

Evidence by signal peptide trap technology for the expression of carbonic anhydrase 6 in rat incisor enamel organs

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Smith CE, Nanci A, Moffatt P. Evidence by signal peptide trap technology for the expression of carbonic anhydrase 6 in rat incisor enamel organs. *Eur J Oral Sci* 2006; 114 (Suppl. 1): 147–153 © Eur J Oral Sci, 2006

During screening of a rat incisor enamel organ cDNA library by signal peptide trap technology, we identified a DNA fragment matching a predicted translation sequence for rat carbonic anhydrase 6 (CA6). This result was unexpected because CA6, to date, has been associated primarily with secretions from glandular tissues. To further characterize this observation, reverse transcription–polymerase chain reaction (RT–PCR) amplifications were carried out on total RNA extracted from freeze-dried secretory and maturation-stage rat incisor enamel organs. A cDNA fragment of the expected size was detected in control samples from rat salivary glands as well as within maturation-stage enamel organ samples. This CA6 RT–PCR fragment was further cloned and sequenced and found to match the nucleotide sequence 770–1079 from clone XM_216584 of GenBank. Northern blot analyses with the rat CA6 cDNA fragment confirmed its expression relative to maturation-stage enamel organ samples. It is at present unclear whether the CA6 expressed by enamel organ cells is secreted into the enamel layer or into the intercellular spaces of the enamel organ itself to assist in neutralizing excess protons arising from the growth of apatite crystals during the maturation stage of amelogenesis.

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Key words: bicarbonate; CA6; carbonic anhydrase; enamel formation; tooth

Accepted for publication October 2005

Prokaryotic and eukaryotic cells require a zinc metallo-enzyme, called carbonic anhydrase (CA) (EC 4.2.1.1), for survival (1). This enzyme catalyzes the simple and reversible hydration reaction of carbon dioxide with water to produce carbonic acid, which rapidly dissociates into hydrogen ions and bicarbonate ions at biological pH ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$) (2). Cells use these ions to make rapid local adjustments in intracellular and pericellular pH and, in the case of H^+ ions, to acidify various intracellular membrane-enclosed compartments, such as lysosomes, which require low pH for function (2). Some cells, in combination with certain solute carrier proteins, ion channels, and energy-driven membrane pumps, have evolved highly efficient methods to exploit the CA reaction for specific purposes. Erythrocytes, for example, use CA to convert waste CO_2 into bicarbonate, which is carried by the blood to the lungs where the erythrocytes change the bicarbonate back to CO_2 for expiration. Gastric parietal cells and osteoclasts use CA to generate large quantities of H^+ ions that they actively pump extracellularly as hydrochloric acid. In contrast, glandular duct cells use CA to produce large amounts of HCO_3^- ions that are excreted extracellularly to alkalinize secretions produced by associated acinar cells (2).

The essential nature of this enzyme is indicated by the simple fact that there is not one gene for CA, but more than 12 genes on widely different chromosomes coding for different proteins known to contain a catalytically active CA domain (2). These isoforms are targeted to three general cellular locations – cytoplasm (five types), mitochondria (two types), or plasma membrane (four types) – and one isoform, CA6, is secreted (2). Mammalian cells usually express a cytoplasmic isoform called CA2 and at least one other isoform, often a membrane-associated one, such as CA4 (2). To date, enamel organ cells and, in particular, ameloblasts, have been shown to express the CA2 isoform, especially during the maturation stage of amelogenesis (3–5).

Carbonic anhydrase 6 (CA6) was originally identified as a protein in the saliva of humans (2). It came to be known as ‘gustin’, because it was suspected that this protein had a protective function in relation to taste buds and taste perception. In 1998, gustin was identified as the same protein independently referred to as CA6 (6). While many bioinformatics databases still refer to CA6 as ‘salivary CA’ and describe it as ‘found only in salivary glands and saliva’ (e.g. Source GeneReport, <http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>),

this protein has been identified in the secretions of many glands, including nasal (7), lacrimal (8), von Ebner's (9), and mammary glands (10), and tentatively in pancreas (11). It has also been identified in human serum (12). Furthermore, there is speculation that salivary CA6 may possess anticariogenic properties in relation to enamel pellicles (13, 14). There are actually two versions of CA6 that cells can produce (15). One, called Type A CA6, is the standard secretory form made in tissues such as salivary glands. The other, called Type B CA6, is a non-secreted cytoplasmic form induced as part of an endoplasmic reticulum (ER) stress response (15). The present study concerns only the secreted Type A form of CA6.

