Expression of Carbonic Anhydrases IX and XII in Embryonic and Adult Mouse Tissues

MASTER'S THESIS
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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Kolmestatoista aktiivisesta hiilihappoanhydraasista CA IX ja XII liittyvät syövän syntyyn. Näiden solukalvolla sijaitsevien proteiinien on ehdotettu osallistuvan syöpäsolujen leviämiseen. Solujen aktiivinen vaellus on myös sikiönkehityksen tyypillinen piirre, joten tutkimuksen tavoitteena oli selvittää, ilmennetäänkö näitä isoentsyymejä hiiren eri-ikäisten sikiöiden kudoksissa.

Tutkimusmenetelmät: CA IX:n ja XII:n lähetti-RNA:n ilmentymistä tutkittiin *in situ* hybridisaatiolla. Tätä varten valmistettiin radioaktiivisesti leimatut RNA-koettimet. CA IX- ja XII-proteiinia tutkittiin immunohistokemiallisesti immunoperoksidaasi-värjäysmenetelmällä.

Tutkimustulokset: CA IX:n ja XII:n lähetti-RNA:n ilmentymistä ei voitu selvittää luotettavasti *in situ* hybridisaatio -tekniikkaan liittyneiden ongelmien takia. Immunohistokemiallinen tarkastelu osoitti, että sekä CA IX:ää että XII:ta ilmennetään useissa hiiren sikiön kudoksissa elinten kehityksen aikana. CA IX:ää löytyi aivoista, haimasta ja maksasta kohtuullisen paljon, kun taas munuainen ja maha antoivat heikot signaalit. CA XII:ta ilmennettiin myös monissa kudoksissa, vaikkakin värjäytyminen oli heikkoa useimmissa tapauksissa. CA XII -proteiinia voitiin havaita aivoissa, joissa huomattavimmin värjäytyi suonipunos, sekä mahassa, haimassa, maksassa ja munuaisessa.

Johtopäätökset: Molempien isoentsyymien havaittiin ilmentyvän sellaisissa sikiökudoksissa, joita vastaavat aikuiskudokset eivät ilmennä näitä proteiineja. Tämä viittaa siihen, että CA IX ja XII osallistuvat tiettyjen kudosten muodostumiseen.

MASTER'S THESIS

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Abstract

Background and aims: Of the thirteen active carbonic anhydrases, CA IX and XII have been linked to carcinogenesis. It has been suggested that these membrane-bound CAs participate in cancer cell invasion. Since active cell migration is a characteristic feature of embryonic development, the aim of the study was to explore whether these isozymes are expressed in mouse embryos of different ages.

Methods: *In situ* hybridization was used to detect the expression of CA IX and XII mRNA. For this, radioactively labelled riboprobes were constructed. CA IX and XII protein expressions were studied immunohistochemically with a peroxidase-antiperoxidase method.

Results: The expression of CA IX and XII mRNA could not be revealed due to problems with the *in situ* hybridization method. Examination by immunohistochemistry showed that both CA IX and XII are present in several tissues of the developing mouse embryo during organogenesis. Staining for CA IX revealed a relatively wide distribution pattern, including the brain, pancreas and liver with moderate signals, and the kidney and stomach with weak signals. The expression pattern of CA XII was also relatively broad, although the intensity was weak in most tissues. The positive tissues included the brain, where the most prominent staining was seen in the choroid plexus, and the stomach, pancreas, liver and kidney.

Conclusions: Since both isozymes were present in some embryonic tissues whose adult counterparts do not express these particular proteins, one could hypothesize that CA IX and XII may have specific roles in the assembly of certain tissues.

CONTENTS

Abbreviations

AE	anion exchanger
BSA	bovine serum albumin
CA	carbonic anhydrase
CA IX	carbonic anhydrase IX
CA XII	carbonic anhydrase XII
CA9	*carbonic anhydrase 9* (refers particularly to the human gene)
CA12	*carbonic anhydrase 12* (refers particularly to the human gene)
Car9	*carbonic anhydrase 9* (refers particularly to the mouse gene)
Car12	*carbonic anhydrase 12* (refers particularly to the mouse gene)
CA-RP	carbonic anhydrase-related protein
CAI	carbonic anhydrase inhibitor
CAM	cell adhesion molecule
ccRCC	clear cell renal carcinoma
cDNA	complementary deoxyribonucleic acid
CRL	crown-rump length
DAB	3,3' -diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
ED	embryonic day
EPO	erythropoietin
GLUT	glucose transporter
HIF	hypoxia-inducible factor
HRE	hypoxia response element
HRP	horseradish peroxidase
IHC	immunohistochemistry
ISH	*in situ* hybridization
mRNA	messenger ribonucleic acid
NRS	non-immune normal rabbit serum
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
p.c.	post coitum
PCR	polymerase chain reaction
PHD	prolyl-4-hydroxylase
p.p.	post partum
PTP	protein tyrosine phosphatase
pVHL	von Hippel-Lindau protein
SSC	standard saline citrate
sCA IX	soluble form of CA IX
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

1. INTRODUCTION

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes, which classically participate in the maintenance of pH homeostasis. The mammalian α-CA gene family includes at least thirteen enzymatically active isoforms with different structural and catalytic properties.

Of the thirteen active isozymes, CA IX and XII have been linked to carcinogenesis. Both are transmembrane proteins. CA IX is a highly active enzyme, and its activity can be efficiently inhibited by sulfonamides (Ilies et al., 2003; Vullo et al., 2003; Abbate et al., 2004; Casey et al., 2004; Vullo et al., 2004). In addition to its enzyme activity, CA IX is a cell adhesion molecule and may also contribute to cell proliferation (Saarnio et al., 1998b; Zavada et al., 2000). There have been studies on the distribution of CA IX in human, rat and mouse tissues (Pastorekova et al., 1997; Hilvo et al., 2004). CA IX is ectopically expressed at relatively high levels and with a high prevalence in tumors whose normal counterparts do not contain this protein. On the other hand, tumors originating from tissues with high natural CA IX expression, such as the stomach and gallbladder, lose some or all of their CA IX upon conversion to carcinomas (Saarnio et al., 2001; Leppilampi et al., 2003).

The tissue distribution of CA XII has not yet been fully characterized. There have been studies on the CA XII protein expression in human and rodent tissues. Its expression has been demonstrated by immunohistochemistry in the normal human kidney, colon, prostate, pancreas, ovary, testis, lung and brain (Ivanov et al., 1998; Ivanov et al., 2001), and the enzyme has been localized to the basolateral plasma membranes of the epithelial cells (Karhumaa et al., 2000; Kivela et al., 2000a; Karhumaa et al., 2001a). In mouse tissues, its expression is at highest in the kidney (Kyllonen et al., 2003) and in the surface epithelial cells of the colon (Halmi et al., 2004). In a recent study, CA XII has been demonstrated in the rat epididymis (Hermo et al., 2005). CA XII also shows a clear association with certain tumors, being overexpressed in renal cancer cells, for example (Tureci et al., 1998).

CA IX and CA XII seem to be regulated by similar mechanisms. The *CA9* and *CA12* genes have been identified as von Hippel-Lindau (VHL) target genes. Wild-type VHL protein down-regulates the transcription of CA IX and XII mRNA, indicating that these isozymes may have a potential role in VHL-mediated carcinogenesis (Ivanov et

al., 1998). In addition, both isozymes are induced in tumors and cultured tumor cells under hypoxic conditions (Wykoff et al., 2000; Ivanov et al., 2001). It has been suggested that these membrane-bound CAs participate in cancer cell invasion, which is facilitated by an acidic tumor cell environment.

One characteristic feature of embryonic development is active cell migration from one place to another. Although this clearly represents a benign process, it has some mechanistic similarities to cancer cell invasion (Derycke et al., 2004; Friedl et al., 2004), e.g. the fact that the moving cells invade through the extracellular matrix. Since it has been suggested that CA IX and XII participate in neoplastic invasion, the aim of this thesis was to study whether these isozymes are expressed in mouse embryos of different ages.

2. LITERATURE REVIEW

2.1 Carbonic Anhydrases (CAs)

2.1.1 General Aspects

The carbonic anhydrases (CAs) are metalloenzymes that exist in three genetically unrelated families of isoforms, α, β and γ, which are present variously throughout virtually all living organisms. Evidence to date suggests that only α genes are present in vertebrates, but that they are also present in many algae and the cytoplasm of green plants and in some eubacteria. β genes have been shown to exist predominantly in bacteria, algae and chloroplasts of both mono- and dicotyledons. Both α and β genes occur together in many plants, lower eukaryotes and invertebrates. The γ carbonic anhydrases are found mainly in archaea and some eubacteria (Chirica et al., 1997; Hewett-Emmett, 2000; Smith et al., 2000; Krungkrai et al., 2001; Supuran et al., 2003). This thesis focuses on α-CAs.

The α-carbonic anhydrases are all monomeric zinc-containing metalloenzymes with a molecular weight of approximately 29-58 kDa. These enzymes catalyze a very simple physiological reaction, the interconversion of carbon dioxide and bicarbonate: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. Thus far, thirteen enzymatically active α-CAs have been reported in mammals: CA I, II, III, VII, and XIII are cytoplasmic (Sly et al., 1995; Lehtonen et al., 2004), CA IV, IX, XII, XIV, and XV are anchored to plasma membranes (Sly et al., 1995; Pastorekova et al., 1997; Tureci et al., 1998; Parkkila et al., 2001; Hilvo et al., 2005), CA VA and VB are mitochondrial (Fujikawa-Adachi et al., 1999), and CA VI is the only secretory form present in saliva and milk (Kivela et al., 1999; Karhumaa et al., 2001b). CAs play important roles in a number of biological processes connected with respiration and the transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions (such as gluconeogenesis and lipid and urea synthesis), bone resorption, calcification, tumorigenity and many other physiological or pathological processes (Hewett-Emmett, 2000; Supuran et al., 2003).

The different α-CAs have very different subcellular localizations and tissue distributions (Table 1). Table 2 shows the catalytic activities of different CA isozymes as well as their affinities for sulfonamide inhibitors.

Table 1. Subcellular localizations and major sites of tissue expression for all the α-CA isoforms. The data on subcellular localizations has been extracted from Supuran (Pastorekova, 2004a; Supuran, 2004), except for CA XV (Hilvo et al., 2005), RPTPβ and RPTPγ (Chegwidden, 2000). The information on the sites of tissue expression has been obtained from Chegwidden (Chegwidden, 2000), except for CA XIII (Lehtonen et al., 2004) and CA XV (Hilvo et al., 2005).

Isozyme	*Subcellular localization*	*Some sites of known tissue expression* [1]
CA I	cytosol	red blood cell, intestine
CA II	cytosol	ubiquitous (certain cells of virtually all tissues)
CA III	cytosol	red muscle, adipose tissue
CA IV	membrane-bound	kidney, lung, gut, brain, eye, probably universally present in capillary endothelium
CA VA	mitochondria	liver (also skeletal muscle, kidney)
CA VB	mitochondria	widespread (except liver)
CA VI	secreted	saliva
CA VII	cytosol	brain, salivary gland, lung, probably widely distributed at low levels
CA-RP VIII	cytosol	brain, especially Purkinje cells of cerebellum, widespread at lower levels
CA IX	transmembrane	various tumors, gastric mucosa
CA-RP X	cytosol	brain (also pineal gland, placenta)
CA-RP XI	cytosol	brain
CA XII	transmembrane	widespread, especially colon, kidney, prostate
CA XIII	cytosol	salivary glands, small intestine, large intestine, pancreas, kidney, testis
CA XIV	transmembrane	widespread, especially kidney and muscle
CA XV	membrane-bound	kidney, brain
RPTPβ	transmembrane	central and peripheral nervous system
RPTPγ	transmembrane	brain, lung

[1] Tissue expression patterns are indications of the current state of knowledge and are not to be considered as the results of definitive studies. In many cases conclusions are based on detection of mRNA.

Table 2. Higher vertebrate α-isozymes, their relative CO_2 hydration activity and affinity for sulfonamide inhibitors. The data has been derived from Supuran (Supuran, 2004).

Isozyme	*Catalytic activity*	*Affinity for sulfonamides*
CA I	low	medium
CA II	high	very high
CA III	very low	very low
CA IV	high	high
CA V	moderate-high [1]	high
CA VI	moderate	medium-low
CA VII	high	very high
CA-RP VIII	acatalytic	[2]
CA IX	high	high
CA-RP X	acatalytic	[2]
CA-RP XI	acatalytic	[2]
CA XII	low	high
CA XIII	moderate	high
CA XIV	high	high
CA XV	n/d [3]	n/d

[1] Moderate at pH 7.4; high at pH 8.2 or higher.
[2] The native CA-RP isozymes do not contain Zn(II), and therefore their affinity for the sulfonamide inhibitors has not been measured.
[3] no data

The Zn(II) ion of CAs is essential for catalysis (Lindskog et al., 2000; Supuran et al., 2003). X-ray crystallographic data show that the metal ion is situated at the bottom of a 15-Å-deep active site cleft, coordinated by three histidine residues and a water molecule/hydroxide ion (Stams et al., 2000). The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Lindskog et al., 2000). This strong nucleophile attacks the CO_2 molecule bound in a hydrophobic pocket in its neighborhood, leading to the formation of bicarbonate coordinated to Zn(II). The bicarbonate ion is then displaced by a water molecule and liberated into solution, forming the acid form of the enzyme, with water coordinated to catalytically inactive Zn(II) (Lindskog et al., 2000; Supuran et al., 2003). The mechanism is schematically represented by Equation 1. The basic form A is regenerated through a proton transfer reaction from the active site to the environment. This reaction might be assisted either by active-site residues or by buffers present in the medium. This is shown in Equation 2.

(1) $EZn^{2+} - OH + CO_2 \longleftrightarrow EZn^{2+} - HCO_3^- \xleftrightarrow{H_2O} EZn^{2+} - OH_2 + HCO_3^-$

(2) $EZn^{2+} - OH_2 \longleftrightarrow EZn^{2+} - HO^- + H^+$

The rate-limiting step in catalysis is the second reaction, i.e., the proton transfer that regenerates the zinc hydroxide species of the enzyme (Lindskog et al., 2000; Supuran et al., 2003).

The two main classes of carbonic anhydrase inhibitors (CAIs) are the metal-complexing anions and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the nonprotein zinc ligand (Equation 3) or by an addition to the metal coordination sphere (Equation 4) generating trigonal-bipyramidal species. Sulfonamides are the most important CAIs binding in a tetrahedral geometry of the Zn(II) ion. Anions might bind either in a tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts, such as the tiocyanate adduct (Stams et al., 2000; Supuran et al., 2003).

(3) $EZn^{2+} - OH_2 + I \longleftrightarrow EZn^{2+} - I + H_2O$ (substitution)

Tetrahedral adduct

(4) $EZn^{2+} - OH_2 + I \longleftrightarrow EZn^{2+} - OH_2(I)$ (addition)

Trigonal-bipyramidal adduct

2.1.2 CA Inhibition as an Approach to Anticancer Therapy

There are no complete data indicating that CA inhibition as a means of tumor pH manipulation perturbs the activity of particular CA isozymes. However, the literature so far clearly indicates that this is a promising avenue toward treating cancer. It has been shown that acetalozamide, a prototypal carbonic anhydrase inhibitor (CAI) of several CA isozymes, reduced the *in vivo* growth of tumors when it was given alone, and it produced additive tumor growth delays when it was administered in combination with various chemotherapeutic agents (Teicher et al., 1993). In another study, the effect of acetalozamide on the invasive capacity of renal carcinoma cell lines was investigated (Parkkila et al., 2000b). It was found that a 10-µM concentration in the culture medium inhibited the relative cell invasion rate through the matrigel membrane by 18 to 74 % depending on the cell line. Based on the levels of CA isozymes, this effect was attributed to the inhibition of CA II or CA XII, or both.

There is also extensive literature showing the *in vitro* antiproliferative activities of CAIs in a broad range of human tumor cell lines. Inhibition of human cancer cell

proliferation by classical sulfonamide CAIs was reported by Chegwidden and Spencer (Chegwidden, 1995), who demonstrated that methazolamide (0.4 mM) and ethoxzolamide (10 μM) inhibited the growth of a human lymphoma cell line U937. Interestingly, only weak inhibition or none at all was observed in cells cultured in a medium containing the nucleotide precursors hypoxanthine and thymidine. This indicates that sulfonamides inhibited the synthesis of nucleotides. This explanation was deduced from the fact CA activity is involved in the production of bicarbonate that is required by carbamoyl phosphate synthetase I for the synthesis of pyrimidines. However, other mechanisms have not been excluded (Chegwidden, 1995).

Supuran and collaborators have synthesized and tested several hundreds of potent sulfonamide CAIs containing the aromatic or heterocyclic moiety, or both (Supuran et al., 2000b, 2000a; Supuran et al., 2001). These compounds were subjected to screening for their ability to inhibit the growth of tumor cells *in vitro* by using a panel of 60 cancer cell lines. The screening led to the identification of lead compounds that exhibited considerably higher inhibitory properties (in the low micromolar range) than did classical sulfonamides (Supuran et al., 2000b, 2000a). These leads were used to design novel classes of derivatives with enhanced antitumor activities by using the tail approach, in which new tails were attached to precursor sulfonamides (Casini et al., 2002). The active compounds showed GI_{50} values (i.e., 50 % inhibition of tumor cell growth after 48 hours of exposure) in micromolar to nanomolar concentrations.

In addition, a new and very potent anticancer sulfonamide E7070 (indisulam) has been discovered through elaborate preclinical screening (Owa et al., 1999). Although it was selected regardless of CA-inhibitory capacity, it has been shown to act as a nanomolar CA inhibitor (Abbate et al., 2004). Its anticancer effects were shown to involve a decrease in the S-phase fraction along with cell cycle perturbations in G1 or G2, or both; downregulation of the cyclins E, A, B1, H, CDK2 and CDC2; reduction of CDK2 activity; inhibition of pRb phosphorylation; and differential expression of many additional molecules that participate in metabolism, the immune response, signaling and cell adhesion (Fukuoka et al., 2001; Yokoi et al., 2002). E7070 has already been successful in Phase II clinical trials for the treatment of colorectal cancer and non-small cell lung cancer (Supuran, 2003). In the future, it will be extremely interesting to examine whether cancer-related or other CA isozymes are among the molecular targets of E7070 in tumor cells.

