

Fig. 2. **Preparation of RNA samples competent for Northern blot analysis from submandibular/sublingual glands.** Along with total liver RNA (lanes 1), RNA samples prepared from submandibular/sublingual glands by the AGPC extraction procedure before (lanes 2) and after (lanes 3) proteinase K treatment were subjected to electrophoresis, followed by ethidium bromide staining (A), and then to Northern blot analysis for detection of arginase I mRNA (B). The positions of 28S and 18S rRNAs are indicated.

that this was the case (Fig. 2A, lane 3). On Northern blot analysis (Fig. 2B), a single band for arginase I mRNA corresponding to that of liver RNA was detected with proteinase K–treated RNA but not with non-treated RNA. In the following experiments, we routinely employed the proteinase K treatment for RNA preparation from submandibular/sublingual glands.

As shown in Fig. 3, arginase I mRNA was detected in the parotid glands, as well as in the submandibular/sublingual glands. Arginase II mRNA was under the detectable level in salivary glands. We also could not detect any apparent signal for arginase II protein on Western analysis (data not shown). We concluded that the major isoform in the salivary glands is arginase I.

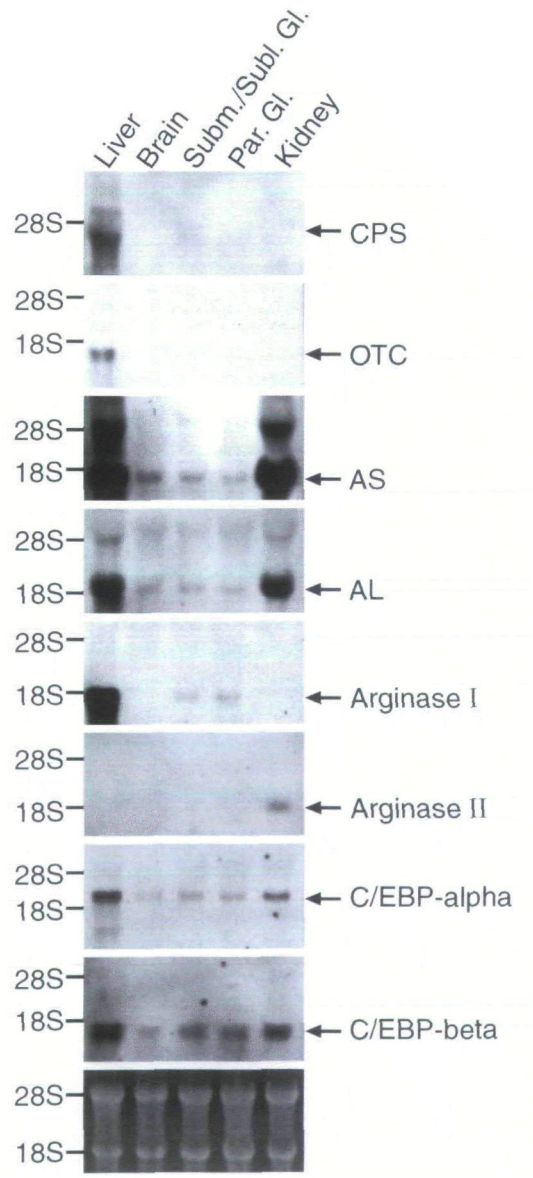

Fig. 3. **Northern blot analysis of mouse salivary glands.** Total RNAs were isolated from the submandibular/sublingual glands, parotid glands, and other organs of wild-type mice, and then subjected to Northern blot analysis for detection of the indicated mRNAs. The positions of 28S and 18S rRNAs are shown. Bottom, ethidium bromide staining of rRNAs is presented.