Our interest in CA6 derives from initial screening of a rat incisor enamel organ cDNA library by signal trap technology in which a clone matching a portion of the sequence of the gene for rat CA6 was identified (16). This finding was of interest from two standpoints: first, CA6 is a secretory protein not currently associated with biological systems other than glandular tissues; and, second, the gene for rat CA6 is not currently well documented. The clone provided an opportunity to test directly the accuracy of hypothetical sequences currently proposed for rat CA6. As will be documented herein, our results indicate that the rat CA6 gene probably codes for a nascent protein comprising 312 amino acid residues, as opposed to the 357- or 287-residue sequences currently listed in existing bioinformatics databases for this protein.

Material and methods

Animal and general tissue preparation

All aspects of the handling, care, and usage of animals were carried out under guidelines specified by federal/provincial governmental agencies and approved by local university animal care committees. A total of 50 male Wistar rats (Charles River Canada, St Constant, QC, Canada), at 100 g body weight, were anesthetized with halothane and killed. Hemimandibles and hemimaxillae were removed, rapidly cleaned of soft tissues, and immersed as quickly as possible in liquid nitrogen where they remained for 5–7 h. Various control tissues, including kidney, liver, parotid and submaxillary salivary glands, were also removed from these animals and snap frozen in liquid nitrogen. All samples were freeze dried at -55°C for 48 h on a 6-l capacity lyophilizer (Labconco, Kansas City, MO, USA). The bony caps at the labial sides of the incisors were removed and the underlying enamel organs were lifted from each incisor, using a curved dental tool, to help shear the freeze-dried cells along the boundary surface between the apical ends of ameloblasts and the enamel layer. On mandibular incisors, a molar reference line (17) was used to prepare separate secretory and maturation-stage enamel organ samples. Microdissected enamel organ samples were placed in small aluminum dishes and weighed on a Mettler MT5 microbalance (Mettler Toledo Canada, Mississauga, ON, Canada), then placed in sterile, RNase-free 15-ml capacity plastic centrifuge tubes. Additional tubes were prepared containing ≈ 100 mg of different control tissues. Total RNA was extracted by homogenizing the freeze-dried tissues in 2.5 ml per tube of Trizol (Invitrogen Canada, Burlington, ON, Canada) using

a Polytron (Brinkmann Instruments, Mississauga, ON, Canada). RNA pellets were resuspended in diethylal poly-carbonate (DEPC)-treated deionized water and stored at -20°C .

Cloning, screening, and sequencing of cDNA clones prepared by signal peptide trapping

The procedures for cloning and sequencing of a DNA fragment by signal peptide trapping have been described in detail previously (16,18). Briefly, a random library was prepared by the oligo-capping method to enrich for 5'-end cDNA fragments. It was then directionally cloned into the viral-based vectors and expression screened in BHK-21 cells. The resulting cDNA fragments encoding a signal peptide were cloned by reverse transcription-polymerase chain reaction (RT-PCR) and identified by automated sequencing and BLASTN search against GenBank.

RT-PCR experiments

Reverse transcription-polymerase chain reaction amplifications were carried out with total RNA using the One Tube Titan Kit (Roche Diagnostics, Laval, QC, Canada), according to the manufacturer's instructions, and oligonucleotide primers were designed for the following two rat CA isoforms:

- (1) CA2 forward ($5' \rightarrow 3'$), TGTGCAGCACCCAGATGG; CA2 reverse, CAGTTCTTCAGCCTCCCC; expected fragment size, 318 bp.
- (2) CA6 forward, ACACCTACCAAGGCTCGC; CA6 reverse, CCGGTTCAATGGCTGTGG; expected fragment size, 310 bp.

Both of these primer pairs were designed to overlap different exons to prevent non-specific amplification on genomic DNA. Reaction mixtures for each tube ($25 \mu\text{l}$) were set up on ice by mixing $0.5 \mu\text{g}$ of total RNA, $5 \mu\text{l}$ of $5\times$ reverse transcription buffer, $0.5 \mu\text{M}$ primer, 0.2 mM dNTPs, 5 mM dithiothreitol, $0.5 \mu\text{l}$ of Titan enzyme mix, and 6 U RNA-Guard (Amersham Biosciences, Baie d'Urfé, QC, Canada). After a 30-min RT step at 50°C , the cDNAs were subjected to the following PCR cycling conditions: 94°C for 30 s, 56°C for 30 s, and 68°C for 45 s. Aliquots of reaction mixture ($8 \mu\text{l}$) were sampled after 25 and 40 cycles, loaded onto 1.8% agarose gels, electrophoresed, and stained with ethidium bromide. Controls for these reactions included (i) using standard oligonucleotide probes for the general housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (ii) using RNA extracted from rat epididymis rather than enamel organ (i.e. expresses CA2 but not CA6) (19), and (iii) running reactions without an RT step.

