Differential expression of novel abundant and highly regionalized mRNAs of the canine epididymis

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Three novel gene products have been cloned by differential screening of a dog epididymis cDNA library as part of a global appraisal of specific gene expression in the epididymis. The predicted proteins were provisionally named CE8–CE10 (for canine epididymal gene products 8–10). Northern blot analyses and *in situ* transcript hybridization confirmed that the cDNAs were all derived from tissue-specific, moderately to highly abundant mRNAs of the epididymal epithelium, showing a distinct regionalized expression pattern within the epididymal duct. Their sequences predict (i) a novel 19 kDa member of the Ly-6-domain protein superfamily (CE8), (ii) an approximately 30 kDa protein with multiple membrane-spanning regions (CE9), and (iii) a novel approximately 13 kDa single whey acidic protein domain protein (CE10). Closely related, cross-hybridizing gene products were abundant in the epididymis of stallions and bulls, but not in rodents or men. Changes in mRNA frequency were observed that specifically correlated with a cryptorchid situation and with the age of the dogs. Gene products restricted to the caput epididymidis were affected by both conditions, while those with a wider regional distribution were not.

Introduction

The majority of mammalian spermatozoa do not have the ability to move progressively or to fertilize an egg when they leave the testis. They require a post-testicular maturation process that takes place in the epididymis to become fertile (Orgebin-Crist, 1967, 1987; Bedford, 1975; Brooks, 1987; Robaire and Hermo, 1988; Blaquier *et al.*, 1989). The molecular basis for this process is poorly understood, but it appears that the expression of tissue-specific proteins by the epididymal duct epithelium plays an important role. The epididymis is a single convoluted duct that can be divided grossly into three major regions: caput, corpus and cauda. These regions can be distinguished by their epithelial cell morphology (for review, see Hamilton, 1990) as well as by their pattern of gene expression (for review, see Cornwall and Hann, 1995; Orgebin-Crist, 1996), possibly reflecting the vectorial and progressive functions of sperm maturation and storage. Rodents have been the primary and sometimes the only animal models in studies of epididymal gene expression, and homologous genes in other mammals, including humans, have often proved difficult to study owing to poor evolutionary conservation. Thus, it remains to be clarified whether all findings made in the rodent epididymis can be broadly extrapolated.

Previous studies have recommended the dog as a useful model in which to study epididymal gene expression. Closely related homologues of a number of human

epididymal gene products, HE1, HE4, HE5/CD52 (Ellerbrock *et al.*, 1994) and HE6 (Osterhoff *et al.*, 1997) have been cloned from the canine epididymis. Thus, of several mammalian species studied, the dog indicated the expression of a relatively high number of abundant, epididymis-specific gene products also expressed in men and offers a convenient and acceptable source of tissue sufficient for *in vitro* studies (Pera *et al.*, 1996). Northern blot analysis (Beiglböck *et al.*, 1998) and *in situ* transcript hybridization (Pera *et al.*, 1994) demonstrated that the relative abundance, as well as the spatial distribution, of the corresponding CE1, CE4, and CE5/CD52 mRNAs are very similar to that observed in the human epididymis (Kirchhoff *et al.*, 1991; Krull *et al.*, 1993). Notably, these similarities did not include the human caput-expressed gene products, HE2 (Osterhoff *et al.*, 1994) and HE3 (Kirchhoff *et al.*, 1994). However, the canine homologue of the epididymal secretory glutathione peroxidase expressed in the caput epididymidis, GPX5, has been cloned and, using our nomenclature, is referred to as CE7, while a corresponding human GPX5 has not been identified (Beiglböck *et al.*, 1998; Hall *et al.*, 1998). These differences in (caput) epididymal gene expression may reflect species differences or differences in epididymal physiology between young, healthy animals and the elderly men suffering prostatic carcinoma whose tissues form the basis of studies in humans.

A global appraisal of specific gene expression in the canine epididymis has been started with the aims of achieving a better understanding of similarities and differences in epididymal gene expression among mammals and developing an alternative model system. A comprehensive

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Received 2 November 1998.

differential screening procedure was applied to a canine epididymal cDNA library, using a similar approach to that adopted for the human epididymis (for review, see Kirchhoff, 1998). The screening of approximately 10 000 independent canine epididymal gene transcripts, in addition to identifying some of the gene products known from other species (Beiglböck *et al.*, 1998), has led to the cloning of three novel, abundant epididymal cDNAs, not observed in other canine tissues by northern blot analysis, and not yet known in other species. The novel mRNAs showed highly regionalized expression patterns within the canine epididymis. Specific changes of mRNA frequency were observed that correlated with the cryptorchid situation and with the age of the animals.