2.2 Functions of the CAs

2.2.1 Carbonic Anhydrases I and II

CA II is one of the fastest known enzymes and appears to be almost universally expressed in some cell types of all major mammalian tissues. In erythrocytes it catalyses the hydration of CO_2 to form HCO_3^- ions, whereas in renal tubules and collecting ducts it eliminates H^+, thereby acidifying the urine. In bones, CA II contributes to the differentiation of osteoclasts and it also provides H^+ for bone resorption in osteoclasts. In metabolic processes CA II provides bicarbonate for pyrimidine synthesis. In the brain, CA II contributes to cerebrospinal fluid production by providing H^+ and regulating pH in the choroid plexus. In the gastric canal, CA II produces H^+ for gastric acid formation in stomach and provides HCO_3^- for bile and pancreatic juice production. In acinar and ductal cells CA II produces HCO_3^- for saliva formation (Chegwidden, 2000). A deficiency of human CA II causes a defined clinical phenotype – osteopetrosis and renal tubular acidosis, in some cases accompanied by mental retardation (Sly et al., 1995). This illustrates the major, crucial roles played by CA II in osteoclasts and in renal tubules. CA II has been reported to bind to the C-terminus of a plasma membrane chloride/bicarbonate anion exchanger, AE1, thus increasing the rate of bicarbonate transport. Similar to CA II-AE1 interaction, CA II has also been shown to bind and function with another type of bicarbonate transporter, the sodium bicarbonate cotransporter (kNBC1: (Gross et al., 2002).

The physiological function of the major red cell isozyme CA I present in concentrations of up to 150 μM in the blood (Supuran et al., 2003) is unknown. The primary sites of *CA1* expression are colonic epithelium and erythrocytes, although low levels are also found in vascular endothelium, myoepithelial cells and cells of several other tissues (Tashian, 1989). In mammalian erythrocytes, CA I appears to contribute 50 % of the CO_2 hydration activity. The gene encoding *CA1* is unusual amongst the carbonic anhydrases in having two cell type specific promoters (Fraser et al., 1989; Brady et al., 1991) separated by a large 35-36 kb intron. The two promoters act in a mutually exlusive manner (Sowden et al., 1993): the proximal promoter transcribes *CA1* in colon epithelia while the more distal promoter is active only in erythroid cells. It is a major challenge to try to understand how transcriptional specificity is achieved at

each *CA1* promoter. Interestingly, individuals who are homozygous for a human *CA1* deficiency gene exhibit no related clinical abnormalities (Sly et al., 1995).

2.2.2 Carbonic Anhydrase III

Hormonally regulated cytoplasmic CA III has very low CA catalytic activity (approximately one hundredth of that of the high activity isozyme, CA II). In addition, this isozyme also has other unique properties: it is relatively resistant to acetazolamide inhibition and it has an unusual tissue distribution. It is present at high levels in all examined mammalian red muscle and, uniquely, is absent from heart muscle. CA III has also been identified at high levels in adipose tissue. Despite intense investigation, the function of CA III has remained obscure over the years but resent results indicate that it has a role as an antioxidant protein, due to the free thiol groups in this molecule. It has been suggested that the two free thiols may scavenge oxygen radicals in skeletal muscle (Cabiscol et al., 1995).

2.2.3 Carbonic Anhydrase IV

This high activity, GPI-anchored membrane isozyme works in tandem with CA II, in both respiration and acid-base regulation. In humans, CA IV is quite abundant in a multitude of tissues. In pulmonary endothelial cells, CA IV catalyses the conversion of plasma bicarbonate to CO_2 for its removal by respiration, whereas in the capillary surfaces of peripheral tissues it catalyses the hydration of CO_2 to bicarbonate to facilitate its removal in the blood (Chegwidden, 2000). In the kidney, this enzyme is highly expressed at the plasma membrane of epithelial cells, where it contributes to the reabsorption of HCO_3^- in the brush border of proximal tubular cells and the thick ascending limb of Henle (Brown et al., 1990). CA IV is also expressed on the apical surfaces of certain epithelial cells of the jejunum, ileum and colon (Fleming et al., 1995). Additionally, immunolocalization studies have shown strong expression in the gallbladder (Parkkila et al., 1996b). Sender et al. (Sender et al., 1994; Sender et al., 1998) have demonstrated abundant expression of CA IV on the plasma face of the capillaries of skeletal muscle and heart muscle. In the brain, cortical capillaries express CA IV on their plasma face (Ghandour et al., 1992). In the eye, the expression of CA

IV is strong in the choriocapillaris but not in the retina (Hageman et al., 1991), suggesting that, along with CA II, it may be a target of CA inhibitors used to reduce intra-ocular pressure in the treatment of glaucoma.

2.2.4 Carbonic Anhydrase V

CA V is a low-activity isoenzyme located in the mitochondrial matrix. cDNA for human mitochondrial CA V was originally cloned from a human liver cDNA library, and its gene was localized to chromosome 16 (Nagao et al., 1993). Later, two laboratories independently characterized another mitochondrial CA and thereafter the two isozymes have been termed CA VA and CA VB (Fujikawa-Adachi et al., 1999; Shah et al., 2000). These proteins have different patterns of tissue-specific distribution, suggesting different physiological roles for the two mitochondrial isozymes. CA VA is specific to human liver, and CA VB is expressed in other tissue types including heart, skeletal muscle, pancreas, kidney, salivary gland, and spinal cord. (Fujikawa-Adachi et al., 1999). Because CA VB is more widely distributed in human tissues than CA VA, CA VA may have arisen from CA VB to play a specific role in the liver. In mitochondria, CA has been shown to provide HCO_3^-, which is required for the initial steps of glyconeogenesis and ureagenesis (Henry, 1996).

2.2.5 Carbonic Anhydrase VI

CA VI is to date the only known secretory isozyme of the CA gene family. In humans, immunohistochemical studies have demonstrated the location of CA VI exclusively in the secretory granules of the acinar cells of the parotid and submandibular glands (Parkkila et al., 1990), from where it is secreted into the saliva. Studies using a time-resolved immunofluorometric assay for CA VI have indicated that the salivary enzyme concentrations follow a circadian periodicity (Parkkila et al., 1995). Independent of the overall CA VI level in the saliva during the day, the enzyme levels are very low during the sleeping period. In addition, low concentrations of CA VI can be detected in human serum, because small amounts leak from the salivary glands or are absorbed from the alimentary canal (Kivela et al., 1997).

It has been proposed that CA VI and II may together form a complementary system regulating the acid-base balance in the mouth and upper alimentary tract (Parkkila et al., 1990; Parkkila et al., 1996a). Leinonen et al. have demonstrated that CA VI binds to the enamel pellicle, which is a thin layer of proteins covering the enamel, and retains its enzyme activity on dental surfaces (Leinonen et al., 1999). In the enamel pellicle, CA VI is located at the optimal site to catalyse the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water. These findings suggest that CA VI may protect teeth by catalysing the most important buffer system in the oral cavity, thus accelerating the removal of acid from the local microenvironment of the tooth surface. CA VI has also been detected in the gastric mucus where it may contribute to the maintenance of the pH gradient on the surface epithelial cells. This view is supported by the observation that CA VI probably maintains its activity in the harsh environment of the gastric lumen and that patients with verified oesophagitis or oesophageal, gastric or duodenal ulcers have a reduced salivary CA VI concentration relative to patients with a non-acid peptic disease (Parkkila et al., 1997).

2.2.6 Carbonic Anhydrase VII

CA VII appears to be the less studied and understood among the cytosolic CAs. It is the most highly conserved of the active CA isozymes, suggesting an evolutionary pressure which may, in turn, imply a significant, but yet unidentified physiological function. Human CA VII, similarly to the (chimeric) murine isozyme, shows high catalytic activity for the hydration of CO_2 (Vullo et al., 2005).

2.2.7 Carbonic Anhydrases IX and XII

The membrane-bound isozymes CA IX and XII are discussed in detail in sections 2.3 and 2.4, respectively.

2.2.8 Carbonic Anhydrase XIII

CA XIII is a recently characterized cytosolic isozyme and its expression has been studied in human and mouse (Lehtonen et al., 2004). CA XIII was found in a number of different tissues in both species, and distinct differences were detected in the distribution of CA XIII between human and mouse tissues.

In the human alimentary tract, CA XIII was found in several tissues including salivary glands, gastric mucosa, duodenum, jejunum, ileum and large intestine. Immunostaining revealed no positive signal for CA XIII in the human liver. The human pancreas also showed weak staining for CA XIII. Additionally, kidney was one of the human tissues positive for CA XIII. CA XIII was highly expressed in the human testis and was also found to be an abundant isozyme in the female reproductive tract.

In mouse, the strongest immunoreaction for CA XIII was observed in the colon. CA XIII expression was also detected in the mouse brain and kidney. No CA XIII-specific staining was detected in the mouse testis, whereas the epithelial cells of the mouse uterus contained CA XIII. Expression for CA XIII was also detected in the mouse lung, where the staining was most abundant in the rounded cells of the alveolar wall.

2.2.9 Carbonic Anhydrase XIV

Transmembrane CA XIV was described in 1999 (Mori et al., 1999). By immunostaining, CA XIV has been shown to be expressed in the human and mouse brain, where the isozyme was found on neuronal membranes and axons in both species. CA XIV is also strongly expressed in the regions of the rodent nephron that have been thought to be important in urinary acidification (Kaunisto et al., 2002). In addition, CA XIV is expressed in the mouse liver, where it is confined to the plasma membrane of hepatocytes (Parkkila et al., 2002). Interestingly, it is located in both the apical and basolateral plasma membranes. In contrast, the other transmembrane isozymes, CA IX and XII, are clearly restricted to the basolateral membranes.

2.2.10 Carbonic Anhydrase XV

A recent study has shown (Hilvo et al., 2005) that mammals have another membrane-bound CA isozyme, CA XV. Three copies of *CA15* were identified in the human chromosome band 22q11.21. However, only two copies were found in the chimpanzee genome, and thus it is possible that one copy of the gene is missing due to incomplete genomic data. Hilvo et al. concluded that in both species, all the *CA15* genes represent pseudogenes, because of frameshifts, insertions, point mutations, and the lack of mRNAs and EST-sequences. In contrast, all the other genomes exhibited only single *CA15* genes, and it was demonstrated that the full-length murine cDNA produced enzymatically active CA XV in COS-7 cells. Therefore, CA XV is the only active CA isozyme thus far known, which is expressed in several vertebrate species but has been lost in humans and chimpanzees.

2.2.11 Acatalytic CA Family Members

Along with active CA isozymes, evolutionally conserved but acatalytic family members have been reported and designated carbonic anhydrase-related proteins (CA-RPs). Three isoforms, CA-RP VIII, CA-RP X and CA-RP XI, have been reported (Tashian et al., 2000). CA-RPs lack one or more histidine residues required to bind the zinc ion, which is essential for CO_2 hydration activity, and are thus believed to be inactive as regards classical CA activity (Hewett-Emmett et al., 1996).

In addition, among a family of protein tyrosine phosphatases (PTPs), two receptor-type protein tyrosine phosphatases, RPTPβ (=PTPξ) and RPTPγ, contain an N-terminal CA-like domain (Barnea et al., 1993; Levy et al., 1993). Because of the absence of two zinc-binding histidine residues in their CA-like domain sequences, these two phosphatases have also been thought to be acatalytic isoforms. The exact biological function of these CA-RPs and CA-RP domains of RPTPs has not been established (Tashian et al., 2000).

2.3 Carbonic Anhydrase IX

2.3.1 General Aspects

CA IX was originally detected in a human carcinoma cell line HeLa as a cell density-regulated membrane antigen named MN (Pastorekova et al., 1992). Later, when the full-length cDNA for the MN protein was cloned, it was found to contain a large CA domain located in the extracellular part of the encoded protein (Pastorek et al., 1994; Opavsky et al., 1996). This CA domain showed 40.8 and 35.8 % identity with secreted isozyme CA VI and cytosolic isozyme CA II, respectively, and contained a perfectly conserved and active enzyme site. Based on the suggestion of Hewett-Emmett and Tashian (Hewett-Emmett et al., 1996), the MN protein was renamed CA IX.

CA IX is expressed at the basolateral plasma membrane of epithelial cells and, in some cases, also in nuclei (Pastorekova et al., 1992). In addition to a central CA domain, a mature CA IX molecule contains a transmembrane anchor followed by a short C-terminal cytoplasmic tail. The N-terminal side of the CA IX molecule is extended with a so-called PG-like region, which is homologous to the keratan sulfate attachment domain of a large proteoglycan aggrecan (Opavsky et al., 1996). This PG-like region is absent from the other CA isozymes known at present. Thus, it is possible that this region of CA IX may play a role in cellular interactions (Zavada et al., 2000; Svastova et al., 2003).

The human *CA9* gene has been mapped to chromosome 17 (Ivanov et al., 1998). Most of the complete CA IX is integrated into the cell membrane as a trimer composed of 54 and 58 kDa monomeric molecules linked together with disulfidic bonds. Body fluids and TC media contain a soluble form sCA IX consisting of 50 and 54 kDa polypeptides (Zavada et al., 2003). It has been proposed that sCA IX may be derived from the complete molecule by the proteolytic cleavage of the extracellular domain from transmembrane anchor and intracytoplasmic tail by membrane-associated proteases. It seems that in the human body, sCA IX is rapidly cleared from the blood. However, until now it has not been shown whether this is due to absorption in unknown deposits, degradation or excretion in urine.

2.3.2 CA IX Expression in Normal Tissues

CA IX has a distinctive expression pattern since it is naturally expressed in few normal tissues, but its ectopic expression is induced in a wide spectrum of human tumors (Table 3). The expression of CA IX has been studied in human, rat and mouse tissues.

In humans, CA IX is predominantly expressed in the gastrointestinal tract (Pastorekova et al., 1997). The most abundant expression has been detected in all major cell types of the gastric mucosa, including the surface pit cells, parietal cells and glandular chief cells. Lower levels of CA IX were expressed in the intestinal epithelium, where it was confined to the cryptal areas composed of cells with the greatest proliferative activity (Saarnio et al., 1998b). Noteworthy, amount of CA IX progressively decreased with increasing distance from the stomach toward the rectum. The CA IX level was also high in the gallbladder mucosa, whereas weak expression has been detected in the epithelia of pancreatic ducts (Kivela et al., 2000b). Variable degrees of CA IX expression have been detected in the lining cells of the body cavity, rete ovarii, rete testis and efferent ducts, in ventricular linings of the central nervous system and in the choroid plexus. Still, most normal tissues have remained negative (Ivanov et al., 2001; Karhumaa et al., 2001a).

In all gastrointestinal epithelia, CA IX is present in the basolateral membranes, suggesting its possible involvement in intercellular communication and in maintaining tissue integrity. This assumption is in accordance with a study describing a phenotype of CA IX-deficient mice constructed by targeted *Car9* gene disruption (Ortova Gut et al., 2002). The knockout mice displayed gastric hyperplasia with aberrant cell lineage development, resulting in an increased number of surface pit cells and a decreased number of glandular chief cells. However, the knockout mice did not show any significant change in gastric pH, hydrochloric acid production or systemic electrolyte status. The hyperplastic phenotype supports the role of CA IX in gastric morphogenesis and in controlling cell proliferation and differentation, although it is possible that other gastric CA isozymes supplement the enzyme activity of CA IX.

In rats, strong expression of CA IX has been detected in the stomach. The reaction was present throughout the gastric mucosa from the gastric pits to the deep gastric glands and confined to the basolateral surface of the epithelial cells. In the intestine, epithelial staining was present in the duodenum and colon but was absent from sections of the jejunum and ileum. The positive reaction was again confined to the basolateral

surface of the epithelium, while no expression was seen at the apical surface. The distribution of CA IX through the mucosal layer is different in the rat and human colon. In the rat colon, the positive signal was strongest in the surface epithelium, whereas the deep colonic glands remained negative or show only a weak reaction. By contrast, in the human colon, the staining intensity increased from the surface towards the base of the crypt. CA IX was also expressed in the rat and human bile ducts, where it was again located at the basolateral surface of the epithelial cells (Pastorekova et al., 1997).

In mouses, the highest expression of CA IX has been detected in the gastric mucosa. A similar phenomenon is seen in human and rat tissues. A strong reaction was seen in the basolateral plasma membrane of the mucus-producing surface epithelial cells, chief cells, and parietal cells. A strong signal was also seen in the plasma membrane of the colon enterocytes and a moderate signal in the pancreatic acini. Lower levels of CA IX were expressed in some other mouse tissues, including the kidney, liver, ileum, and spleen (Hilvo et al., 2004).

Table 3. A schematic overview of CA IX distribution in normal human tissues and derived tumors. The intensity of the gray tone in the rectangles corresponds to both the level and the frequency of expression: darker tones indicate strong expression and the white indicates no expression. The data has been derived from Pastorekova and Pastorek (Pastorekova, 2004a).

Normal tissues	Expression	Tumor tissues
CNS - neurons		Glioma/ependynoma
CNS - choroid plexus		Choroid plexus tumor
Body cavity linings		Mesothelioma
Salivary glands		Papillary/follicular carcinoma
Esophagus		Head/neck carcinoma
Respiratory tract		Lung carcinoma
Stomach/duodenum		Stomach carcinoma
Colon		Colon carcinoma
Gallbladder		Biliary carcinoma
Pancreas		Pancreatic carcinoma
Kidney		Renal cell carcinoma
Prostate		Prostate carcinoma
Testis		Germ cell tumor
Uterine cervix		Carcinoma of cervix uteri
Endometrium		Endometrial carcinoma
Breast		Breast carcinoma
Skin		Squamous/basal cell carcinoma

2.3.3 CA IX Expression in Cancers

In contrast to the relatively limited presence of CA IX in normal tissues, the spectrum of cancers expressing CA IX has successively expanded to various types of benign and malignant tumors. These include tumors derived from the kidney, esophagus, colon, lung, pancreas, liver, endometrium, ovary, brain, skin and breast (Liao et al., 1997; McKiernan et al., 1997; Turner et al., 1997; Saarnio et al., 1998a; Vermylen et al., 1999; Kivela et al., 2000b; Ivanov et al., 2001; Saarnio et al., 2001; Bartosova et al., 2002). Tumors originating from tissues with high natural expression, such as hepatobiliary epithelial tumors, have revealed decreasing levels of CA IX with increasing grades of dysplasia and carcinoma (Saarnio et al., 2001). A similar phenomenon has been observed in gastric carcinomas (Pastorekova et al., 1997), which is in accordance with the proposed involvement of CA IX in the differentation of gastrointestinal epithelia. Nevertheless, tumors originating from CA IX-negative tissues showed its ectopic activation (Table 3).