Cloning and sequencing of the RT-PCR DNA fragment

The CA6 DNA fragment was purified from agarose gels and cloned at the *EcoRV* site of pBluescriptKS(+) (Stratagene, La Jolla, CA, USA). The plasmid DNA was sequenced on both strands with the T3 and T7 primers using the Dye Terminator Cycle Sequencing Kit (Beckman Coulter Canada, Mississauga, ON, Canada). The sequencing reactions were run and analyzed using a Beckman Coulter CEQ2000 automated DNA sequencing system.

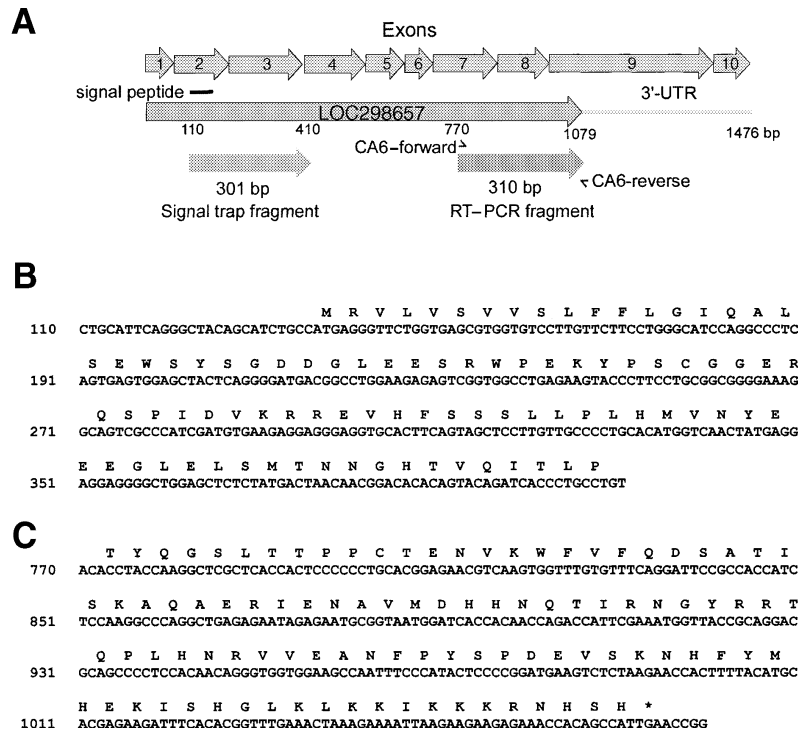


Fig. 1. (A) Schematic representation of the 1479-base pair (bp) hypothetical cDNA transcript, XM_216584, at GenBank, for rat carbonic anhydrase 6 (CA6) and its exon structure at locus 298657 on chromosome 5. The regions where the 301-bp signal trap fragment (B) at the 5' end, and the 310-bp reverse transcription–polymerase chain reaction (RT–PCR) fragment (C) at the 3' end, were cloned, and their sequences, are indicated. (B) Sequence obtained for the signal trap fragment from rat incisor enamel organ RNA. It matches exactly with a portion of the XM_216584 transcript, extending from positions 110–410 of exons 2–4. (C) Sequence obtained for the cloned RT–PCR fragment (Fig. 2). It corresponds exactly along its length to positions 770–1079 of transcript XM_216584. UTR, untranslated region.

Northern blotting with the RT–PCR fragment

Total RNA from microdissected rat S- and M-stage enamel organs (each with 1 or 10 μ g per lane) were separated by agarose-gel electrophoresis and visualized with ethidium bromide staining, according to standard protocols (20). After capillary transfer onto a nylon membrane (Osmonics, Westborough, MA, USA) and ultraviolet (UV) crosslinking, the blot was prehybridized at 65°C in Church buffer for 2 h. Hybridization was carried out under the same conditions overnight with the CA6 310 bp cDNA fragment (Fig. 1) radiolabeled with 32 P-dCTP by random priming. Stringent washes were performed at 65°C with 0.2 \times salted sodium citrate buffer (SSC)/0.1% sodium dodecyl sulfate, and the blot was then autoradiographed.