Materials and Methods

Tissues

Canine testes and epididymides were obtained from local veterinary practices, where dogs were being castrated for behavioural disturbances. Preliminary studies involving tissues from a wide variety of breeds and mongrels showed that epididymal tissue can probably be taken from any breed of dog with the expectation of comparable results (Ellerbrock *et al.*, 1994). Nevertheless, whenever possible, preference was given to medium-sized, outbred (mongrel) dogs. Sexually mature animals (>3 years old) were chosen for most experiments. Dogs of different strains from the ages of 7 months to 9 years (7 months: Jack Russell; 1 year: Australian shepherd; 18 months: Rottweiler; 2 years: mongrel; 3 years: Beagle; 5 years: Boston terrier; 7 years: mongrel; 8 years: Maltese; 9 years: mongrel) were included to study age-related differences. Tissues from animals were removed, the epididymides cleaned of irrelevant tissue and snap-frozen in liquid nitrogen, either as entire organs or separated according to gross morphology into caput, corpus, cauda and vas regions. In addition, scrotal and abdominal epididymides from two unilateral cryptorchid dogs (4-year-old littermate Huskies) were included in the analysis. Epididymal tissues from bull, boar, and stallion had been obtained at operation, as described by Uhlenbruck *et al.* (1993). Rodent epididymides were taken from freshly killed laboratory animals. Human epididymides were obtained from local hospitals where patients with prostatic carcinoma were undergoing orchidectomy. The requirements of the Helsinki Declaration were observed in all cases (World Medical Association, 1997).

Differential screening of a canine epididymal cDNA library

A canine epididymal cDNA library that had been constructed in the bacteriophage vector Lambda UniZAP-XR (Stratagene, Heidelberg, Germany; Ellerbrock *et al.*, 1994) was screened using a differential hybridization strategy essentially as described by Kirchhoff *et al.* (1990). Approximately 10 000 independent cDNA clones were plated at low density (approximately 500 plaque-forming

units per plate) and screened by a multistep differential procedure, using ³²P-radiolabelled single-stranded cDNA pools from canine epididymis to provide positively hybridizing signals, and from liver, testis and lung as negative controls. Epididymis-specific plaques were isolated and purified by successive dilution, and the inserted cDNA sequence was recovered by excision *in vivo* as described by Ellerbrock *et al.* (1994). The resulting recombinant plasmid DNAs were amplified and purified using standard procedures, and replicate cDNA clones were subjected to DNA sequence analysis.

Generation of 5' cDNA ends by 5' inverse PCR

Completion of the missing 5' end of the CE8 cDNA was achieved by an inverse PCR technique modified as described by Barth *et al.* (1997). Specifically, a gene-specific antisense primer (5'-AAGCCGGGCAAAGAAGC-3') located in the known sequence of the partially cloned CE8 cDNA was used for reverse transcription of 5 µg total canine epididymal RNA with SuperscriptTM reverse transcriptase (Gibco-BRL, Karlsruhe); second strand synthesis was performed with *Escherichia coli* DNA polymerase (Stratagene, Heidelberg) after nicking with RNase H (Stratagene). Blunt ends were generated with T₄ DNA polymerase (Biolabs, Schwalbach-Taunus) and ligated by T₄ DNA ligase (Boehringer, Mannheim) to concatemerize or circularize the DNA. Inverse PCR was then carried out using the sense primer, 5'-TGTCTCTTAGTTGACACC-3', and the antisense primer, 5'-AAATCAAGAAGATGGATG-3', arranged in a back-to-back orientation opposite to that used for conventional PCR.

RNA preparation, hybridization probes and northern analysis

RNA from various tissues was extracted into 15–20 volumes of chaotropic solution as described by Pera *et al.* (1996). Depending on the type of experiment, 5–10 µg total RNA per lane was separated by denaturing agarose gel electrophoresis and transferred to Hybond N (Amersham-Buchler, Braunschweig) nylon membranes. Equal RNA loading was ascertained by ethidium bromide staining of gels before blotting. Digoxigenin-labelled cDNA probes were prepared by PCR amplification of purified fragments following the instructions of the supplier (Boehringer). Sequences of oligonucleotide primer pairs were as follows: CE1, 730 bp product: AAGCAGCAG AGCTGGAG / AATGTCACTTCACCAG; CE4, 370 bp product: ACGCAGGAGTGCCTCTCGGA / AGCGGTCAC TTTATTGGTTG; CE5, 400 bp product: CAAAATGA AGCGCTTCCTCT / ACAGGACATGTTTATTGACC; CE7, 330 bp product: ACTAGTCATGACTGCATGG / TGC TTCCCAAATTGGTTGC; CE8, 200 bp product: AAGGTAG CCGGAGTATGGGCG / GGCGAGGCAGATGTCAGATCC; CE9, 390 bp product: AGAGCTCGCATAACGTACATG / CCAGCAGAAGAAGGTGG; CE10, 400 bp product: CC AGTGACAGGAAGTATG / AATAGGAGAGACATTGC; glyceraldehyde dehydrogenase (GAPDH) mRNA, 200 bp