Especially striking is the very high proportion of CA IX-positive specimens among cervical, renal and lung cancers. The CA IX immunoreactivity with M75 has been observed in virtually all cervical carcinomas and the majority of cervical intraepithelial neoplasia (Liao et al., 1994). The diffuse CA IX-positive staining signal in normal cervical tissues was only found in the concurrent presence of dysplasia or carcinoma. Thus, it can be useful as an early diagnostic indicator of cervical neoplasia in Pap smears (Liao et al., 1996). In kidney cancer, CA IX protein expression was selectively linked with the most frequent carcinomas of renal clear cell type (ccRCC). High levels of CA IX were seen in primary, cystic and metastatic ccRCCs, but not in benign lesions (Liao et al., 1997). In lung cancer, CA IX was not found in preneoplastic lesions, but it was present in 80 % of malignant tumors (Vermylen et al., 1999). Some normal-looking bronchial and alveolar epithelia in close vicinity to the tumors contained CA IX-positive cells, whereas all other normal lung specimens sampled at a distance from the tumor were negative. Aberrant expression of CA IX has also been detected in colorectal tumors, wherein it correlated with proliferation evaluated according to the Ki-67 index, on which basis it has been proposed that it serves as a marker of increased proliferation in the colorectal mucosa (Saarnio et al., 1998a).

2.3.4 Intratumoral Expression Pattern

CA IX expression pattern *in vivo* clearly mirrors a distribution of hypoxic areas. The protein is localized in the perinecrotic regions of various solid tumors, including carcinomas of the breast, skin, ovary, cervix uteri, head and neck, lung, and bladder (Wykoff et al., 2000; Chia et al., 2001; Giatromanolaki et al., 2001; Koukourakis et al., 2001; Loncaster et al., 2001; Olive et al., 2001). According to the measurements in head and neck carcinomas, CA IX expression started at a distance of 40-140 μm (median 80 μm) from a blood vessel and continued toward necrosis (Beasley et al., 2001). A similar spatial relationship of CA IX to microvessels was found in bladder and lung cancer (Turner et al., 2002; Swinson et al., 2003). When compared to the distribution of HIF-1α and the chemical marker of hypoxia EF5 investigated in an independent study (Vukovic et al., 2001), CA IX expression began at a greater distance than HIF-1α, but at a lesser distance than EF5. This finding might suggest that CA IX induction requires lower oxygen levels than HIF-1α and that it occurs in a perinecrotic zone, which is larger than the zone labeled by EF5. In addition, Olive et al. (Olive et al., 2001) found that CA IX staining extends beyond the regions binding another chemical marker, pimonidazole, in cervical carcinomas. Moreover, they demonstrated that CA IX-expressing cells isolated from tumor xenografts are viable, clonogenic and resistant to killing by ionizing radiation. These important findings indicate that at least a fraction of the tumor cells that express CA IX is intermediate in oxygenation and may represent a potential source of metastases.

However, the intratumoral distribution of CA IX implies that hypoxia is not the only factor driving its expression. Immunohistochemical studies often refer to a certain proportion of tumors that do not show signs of hypoxia (such as the presence of necrotic areas, expression of HIF-1α, VEGF, and/or GLUT-1, incorporation of pimonidazole), but still do express CA IX and *vice versa*, some tumors with apparent hypoxic regions and absence of CA IX (Chia et al., 2001; Wykoff et al., 2001; Swinson et al., 2003). In some tumors, CA IX is coexpressed with proteins involved in angiogenesis, apoptosis inhibition and cell-cell adhesion disruption, including oncoproteins EGFR and c-ErbB2. Therefore, it is plausible that CA IX might also be regulated by the oncogenic pathways (Giatromanolaki et al., 2001; Bartosova et al., 2002), but these observations require further proof. Finally, the specific expression pattern of CA IX might be related to its high posttranslational stability in reoxygenated

cells, which has been proposed in tumor studies as a reason for the lack of full overlap with other hypoxic markers that are either short-lived (e.g., HIF-1α) or secreted (e.g., VEGF; (Turner et al., 2002). Pulse-chase analysis determined the CA IX protein half-life to be ca. 38 hours and showed that it is independent of the duration of hypoxia (Rafajova et al., 2004). This high protein stability allows for the long persistence of CA IX in reoxygenated tumor areas and might contribute to the adaptation of tumor cells to reoxygenation.

Although hypoxia is the major factor underlying CA IX expression in non-VHL human tumors, these adverse regulatory pathways can affect its overall distribution pattern considerably. Thus, understanding them can have important implications for the clinical interpretation of immunohistochemical data as well as for the use of CA IX as a therapeutic target.

2.3.5 Regulation of CA IX Expression

The broad carcinoma-related distribution indicated that expression of CA IX might represent a more general attribute of tumor tissues or that it might be regulated by a mechanism or pathway common to many tumor types. However, a sequence comparison between *CA9* cDNA derived from HeLa carcinoma cells and that from the normal human stomach did not show any mutation in the coding region, suggesting that mutations do not play any role in the differential expression of CA IX. Therefore, it has been proposed that other regulatory events, e.g. tumor-specific transcription factors, are involved (Pastorekova et al., 1997). Experimental evidence that the increased cell density can influence CA IX expression through promoter activation redirected the attention to a transcriptional regulation of the *CA9* gene (Lieskovska et al., 1999). A *CA9* promoter analyzed under conditions of high cell density was shown to possess five regulatory regions containing several cis-acting elements (Kaluz et al., 1999). An additional study has shown that synergistic cooperation between SP and AP-1 transcription factors is necessary for the basic transcriptional activation of *CA9* (Kaluzova et al., 2001). However, the most important regulatory element of the CA9 promoter is localized on the antisense strand between the SP-1 binding site and the transcription start at position -10/-3. It consists of the nucleotide sequence 5'-

TACGTGCA-3' corresponding to a hypoxia response element (HRE) (Wykoff et al., 2000).

The first clue to a major pathway involved in CA IX control was given by a demonstration of its downregulation by the wild-type von Hippel-Lindau (VHL) tumor suppressor protein (Ivanov et al., 1998). CA IX expression was not suppressed by the mutated variants of pVHL that lacked the elongin-binding domain, which is required for the interaction of pVHL with elongin C and integration within a ubiquitin ligase complex (Iwai et al., 1999; Lisztwan et al., 1999). This finding explained the overexpression of CA IX in the majority of renal cell carcinomas (RCCs) that frequently carry an inactivating mutation in the VHL gene (Gnarra et al., 1994). Additionally, CA IX has been demonstrated as a very early sign of premalignant lesions in VHL patients (Mandriota et al., 2002).

In tumors other than RCC, pVHL plays a critical role as an upstream negative regulator of an α-subunit of the hypoxia-inducible transcription factor or HIF (Maxwell et al., 2001). The mechanism of hypoxia-induced gene expression mediated by HIF transcription factor is described in Figure 1. In normoxic tumor cells with an adequate supply of oxygen, prolyl-4-hydroxylases (PHDs) hydroxylate two conserved proline residues of HIF-α. The von Hippel-Lindau protein (VHL) binds hydroxylated HIF-α and targets it for degradation by the ubiquitin-proteasome system and thus abrogates its functioning in the transcriptional activation of downstream genes (Ivan et al., 2001; Jaakkola et al., 2001). In hypoxia, which frequently occurs in tumors as a result of aberrant vasculature, HIF-α is not hydroxylated, because PHDs are inactive in absence of dioxygen. Nonhydroxylated HIF-α is not recognized by the VHL protein; instead, it is stabilized and it accumulates. As a result, HIF-α translocates to the nucleus and dimerizes with the HIF-β constitutive subunit to form the active transcription factor. The HIF transcription factor then binds to the hypoxia response element (HRE) in the target genes and activates their transcription. The target genes include glucose transporters (GLUT-1 and GLUT-3), which participate in glucose metabolism; vascular endothelial growth factor (VEGF), which triggers neoangiogenesis; erythropoietin (EPO-1), which is involved in erythropoiesis; CA IX, which is proposed to contribute to pH regulation; and additional genes with functions in cell survival, proliferation, metabolism and other processes (Maxwell et al., 2001; Semenza, 2001).

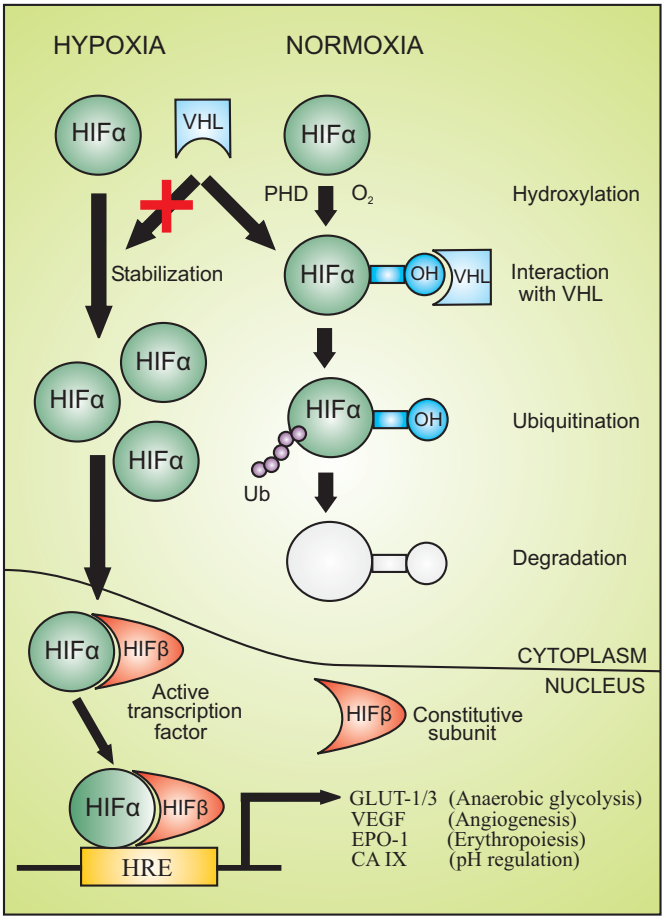

Figure 1. The mechanism of hypoxia-induced gene expression mediated by the HIF transcription factor. The mechanism illustrated in this figure is explained in the text. The figure is adapted from Pastorekova and Pastorek (Pastorekova, 2004a).

It is possible that CA IX expression is also regulated at higher stages of the biosynthetic trail, similarly to some other hypoxia-induced genes. There are several indications that support this theory, including the presence of consensus phosphorylation sites in the intracytoplasmic tail, which might affect the functional performance of CA IX, and the shedding of soluble CA IX, which might control the amount of the plasma-membrane associated protein (Zavada et al., 2003). However, these assumptions require further investigation.

2.3.6 Functions of CA IX in Tumors

It has been proposed that CA IX, similarly as other active CA isoforms, plays a role in pH regulation. This proposal seems meaningful especially in relation to anaerobic tumor metabolism that generates an excess of acidic products, such as lactic acid and H^+, which have to be extruded from the cell interior in order to maintain the neutral intracellular pH and protect the cells from death. The extrusion of metabolic waste and its poor clearance by inadequate tumor vasculature creates an acidic extracellular microenvironment that is more permissive for tumor cell growth and invasion (Stubbs et al., 2000). Nevertheless, lactic acid is not the only cause of acidosis, and the studies of intratumoral physiological parameters have indicated that CO_2 is a significant contributing factor (Helmlinger et al., 1997; Helmlinger et al., 2002). A role for CA IX in this process appears to involve a catalytic conversion of CO_2 to bicarbonate and proton at the extracellular side of the plasma membrane and facilitation of the bicarbonate transport to the cell cytoplasm. In analogy to another extracellular isozyme, CA IV, which physically interacts with bicarbonate transporters such as anion exchangers (AE) to form a transport metabolon in differentiated cells (Sterling et al., 2002), CA IX may cooperate directly with AE in tumor cells and assist in neutralizing their intracellular space. At the same time, the protons produced by CA IX from the hydration of CO_2 may remain outside and improve the acidosis of the microenvironment.

The proposed involvement of CA IX in the pH regulation in tumors is illustrated in Figure 2. The transport metabolon is composed of AE and CAs, and is analogous to the CA IV-AE-CA II metabolon (Sterling et al., 2002). As an extracellular component of the metabolon, CA IX hydrates carbon dioxide and provides bicarbonate anions to AE, which transports them to the cytoplasm in exchange for chloride anions. At the intracellular side, CA II converts bicarbonate to carbon dioxide, which diffuses out through the plasma membrane. In addition, extracellular CA IX activity generates protons that contribute to the acidification of external pH, whereas cytoplasmic CA II activity allows for the consumption of intracellular protons and contributes to the neutralization of internal pH.

It is well known that an acidic extracellular milieu induces the production of growth factors, increases genomic instability, perturbs cell-cell adhesion, and facilitates tumor spread and metastasis (Stubbs et al., 2000). Evidence for CA IX as a causal factor of tumor acidosis may thus support its functional involvement in these processes.

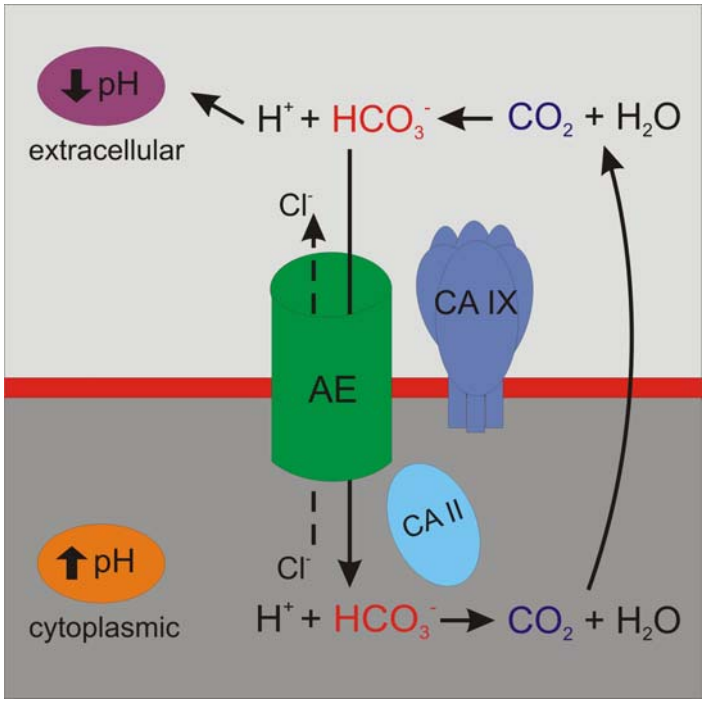

Figure 2. A Model of a transport metabolon composed of an anion exhanger (AE) and CAs. This model illustrates the proposed involvement of CA IX in pH regulation in tumors. The mechanism illustrated in this figure is explained in detail in the text. The figure is adapted from Pastorekova and Zavada (Pastorekova, 2004b).

CA IX is also a cell adhesion molecule (CAM), which can mediate the attachment of cells to non-adhesive solid support, suggesting its possible role in cell-matrix interactions (Zavada et al., 2000). This activity resides in the N-terminal end of the molecule, in the proteoglycan-like domain. The adhesion site of CA IX overlaps with the epitope for M75 monoclonal antibody, PGEEDLP, since M75 blocks the adhesion of cells to the immobilized CA IX protein. The PG region contains three identical repeats of the motif GEEDLP and four modified repetitions.

One significant feature of the PG segment of the CA IX molecule is a high dicarboxylic amino acid content (24 D + E out of total 59 residues) and at the same time, a low basic amino acid content (4 R + K). The acidic character of the PG is reflected by the fact that CA IX dissociates easily from the complex formed with the

M75 antibody or with the cell surface receptor, even at only slightly acidic pH levels. This property might facilitate the release of cells from the tumors acidified by the products of hypoxic metabolism. The cells might then attach elsewhere in the organism where the pH is neutral or slightly basic, and start a metastatic growth (Pastorekova, 2004b). In addition, CA IX appears to play a role in intercellular adhesion. In polarized epithelial MDCK cells transfected with the human *CA9* cDNA, the CA IX protein colocalizes with a key adhesion molecule E cadherin and destabilizes E cadherin-mediated cell-cell contacts via a mechanism that involves competitive interaction with β-catenin (Svastova et al., 2003). This capability of CA IX is reminiscent of some oncoproteins (EGFR, c-ErbB2, MUC-1) and makes it a candidate contributor to tumor invasion that is known to require a diminished intercellular adhesion.

2.3.7 CA IX as a Marker of Tumor Hypoxia

The discovery that CA IX is a HIF target started a new era in CA IX research, since hypoxia is a clinically important tumor parameter that has a significant impact on the treatment outcome and disease progression (Hockel et al., 2001). Many studies of CA IX expression in hypoxic tumors have been performed in a hope that it might potentially serve as an intrinsic marker of tumor hypoxia and possibly also as a therapeutic target. CA IX distribution has often been examined in relation to the extent of necrosis as an indicator of severe hypoxia, to microvascular density (MVD) as a measure of angiogenesis, and to tumor stage and disease progression.

In breast tumors, CA IX was associated with necrosis and a high grade of ductal carcinomas *in situ* (Wykoff et al., 2001), negative estrogen receptor status (Span et al., 2003), higher relapse rate and worse overall survival of patients with invasive carcinomas (Chia et al., 2001). CA IX was also associated with necrosis, MVD, advanced stage and poor response to chemoradiotherapy in head and neck carcinomas (Beasley et al., 2001; Koukourakis et al., 2001). Additionally, CA IX expression correlated with the level of hypoxia measured by needle electrodes in cervical tumors, wherein it was a significant and independent prognostic indicator of overall survival and metastasis-free survival after radiation therapy (Loncaster et al., 2001). In the non-small cell lung cancer, CA IX was a significant factor of poor prognosis independent of angiogenesis (Giatromanolaki et al., 2001) and its stromal expression was associated

with advanced tumor stage (Swinson et al., 2003). In bladder cancer, CA IX was found predominantly on the luminal surface and in surrounding areas of necrosis. It was expressed more in superficial than in invasive tumors, and although it did not predict outcome in superficial disease, its luminal expression deserves further investigation (Turner et al., 2002). The examination of biopsies from patients with a locally advanced nasopharyngeal carcinoma treated by chemoradiation showed that tumors with a positive hypoxic profile (defined as high expression of both CA IX and HIF-1α) were associated with a worse progression-free survival (Hui et al., 2002).