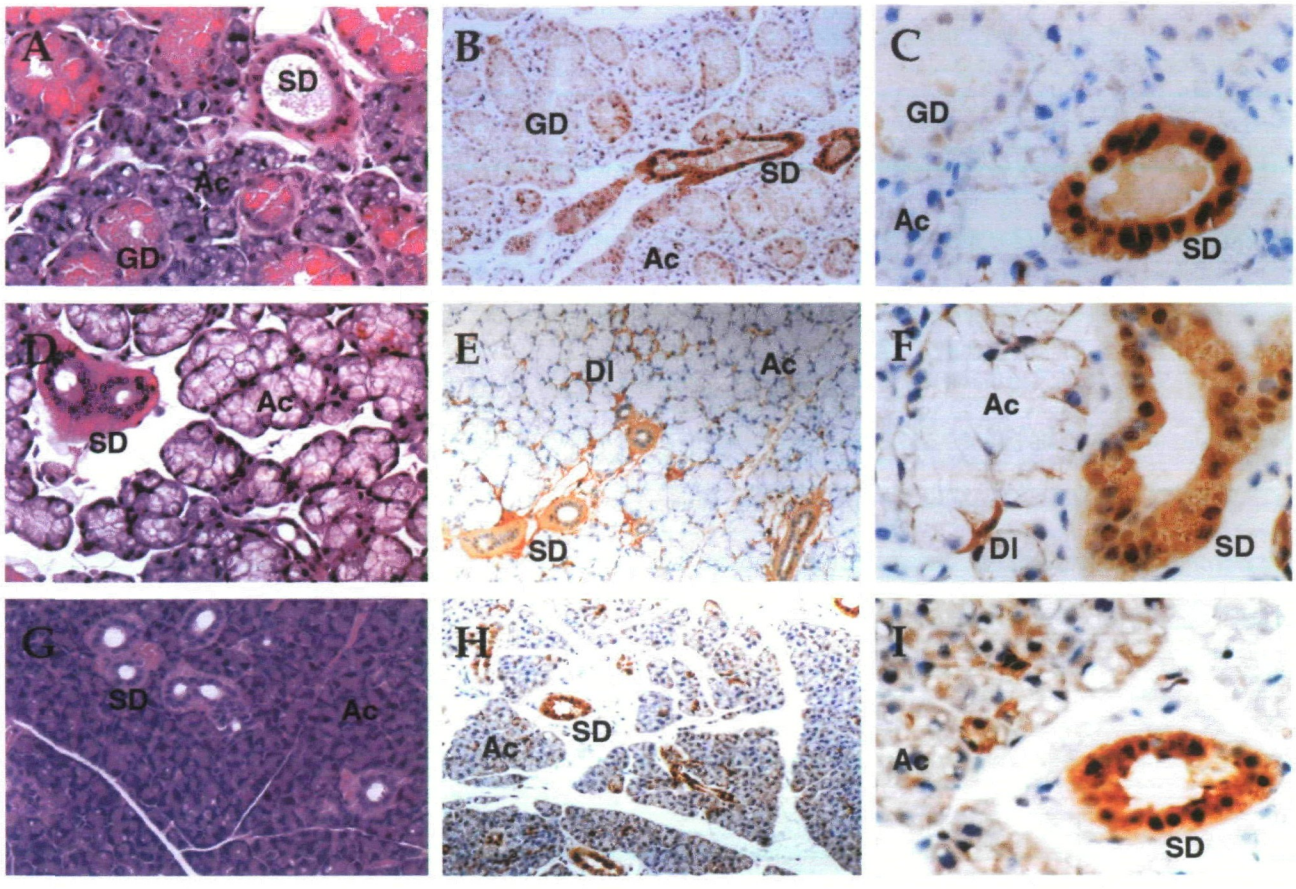

Fig. 4. **Immunohistochemical analysis of the distribution of arginase I protein in salivary gland.** A–C: submandibular glands. D–F: sublingual glands. G–I: parotid glands. Alongside hematoxylin-eosin staining (A, D, G), low (B, E, H), and high (C, F, I) magnification views of the histochemical staining are shown. Abbreviations: Ac, acinus; Dl, demilune; GD, granulated duct; SD, striated duct.