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Table 1. Characterization of CE8–CE10 gene products isolated from dog epididymis

Gene product	Frequency (% of dog epididymal cDNA library)	Approximate length of mRNA	Size of predicted protein precursor	Putative protein structure
CE8	0.03%	0.9 kb	19 kDa	Single Ly-6 domain
CE9	0.06%	1.2 kb	30 kDa	Multiple transmembrane domains
CE10	0.3%	0.7 kb	13 kDa	Single WAP domain

WAP, whey acidic protein.

detection reactions were performed following the instructions of the supplier. Negative controls were performed in parallel on adjacent sections, using the corresponding digoxigenin-labelled sense-strand cRNAs. Sections were analysed by brightfield microscopy without counterstaining.

Results

Molecular cloning and sequence analysis of CE8–CE10 cDNAs and predicted proteins

Differential screening of a canine epididymal cDNA library (Ellerbrock *et al.*, 1994) was performed as described by Beiglböck *et al.* (1998), using complex single-stranded, ³²P-labelled cDNA pools from canine epididymis as positive probes, and from canine liver, testis and lung cDNAs as negative probes. Approximately 10 000 independent cDNA clones from the epididymal library were included in this procedure. Since many clones were likely to represent the common CE1, CE4, and CE5 cDNAs, the filter replicas were rehybridized against a mixed probe specific for these cDNAs. After this step, 24 epididymis-positive clones were identified, constituting seven hitherto unknown, independent groups of epididymis-positive cDNAs. Eight of these 24 clones represented the CE7–GPX5 family of epididymis-specific, secretory glutathione peroxidase, GPX5 (Beiglböck *et al.*, 1998).

Three novel cDNA clone families were selected for closer examination. These families represented abundant canine epididymal gene products, tentatively named CE8–CE10 (Table 1; Figs 1 and 2). Northern blot hybridization confirmed that the corresponding mRNAs were abundant in the epididymis but not in other canine tissues (Fig. 3). Rescreening of approximately 100 000 clones of the original canine epididymal cDNA library showed that the CE8–CE10 cDNAs were present at different, though relatively high, frequencies in this library (Table 1). cDNA clones representative of each family of clones, and containing the longest inserts, were sequenced in both directions and their sequences were compiled by DNASTAR™ software to obtain 'near full-length' sequence information (Fig. 1). For the CE8 cDNA, a comparison of the sequence information obtained with the result of northern hybridization indicated that approximately 200 nucleotides from the 5' terminus were still unaccounted for. The 0.7 kb and 1.2 kb hybridizing mRNAs on northern blots (Fig. 3) for the CE9 and CE10 gene transcripts, respectively, implied that the sequence information available for these two transcripts was 'near full-length'.

An inverse PCR protocol was applied for CE8-encoding gene transcripts to obtain additional sequence information for the 5' end. A complex canine epididymal cDNA preparation from another individual animal was used for this step. Three independent CE8-encoding PCR products were obtained with mutually co-linear sequences that all terminated at the same 5' location. However, within the sequence, there appeared to be small deletions or insertions (Fig. 2), which indicated microheterogeneity. The length heterogeneity of the CE8 mRNA was apparent also on northern blots. The longest compiled CE8 cDNA sequence (Fig. 1; CE8) comprised 800 nucleotides followed by a short poly(A) tail. The sequence was novel and included a 426 bp open reading frame (ORF, nucleotides 2–424), with the first in-frame methionine at nucleotides 23–25. The ORF was followed by a TAG stop codon and a 361 bp 3' untranslated region (UTR). This 3' UTR contained two putative polyadenylation signals at positions 461–466 and 780–785. It would appear from the length of the hybridizing mRNA(s) that the second signal is the one generally used. The two shorter PCR-derived sequence variants of CE8 showed deletions within the apparent signal peptide-coding region, although these did not influence the reading frame of the predicted mature protein (Fig. 2). Assuming that the first in-frame methionine of the longest variant CE8V1 codon is the true translation start site (Fig. 1; CE8), the variant transcripts will encode polymorphic protein precursors, differing in length from the longest form by 10 or 20 amino acids at the N-terminus (Fig. 2). A hydrophobicity plot (data not shown) indicated a classical signal peptide pattern in the two longer variants, CE8V1 and CE8V2, with strongly hydrophobic core sequences flanked by polar residues after the presumed methionine start codons, but no typical signal peptide in the shortest PCR-derived variant, CE8V3. Comparing RT-PCR- and cDNA library-derived clones, another polymorphism was detected near the predicted C-terminus, leading to an exchange of two amino acids in CE8V4 (Fig. 2). BLAST protein database (NCBI, NIH, Bethesda, MD) searches revealed that the predicted spacing of ten cysteine residues was similar to that described for a number of proteins belonging to the Ly-6 superfamily, including the snake venom neurotoxins and the urokinase plasminogen activator receptors (for review, see Palfree, 1996). The alignment showed that the predicted mature CE8 protein, like most members of this family, contained a single Ly-6 domain, but no glycosyl-phosphatidylinositol (GPI) anchor signal. There was also significant similarity to the caltrin protein from mouse seminal vesicle (Coronel *et al.*, 1992; Fig. 2).