In an independent study of mechanisms involved in the adaptation of tumors to hypoxia, using a serial analysis of gene expression in human glioblastoma cells, *CA9* displayed the highest magnitude of induction among 32 identified hypoxia-responsive genes (Lal et al., 2001). Of the 12 genes selected, *CA9* was induced in the highest number of tumor cell lines and was the most consistently induced gene in human solid tumors.

An analysis of spheroids and tumor xenografts generated from human cervical carcinoma and glioma cells confirmed that CA IX-expressing cells are clonogenic, more likely to be resistant to killing by ionizing radiation and bind significantly more pimonidazole, a chemical marker of hypoxia, than do cells that express little or no CA IX (Olive et al., 2001).

Thus, CA IX has potential clinical utility as an intrinsic marker of hypoxia in a wide variety of tumors. However, its further investigation as a prognostic indicator and therapeutic target is required.

2.4 Carbonic Anhydrase XII

2.4.1 General Aspects

The cDNA sequence of CA XII was published in 1998 by two independent groups and allowed the classification of CA XII as a CA IX/CA IV-related transmembrane protein with an extracellularly exposed enzyme domain containing all three histidines needed for catalytic activity (Ivanov et al., 1998; Tureci et al., 1998). Türeci et al. (1998) identified CA XII in a human renal cell carcinoma by serological expression screening with autologous antibodies. They cloned and sequenced the corresponding cDNA and

proved that its mRNA is overexpressed in ca. 10 % of RCC patients. Ivanov et al. (1998) cloned CA XII as a novel pVHL target by using RNA differential display. They showed that the expression of *CA12* mRNA is strongly inhibited by the wild-type pVHL in RCC cell lines, which suggests that it is subjected to similar regulation as *CA9*.

The *CA12* gene has been mapped to chromosome 15, and its amino acid sequence includes a 29-amino acid signal peptide, a 261-amino acid CA domain, an additional short extracellular segment, a 26-amino acid hydrophobic transmembrane domain, and a 29-amino acid C-terminal cytoplasmic tail containing two potential phosphorylation sites. The extracellular CA domain has three zinc-binding histidine residues found in active CAs and two potential sites for asparagine glycosylation (Tureci et al., 1998). CA XII has a sequence identity of 30-42 % to other CAs. The reported molecular weight of CA XII produced in transfected COS cells is 43-44 kDa. It is reduced to 39 kDa by PNGase F digestion, which is consistent with the removal of two oligosaccharide chains (Tureci et al., 1998). The recombinant CA XII protein is an active isozyme, and its catalytic properties are similar to those of the high-activity membrane-associated CA IV (Ulmasov et al., 2000).

2.4.2 CA XII Expression in Normal Tissues

The tissue distribution of CA XII has not yet been fully characterized. The first studies showed that *CA12* mRNA is expressed at very low levels in the normal adult kidney, pancreas, colon, prostate, ovary, testis, lung, and brain (Ivanov et al., 1998; Tureci et al., 1998). This is shown in Table 4. The CA XII protein expression has been studied in human and rodent tissues.

In humans, the CA XII protein expression has been demonstrated in normal endometrium, where it was confined to the basolateral plasma membrane of epithelial cells. The function of CA XII in the human endometrium is not known, but it has been suggested that it may play a role in the reproductive functions of the uterus by contributing to bicarbonate production at this site. CA XII may also be functionally linked to the pH-dependent events in spermatozoa that precede fertilization (Karhumaa et al., 2000).

In the human intestine, CA XII was absent from the small intestine but was expressed in all segments of the normal human large intestine. The positive signal was confined to the basolateral plasma membranes of the epithelial cells of the surface epithelial cuff (Kivela et al., 2000a). The active center of CA XII was located on the cell exterior, beneath the basolateral plasma membrane (Ivanov et al., 1998; Tureci et al., 1998), where it may be functionally involved in transcellular water transport. Through its enzymatic activity, CA XII could convert extracellular water and CO_2 to bicarbonate and a proton, resulting in net acidification and a concentration of the extracellular fluid (Kivela et al., 2000a).

In the human kidney, CA XII was strongly expressed in the basolateral plasma membrane of the epithelial cells in the thick ascending limb of Henle and distal convoluted tubules, and in the principal cells of the collecting ducts. A weak basolateral signal was detected in the epithelium of the proximal convoluted tubules (Parkkila et al., 2000a). CA XII expression has also been detected in the pancreatic epithelium, the expression being confined to the basolateral plasma membranes of acinar and ductal cells (Kivela et al., 2000b). In addition, CA XII has showed a weak immunoreaction in normal gastric mucosa (Leppilampi et al., 2003) and strong expression in the male excurrent ducts (Karhumaa et al., 2001a).

In mouses, a strong positive signal for CA XII mRNA has been detected in the kidney, and weak signals have been obtained in the testis and lung (Halmi et al., 2004). Kyllönen et al. (Kyllonen et al., 2003) have studied the localization of the CA XII protein in the mouse and rat kidney. In the mouse kidney, CA XII was present in the proximal tubules and intercalated cells of the collecting ducts. In the medulla of the mouse kidney, a strong immunoreaction was seen in the collecting ducts. In the proximal tubules, CA XII immunostaining was intense in the S1 segment and decreased towards the S2 segment, whereas S3 proximal tubules were negative. The labelling was restricted to the basolateral plasma membrane, while the luminal brush border membrane was negative. In the rat kidney, the staining pattern was similar, although the signal was weaker in proximal tubules.

In addition to kidney, CA XII is expressed in other mouse tissues, which has been shown by Halmi et al. (Halmi et al., 2004). In the gastrointestinal tract, CA XII was not expressed in the stomach, duodenum, and jejunum. The enterocytes of the ileum showed a faint positive signal, and the reaction became much stronger in the colon and rectum. In the large intestine, the staining was most intense in the surface epithelial cuff

region. The highest expression was seen on the basolateral surfaces. Weak staining was detected in the developing sperm cells. No specific staining for CA XII was found in the mouse liver and pancreas or in the psoas and heart muscle. In another study, it has been shown that CA XII is expressed in the mouse endometrium (Hynninen et al., 2004). The isozyme was detected in the epithelial cells of the mouse endometrium, and it was more intensely stained in the deeper endometrial glands. However, CA XII was also clearly expressed in the surface epithelial cells, but the staining intensity was weaker compared to the glands. The strongest reaction for CA XII was associated with the basolateral plasma membrane, as expected. Since CA XII is expressed in the endometrium of different species (mouse and human), this isozyme can be hypothesized to have a role in reproductive physiology.

Table 4. A schematic overview of CA XII distribution in normal human tissues and derived tumors. The intensity of the gray tone in the rectangles corresponds to both the level and the frequency of expression: darker tones indicate strong expression and the white indicates no expression. The data has been derived from Pastorekova and Pastorek (Pastorekova, 2004a).

Normal tissues	Expression		Tumor tissues
CNS - neurons			Glioma/ependynoma
CNS - choroid plexus			Choroid plexus tumor
Body cavity linings			Mesothelioma
Salivary glands			Papillary/follicular carcinoma
Esophagus			Head/neck carcinoma
Respiratory tract			Lung carcinoma
Stomach/duodenum			Stomach carcinoma
Colon			Colon carcinoma
Gallbladder			Biliary carcinoma
Pancreas			Pancreatic carcinoma
Kidney			Renal cell carcinoma
Prostate			Prostate carcinoma
Testis			Germ cell tumor
Uterine cervix			Carcinoma of cervix uteri
Endometrium			Endometrial carcinoma
Breast			Breast carcinoma
Skin			Squamous/basal cell carcinoma

Recently, CA XII has been demonstrated in the rat epididymis (Hermo et al., 2005). CA XII appeared to be maximally expressed in the corpus and proximal cauda epididymis, where it was localized to the basolateral plasma membranes of adjacent

principal cells, corresponding to similar localizations in analogous regions of the kidney (Parkkila, 2000).

2.4.3 CA XII Expression in Cancers

CA XII is widely expressed in several different tumor types including renal (Parkkila et al., 2000a), colorectal (Kivela et al., 2000a; Kivela et al., 2005), pancreatic (Kivela et al., 2000b), gastric (Leppilampi et al., 2003) and breast tumors (Watson et al., 2003). This is shown in Table 4.

CA XII is expressed not only in the normal human kidney but also in most clear-cell carcinomas and oncocytomas (Parkkila et al., 2000a). Parkkila et al. investigated the expression of CA XII in 31 renal tumor specimens. CA XII showed moderate or strong plasma membrane-associated expression in most oncocytomas and all clear-cell carcinomas. The mean staining intensity varied from moderate to strong in all tumor categories except for angiomyolipoma and Wilms' tumor, the latter showing much weaker immunoreaction compared to other tumors ($P<0.001$). In clear-cell carcinomas, the immunoreaction showed a trend of correlating with the histological grade, being slightly weaker in well-differentiated carcinomas. It is notable, however, that this difference did not reach statistical significance because of the small number of grade 1 tumors.

The distribution of CA XII in colorectal tumors clearly differs from the normal expression in the colon (Kivela et al., 2000a). The most dramatic change was observed in the deep parts of the adenomatous mucosa, where the positive immunoreaction clearly increased with the grade of dysplasia. The adenomas with severe dysplasia and the carcinomas showed a diffusely spread staining pattern that seemed to be associated with the malignant behavior of the colorectal tumors. These results suggest that, in addition to CA IX (Saarnio et al., 1998a), CA XII is the second transmembrane isozyme that might be useful in the histopathological diagnosis of colorectal tumors.

CA XII is expressed in the normal human pancreas as well as in pancreatic tumors (Kivela et al., 2000b). However, the expression of CA XII was very weak or weak in most carcinomas (n=21). A moderate or strong immunoreaction for this isozyme was observed only in 3 out of 29 malignant tumors of exocrine pancreas, while 5 of them were completely negative. In ductal carcinomas, the positive signal for CA XII was

present in 0-75 % of the epithelial cells. No correlation was observed between the staining extent or intensity and the grade of differentation. From the benign tumors of exocrine pancreas, only one mucinous cystadenoma and one microcystic adenoma showed a strong positive signal for CA XII. Three endocrine tumors remained unstained and one glucagonoma showed a weak positive reaction. The expression of CA XII in a relatively low number of malignant tumor specimens suggests that it has a limited value in the diagnostic evaluation of pancreatic carcinoma.

CA XII is not expressed in considerable amounts in the normal gastric mucosa. Instead, the staining indices increase significantly in hyperplastic and adenomatous lesions as well as in grade I and II adenocarcinomas and metastases. The indices were only very slightly increased in grade III adenocarcinomas and diffuse carcinomas, but the difference was not statistically significant. Since CA XII did not show any prominent changes in different tumor categories, its clinical significance may be limited in this area (Leppilampi et al., 2003).

Watson et al. (Watson et al., 2003) have studied the expression of CA XII in a series of 103 cases of invasive breast cancer and examined any association with recognized prognostic factors or relation with the outcome. CA XII was present in 77 out of 103 (75 %) cases and was associated with a lower grade ($P=0.001$), positive estrogen receptor status ($P<0.001$), and a negative epidermal growth factor receptor status ($P<0.001$). Furthermore, although CA XII expression was associated with an absence of necrosis ($P<0.001$), expression in some high-grade tumors was induced in regions directly adjacent to morphological necrosis. In addition, using univariate analysis, CA XII positive tumors were associated with a lower relapse rate ($P=0.04$) and better overall survival ($P=0.01$). The authors concluded that CA XII expression is influenced by factors related to differentiation and hypoxia in breast cancer *in vivo*, and CA XII expression is associated with a better prognosis in an unselected series of invasive breast carcinoma patients.

2.4.4 Regulation of CA XII Expression

It has been demonstrated by Ivanov et al. (Ivanov et al., 1998) that CA XII is a von Hippel-Lindau (VHL) target. They have shown that the expression of CA XII is strongly inhibited by the wild type VHL in diverse RCC cell lines, also suggesting that

it is subject to similar regulation as the gene encoding the other tumor-associated CA, that is, *CA9*. However, the suppression of *CA12* requires both the central VHL domain involved in the hypoxia-inducible transcription factor 1α (HIF-1α) binding and the C-terminal elongin-binding domain, whereas only the latter is needed for the negative regulation of *CA9*. Thus, CA XII is regulated by hypoxia, similarly with CA IX, but by means of diverse biochemical pathways (Wykoff et al., 2000; Ashida et al., 2002).

In addition, the *CA12* gene does not contain any HRE element that would correspond to the HRE of *CA9* present in the promoter region close to the transcription initiation site (and found also in the mouse *Car9* gene). In fact, there are additional candidate core HRE sequences in the *CA9* 5' upstream region that are more distant from the transcription strart (Table 5).

Table 5. The consensus sequences of hypoxia response elements (HREs) in the 5' regions of the human *CA9* gene, its mouse *Car9* counterpart and the human *CA12* gene [1]. The data has been obtained from Pastorekova (Pastorekova, 2004a).

	TGCACGTA	CACGY	RCGTG
CA9	-10/-3	-8/-4	-1410/-1406
			-1711/-1707
			-2129/-2125
Car9	-1/+7	+1/+6	-181/-177
		-1349/-1345	-879/-875
			-1533/-1529
CA12		-515/-511	-1046/-1042
		-245/-241	-417/-413
		+28/+32	

[1] Positions of HRE are numbered with respect to the transcription initiation site. The first column marks the sequence of the functional *CA9* HRE that is localized on the negative strand close to the transcription start site. It is found also in the mouse *Car9* gene. The putative core HRE sequences are present more upstream on both strands of all three genes (second and third column).

The upstream region of the *CA12* gene possesses several putative HRE elements with a core HIF-binding sequence, but their functionality has not been examined so far. According to tissue distribution and *in vitro* experiments, CA XII does not appear to be

as tightly regulated by the hypoxia/pVHL pathway and as strongly linked to cancer as CA IX (Wykoff et al., 2000; Ivanov et al., 2001).

2.5 Carbonic Anhydrases in Embryonic Development

2.5.1 CA I, II and III

It has been shown that the human CA I gene product appears in a developmental stage-specific manner (Brady et al., 1990). When red cells from different stages of ontogeny were analyzed, virtually no CA I protein was detectable in fetal red cells prior to birth. Nonetheless, at about the time of normal delivery (40 weeks gestation) CA I production was switched on.

Expression of the carbonic anhydrase II gene and protein in early mouse brain cells has been studied by *in situ* hybridization and immunohistochemistry (De Vitry et al., 1989). In this study, hypothalamic cells of embryonic day (ED) 12-14 mice were cultured for various periods, and the chronologic appearance of CA II mRNA and protein was studied. The *CA II* gene transcripts were detectable as early as ED 12-13, although the protein they encode was not detectable until ED 17-18. Gene expression was restricted to 0.1 % of the total population. At postnatal stage, a majority of glial cells expressed both the CA II mRNA and the protein.

CA II localization has also been studied in mouse embryonic and fetal hearts (Vuillemin et al., 1997). In the earliest stages studied, 10, 11 and 12 ED, a sharp decrease of labelled cells was observed in the endocardium from which cushion-tissue mesenchyme is derived. During the same period, differences in the decreasing frequencies of labelled cells were also observed between three different cushion-tissue mesenchyme localizations: immunostained cells were abundant in the atrioventricular cushions, less numerous in the proximal part of the conotruncal ridges and rare in their distal part. From 13 ED their repartition was more regular along the conotruncus. From 13 to 16 ED the signal was also present in a peculiar region of the myocardium: the anterior and left walls of the left ventricle. At the 18 and 20 ED labelling was found only in some endothelial cells of coronary vessels, particularly in the interventricular septum. On the basis of this expression pattern, Vuillemin et al. suggested that CA II can be a useful marker for a subpopulation of endothelial cells and cells derived from

this endothelium that morphologically express signs of active cell behavior (e.g., invasion, migration, proliferation).

In addition, CA II is expressed during the development of the choroid plexus in the human fetus (Catala, 1997). Choroid plexuses between 9 and 34 weeks of gestation were included in the study. The CA II protein was present as early as the 9^{th} week of gestation. Thus, it is possible that this isozyme could account for the secretion of cerebrospinal fluid during fetal life.

The appearance of carbonic anhydrase isoenzymes II and III in rat liver and skeletal muscle during fetal and postnatal development has been demonstrated by Laurila et al. (Laurila et al., 1989). They showed that in the 12-day fetus the early strong expression of CA I in hepatocytes was partially replaced by the expression of CA II and CA III during the late prenatal development. In the 20-day fetus the staining intensity of CA III was equal to that of a mature female rat. In the male, the staining intensity in hepatocytes clearly increased during sexual maturation. Immunoelectron microscopy showed diffuse cytoplasmic and nucleoplasmic staining of CA III in hepatocytes. The authors suggested that the time-dependent expression of the isoenzymes in hepatocytes may reflect a different metabolic function of these structurally closely related isozymes. In skeletal muscle, CA III was the only isozyme detected during development. Furthermore, in late prenatal and early postnatal stages all muscle fibers contained roughly equal amounts of CA III.

The expression of the CA II and III protein has also been studied in bovine parotid glands during fetal development (Asari et al., 1994). In a 3-month-old fetus of a crown-rump length of (CRL) 17 cm, the expression of CA II in undifferentiated epithelial cells was noted, whereas immunostaining for CA III remained negative. At 26 cm CRL (4-5 months old), weak expression of CA III was seen in large ductal epithelial cells. The accumulation of secreted granules in primary acinar cells was initially observed at this stage. In a newborn calf, anti-CA II reactivity almost disappeared from most duct segments. Asari et al. suggested that the time-dependent expression and distribution of the isozymes in parotid glands may reflect the different biological functions of these structurally closely related isozymes. Thus, the bovine parotid acinar cells of fetuses would appear to possess all the cellular structures and immunohistochemical properties at 4 and 5 months of gestation.

Lyons et al. (Lyons et al., 1991) have shown that CA III mRNA is expressed in embryonic mouse skeletal muscle and notochord. They studied mouse embryos and

fetuses from 7.25 days to 17.5 days post coitum (p.c.). CA III mRNAs were first detected in the myotomes of somites between 9.5 and 10.5 days p.c. At 15.5 days p.c., CA III began to be restricted to developing slow muscle fibers. By two weeks post partum (p.p.), CA III mRNAs were detected mainly in slow muscle fibers. At the developing notochord, CA III transcripts were seen at an earlier stage (7.25 days p.c.). In addition, CA III was expressed at a much higher level in the notochord than in the developing skeletal muscle.