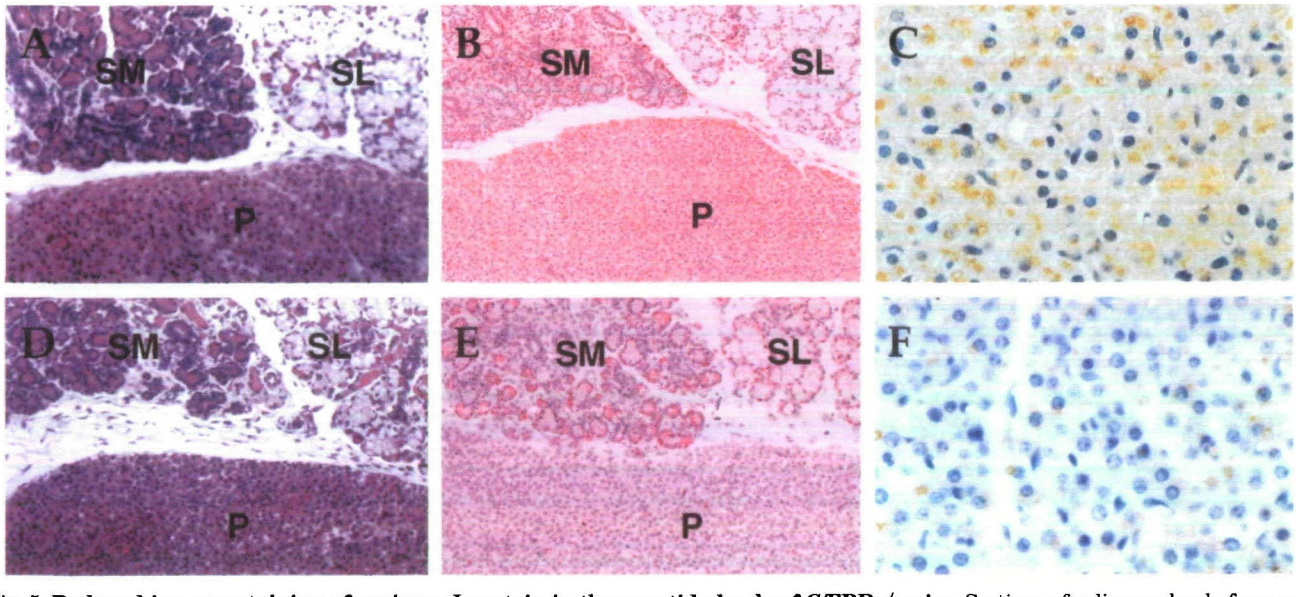

Fig. 5. **Reduced immunostaining of arginase I protein in the parotid glands of C/EBPα$^{-/-}$ mice.** Sections of salivary glands from newborn C/EBPα$^{+/+}$ (A–C) and C/EBPα$^{-/-}$ (D–F) mice were subjected to hematoxylin-eosin staining (A, D), and immunohistochemical staining for arginase I, low magnification (B, E) and high magnification (C, F) for the parotid gland. Abbreviations: SM, submandibular gland; SL, sublingual gland; P, parotid gland.

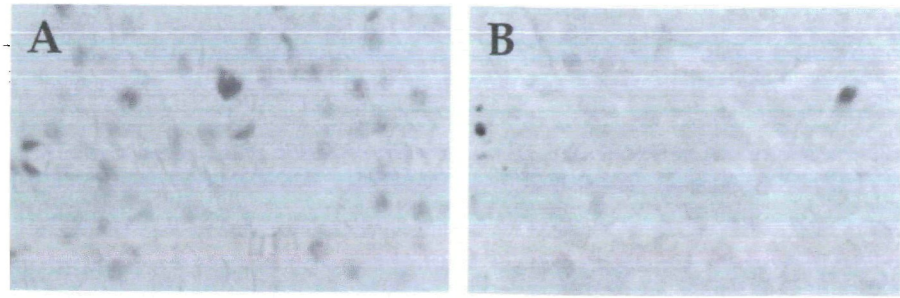

Fig. 6. **Enhanced immunostaining of PCNA in the parotid gland of C/EBPα$^{-/-}$ mice.** Sections of salivary glands from newborn C/EBPα$^{-/-}$ (A) and C/EBPα$^{+/+}$ (B) mice were subjected to immunohistochemical staining for PCNA, followed by counterstaining with methyl green.

As for other ornithine cycle enzymes, mRNAs for CPS and OTC were under the detectable levels in salivary glands. mRNAs for AS and AL were detected in salivary glands. Taking the above together with the results of Western analysis (Fig. 1), we concluded that among the five ornithine cycle enzymes, at least AS and AL are present in salivary glands, in addition to arginase I. AS and AL in extrahepatic tissues are generally involved in arginine synthesis, converting blood-derived citrulline into arginine (*38, 39*). Therefore, salivary glands also seem to be able to synthesize arginine, the substrate of arginase.

mRNAs for C/EBPα and C/EBPβ, which are transcription factors essential for regulation of the arginase I gene in the liver (*18, 19*), were also detected in salivary glands. Involvement of these transcription factors in expression of the arginase I gene in salivary glands will be investigated below.