The compiled CE9 cDNA sequence consisted of 1165 bp, comprising an 800 bp ORF and a 365 bp 3' UTR, including a

CE8V1	MGA EY GCLPSTSQALYVILLIVLRMSLVFG
CE8V2	MGA EY GCLPSTSQALYVILLIVLRMSLVF-
CE8V3	MGA FPA-----PLKPC
CD59 (monkey)	MGIQGG-----SVLFGLLLVLA-----VFC
CE8V1	NFDPEFPHPVQFPKYLR CYRCLL-ETKELGCLL
CE8V2	-----VQFPKYLR CYRCLL-ETKELGCLL
CE8V3	NFDPEFPHPVQFPKYLR CYRCLL-ETKELGCLL
CD59	-----HSGNS-----LQCYSCPLPTMESMECT-
Caltrin (mouse)	LI CNSC--KS-RDSRCTM
CE8V1	GSDICLAPPGSS CMTL--LIKNSSSGSDIMVS-
CE8V2	GSDICLAPPGSS CMTL--LIKNSSSGSDIMVS-
CE8V3	GSDICLAPPGSS CMTL--LIKNSSSGSDIMVS-
CE8V4	DICLAPPGSS CMTL--LIKNSSSGSDIMVS-
CD59	ASTNCTSNL-DSC-----LIAKA--GSG-VYY-
Caltrin	SQSR CVAKPGESCSTVSHFVGTKHVYSKQMCSP
CE8V1	DCRHKEQMSDCSYTR-SSPVFGFWIFSR CCLR
CE8V2	DCRHKEQMSDCSYTR-SSPVFGFWIFSR CCLR
CE8V3	DCRHKEQMSDCSYTR-SSPVFGFWIFSR CCLR
CE8V4	DCRHKEQMSDCSYTR-SSPVFGFWIFSR CCLR
CD59	RCW---KFDDCSFKRISNQLSETQLKYHCCKK
Caltrin	QC----KEKQ
CE8V1	EFCNNPQNRVFYIP
CE8V2	EFCNNPQNRVFYIP
CE8V3	EFCNNPQNRVFYIP
CE8V4	RSGNNPQNRVFYIP
CD59	NLCN-anchor

Fig. 2. Amino acid alignment of four putative canine epididymal gene product CE8 peptide sequences, as predicted from cDNA sequence variants CE8V1–V4 with two related Ly-6 domain proteins (squirrel monkey CD59, accession number P47777, and mouse caltrin, accession number Q09098). The CE8V4 sequence is truncated at the N-terminus; the known mouse caltrin peptide sequence does not comprise a signal peptide. Conserved cysteines of the Ly-6 motif are accentuated in italics. Gaps are introduced to allow alignment.

polyadenylation signal at nucleotides 1145–1150, followed by a poly(A) tail (Fig. 1; CE9). The ORF of CE9 contained 13 ATG (methionine) codons. The first in-frame methionine was at nucleotides 31–33. However, this is not part of a typical Kozak consensus and is not followed by a typical signal peptide motif. BLAST protein database searches revealed significant sequence similarity to a multiple membrane-spanning protein of mice, 19.5, which was originally cloned from T cells and is also found in the ovary, but not in testis (MacLeod *et al.*, 1990). It was inferred from the alignment of the amino acid sequences that the first in-frame methionine represents the translation start site of CE9. As a result, the predicted CE9 protein should consist of 257 amino acids, of which 117 are hydrophobic.

The CE10 cDNA sequence is very short, comprising only 552 bp, and including a 357 bp ORF and a 198 bp 3'

Fig. 3. Northern blot analysis of the tissue distribution of mRNAs encoding canine epididymal gene products, CE8–CE10. Approximately 10 µg of total RNA per lane, as extracted from a panel of different dog tissues (Ep: epididymis; Te: testis; Li: liver; Lu: lung; Di: diaphragm; Ov: ovary; Ut: uterus; Sp: spleen; Mu: muscle; Ly: lymph node) was loaded. ³²P-labelled CE8–CE10 cDNA fragments were hybridized to blots (a,b). Equal loading and integrity of ribosomal RNA (28S and 18S) was ascertained by ethidium bromide staining of the gels before blotting (c).