Bioinformatics

Information about CA6 genes and their sequences in rat (chromosome 5), mouse (chromosome 4) and human (chromosome 1) were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>), Ensembl (<http://www.ensembl.org/>), and RGD (the Rat Genome Database) (<http://rgd.mcw.edu/>).

Results

Figure 1A shows a schematic drawing of the cDNA proposed to represent CA6 (ID: 246357) in segment q36

of chromosome 5 of rat, based on predictions from automated computational analyses at GenBank (transcript XM_216584). The cDNA from locus 298657 is 1,476 bp long and comprises 10 exons. The coding region of the transcript is proposed to begin at position 1, from an ATG start codon in exon 1, and to extend for 1,079 bp. The hypothetical protein translated from this sequence is proposed to contain 357 amino acids beginning with the sequence 'MKISFKH' at its N-terminal end.

Figure 1B shows the nucleotide and amino acid sequences for the 301-bp signal peptide trap fragment obtained from rat enamel organ RNA. The nucleotide sequence was identical, along its entire length, to a portion at the 5' end of the XM_216584 transcript stretching from location 110–410 bp. This fragment did not contain the ATG start codon of exon 1 in the XM_216584 transcript, but it did contain a second ATG codon present at the end of exon 2 (Fig. 1A) coding for a 91 amino acid sequence beginning with 'MRVLVSV' (Fig. 1B).

Figure 2 shows the results of RT–PCR reactions for various primer pairs using RNA extracted from rat enamel organs and submaxillary salivary glands (only the 40-cycle data are presented). As indicated, specific amplification bands were obtained for GAPDH, for CA2 in secretory and maturation-stage enamel organ samples, and for CA6 in salivary glands (Fig. 2). Specific amplification bands for CA6 were observed only within maturation-stage RNA extracts (Fig. 2). Figure 3 shows the

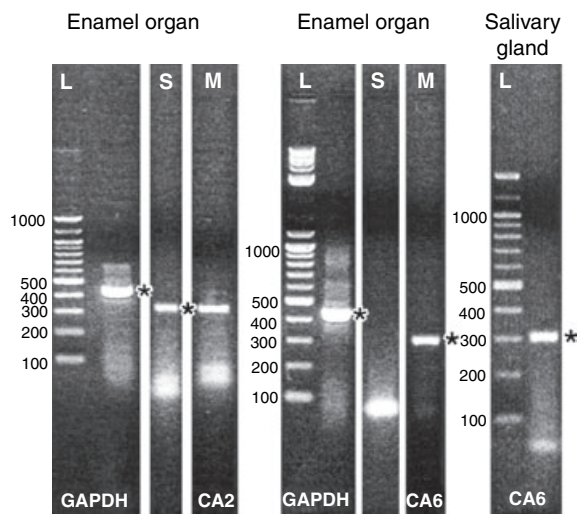


Fig. 2. Ethidium bromide-stained agarose gels showing amplification fragments (asterisks) obtained following 40 reverse transcription-polymerase chain reaction (RT-PCR) cycles with total RNA isolated from rat incisor enamel organ or rat submaxillary salivary gland and oligonucleotide probes specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), carbonic anhydrase 2 (CA2), or CA6. L, molecular weight ladders; S, secretory-stage extracts; M, maturation-stage extracts.

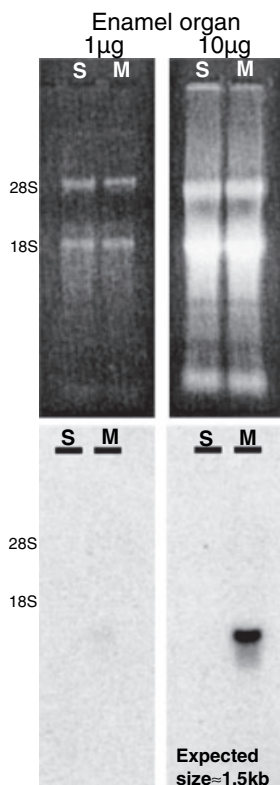


Fig. 3. Top: ethidium bromide-stained agarose gels showing total RNA in 1- and 10- μ g samples of secretory (S) and maturation (M) stage enamel organ extracts; the positions of ribosomal RNA are indicated at the left of the figure. Bottom: northern blot after hybridization with the cloned carbonic anhydrase 6 (CA6) reverse transcription-polymerase chain reaction (RT-PCR) amplification fragment (Fig. 2) radiolabeled with 32 P-dCTP.