Moreover, the expression of CA III has been demonstrated in extraocular muscles of human embryos (Carnegie stages 13-23) (Oguni et al., 1992). At stage 20, CA III immunoreactivity appeared in some muscle fibers of extraocular muscles. From stage 21 to stage 23, CA III-immunoreactive fibers increased. It can be suggested that CA III-immunoreactive type 1 fibers appear in the late stage of myogenesis compared with beta-enolase-immunoreactive type 2 fibers, which appear at stage 18.

2.5.2 CA VI

CA VI expression has been demonstrated in the ovine parotid and submandibular glands (Penschow et al., 1997). CA VI was detectable by immunohistochemistry in parotid excretory ducts from 106 days gestation (the term is 145 days), in striated ducts from 138 days and in acinar cells from 1 day postnatal. The duct cell content of CA VI declined as the acinar cell population increased. The production of CA VI by submandibular duct cells was detectable initially at 125 days gestation, and acinar production was not seen until 29 days post-natal. Thus, there was a parallel pattern of CA VI expression during the development of these major salivary glands.

Furthermore, CA VI is expressed in the developing bovine parotid gland (Asari et al., 2000). In the 26-cm CRL fetus, estimated to be 4-5 months of fetal age, a few immature epithelial cells expressed CA VI weakly. These cells eventually differentiated into ductal and acinous epithelial cells of the ductal and terminal regions. In the 52-cm CRL fetus, estimated to be 7 months of fetal age, most acini (matured terminal tubules) and ductal epithelial cells were intensely positive for anti-CA VI. Both acini and ductal cells possessed CA VI throughout prenatal development. Following birth, the expression of CA VI gradually began to disappear from all small (intercalated) and large (interlobular) duct segments. In the end, the immunoreaction had almost

completely disappeared from the entire ductal cell region. Instead, the immunoreaction found in the acinar cells from 1 or 5 months of age was strong and the expression pattern was almost indistinguishable from that of the adult.

2.6 Principles of the Methods

2.6.1 *In Situ* Hybridization

In situ hybridization (ISH) is based on the process where labelled single-stranded fragments of DNA or RNA containing complementary sequences (probes) are hybridized to cellular DNA or RNA under conditions that are appropriate for forming stable hybrids. ISH was first introduced in 1969 (Höfler, 1990) and it has become an important technique in a number of fields, including the diagnosis of chromosomal rearrangements, the detection of viral infections, and the analysis of gene function during embryonic development (Wilkinson, 1999). The essential features of ISH are good cellular spatial resolution and specificity. ISH can also provide information on the timing of expression.

In situ hybridization involves the generation of a nucleic acid probe, which must be labelled to enable subsequent detection (Wilkinson, 1999). Probes can be as small as 20-40 base pairs, up to 1000 base pairs. There are essentially four usable probe types, which include oligonucleotide probes, single and double stranded DNA probes, and single stranded RNA probes or riboprobes (Höfler, 1990).

Oligonucleotides make applicable hybridization probes because they can be designed to detect specific groups of genes, specific genes, or indeed specific alleles (genetic disease diagnosis) or serotypes (pathogene detection) (Lathe, 1990). Oligonucleotide probes are prepared conveniently by an automated chemical synthesis and they are small, generally from 20 to 40 base pairs. The small size allows easy penetration into the cells or tissue of interest. On the other hand, they cover less target. The main disadvantage of this technique is the low specific activity of the probes.

Single stranded DNA probes have similar advantages to the oligonucleotide probes but they are much larger, approximately in the 200-500 base pair size range. Compared to double stranded DNA, which is denatured prior to hybridization, single stranded probes have the theoretic advantage that re-annealing of the probe to the

second strand cannot occur (Höfler, 1990). Double stranded DNA probes are generally less sensitive because of the tendency of the DNA strands to rehybridize to each other.

The use of RNA probes for ISH has been pioneered largely by Angerer and colleagues (Cox et al., 1984). Riboprobes are single-stranded RNA molecules produced from a cloned cDNA that has been introduced into a specifically designed plasmid reverse-transcription system. Unlike double-stranded DNA probes, riboprobes do not re-anneal in solution, so a greater percentage of the probe is available for hybridization, giving stronger signals than cDNA probes (Cox et al., 1984). Another advantage of the riboprobes is that they can be synthesized to high specific activity. RNA probes can be readily created using SP6, T3, or T7 promoters in both sense and antisense orientations to provide specific and control probes (Wilson et al., 1997). In addition, the cRNA-mRNA hybrids are more stable than corresponding cDNA-mRNA hybrids (Gibson, 1990). However, RNA probes are sometimes 'stickier' than DNA probes, producing a higher degree of non-specific binding to tissue. This problem can be circumvented by the use of enzymes in the post-hybridization solution, which reduces the possibility of background staining (Gibson, 1990). The disadvantages of the riboprobes include the fact that they are very sensitive to RNases, hence strict adherence to RNase-free precautions is very important during most of the protocol.

Probes for ISH can be labelled with a radioisotope when the product is detected by autoradiography (Bhatt, 1990). On the other hand, non-radioactive labels such as digoxigenin or biotin are detected by immunocytochemical methods. Radioactively labelled probes are widely used for several reasons. First, the efficiency of probe synthesis can be monitored more easily. In addition, radioisotopes are readily incorporated into the synthesized DNA and RNA using most enzymes. Finally, results can be interpreted sensitively with autoradiography (Höfler, 1990). The most generally used radioactive isotopes are ^{33}P, ^{32}P, and ^{35}S.

The combination of radioisotopes and detection via a contact emulsion has been succesfully used for ISH for many decades. The detection of beta particles in an emulsion is due to the ionization that occurs from the passage of fast electrons (β particles) in matter. A large excess of energy is deposited locally from each interaction of a fast electron with the atoms in the emulsion. This energy causes the reduction of Ag^+ ions to metallic silver, which subsequently aggregate to form a latent image. The latent image is then developed and fixed by normal photographic procedures. The results can be interpreted using a dark-field microscope when image centers are seen as

white specks or using a light-field microscope when image centers are seen as black specks. The combination of radiolabel, sample, and emulsion thickness can be chosen to yield either a high-sensitivity result with relatively poor resolution, or alternatively a high-resolution result with the sacrifice of time (Brady, 1990).

There are many aspects in tissue fixation and preparation that have to be considered for ISH. Optimal fixation and tissue preparation should retain the maximal level of cellular target DNA or RNA while maintaining optimal morphological details and allowing sufficient accessibility of the probe. In contrast to the rather stable DNA, mRNA is steadily synthesized and degraded enzymatically. Therefore, tissue prepared for RNA localization should be fixed or frozen as soon as possible after surgical excision. For the localization of DNA, the type and concentration of the fixative is not of major significance. On the other hand, for RNA localization, the type, time and concentration of the fixative are significant, if loss of RNA is to be minimized (Höfler, 1990).

ISH is a powerful method for specifically localizing DNA or RNA in cells. For optimal results, however, an appropriate system of probe construction, labelling and signal detection has to be chosen. In addition, the degree of specificity of the hybridization reactions can be controlled accurately by varying the reaction conditions (Höfler, 1990).

The main steps of the ISH procedure are summarized below.

1. Tissue treatment
    - preparation of chromosome spreads or fixation of tissues (which can be sectioned)
2. Constructing a nucleic acid probe
3. Probe labelling to enable subsequent detection
4. Pre-treating tissues to increase probe penetration and accessibility to target nucleic acid
5. Hybridization of the labelled probe to chromosomes or tissues
6. Post-hybridization treatments to remove non-hybridized probe
7. Detection of the labelled probe, revealing the location of the target cellular nucleic acid
    - isotopic probes: X-ray film-emulsion dipping
    - non-isotopic probes: enhancing detection systems

2.6.2 Peroxidase-antiperoxidase Method

The peroxidase-antiperoxidase method (PAP) is a soluble enzyme immune complex technique, sometimes also called unlabelled antibody method (Boenisch, 2001). The PAP method is illustrated in Figure 3. The staining procedure of this method consists of the use of an unconjugated primary antibody, a secondary antibody, the soluble enzyme-anti-enzyme complex and substrate solution. The primary antibody and the antibody of the enzyme immunocomplex must be made in the same species. The secondary antibody must be directed against immunoglobulins of the species producing both the primary antibody and the enzyme immunocomplex. The secondary antibody is added in excess so that one of its two Fab sites binds to the primary antibody leaving the other site available for binding the antibody of the enzyme immunocomplex.

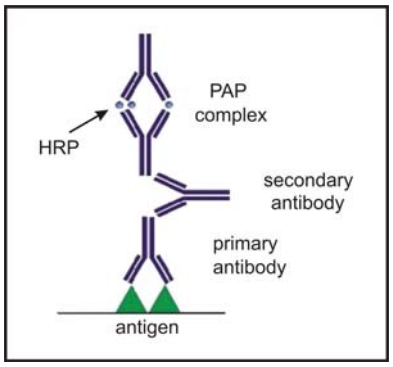

Figure 3. PAP method. This method includes the use of an unconjugated primary antibody, a secondary antibody, the PAP complex and substrate solution. PAP method is explained in detail in the text.

The PAP complex consists of three molecules of horseradish peroxidase (HRP) and two antibodies. The enzyme activity of HRP is used to detect the sites containing the studied antigen. HRP activity in the presence of an electron donor first results in the formation of an enzyme-substrate complex, and then in the oxidation of the electron donor. The electron donor provides the "driving" force in the continuing catalysis of H_2O_2, while its absence effectively stops the reaction. There are several electron donors that, when oxidized, become colored products, and hence are called chromogens. One electron donor of that kind is DAB (3,3' -diaminobenzidine tetrahydrochloride), whicproduces a brown end product.

3. AIMS OF THE RESEARCH

The present study had two aims:

1. To identify the tissues that express CA IX and CA XII in mouse embryos of different ages.
2. To compare the expression patterns of CA IX and XII between embryonic and adult mouse tissues.

The expression of CA IX and XII mRNAs was examined by *in situ* hybridization, and immunohistochemistry was used to study the protein expression.

4. MATERIALS AND METHODS

4.1 Tissue Samples

Adult and newborn mouse tissues for *in situ* hybridization were obtained from Balb/c mice. Samples from the adult mouse were serving as positive controls and included kidney, spleen, pancreas, liver, brain, stomach, duodenum, colon, skeletal muscle, heart, thymus and lung. The tissue specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Mouse embryos were obtained by mating male and female NMRI mice. The noon on the day of which the copulation plug was found was considered to represent 0.5 days p.c. 7.5, 8.5, 9.5, 10.5, 11.5, 12.5 p.c. embryos with or without extraembryonic tissues were briefly washed with PBS, fixed with 4 % paraformaldehyde and embedded in paraffin. Adult and embryonic mouse tissue sections were cut at 5-8 μm and placed on SuperFrost® Plus microscope slides (Menzel, Braunschweig, Germany).

For immunohistochemistry, tissue samples of the stomach, heart, brain, liver, kidney, and pancreas were obtained from an adult Balb/c mouse to serve as positive controls. The tissue specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Sections were cut at 5 μm and placed on SuperFrost® Plus microscope slides. The 7.5, 11.5, 12.5, and 13.5 p.c. embryos were obtained by mating male and female NMRI mice and processed as described above. The procedures were approved by the institutional animal care committees (University of Tampere and University of Helsinki**)**.

4.2 Cloning of the Mouse CA IX and XII cDNAs

In order to detect CA IX and CA XII RNA from tissues of interest, cDNA templates were prepared by creating plasmid constructs. cDNA templates for CA IX and XII were synthesized by PCR, and kidney from a commercial cDNA kit (Mouse MTC Panel I, BD Biosciences, Palo Alto, CA) was serving as a source of cDNA. The primers for the PCR were designed using the published information on the mouse CA IX and XII

mRNA sequence (GenBank NM_139305 and NM_178396, respectively) and commercial Primer designer -software (Primer designer, version 1.01, Scientific and Educational Software). A 565-bp CA IX cDNA was synthesized using the forward primer 5'-GGAGGCCTGGCAGTTTTGGC-3' (nucleotides 794-814) and the reverse primer 5'-CCGTCTCTACTGTCTTTGACCTC-3' (nucleotides 1336-1358). To produce a CA XII amplification product of 574 bp, the forward primer was 5'-AGAACTGGTCCAAGAAGTAC-3' (nucleotides 229-248) and the reverse primer was 5'-GACTGATGTGGAGGAACGAT-3' (nucleotides 783-802). The primers were produced by Sigma Genosys (Cambridge, UK).

2-3 ng of the cDNAs were used as templates for the PCRs. The PCR was carried out in a thermal cycler (Biometra, Göttingen, Germany) using BD Advantage 2 polymerase (BD Biosciences, San Jose, CA, USA), and the cycling protocol consisted of the following steps:

CA IX	CA XII
Denaturation 94 ^{o}C 1 min	Denaturation 94 ^{o}C 1 min
31 cycles of	*31 cycles of*
Denaturation 94 ^{o}C 30 sec	Denaturation 94 ^{o}C 30 sec
Annealing 60 ^{o}C 30 sec	Annealing 51 ^{o}C 30 sec
Elongation 72 ^{o}C 1 min 30 sec	Elongation 72 ^{o}C 1 min 30 sec
Final extension 70 ^{o}C 10 min	Final extension 70 ^{o}C 10 min

The PCR products were analyzed by electrophoresis on 1.0 % agarose gel containing 0.1 µg/ml ethidium bromide with 100 bp DNA standard (GeneRuler™ 100 bp DNA Ladder Plus, Fermentas Inc., USA). The PCR products were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK).

The PCR products were ligated into commercial pGEM-T Easy Vector (Promega Corp.) and the resulting plasmid constructs were transformed into OneShot™ TOP10 chemically competent *E.coli* (Invitrogen Corp.). Plasmid DNAs were isolated using QIAprep Miniprep Kit (Giagen, Hilden, Germany).

The same templates were used to transcribe both the antisense and the sense probe, and hence the orientation of the inserts was confirmed using PCR. For CA IX,

primers T7 and Sp6 according to the pGEM-T Easy Vector sequence and one primer designed according to the CA IX sequence (sense primer) were used. Primer T7 and two complementary primers designed according to the CA XII sequence were used for CA XII (sense and antisense). All primers were produced by Sigma. The primers are shown in Table 6.

Table 6. The primers used to confirm the orientation of the inserts in the vector.

Primer	Sequence
CA IX sense	5'-GCTCCAAGATTGAGATCC-3' (894-911)
T7	5'-TAATACGACTCACTATAGGGCG-3' (2999-3015)
Sp6	5'-ATTTAGGTGACACTATAGA-3' (140-158)
CA XII sense	5'-GTTCCAAGGTTACAACGTGT-3' (332-351)
CA XII antisense	5'-ACACGTTGTAACCTTGGAAC-3' (332-351)
T7	5'-TAATACGACTCACTATAGGGCG-3' (2999-3015)

The PCR was carried out using ReddyMix™ PCR Master Mix (Abgene House, UK) in a thermal cycler (Biometra) according to the following protocol:

Denaturation 95 ^{o}C 5 min

31 cycles of

Denaturation 95 ^{o}C 15 sec

Annealing 60 ^{o}C (for primer T7) or 48 ^{o}C (for primer Sp6) 30 sec

Elongation 72 ^{o}C 30 sec

Final extension 72 ^{o}C 5 min

The results of the PRC reaction were analyzed by agarose gel as described above.

4.3 In Situ Hybridization

In situ hybridization was performed according to the protocol described previously with slight modifications (Arceci et al., 1988). Riboprobes were created by *in vitro*

transcription using commercial Riboprobe™ *in vitro* Transcription System (Promega Corp.). The antisense riboprobe for CA IX was prepared by linearizing the plasmid with NcoI (New England Biolabs Inc., Beverly, MA, USA), followed by transcription with Sp6 polymerase in the presence of [α-^{33}P]-UTP (~2500 Ci/mmol, Amersham Life Sciences, Buckinghamshire, UK or Perkin Elmer Inc,. Wellesley, MA, USA). For control studies, ^{33}P-labelled sense riboprobe was prepared by linearizing this same plasmid with SalI (Fermentas Inc.), followed by transcription with T7 polymerase in the presence of [α-^{33}P]-UTP. For CA XII, the riboprobes were generated as described above, except that the antisense riboprobe was prepared by linearization with SalI (Fermentas) and transcription with T7; the sense riboprobe was linearized with SacII (Fermentas) and transcibed with Sp6.

Prior to *in vitro* transcription, the linearized vectors were purified using GIAquick Nucleotide Removal Kit (Qiagen) and ethanol precipitated in order to concentrate the products. After labelling with ^{33}P, the transcription products were purified using GIAquick Nucleotide Removal Kit (Giagen).

Prehybridization

1. The tissue sections were deparaffinized, rehydrated, immersed in phosphate-buffered saline (PBS) for 5 minutes and digested with proteinase K (10 µg/ml) in 0.05 M EDTA/0.1 M Tris-HCl (pH 8) at 37 ^{o}C for 90 minutes. Proteinase K is a non-specific endopeptidase, which attacks all peptide bonds, is active over a wide pH range and is not easily inactivated. Pretreatment with proteinase K is a standard procedure for increasing probe penetration and accessibility (Lawrence et al., 1985). Proteinase K does this by removing protein that surrounds the target sequence.
2. The slides were soaked in DEPC-treated water for 2 minutes and incubated in 0.1 M triethanolamine (pH 8) for 3 minutes. Then the slides were acetylated in a solution containing acetic anhydride (0.25 %) in 0,1 M triethanolamine (pH 8) for 10 minutes. The acetylation is done in order to decrease background, but it also appears to inactivate RNases and may help in producing a strong signal.
3. The slides were immersed in 2 x standard saline citrate (SSC) twice for 2 minutes, subjected to an increasing ethanol series and dried in a vacuum.

Hybridization

1. The riboprobe was added to a solution that contained hybridization mix, 40 µg of Sheared Salmon Sperm DNA (Ambion Inc., Austin, TX, USA) and DEPC-treated water.

   The hybridization mix included the following substances:

   - 0.2 vol 50 % dextran sulphate
   - 5 x Denhardt's solution
   - 0.5 vol formamide
   - 10 mM Tris-HCl
   - 1 mM EDTA
   - 0.3 M NaCl

   Sheared Salmon Sperm DNA is a tRNA that acts as a carrier RNA. Dextran sulphate is added to increase hybridization efficiency (Wahl et al., 1979). Denhardt's solution decreases the chance of nonspecific binding of the probe. Formamide is an organic solvent which reduces the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature. EDTA is a chelator which removes free divalent cations from the hybridization solution that strongly stabilize duplex DNA.