UTR, with a polyadenylation signal at nucleotides 537–542 (Fig. 1; CE10). The first in-frame methionine at nucleotides 30–32 is followed by a characteristic signal peptide coding sequence of 20 amino acids. The putative mature CE10 protein should thus represent a small secretory peptide of 90 amino acids only. The pattern of cysteine residues indicates that CE10 is a four-disulphide core protein, similar to the epididymal CE4 protein (Ellerbrock *et al.*, 1994) but with only a single whey acidic protein (WAP)-domain. BLAST database searches revealed significant similarity to a number of WAP-domain proteins, among them several secretory proteinase inhibitors.

Tissue distribution of CE8–CE10 mRNAs as revealed by northern blot analysis and in situ transcript hybridization

Northern blot analyses were performed with total RNA extracts from various canine tissues to assess the abundance of CE8–CE10 mRNAs in epididymis relative to other organs (Fig. 3). High stringency hybridization using ³²P-labelled CE8–CE10 cDNA fragments revealed that all three mRNAs were abundant in the epididymis, but not in the other canine

Fig. 4. Transcript localization of canine epididymal gene products, CE8–CE10, in tissue sections of the dog epididymis by non-radioactive *in situ* hybridization analysis. Sections hybridized with digoxigenin-labelled CE8, CE9 and CE10 cRNA antisense probes are shown (a,c,e,g,i). Adjacent sections were hybridized with the corresponding sense cRNAs (b,d,f,h,j). Hybrids were detected by immunostaining using anti-digoxigenin antibodies and visualization by standard brightfield microscopy. CE8 and CE9 antisense probes revealed epithelial staining within restricted areas of the caput region (a–d); a CE10 antisense probe revealed epithelial staining in the cauda epididymidis and in the vas (e–h). Scale bar represents 80 μ m. At a higher magnification, a characteristic checkerboard-like staining pattern was observed in areas of the cauda epididymidis. Scale bar represents 30 μ m.

Fig. 5. Interspecies cross-hybridization of cDNAs encoding canine epididymal gene products, CE8–CE10, on northern blots. Total epididymal RNA extracts (10 µg per lane) from six mammalian species were hybridized under non-stringent conditions with either radioactive ^{32}P -labelled (left panels) or non-radioactive digoxigenin-labelled 'near full length' cDNAs (right panels). In the case of the non-radioactive probes, two CE cDNAs were used simultaneously as indicated. The stallion epididymis included in this study had been separated into distinct regions before RNA extraction (St_{cap} : caput epididymidis; St_{cor} : corpus epididymidis).

tissues investigated. Strong hybridization signals were observed corresponding to transcripts of approximately 0.9 kb for CE8, 1.2 kb for CE9, and 0.7 kb for CE10, corroborating the assumption that the CE8–CE10 cDNA sequences shown (Fig. 1) were 'near full-length'. When CE9 was used as a probe, a second, very faint hybridization signal was observed at a higher electrophoretic mobility (approximately 0.9 kb). It is not known whether this signal corresponds to an alternatively spliced CE9 mRNA or to a CE9-related but different gene product of the canine epididymis.

The CE8–CE10 transcripts were localized within the epithelium of the dog epididymis by non-radioactive *in situ* hybridization, using digoxigenin-labelled antisense CE8–CE10 cRNAs as probes (Fig. 4a–j). The specificity of the labelling reactions was confirmed by comparison with adjacent sections through the same tissue that had been hybridized with the corresponding sense riboprobes as negative controls. *In situ* hybridization signals obtained with the CE8 (Fig. 4a,b) and, especially, with CE9 (Fig. 4c,d) antisense probes were relatively weak and impaired by background problems. A specific epithelial staining was

observed only in restricted regions of the caput. In comparison, and reflecting the much higher frequency of the CE10 mRNA (Table 1), the CE10 antisense probe showed a very strong epithelial labelling in the distal segments, that is, the cauda epididymidis (Fig. 4e,f) and vas deferens (Fig. 4g,h), while the caput and corpus sections were negative (data not shown). In some areas of the cauda epididymidis, a characteristic 'checkerboard' pattern of epithelial labelling was observed (Fig. 4i,j), indicating that, within the same cross-section, most epithelial cells exhibited strong labelling, whereas a few directly adjacent cells displayed significantly lower or even no labelling.