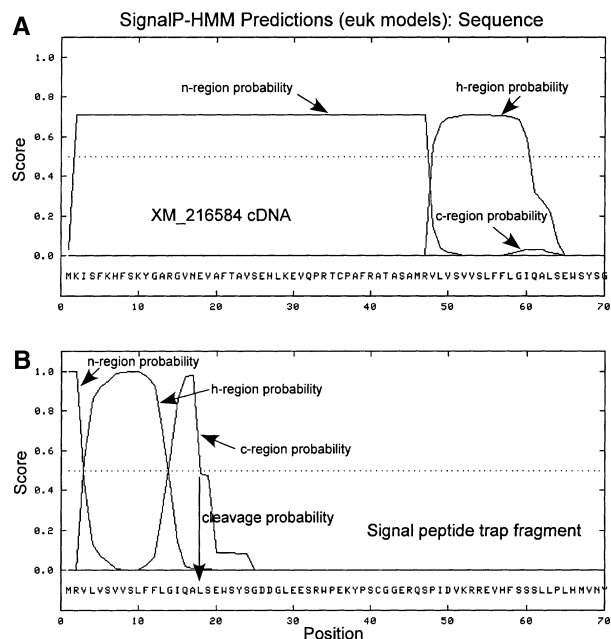


Fig. 4. Prediction of signal peptide probabilities by the SignalP Server (24) for the hypothetical XM_216584 carbonic anhydrase 6 (CA6) GenBank transcript (A) vs. the CA6 signal trap fragment obtained in this study (B) (see also Fig. 1B). This server uses hidden Markov model (HMM) analyses to predict (rated as a 'score' on the y-axis) whether a eukaryotic (euk) protein (sequence of amino acids along the x-axis) is cytoplasmic (low n-, h- and c-region probabilities), membrane-spanning (high n- and h-, and low c-, region probabilities), or possess a signal peptide (variable length and high n-, h-, and c-region probabilities). (A) The SIGNALP software predicts that rat CA6 might be a Type 2 membrane-embedded protein based on the hypothetical GenBank sequence (which it is not). (B) Based on the sequence obtained from the cloned signal trap fragment (Fig. 1B), the software predicts that rat CA6 probably has a signal peptide of 17 amino acids in length (high cleavage probability between residues 17-18) and is secreted, as occurs in human and mouse CA6 (see Fig. 5).

results from northern blotting with a radiolabeled rat CA6 RT-PCR cDNA fragment; only RNA extracts from maturation-stage enamel organ samples were reactive with this probe. Figure 1C shows the nucleotide and amino acid sequences obtained for a 310-bp fragment cloned from the maturation-stage enamel organ RT-PCR amplification band (Fig. 2). The nucleotide sequence for this DNA fragment was identical, along its entire length, to an area at the 3' end of the XM_216584 transcript at location 770-1079. This corresponded to the last 100 amino acid residues at the C-terminal end of rat CA6 (Fig. 1A,C).

Discussion

The finding that enamel organ cells express CA6 during the maturation stage of amelogenesis was surprising, considering the heretofore association of this secretory protein with primarily glandular tissues and, in particular, salivary glands (2,21). This finding was also