2. The slides were incubated with 1 x 10^6 cpm of [α-^{33}P]-labelled antisense or sense riboprobe in a total volume of 80 µl. The sections were covered with coverslips and incubated at 60 ^{o}C for 24 hours in a humid chamber.

Posthybridization

1. The coverslips were removed, and washing steps were performed in decreasing consentrations of SSC in order to reduce non-specific binding (i.e. increasing stringency). The slides were washed in 4 x SSC once for 20 minutes and five times for 5 minutes at room temperature (RT).

2. Non-specifically hybridized riboprobes were removed by treatment with RNase A (20 µg/ml) in 0.5 M NaCl/10 mM Tris-HCl/1mM EDTA (pH 8) at 37 ^{o}C for 30 minutes.
3. Washes in SSC were done as follows:

   2 x SSC twice for 5 minutes at RT

   1x SSC for 10 minutes at RT

   0.5 x SSC for 10 minutes at RT

   0.1 x SSC for 30 minutes at 60 ^{o}C
4. The slides were subjected to graded concentrations of ethanol and air-dried.

Emulsifying, developing and counterstaining

1. The slides were emulsified with Ilford K5 nuclear emulsion (ILFORD Imaging Corp., Cheshire, UK) for autoradiography. The slides were developed after 12 days using Ilford Phenisol developer.
2. The sections were counterstained with Mayer's hematoxylin.
3. The slides were examined and photographed with a Zeiss Axioskop 40 light/darkfield microscope (Carl Zeiss, Göttingen, Germany).

4.4 Immunohistochemistry

The tissue samples for immunohistochemistry have been described in section 4.1. For control purposes tissue samples of the stomach, heart, brain, liver, kidney, and pancreas were used. Polyclonal rabbit antibodies to mouse CA IX and CA XII have been described by Ortova Gut et al. (Ortova Gut et al., 2002) and Kyllonen et al. (Kyllonen et al., 2003), respectively. Non-immune normal rabbit serum (NRS) was used in the control stainings instead of the specific antisera.

Immunoperoxidase staining for adult and embryonic mouse tissues was performed using automated Lab Vision Autostainer 480 (ImmunoVision Technologies Co., Brisbane, CA, USA) and Power Vision+™ Poly-HRP IHC Kit (ImmunoVision Technologies, Co.) reagents. Immunostaining was performed according to the following protocol:

1. The slides were rinsed in wash buffer.

2. Endogenous peroxidase was blocked with 3 % H_2O_2 in double-distilled water for 5 minutes, and sections were rinsed in wash buffer.
3. Non-specific binding of the antibody was blocked using Universal IHC Blocking/Diluent for 30 minutes, and the samples were rinsed in wash buffer.
4. The specimens were incubated with the primary antibody (rabbit anti-mouse IX or XII) or NRS diluted 1:2000 in Universal IHC Blocking/Diluent for 30 minutes.
5. The sections were soaked in wash buffer three times for 5 minutes.
6. The specimens were incubated in Poly-HRP-conjugated anti-Rabbit IgG for 30 minutes and soaked in wash buffer three times for 5 minutes.
7. The slides were incubated in DAB (3,3' -diaminobenzidine tetrahydrochloride) solution (one drop DAB solution A and one drop DAB solution B with 1 ml double-distilled water) for 6 minutes. DAB is a chromogen which produces a brown end product when it is oxidized by horseradish peroxidase (HRP).
8. The sections were rinsed with double-distilled water.
9. $CuSO_4$ treatment for 5 minutes was performed to enhance the signal.
10. The slides were rinsed with double-distilled water.

All procedures were carried out at room temperature. The sections were mounted in Entellan Neu (Merck, Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 microscope.

As this automated immunostaining method produced some nonspecific labelling of the nuclei in the embryonal tissues, immunostaining was repeated using a less sensitive but more specific peroxidase-antiperoxidase complex method (manual PAP) to confirm the validity of the results. The PAP method is described in section 2.4.2. The immunostaining by the PAP method included the following steps:

1. The sections were treated with 3 % H_2O_2 in methanol for 5 minutes and washed in PBS for 5 minutes.
2. Treatment with undiluted cow colostral whey (Biotop) for 30 minutes was performed and the slides were rinsed in PBS.
3. The slides were incubated with the primary antibody (rabbit anti-mouse IX or XII) diluted 1:100 in 1 % bovine serum albumin (BSA) in PBS for 1 hour and washed in PBS 3 times for 10 minutes.

4. The specimens were treated with undiluted cow colostral whey for 30 minutes and rinsed in PBS.
5. The slides were incubated with the secondary antibody (swine anti-rabbit IgG, DAKO, Glostrup, Denmark) diluted 1:100 in 1 % BSA in PBS for 1 hour and washed in PBS 3 times for 10 minutes.
6. The sections were incubated with peroxidase-antiperoxidase complex (PAP-rabbit, DAKO) diluted 1:100 in PBS for 30 minutes and washed in PBS 4 times for 5 minutes.
7. Incubation for 2½ minutes in DAB solution (6 mg 3,3' -diaminobenzidine tetrahydrochloride, Sigma, St Louis, MO) in 10 ml PBS plus 3,3 µl 30 % H_2O_2 was performed.

All incubations and washings were carried out at room temperature. The sections were mounted in Entellan Neu and finally examined and photographed with a Zeiss Axioskop 40 microscope.

5. RESULTS

5.1 Cloning of the Mouse CA IX and XII cDNAs

The cDNA templates for the synthesis of the riboprobes were amplified from the commercial cDNA kit using PCR. For CA IX and XII the amplification products were 565-bp and 574-bp, respectively. The obtained products were analyzed by agarose gel electrophoresis and are shown in Figure 4A and 4B.

The correct PCR products were cloned into pGEM-T Easy Vector by TA-cloning and the orientation of the inserts was confirmed by PCR using primers which are described in Table 6 (section 4.2). For CA IX, the template had right orientation if the PCR product was 558-bp and reverse orientation if the PCR product was 532-bp. For CA XII, the orientation was right when a 191-bp template was produced and reverse when a 539-bp template was produced. The analysis was done by agarose gel electrophoresis and the results are shown in Figure 5. For CA IX, every template had a right orientation (Figure 5A) and for CA XII, every template had a reverse orientation (Figure 5B).

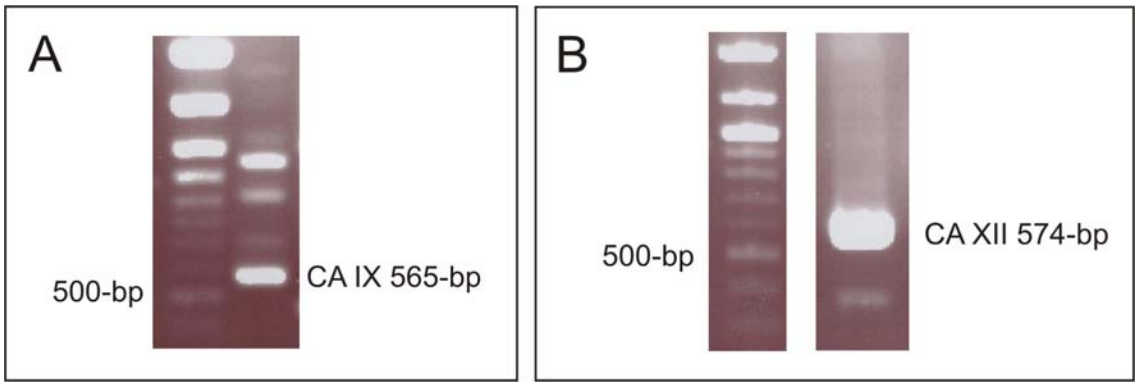

Figure 4. Agarose gel electrophoresis analysis of the amplified cDNA templates. For CA IX, a 565-bp product was obtained (A) and a 574-bp product was produced for CA XII (B).

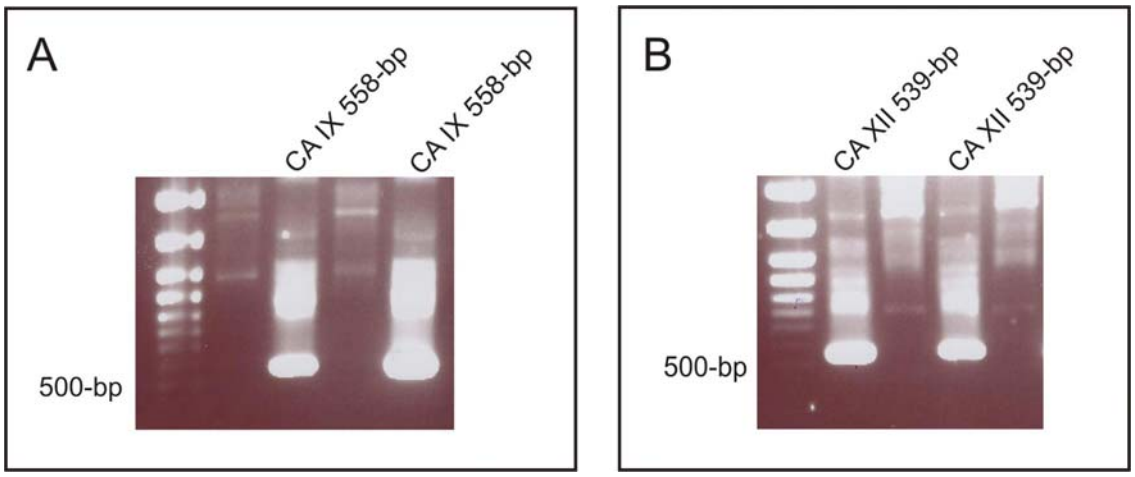

Figure 5. Agarose gel electrophoresis analysis of the orientations of the templates. For CA IX, the 558-bp sized band represents right orientation (A) and for CA XII, the 539-bp sized band is in reverse orientation.

The plasmids containing the templates were linearized with restriction enzymes as described in section 4.3. The antisense and sense riboprobes for CA IX were produced by linearizing the plasmid with NcoI and SalI, respectively (Figure 6A). For CA XII, the antisense and sense riboprobes were generated by linearization with SalI and SacII, respectively (Figure 6B). The templates for in vitro transcription were purified and ethanol-precipitated in order to concentrate products. The Figure 7 shows the templates analyzed by agarose gel electrophoresis after purification and precipitation.

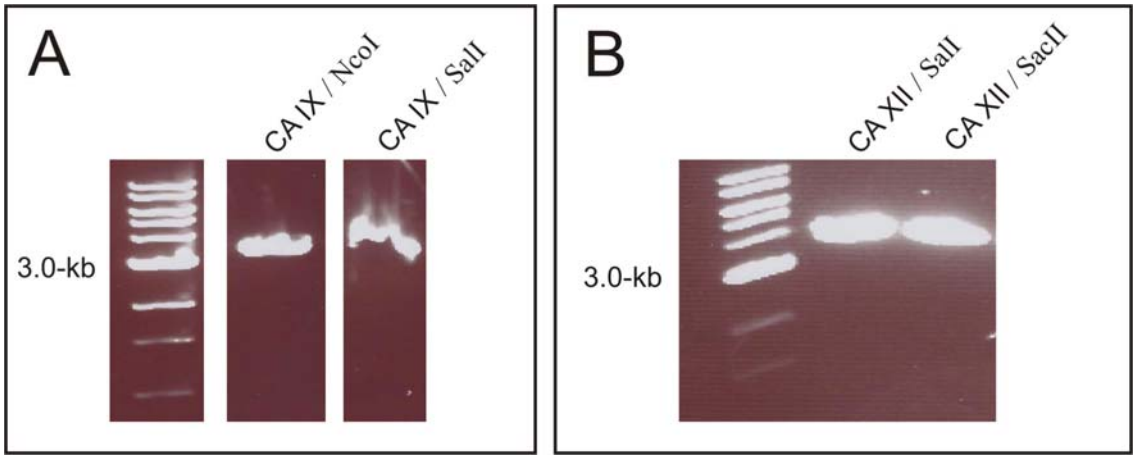

Figure 6. Agarose gel electrophoresis analysis of the linearized plasmids containing the templates. The plasmid was linearized with NcoI and SalI to produce the antisense and sense riboprobes for CA IX, respectively (A). For CA XII, the antisense and sense riboprobes were produced by linearization with SalI and SacII, respectively (B).

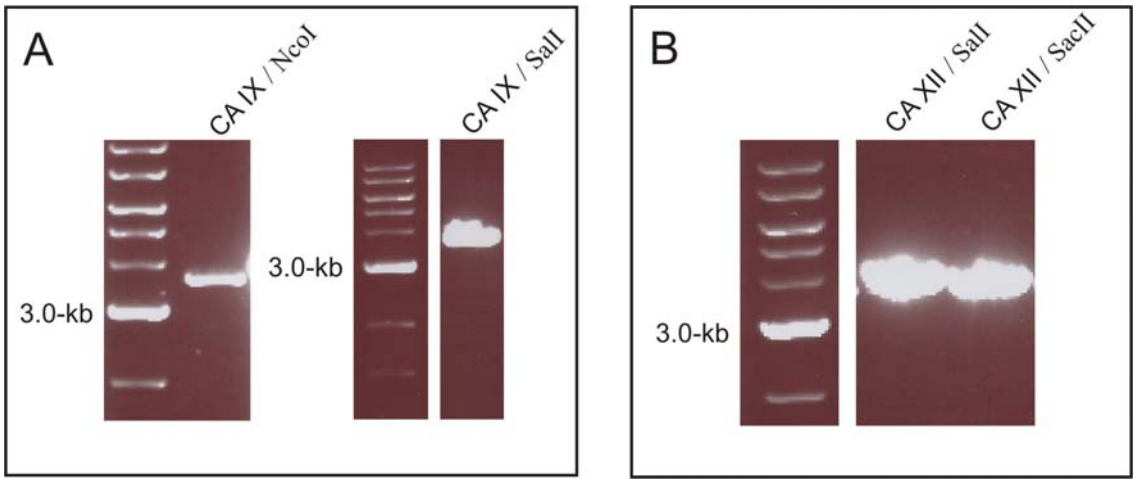

Figure 7. Purified and ethanol-precipitated templates for *in vitro* transcription. CA IX is shown in panel A and CA XII in panel B.

5.2 In Situ Hybridization

The expression of CA IX mRNA in mouse embryos of different ages and in a newborn mouse was examined by ISH analysis. Tissue specimens from an adult mouse served as positive controls and showed that the ISH method was not functioning reliably. Moreover, in most tissues, the signal was approximately equal in intensity when using CA IX sense or antisense probes.

CA IX expression in the stomach and duodenum of an adult mouse is shown in Figure 8. A positive signal was seen in the stomach mucosa as expected. A weak signal was also detected in the stomach submucosa, although it is known to be negative for CA IX protein expression (Pastorekova et al., 1997). The duodenum showed a weak positive reaction, which was primarily seen in the villi. In these tissues, only background signals were detectable when the control probes were used.

Similar to CA IX, the specificity of the CA XII ISH results was also questionable. For example, CA XII mRNA showed only faint positive labelling in the adult mouse kidney (Figure 9), although the CA XII protein is known to be expressed strongly in the kidney (Parkkila et al., 2000a). A weak signal was detected in the colonic mucosa (Figure 9), as expected. Weak signals were also seen using a control sense probe on kidney and colon.

The embryonic tissues also showed an excessively high level of unspecific labelling by ISH (data not shown). Therefore the subsequent studies were performed using immunohistochemistry, which turned out to be more accurate for the analysis of these isozymes.

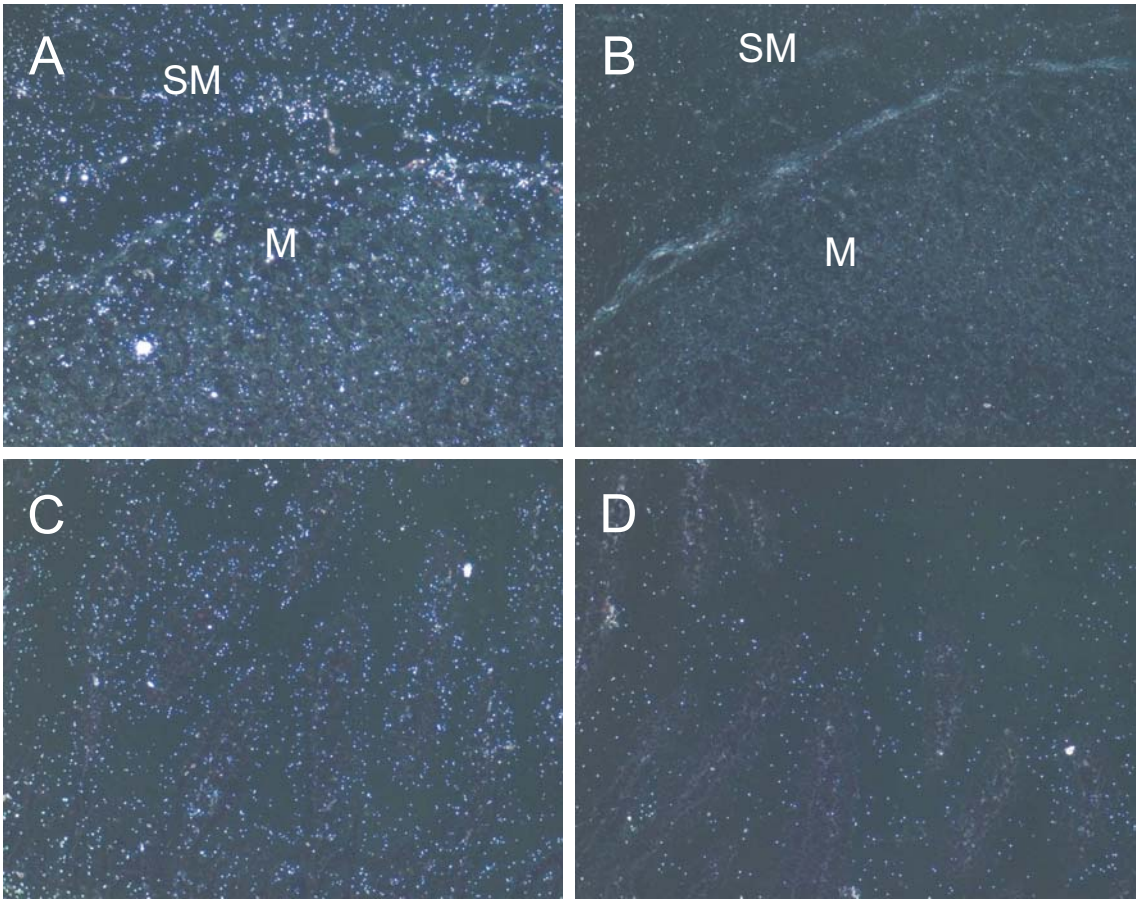

Figure 8. *In situ* hybridization for CA IX mRNA in the adult mouse stomach and duodenum. CA IX shows a positive reaction in the stomach (A), with the signal mainly located in the mucosa and submucosa. A positive signal is also detected in the villi of the duodenum (C). A background signal is detectable in the stomach (B) and duodenum (D) after hybridization with a control CA IX sense probe. Abbreviations: M=mucosa, SM=submucosa. Original magnifications: x 200.