Interspecies conservation of epididymal CE8–CE10 mRNA expression among mammals

Low stringency northern blot analyses were performed, using ^{32}P - as well as digoxigenin-labelled CE8–CE10 cDNA fragments as probes, to assess the degree of conservation in different mammalian species. RNA extracts from

Fig. 6. Region-dependent alterations of epididymal mRNA concentrations in response to cryptorchidism as revealed by northern blot analysis. Abdominal and scrotal epididymides of two unilateral cryptorchid dogs were compared. RNA extracts from whole abdominal (ab) and corresponding scrotal organ (sc); RNA extracts from abdominal organ and scrotal organ after separation of this pair of epididymides into caput (ca), proximal corpus (co1), distal corpus (co2), and cauda (cu). 32 P-labelled canine epididymal gene products, CE8, CE9, CE10, CE4 and GAPDH cDNA fragments were hybridized successively to the same northern blot (upper panels). Equal loading (5 μ g per lane) and integrity of ribosomal RNA (28S and 18S) was ascertained by ethidium bromide staining of the gel before blotting (bottom panel).

epididymides of various species, including humans, were hybridized either against one CE cDNA probe alone, or against a mixture of two different CE probes, the combination depending on the lengths of the hybridizing mRNAs. Cross-hybridization resulted in the detection of CE8- and CE9-related mRNAs in a few other domestic animal species, particularly bulls and the stallions, but not in humans or in rodents (Fig. 5). No closely related counterparts

were identified when the near full-length CE10 cDNA was used as a probe.

Regionalization within the epididymis and modulation of CE8–CE10 mRNA expression by cryptorchidism

The CE8–CE10 mRNAs were analysed for their expression patterns along the length of the canine epididymis using standard northern blot analyses (Fig. 6). GAPDH and CE4 mRNA, which are expressed in all regions of the organ (Pera *et al.*, 1994; Beiglböck *et al.*, 1998), served as controls. The CE8–CE10 mRNAs, in comparison, showed a specific spatially restricted pattern of distribution, corroborating the results obtained by *in situ* transcript hybridization: CE8 mRNA and CE9 mRNA were restricted to the caput region and were not detected in distal portions of the canine epididymis. However, CE10 mRNA was expressed only in the distal parts of the organ, excluding the caput region. Maximum CE10 mRNA concentrations were observed in the distal corpus and cauda epididymidis.

In the unilateral cryptorchid condition, in which one testis plus epididymis is retained in the abdomen, normal amounts and expression patterns of mRNAs were observed in the scrotal epididymides which, thus, served as internal controls (Fig. 6). However, a marked reduction in the amounts of CE8–CE10 mRNAs was observed in the abdominally located organs, while CE4 and GAPDH mRNA concentrations were only mildly affected. When different regions of the epididymis were compared, a reduction of mRNA concentrations in the abdominal organ was most obvious for gene products that were spatially restricted. GAPDH and CE4 mRNAs, which were found in all parts of the normal canine epididymis, were also reduced, but only in the caput region. In the more distal parts of the organ, the expression of these transcripts was not affected or even slightly increased (Fig. 6).

Correlation of epididymal mRNA concentrations with age

Tissues included in this study were from nine dogs aged between 7 months and 9 years, each animal belonging to a different breed. In agreement with previous results (Ellerbrock *et al.*, 1994), CE1, CE4, and CE5 mRNA concentrations did not vary greatly, indicating that there was no difference in their expression pattern related to breed or age. Therefore, these mRNAs served as internal controls for the study of possible variations of CE7–CE10 mRNA concentrations. Higher inter-individual variability was observed for the concentrations of CE7–CE10 mRNAs (Fig. 7a–d). Moreover, this variability seemed to be related to the age of the animals investigated. Age-related reduction of mRNA concentrations was most obvious for the caput-expressed CE7–CE9 mRNA species (Fig. 7a–c), while the cauda-expressed CE10 mRNA seemed to increase with age (Fig. 7d). The ethidium bromide staining of the gels and the uniform amounts of the control mRNAs, respectively, ruled out any marked effect due to possible RNA degradation or uneven loading.

Arranging the animals according to their age into three

Fig. 7. Age-related alterations of concentrations of mRNA encoding canine epididymal gene products, CE1–CE10, as revealed by northern blot analysis. Total RNA (5 µg per lane) extracted from whole epididymides of dogs of increasing age were loaded (RNA extracts were from a 7-month-old dog (7 m); a 12-month-old dog (12 m); an 18-month-old dog (18 m); a 2-year-old dog (2 y); a 3-year-old dog (3 y); a 5-year-old dog (5 y); a 7-year-old dog (7 y); an 8-year-old dog (8 y); and a 9-year-old dog (9 y)). Blots were hybridized with two different CE cDNA probes simultaneously (a: CE7–CE5; b: CE7–CE8; c: CE9–CE4; d: CE1–CE10). The integrity of RNA was ascertained by ethidium bromide staining of gels; 28S and 18S RNA are visualized (lower panels).