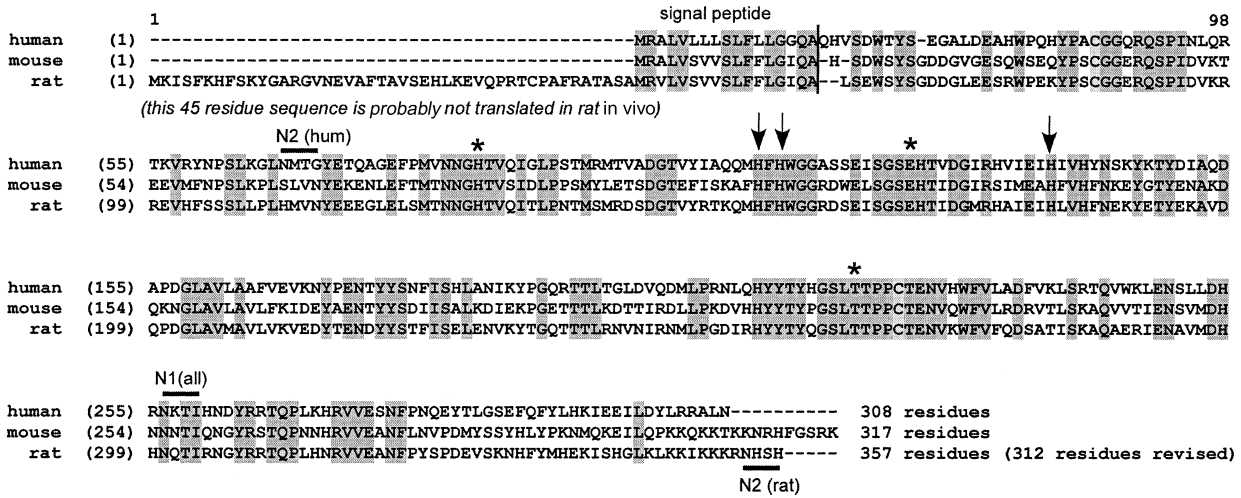


Fig. 5. Aligned sequences for human, mouse, and rat carbonic anhydrase 6 (CA6). Amino acids that are identical in all three sequences are indicated in gray. The signal peptide and predicted cleavage points in each sequence are indicated by the vertical line. The triad of histidine residues that comprise the catalytic zinc-binding site in CA6 are indicated by arrows, and the highly conserved histidine (H), glutamate (E), and threonine (T) residues that assist the catalytic reaction are highlighted by the asterisks (*). Carbonic anhydrase 6 from all three species contain one common potential N-linked glycosylation site (N1), whereas human and rat CA6 have a second potential N-linked glycosylation site in different regions of the protein (N2; human is N-terminally and rat is C-terminally located). We conclude that the translated version of rat CA6 is 312 amino acid residues in length and begins with the sequence 'MRVLVSVV', analogous to sequences seen in human and mouse CA6.

fortuitous in light of results from a recent study where we estimated that maturation-stage enamel organ cells probably have to provide considerable 'on site' (local) buffering with bicarbonate ions generated intracellularly by CA2 in order to compensate for the large quantities of H⁺ ions (acid) formed as enamel crystals mature (22). It is easy to conceive, by analogy to the anticariogenic role that salivary CA6 is believed to perform in enamel pellicles (13), that a situation could exist during amelogenesis where maturation-stage ameloblasts secrete CA6 extracellularly to provide additional local buffering capacity by forming bicarbonate ions or by recycling excess carbonic acid back into CO₂ and H₂O. What remains to be determined by *in situ* hybridizations and immunolocalizations in the enamel organ are the cell types that express the CA6 (ameloblasts and/or papillary layer cells), and where CA6 is located following its secretion (in the enamel layer and/or intercellular spaces of the enamel organ). It will also be of interest to determine if enamel organ cells also express the Type B intracellular version of CA6 (15), considering the major low-pH stress they are under during the maturation stage (22), and the fact that enamel organ cells can express the Type B isoform under certain conditions, for example following exposure to high doses of fluoride (23). Irrespective of this, it is evident from the findings of this study that CA6 can no longer be viewed as an isoform associated solely with salivary glands or glandular tissues in general.

The results of this study agree in principle with the conclusion reached by previous workers that substantial CA activity is present in enamel organs, especially relative to the maturation stage of amelogenesis (3-5). The results are also consistent with the conclusion that

one of the main isoforms expressed by enamel organ cells is CA2 (5). A consistent finding of these previous studies was the apparent absence of any CA activity or CA2 antigen in secretory-stage cells (3-5). At present we do not have a good explanation for why we were able to detect mRNA signals for CA2 in the secretory-stage enamel organ cells by RT-PCR (Fig. 2). While not quantitative, the intensity of amplification bands from secretory-stage RNA was much less than for equivalent amounts of maturation-stage RNA and was more evident after 25 cycles of RT-PCR than after the 40 cycles shown in Fig. 2. Assuming that the CA2 mRNA detected in this study is not an artifact arising from contamination from adjacent tissues/cells prior to freezing, it is possible that CA2 mRNA is transcribed by secretory-stage cells but held in reserve and not routinely translated into actual protein unless local conditions place demands for increased buffering capacity by bicarbonate. It is also possible that more CA2 mRNA is made than actually becomes translated into real protein, and fixative procedures used in previous studies masked the low levels of the activity and presence of the protein in these cells.