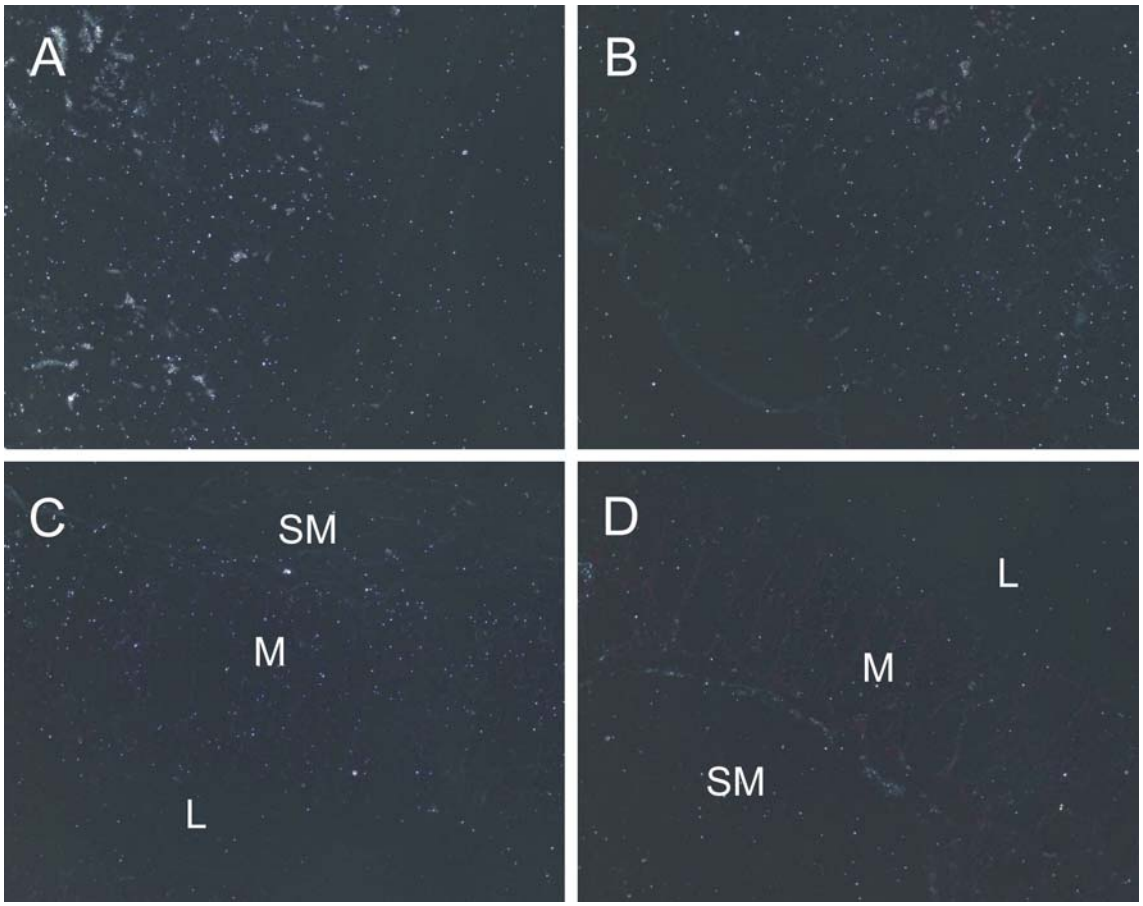

Figure 9. *In situ* hybridization for CA XII in the adult mouse kidney and colon. A very weak signal is seen in the kidney (A). In the colon, a weak positive signal is detected in the mucosa (C). Hybridizations with a control CA XII sense probe on kidney (B) and colon (D) are also weakly positive. Abbreviations: L=lumen, M=mucosa, SM=submucosa. Original magnifications: x 200.

5.3 Immunohistochemistry

The immunohistochemical staining of CA IX revealed a relatively wide distribution pattern, although the signal intensity remained quite low or moderate at its maximum. The E7.5 embryos, representing a gastrulation stage, were completely negative (Figure 10). CA IX expression in the various tissues during organogenesis is summarized in Table 7. The protein was present in the developing brain at all ages studied (Figure 11). The brain tissue was stained moderately, and some positivity was also occasionally observed in cells present in the mesenchyme beneath the developing brain (data not shown). Moderate staining was also seen in the nerve ganglia and choroid plexus

(Figure 11). No immunoreaction for CA IX was detected in the kidney at E11.5, whereas a weak positive signal appeared at E12.5 (Figure 12). The developing pancreas showed a moderate positive reaction at E12.5, which was primarily seen in the basolateral plasma membrane and intracellular compartment of the epithelial cells (Figure 13). Weak staining for CA IX was present in the stomach at all ages studied (Figure 12). The liver also showed positive immunostaining in scattered cells (Figure 14). Positive labelling was seen in certain tissues which do not express the protein in the adult mouse, including the heart and lung (data not shown). The immunoreaction in the heart was slightly stronger in the atrium than in the ventricle.

Table 7. Distribution of CA IX in mouse embryonic tissues of different ages.*

Organ	E11.5	E12.5	E13.5
Brain	++	++	++
Heart (ventricle/atrium)	+ / ++	++ / ++	+ / ++
Lung	ND	++	+
Kidney	-	+	+
Pancreas	ND	++	ND
Liver	+	++	++
Stomach	+	+	+
Intestine	+	++	+

* Scores in immunohistochemistry: strong reaction (+++), moderate reaction (++), weak reaction (+), no reaction (-), not done (ND).

The expression pattern of CA XII in embryonic tissues was also relatively broad, although the intensity was weak in most tissues. The E7.5 embryos showed no immunoreaction (Figure 10). Results at later stages are summarized in Table 8. The CA XII protein was expressed in the brain and nerve ganglia at every subsequent age during organogenesis (Figure 11), most prominently in the choroid plexus at E12.5 and E13.5 (Figure 11), the time when the developing choroid plexus usually becomes visible. Interestingly, a weak signal for CA XII was detected in several embryonic tissues, including the stomach (Figure 14), pancreas (Figure 13) and liver (Figure 14), which have previously been reported to be negative in adult mice (Halmi et al., 2004) and showed no immunoreaction in the present control stainings with adult tissues (data not shown). No staining was detected in the stomach at E11.5, while a weak positive signal appeared there at E12.5. The liver showed weak or moderate staining for CA XII

during organogenesis. The embryonic kidney showed a weak signal (Figure 12). Weak immunostaining was also seen in the pancreas, where just a few of the developing ducts were positive (Figure 13). In the heart, the staining became stronger during mouse development (data not shown). The control stainings using normal rabbit serum instead of the anti-CA IX or anti-CA XII serum gave no positive signals.

Table 8. Distribution of CA XII in mouse embryonic tissues of different ages.*

Organ	E11.5	E12.5	E13.5
Brain	+	+ (CP +++)	++ (CP +++)
Heart (ventricle/atrium)	+ / +	++ / ++	++ / ++
Lung	ND	+	+
Kidney	ND	+	+
Pancreas	ND	+	+
Liver	+	+	++
Stomach	-	+	+
Intestine	+	+	+

* Scores in immunohistochemistry: strong reaction (+++), moderate reaction (++), weak reaction (+), no reaction (-), choroid plexus (CP), not done (ND).

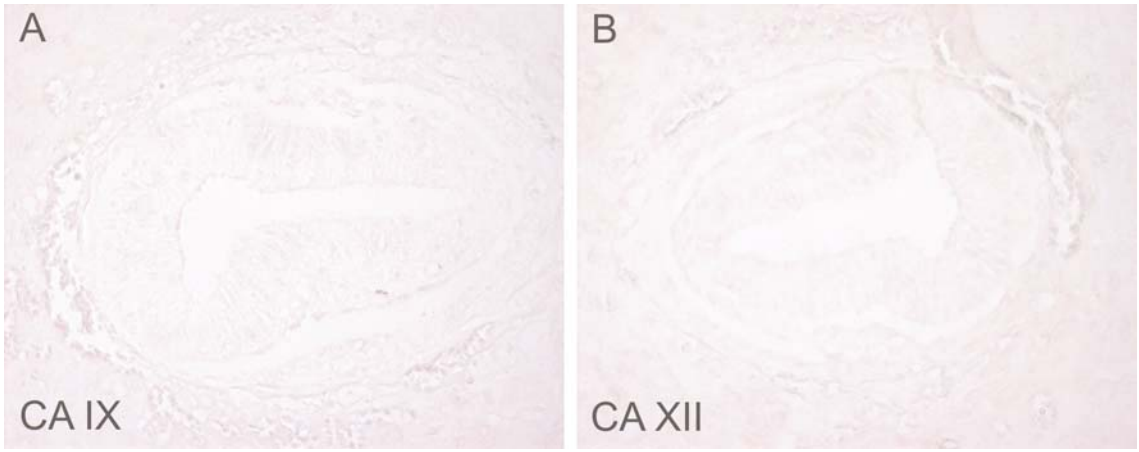

Figure 10. Immunostaining of CA IX and CA XII in the E7.5 embryos. No immunoreaction is detected for CA IX (A) or CA XII (B). Manual PAP staining in both panels. Original magnifications: x 400.

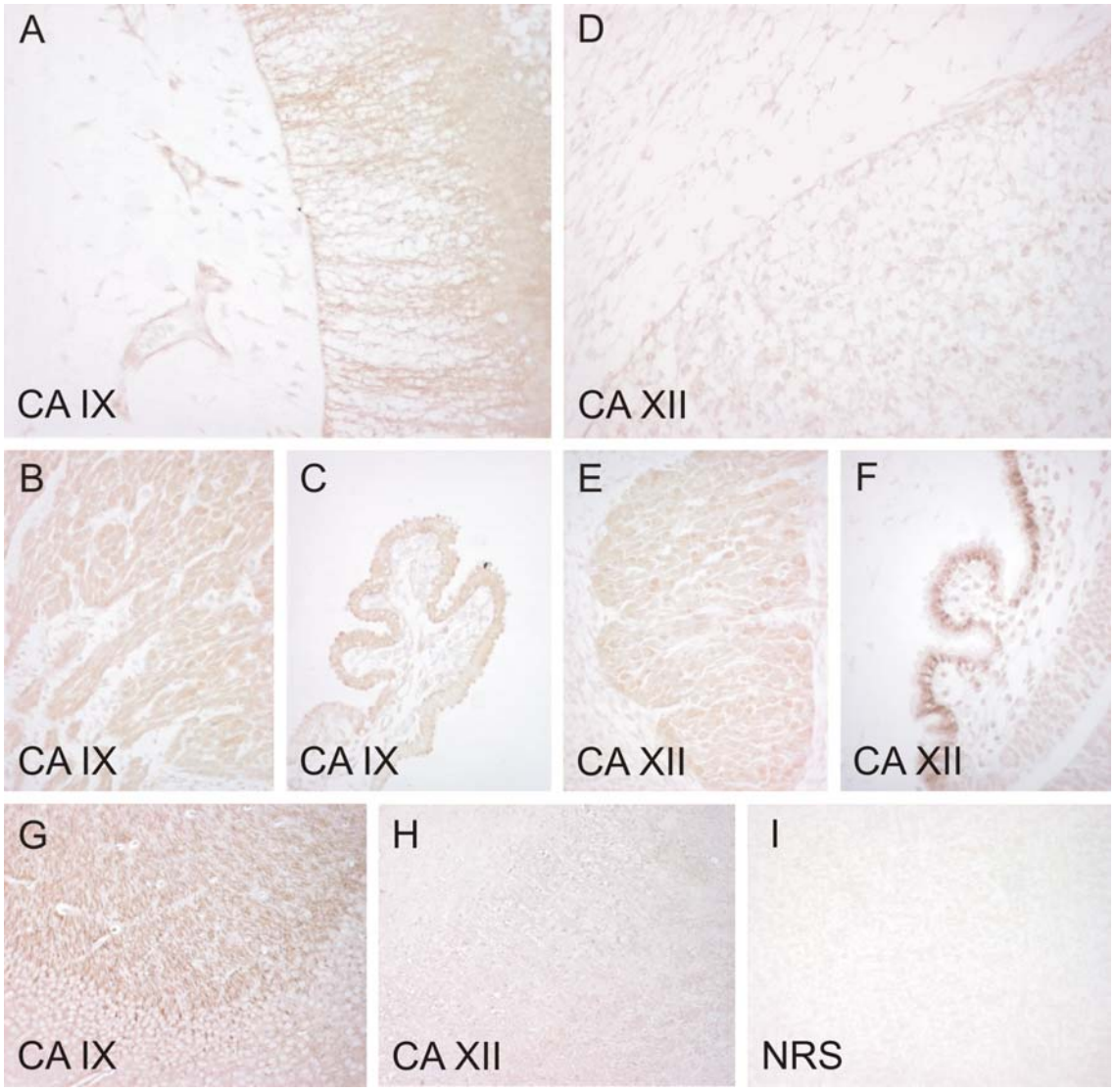

Figure 11. Immunostaining of CA IX and CA XII in embryonic and adult mouse nervous tissue. All embryos are aged E12.5 except the choroid plexus for CA XII, which is aged E13.5. CA IX shows moderate staining in the embryonic brain (A), with the signal mainly located in the neurons. CA IX is also present in the trigeminal ganglion (B) and the choroid plexus (C). Panel G shows strong positive staining for CA IX in the adult brain. CA XII gives weak staining in the embryonic brain (D), but panel E shows moderate staining in the trigeminal ganglion. The strongest immunoreaction is located in the chroid plexus (F). No specific signal for CA XII is detectable in the adult brain except for the choroid plexus (data not shown) (H). The control immunostaining of the embryonic brain with normal rabbit serum is negative (I). Manual PAP staining in panels A-E and I, automated immunostaining in panels F-H. Original magnifications: A-E, I x 400, F x 630, G-H x 200.

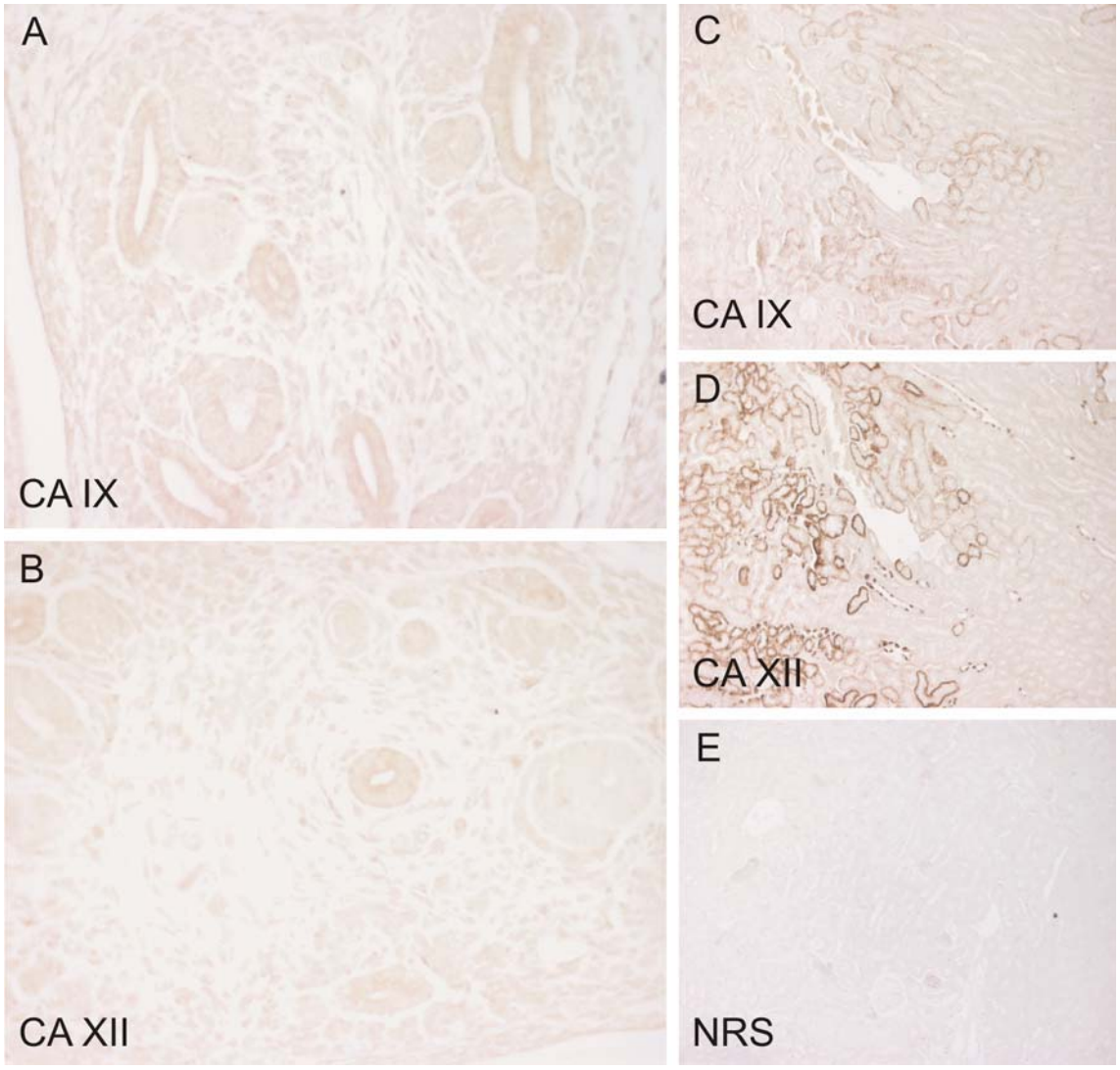

Figure 12. Immunostaining of CA IX and CA XII in the kidney at E12.5 mouse embryos and in the adult mouse kidney. Both CA IX (A) and CA XII (B) show weak staining in the ductal epithelium of the embryonic tissue, and a positive immunoreaction is seen for both isozymes in the adult mouse renal tubules (C,D), with CA XII also located in the collecting ducts. Control immunostaining of an adult mouse kidney with NRS shows no positive signal (E). Manual PAP staining in panels A-B and E, automated immunostaining in panels C-D. Original magnifications: A-B x 400, C-E x 100.