arbitrary groups, aged < 2, 2–5, and > 5 years, respectively, allowed a simple statistical analysis of these observations (Fig. 8). A total of three individuals per group was taken for this analysis, which confirmed the observed tendency for age-related differences in the expression of the caput-restricted CE7–GPX5 and CE8 mRNAs. These concentrations decreased significantly ($P < 0.03$; ANOVA), in correlation with the increasing age of animals (Fig. 8). This correlation with increasing age was independent of the substantial variations among individual epididymal mRNAs that may have been related to breed. A similar tendency, although not statistically significant, was observed for the CE9 mRNA concentrations (Figs 7c and 8). However, CE10 mRNA concentrations, although highly variable and not statistically significant, appeared to increase with increasing age (Figs 7d and 8). Most mRNAs (with the exception of CE10) showed maximum concentrations in tissue samples from the group

of the youngest animals (< 2-year-old dogs), which had recently attained sexual maturity.

Discussion

A comprehensive assessment of gene expression in the canine epididymis was started to extend understanding of epididymal physiology in different mammalian species (for review, see, Ivell *et al.*, 1998). As part of this study, three novel, abundant tissue-specific gene products are described: CE8, CE9 and CE10. Differential analysis of 10 000 independent cDNA clones indicated that there are at least three further novel epididymis-specific genes, expressed at relatively high frequency in this tissue (K. Ellerbrock and C. Kirchhoff, unpublished). As shown by *in situ* transcript hybridization, the CE8–CE10 mRNAs are all transcribed by

Fig. 8. Quantitative alterations in epididymal gene expression in dogs of increasing age. Quantitation is based on phosphorimager analysis of northern blots shown (Fig. 7). Concentrations (in arbitrary units) of mRNA encoding canine epididymal gene products, CE1–CE10, were measured in three age groups (□, < 2; ■, 2–5; and ■, > 5 years). Maximum concentrations for each mRNA (mean FSD for $n = 3$ dogs) were set as 100. Asterisks indicate significant differences in mRNA concentrations among age groups (ANOVA; $P < 0.03$).

the epithelial cells of the epididymal duct, although in different regions, but are not detected in the other canine tissues included in the present analysis.

As predicted from the corresponding cDNA clones, the CE8 and CE10 mRNAs encode novel members of two different, well-defined superfamilies of secretory proteins, respectively, while CE9 has not yet been assigned to a known family. CE9 may represent a membrane protein of the caput epididymidis, but is not related to the 'CE9' transmembrane protein of rodent spermatozoa. The spacing pattern of cysteines within its mature peptide-coding region indicates that CE8 represents a novel, approximately 19 kDa single-domain member of the Ly-6-domain superfamily of proteins, not homologous to any of its known members. The majority of Ly-6 domain proteins are cell surface molecules, anchored in the plasma membrane through C-terminal GPI attachment (for review, see Palfree, 1996). However, there is no indication of GPI anchoring in the four CE8 cDNA sequences analysed in the present study. This finding puts CE8 into the smaller group of non-GPI-anchored, soluble members of the Ly-6 superfamily, which also includes the snake venom neurotoxins, the snake PLA-2 inhibitors, and the sperm-specific antigen, SP-10 (Freemerman *et al.*, 1994, 1995). It is not known whether the different CE8 sequence variants cloned from canine epididymides produce processed proteins *in vivo*, and whether any of these proteins are secreted.

The CE10 cDNA sequence predicts a small, 10 kDa protein, consisting of a single WAP domain. Aside from the 'four disulphide-core' pattern shared with numerous other proteins, its amino acid sequence is novel, and is not closely related to any of the known members of this large group of secretory proteins. Thus, CE10 represents a second novel

WAP motif protein of epididymal origin, in addition to CE4, which has been identified as the HE4-homologous, two-domain WAP protein of the canine epididymis (Kirchhoff *et al.*, 1991; Ellerbrock *et al.*, 1994). Many WAP motif proteins have been described to function as extracellular proteinase inhibitors, and HE4/CE4 shows close structural and genetic relationship to the secretory leucocyte proteinase inhibitor HUSI-1/SLPI (Kirchhoff *et al.*, 1991), which has also been implicated in the innate mucosal defence against microbial attack (Jin *et al.*, 1997). Whether CE10 exhibits similar functions remains to be clarified.

However, CE9 does not appear to represent a secretory protein. The deduced amino acid sequence contains no typical signal peptide and consists of almost 50% hydrophobic residues arranged in stretches that may represent membrane-spanning regions. Moreover, the predicted peptide sequence of about 30 kDa shows 27% identity to a 33 kDa protein with four putative transmembrane-spanning regions, 19.5, which has been cloned from mouse T cells (McLeod *et al.*, 1990). However, the function of this protein, which is also expressed in mouse ovary, is unknown.