It is a basic premise of the signal peptide trap methodology that only mRNAs coding for proteins targeted to the ER will eventually form clones by the procedures used (false positives are low frequency) (18). This approach does not distinguish, however, between proteins that will be secreted vs. those that will be embedded in cell membranes. If a signal peptide cleavage prediction is run using the XM_216584 transcript for CA6 that is on file at GenBank, the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) (24) predicts that there is no signal peptide in

this protein (Fig. 4A, low c-region probability) but a transmembrane signal anchor (Type 2), consisting of a N-terminal cytoplasmic region about 48 amino acids long (Fig. 4A, high n-region probability) and a membrane-spanning region about 17 amino acids long (Fig. 4A, high h-region probability), are present. However, if the sequence for the signal trap fragment is used (Fig. 1B), then the SignalP Server predicts, with high probability, that there is a signal peptide targeting this protein for secretion (Fig. 4B, c-region probability), and this peptide is approximately 17 residues in length, just like other mammalian CA6 isoforms (Fig. 5). When sequences for rat, mouse, and human CA6 are compared (Fig. 5), the three sequences show 83.5% similarity if the 45-residue region of the XM_216584 transcript from GenBank (see the updated information detailed in the Addendum) is ignored, and the start sequence of the signal peptide begins with 'MRVLV' from the signal trap fragment (Figs 1B and 5). For these reasons, we conclude that the translated form of rat CA6 probably contains 312 residues and not 357 residues, as proposed in GenBank for transcript XM_216584 (Fig. 5). The secreted form of rat CA6 probably has 295 residues with a base molecular weight of 34,283 Da and a pI of 6.22. The actual molecular weight of the secreted protein is likely to be higher than this, as rat CA6 shows two potential N-linked glycosylation sites, as found in human CA6 (Fig. 5).

An important issue that remains to be determined is whether CA6 is secreted into the enamel layer, or if it resides primarily within the intercellular spaces of the enamel organ (or both). We suspect that the latter destination is more likely, for two reasons. First, proteins have no survival potential within the proteolytic-enriched environment of maturing enamel (25), and the need for major bicarbonate buffering does not arise until most proteins in the enamel layer have been destroyed (22,25). In addition, sensitive analytical techniques have provided little evidence for any 30–40 kDa proteins in the enamel once apatite crystals are expanding at their fastest rates (25). Second, theoretical models that have been proposed for bicarbonate buffering by CA2 during the maturation stage envision a scheme where ameloblasts need to 'unload' the excess intracellular hydrogen ions left as a byproduct of bicarbonate excretion in a direction toward the basolateral intercellular space between ameloblasts and papillary layer cells (25). This is carried out to transfer the effective hydrogen ion load to the blood vessels for terminal buffering by replenishable bicarbonate present in tissue fluids and blood. The intercellular spaces of the enamel organ would seem to comprise a very suitable location to provide extra buffering capacity potentially available through the activity of CA6.

Lastly, it is interesting to note that WINSNES *et al.* (26) presented a clinical case report of congenital persistent proximal type renal tubular acidosis in two brothers. These brothers showed severe and persistent hyperchloraemic metabolic acidosis (capillary blood pH 7.07–7.15) owing to a low renal bicarbonate threshold at 11 mmol l⁻¹. The maximum tubular capacity for bicar-

bonate reabsorption was reduced to about half the normal. Among the numerous clinical characteristics observed were enamel defects of the permanent teeth. It is uncertain from the report if the enamel defects were posteruptive or caused by an underlying defect during amelogenesis. Such case reports, albeit rare, may serve to support a critical role for bicarbonate buffering during amelogenesis.

Acknowledgements – Part of this research was funded by NIDCR Program Project grant DE013237 and by CIHR grant MOP68826.

Addendum added after submission

The GenBank-predicted mRNA sequence for rat CA6 described in this report refers specifically to transcript XM_216584.2 (GI:34872489), which was the latest deduced sequence on file at the time of submission of our manuscript. In mid-April (2005), GenBank revised their prediction (to transcript XM_216584.3; GI:62650059) and they now indicate that rat CA6 mRNA has a start sequence identical to the one described in this paper for the signal trap fragment (as 'MRVLVSV').

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