Distribution of the Arginase I Protein in Salivary Glands—To determine the precise localization of the arginase I protein, immunohistochemical staining was performed on tissue sections of salivary glands (Fig. 4). Two independent rabbit antisera against arginase I gave almost identical immunostaining patterns, while a control nonimmune serum gave no apparent signal (data not shown). Arginase I immunoreactivity was detected in all three salivary glands. In the submandibular gland, strong signals were detected in the striated duct. Less intense staining was seen in the granulated duct. In the sublingual gland, strong signals were detected in the demilune surrounding the acinus, as well as in the striated duct. In the parotid gland, strong and moderate signals were observed in the striated duct and acinus, respectively.

Impaired Expression of Arginase I in the Parotid Gland of C/EBPα-Deficient Mice—Our previous studies showed that mice with disruption of the C/EBPα gene suffer from impaired expression of the arginase I gene in the liver just after birth (*18*), and that primary-cultured hepatocytes derived from C/EBPβ-deficient mice lack inducibility of the arginase I gene in response to glucocorticoids and glucagon (*19*). Here, we examined whether expression of the arginase I gene is impaired or not in the salivary glands of mice with disruption of the C/EBPα or C/EBPβ gene. Because C/EBPα$^{-/-}$ mice die within 8 h of birth, and because it is difficult to dissect salivary glands from newborn mice, we performed immunohistochemical staining of head and neck sections to examine the expression of arginase I protein (Fig. 5). A dramatic decrease in immunoreactivity was observed in the parotid glands of C/EBPα$^{-/-}$ mice compared to in C/EBPα$^{+/+}$ mice (Fig. 5, B and E, higher magnification views in C and F). On the other hand, no apparent difference was seen in immunostaining of the submandibular and sublingual glands between C/EBPα$^{+/+}$ and C/EBPα$^{-/-}$ mice (Fig. 5, B and E), which was confirmed on higher magnifications (data not shown). These results were reproduced each in four animals. Therefore, C/EBPα is essential for high-level expression of arginase I in the parotid gland, as well as in the liver, in a tissue-specific manner. As for C/EBPβ-deficient mice, no apparent change was observed in the protein and mRNA levels of arginase I in any salivary gland (data not shown).

Hepatocytes of new-born C/EBPα$^{-/-}$ mice have been shown to be proliferating actively (*40, 41*), by demonstrating an increased frequency of hepatocytes that express PCNA, an S-phase–specific protein. We performed here immunostaining of the parotid gland for PCNA using newborn C/EBPα$^{-/-}$ mice. As shown in Fig. 6, the number of PCNA-positive cells increased dramatically in C/EBPα$^{-/-}$ mice compared to in C/EBPα$^{+/+}$ mice. No apparent change in PCNA immunostaining was observed in the submandibular and sublingual glands between C/EBPα$^{+/+}$ and C/EBPα$^{-/-}$ mice (data not shown). Seemingly, parotid cells of C/EBPα$^{-/-}$ mice are in an immature proliferating stage, and this may be related to the decreased expression of the arginase gene that is expressed in terminally differentiated cells in a cell type-specific manner.

Studies on transcriptional regulation in the liver revealed that C/EBP family members activate the arginase I gene through direct binding to regulatory regions such as the promoter and enhancer (*14–17*). Similar mechanisms may also occur in salivary glands. The presence of mRNAs for C/EBPα and C/EBPβ in salivary glands (Fig. 3) coincides with this notion. However, it remains to be determined if arginase I mRNA is colocalized with C/EBPα and/or C/EBPβ proteins in the same cell populations of salivary glands. Such a study will also serve as an initial step for elucidating why the expression of arginase I is impaired specifically in the parotid gland among the three salivary glands of C/EBPα$^{-/-}$ mice, and whether C/EBPα and C/EBPβ compensate, at least in part, for a lack of the other, as has been proposed for the regulation of target genes in hepatocytes (*19, 42*).

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