The epididymis of dogs has been shown to represent a good model with relevance to humans (Ellerbrock *et al.*, 1994; Pera *et al.*, 1994). The canine homologues of four abundant, epididymis-specific human gene products, CE1, CE4, CE5 and CE6, have been identified (Ellerbrock *et al.*, 1994; Osterhoff *et al.*, 1997) and outnumber the human homologues found in rodents. A canine gene not expressed in humans but expressed in abundance in the epididymides of most other mammals, CE7–GPX5, has been identified (Beiglböck *et al.*, 1998). Analysis of various species, including humans, for cross-hybridization of epididymal RNA on northern blots with CE8–CE10 cDNA probes showed that closely related epididymal mRNAs were not commonly expressed among mammals. As well as in dogs, CE8- and CE9-homologous mRNAs were identified only in the bovine and stallion epididymis; CE10 was restricted to the dog epididymis, and closely related human or rodent counterparts were not observed. Poor evolutionary sequence conservation, as well as qualitative and quantitative species differences in epididymal gene expression, may explain this. Considerable sequence divergence of Ly-6 genes among species has been reported, and direct cloning of human homologues through cross-hybridization has been unsuccessful (for review, see Palfree, 1996). Accelerated progress of evolution has also been suggested for WAP-domain proteins. The elafin proteinase inhibitor family members show extreme divergence in their WAP motif (Tamechika *et al.*, 1996). Moreover, cross-hybridization with a probe of the caput-expressed CE8 with stallion epididymal mRNA was restricted to corpus RNA extract, indicating that, in contrast to earlier observations (Pera *et al.*, 1994), the spatial distribution of some epididymal mRNAs varies among species.

The importance of having a number of such tissue-specific marker genes available was illustrated by a study of the direct effect of temperature on canine epididymal epithelial cells in culture (Pera *et al.*, 1996), in which it was shown that some transcript concentrations more than others were

negatively affected by increased temperature and by cryptorchidism. Here, we have been able to extend our studies on the degree to which the epididymal phenotype can be differentiated as part of the response to cryptorchidism. The novel gene transcripts, CE8, CE9 and CE10, are all markedly reduced in the abdominal epididymis, while the CE4 and GAPDH control mRNAs are only mildly affected, depending on the epididymal region studied. Moreover, the new gene transcripts also allow a more refined spatial differentiation of the epididymal duct. Results from the present and previous studies (Pera *et al.*, 1994; Beiglbock *et al.*, 1998) allow each segment of the normal epididymis of the sexually mature dog to be defined by its specific pattern of gene expression.

Although the present results are based on a relatively small number of dogs, it appears that a specific mRNA phenotype also reflects the age of the animal, and hence may be suited to defining the effect of ageing on sperm maturation and storage. The majority of dogs being castrated for behavioural disturbances are sexually mature animals between 3 and 5 years of age, and tissues from younger or older animals are relatively rare. Thus, only a small group of animals was included in the present study. Nevertheless, the present results indicate that the concentrations of caput-expressed CE7-GPX5 and CE8 mRNAs significantly decrease with increasing age. Canine serum testosterone concentration also varies significantly with age (Günzel-Apel *et al.*, 1990). Androgens are critical for epididymal gene expression and act at the transcriptional level on the mouse GPX5 gene (Lareyre *et al.*, 1997). However, whether the decreasing CE7-GPX5 and CE8 mRNA concentrations directly reflect age-related testosterone decline remains unclear. Viger and Robaire (1995) showed that, in brown Norway rats, epididymal gene expression is specifically altered with age, and that the age-related effects vary depending upon the region of the epididymis studied. In particular, 5 α -reductase type 1 and type 2 mRNA concentrations decrease significantly in the proximal epididymis of aged animals, but not in the cauda epididymidis.

Age-related alterations of epididymal gene expression, in combination with accelerated evolution of genes expressed specifically in the epididymis, provide an explanation for the apparent species differences observed, and for the difficulty in detecting counterparts of a number of epididymal gene products that have long been known from other species in human epididymides (all of which were obtained from elderly men with prostatic carcinoma). Future studies will extend the number of epididymis-specific gene products available, increasing the resolution of phenotypic differentiation possible, and apply these phenotypic indicators in more detailed studies of epididymal physiology and pathology. Furthermore, these genes may be used to probe the specific temporal and spatial expression patterns of the epididymis at the various levels of gene regulation.

The authors gratefully acknowledge the assistance of A. Beiglbock, M. Balvers and B. Harms in various parts of this study, as well as the considerable support by the veterinary community in and around Hamburg. This investigation was financially supported

by the German Research Association (DFG contract number Ki 317/5-1), and by a stipendium of the Ernst Schering Foundation to K. Ellerbrock. The authors would also like to thank F. Leidenberger for provision of excellent facilities and his continued support throughout this project.

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