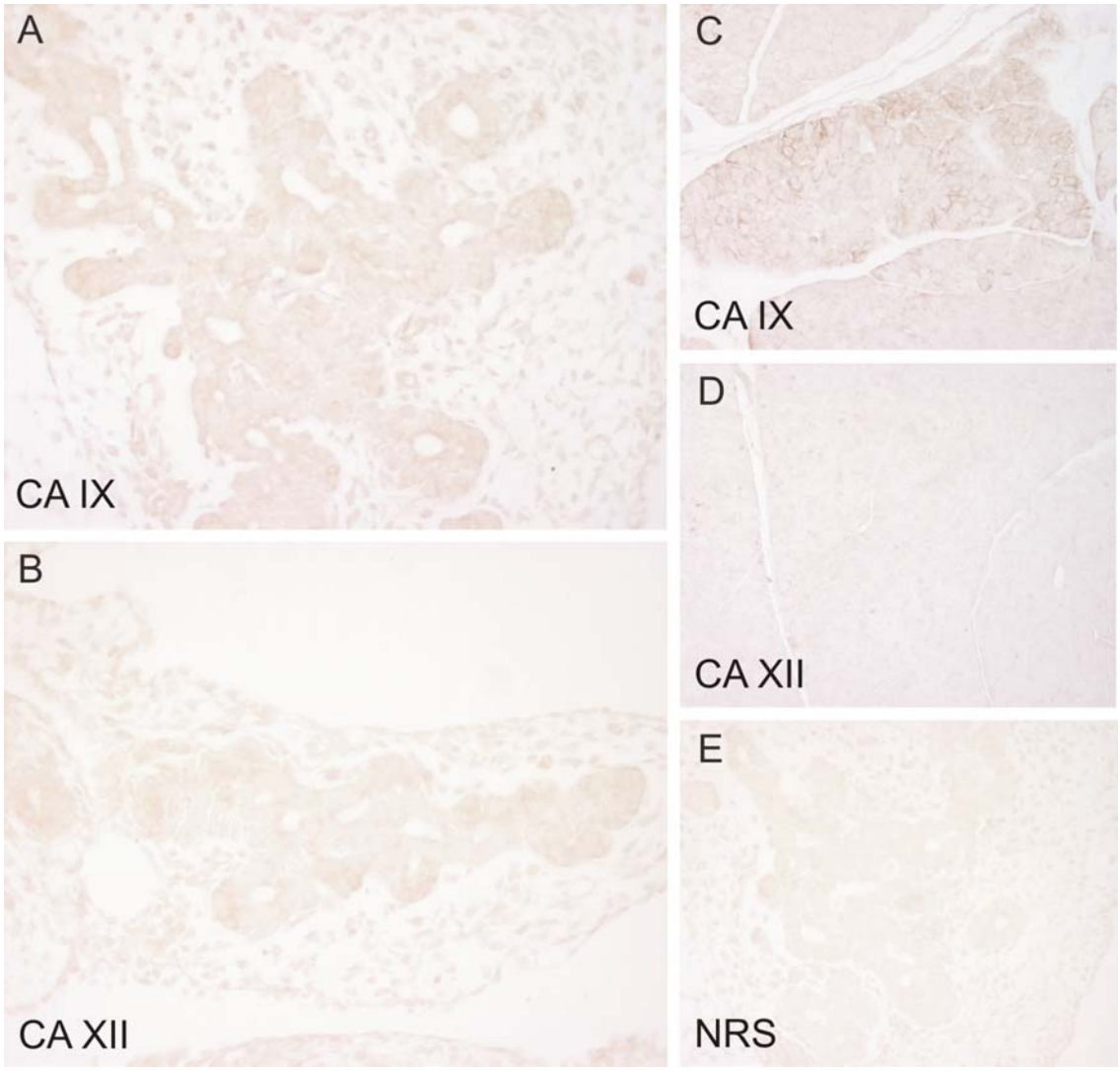

Figure 13. Immunostaining of CA IX and CA XII in the embryonic (E12.5) and adult mouse pancreas. The reaction for CA IX is moderate in the embryonic tissue, with the most intense staining in the basolateral plasma membrane of the epithelial cells (A). CA XII gives weak staining in the embryonic tissue (B). A fairly strong but focal signal is seen for CA IX in the acinar cells of the adult pancreas (C), while no immunoreaction is detected for CA XII (D). The control immunostaining of the mouse embryonic pancreas is negative (E). Manual PAP staining in panels A-B and E, automated immunostaining in panels C-D. Original magnifications: A-B, E x 400, C-D x 100.

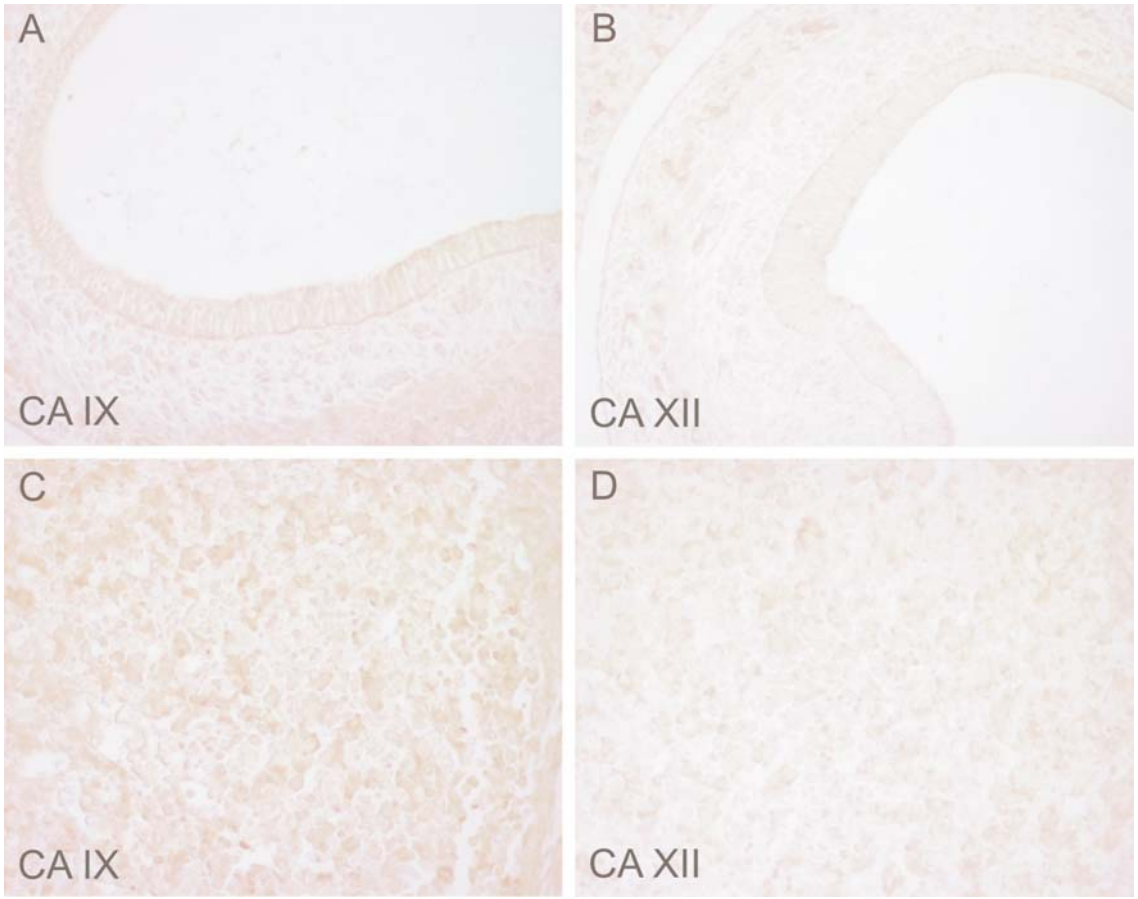

Figure 14. Immunostaining of CA IX and CA XII in the embryonic (E12.5) mouse stomach and liver. Both CA IX (A) and CA XII (B) show weak immunoreaction in the stomach (CA XII barely detectable). CA IX gives moderate staining in the liver, the signal being seen in scattered cells (C). Panel D shows a weak positive signal of CA XII in the liver (D). Manual PAP staining in panels A-D. Original magnifications: x 400.

6. DISCUSSION

During embryonic development the fertilized egg gives rise to a number of tissues, each comprised of many cell types organized in a characteristic spatial arrangement. Three types of processes that occur during the development can be distinguished. First, the generation of body morphology is accomplished through morphogenetic movements, such as the transient migration of certain cell populations and the folding and fusion of epithelial sheets. These processes involve changes in cell behavior; for example, in their interactions with other cells and with the extracellular matrix. Second, cells become progressively more restricted in their potential fate so that eventually they are committed to differentiating into a specific cell type. Third, differentiated cells and organs arise with a defined spatial relationship, a phenomenon known as pattern formation. It is obvious that all of these processes must involve the temporal and spatial control of gene expression (Wilkinson, 1990).

There are two major ways of studying the spatial distribution of gene products: to detect the protein through the use of antibodies or to detect mRNA by *in situ* hybridization. An essential advantage of ISH is the ease with which specific probes can be generated from genomic or cDNA clones. This is an important consideration given the vast amount of genes with potential roles in development that are being studied. In addition, it can be time-consuming and difficult to raise specific antibodies. However, it is advantageous to study both RNA and protein accumulation since this information can contribute significantly to the understanding of their developmental role. Therefore, both methods were utilized in this thesis.

6.1 Methodological Aspects

In this study, the original main purpose was to study the expression of *Car9* and *Car12* genes at mRNA level using *in situ* hybridization. These results were to be complemented with the study of protein expression by immunohistochemistry in the same samples. Since background staining turned out to be a problem in ISH, the study of protein expression became the main objective of the thesis.

6.1.1 *In Situ* Hybridization

In situ hybridization is considered a powerful method for specifically and sensitively studying mRNA expression on tissue sections *in situ*. However, before initiating an experiment, one has to choose the appropriate system of probe construction, labelling and signal detection. Furthermore, many optimizations are required for the experiment to succeed sufficiently. For example, the sensitivity of ISH depends on several variables. Tissue fixation and preparation affects retention and accessibility of target DNA or RNA. The type of probe construct, efficiency of probe labelling and sensitivity of the method used for signal detection are also to be considered. Finally, the efficiency of the hybridization depends on the hybridization conditions (Höfler, 1990).

In the present study, background seemed to be a problem to the extent that it was not possible to interpret the results reliably. Background staining may be due to a number of factors, including the formation of imperfect duplexes with non-homologous nucleic acids, electrostatic interactions among charged groups, physical entrapment of the probe in the three-dimensional lattice of the tissue section, and artefacts of the detection system (Gibson, 1990). However, many attempts were made during the *in situ* hybridization protocol to reduce the background.

The first attempt, during the prehybridization step, was a 0.25 % acetic anhydride treatment, which is designed to reduce tissue 'stickiness'. The 'stickiness' of tissue may depend in part on electrostatic attraction between the hybridization probe and basic proteins in the tissue. 0.25 % acetic anhydride reduces background by blocking basic groups by acetylation. Second, posthybridization washes at increasing stringencies were done to ensure the dissociation of imperfect hybrids, since the hybridization step is performed under low-stringency conditions that permit nonspecific adherence of probe molecules to various tissue components and background enhancement (Gibson, 1990). Third, an RNase treatment was carried out during posthybridization to remove non-specifically bound RNA probes. One possibility would have been to replace the 0.25 % acetic anhydride in the prehybridization mix with a component that saturates sites in the tissue section that might otherwise bind to nucleic acid unspecifically. These include ficoll, bovine serum albumin, and polyvinyl pyrrolidone. Moreover, sodium pyrophosphate and nucleic acids can be added to decrease nonspecific nucleic acid interactions (Gibson, 1990).

One highly likely cause of background may lie in the inadequate probe specificity. Adult mouse tissues with known protein expression were used as positive controls and they revealed, indeed, that ISH did not yield the expected results for mRNA distribution. In addition, the optimal probe concentration is difficult to predict, but the criterion should be that it gives the greatest signal-to-noise ratio. Since background is linearly related to probe concentration, it is best to use the lowest concentration required to saturate the target nucleic acids (Gibson, 1990). In this study an ISH protocol was utilized which was provided by our collaborator group and which had been used succesfully in several previous studies. Thus, there was no need to optimize the probe concentration.

6.1.2 Immunohistochemistry

Initially, protein expression was studied by an automated immunostaining method which provides a high sensitivity and repeatability. However, this method produced some nonspecific labelling of the nuclei in embryonal tissues. Since it was known that the antibodies used in the immunostainings have worked properly in several previous studies, the presumable reason for the labelling of the nuclei was the automated staining method. Thus, the validity of the results was confirmed by performing the staining again with the peroxidase-antiperoxidase (PAP) complex method which is less sensitive but more specific. Indeed, the PAP method came through with results of good quality.

6.2 Expression of CA IX and XII mRNA and Protein

CA IX and XII are distinct CA isozymes in that they are overexpressed in certain tumors and subjected to regulation by the von Hippel Lindau tumor suppressor protein/hypoxia pathway (Wykoff et al., 2000; Ivanov et al., 2001). The high catalytic activities of these two CA isoforms support their role in acidification of the tumor microenvironment, which in turn may facilitate the migration of tumor cells through the extracellular matrix. Since active cell migration is a characteristic feature of embryonic development, we set out to explore whether these isozymes are expressed in mRNA and protein level in mouse embryos of different ages. Some adult mouse tissues were also included in the study for control purposes.

6.2.1 Expression of mRNA

The problems of the *in situ* hybridization method were demonstrated by showing CA IX mRNA expression in the stomach and duodenum of an adult mouse. A positive signal was seen in the stomach mucosa as expected. A weak signal was also detected in the stomach submucosa although it is known to be negative for CA IX protein expression. The duodenum showed a weak positive reaction, which was primarily seen in the villi. However, the CA IX protein is known to be expressed especially in the crypts of the duodenum. Therefore, these results are to be interpreted as unreliable.

CA XII mRNA was expressed faintly in the adult mouse kidney. Nonetheless, the CA XII protein is known to be expressed strongly in the kidney. A weak signal was detected in the colonic mucosa, as expected. Unfortunately, weak signals were also seen using a control sense probe in the kidney and colon. Thus, the specificity of the signal is highly questionable in these organs.

6.2.2 Protein Expression

Examination by immunohistochemistry showed that both CA IX and XII are present in several tissues of the developing mouse embryo during organogenesis. Staining for CA IX revealed a relatively wide distribution pattern, including the brain, pancreas and liver with moderate signals and the kidney and stomach with weak signals. CA IX was expressed in the developing brain at all ages studied, most clearly in the nerve ganglia and choroid plexus. The positive staining in the developing pancreas was primarily seen in the basolateral plasma membrane and intracellular compartment of the epithelial cells. Previously, moderate staining has been demonstrated in the pancreatic acini of an adult mouse pancreas (Hilvo et al., 2004). A weak immunoreaction for CA IX was present in the stomach at all ages studied. This is in accordance with the finding that CA IX is functionally important for a normal gastric histological structure, as Ortova Gut et al. have shown previously (Ortova Gut et al., 2002). Positive expression was seen in certain tissues which do not express the protein in the adult mouse, including the heart and lung. It is notable, however, that the adult heart tissue also gave a slight positive signal with the automated immunostaining method, even though it has previously been considered negative for CA IX (Hilvo et al., 2004).

The expression pattern of CA XII in the embryonic tissues was also relatively broad, although the intensity was weak in most tissues. The positive tissues included the brain, where the most prominent staining was seen in the choroid plexus, and kidney. It is notable that even though CA XII is highly expressed in the adult mouse kidney, the embryonic kidney showed only a weak signal. As with CA IX, CA XII expression was detected in several embryonic tissues which have previously been reported to be negative in adult mice (Halmi et al., 2004). These included the stomach, pancreas and liver. In the heart, the staining became stronger during mouse development, but as with CA IX, the specificity of CA XII immunostaining is questionable in this particular organ.

The present results provide no functional evidence that CA IX or XII is involved in cell migration during embryogenesis, but they do indicate that several cell types in the mouse embryo express these isozymes. Interestingly, both isozymes were present in some embryonic tissues whose adult counterparts do not express these particular proteins. Therefore, one could hypothesize that CA IX and XII might have specific roles in the assembly of certain tissues but that these functions are attenuated during later development or in the postnatal period as enzyme expression is downregulated. Thus, it would be valuable to study the expression of these isozymes at every subsequent age after E13.5.

CA IX and XII are subjected to regulation by the von Hippel Lindau tumor suppressor protein/hypoxia pathway. In developing embryo, the expression patterns of CA IX and CA XII may also be related to the presence hypoxia, which is considered essential for proper morphogenesis of various tissues (Chen et al., 1999). Hypoxia appears important particularly for development of the brain, myocardial vascularisation, lung branching morphogenesis, formation of mesoderm and establishment of various progenitor cells (Gebb et al., 2003; Tomanek et al., 2003; Ramirez-Bergeron et al., 2004).

As membrane-bound CAs with an extracellular active site, CA IX and XII may represent key enzymes in the maintenance of an appropriate pH in the extracellular milieu in various embryonic tissues. Future studies should therefore be focused on exploring how strictly pH homeostasis is regulated in a developing embryo and what the possible structural or functional consequences are if this homeostasis is disrupted. Additionally, it would be interesting to study the contribution of each CA isozyme to embryonic development.

7. CONCLUSIONS

It was shown by immunohistochemistry that both CA IX and XII are present in a number of tissues of the developing mouse embryo during organogenesis. CA IX was expressed moderately in the brain, pancreas and liver and weakly in the kidney and stomach. The expression pattern of CA XII was also relatively broad, although the intensity of staining was weak in most tissues. The positive tissues included the brain, stomach, pancreas, liver and kidney. Interestingly, both isozymes were present in some embryonic tissues whose adult counterparts do not express these particular proteins. One could thereby hypothesize that CA IX and XII may have specific roles in the assembly of certain tissues. The focus of future studies should be on exploring how CA IX and XII contribute to the maintenance of an appropriate extracellular pH in several embryonic tissues and what the consequences are if this pH homeostasis is disrupted.

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