

Regional variation of specific gene expression in the dog epididymis as revealed by in-situ transcript hybridization

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Summary

In-situ transcript hybridization was used to characterize the regional distribution of three marker gene transcripts which are expressed abundantly in the canine epididymis. The gene products CE1, CE4 and CE5, which are the canine equivalents of the human homologues HE1, HE4 and HE5, are shown to be expressed in a tissue-specific and regionally characteristic pattern in the epididymal epithelium. CE1 mRNA was expressed very weakly in the efferent ducts but was expressed at a high level throughout the caput and corpus regions of the epididymis, decreasing somewhat in the distal cauda region. CE4 mRNA was not detectable in the efferent ducts and was only expressed moderately in the remainder of the epididymis, with greatest levels in the caput and proximal cauda regions, and decreasing in the distal cauda. CE5 mRNA showed the most marked regional variation in levels with little or no mRNA detectable in the caput and proximal corpus regions, but increasing dramatically in the distal corpus and cauda. In the transition region of the central corpus, the CE5 mRNA appeared to be expressed intermittently, giving a mottled signal appearance over the epididymal epithelium. The patterns of mRNA distribution for the three marker genes in the dog epididymis were, therefore, essentially similar to those for the equivalent human homologues, providing further support for the suitability of the dog epididymis as a model for the human.

Keywords: epididymis, canine, secretory proteins, gene expression, in-situ hybridization

Introduction

Andrological research has focused most attention logically on the testis as the site of gamete generation. However, though morphologically having the characteristic features of ejaculated spermatozoa, such gametes on leaving the testis are still unable to fertilize an oocyte. These final differentiation steps appear to take place largely within the epididymis, where sperm acquire the mature phenotype and are stored prior to ejaculation (Bedford *et al.*, 1973; Cooper, 1986). The extent to which this process is controlled by

extrinsic biological signals determined by the milieu of the epididymal lumen is unclear. One potentially important aspect of sperm maturation is that specialized glycoproteins synthesized by the epididymal epithelium are shed into the lumen and may interact with the sperm surface during epididymal transit (for reviews see Brooks, 1987; Orgebin-Crist, 1987; Cooper, 1992). These products are released from the apical surface of a highly differentiated and polarized epithelium.

It has been shown in several species for a number of metabolites, hormones, enzymes and other proteins, that these are not distributed uniformly along the epididymal duct, but occur rather in a spatially distinct pattern (reviewed

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in Robaire & Hermo, 1988). Furthermore, the profile of sperm surface antigenicity also changes as spermatozoa mature during their transit through the epididymis, indicating that proteins influencing the sperm surface may be secreted in specific regions of the epididymal tubule (for review see Jones, 1989). Indeed, the appearance of additional surface glycoproteins may coincide with the sperm acquiring their most important maturation properties; such proteins have been referred to as maturation antigens (Zeheb & Orr, 1984; Moore *et al.*, 1989; Eccleston *et al.*, 1994).

Segment-specific differences in mRNA levels for different transcripts have been reported in the rodent epididymis (Garrett *et al.*, 1991; Cornwall *et al.*, 1992; Winer *et al.*, 1993). Tissue-specific gene probes, isolated from a human epididymal cDNA library, have also been employed to demonstrate specific gene expression within the epithelium of the human epididymis in a regionally specific manner (Kirchhoff *et al.*, 1991; Krull *et al.*, 1993). Since these probes represented both very common as well as highly tissue-specific transcripts, this pattern of gene expression probably reflects a regional specialization of epididymal function.

Detailed histological and histochemical studies of the epididymis of several mammalian species concur in demonstrating a monolayer epithelium lining the epididymal duct throughout its entire length. However, the morphology of the principal cells of this epithelium is regionally variable, from tall columnar cells to squat rhomboid types. All cells appear to have cytological characteristics indicating active protein synthesis and secretion. The dog was one of the first mammals in which conspicuous regional differences of the epididymal epithelium were reported (Hammar, 1897), with several histochemical and ultrastructural studies emphasizing regional differences in protein and enzyme secretion, as well as in sperm transport, resorption, and storage within the epididymal duct (Sinowatz *et al.*, 1979; Chandler *et al.*, 1981).

Based on histological findings, Reid & Cleland (1957) have demonstrated as many as eight different regions of the mammalian epididymis. This nomenclature, however, seems of questionable value for a generalized description of important changes in function along the length of the epididymal duct (Glover & Nicander, 1971). The study described here set out to assess changes in function along the epididymis by studying the regionally specific variation of gene expression in the dog epididymis. Levels of tissue-specific mRNAs were detected by in-situ transcript hybridization, employing three gene probes cloned from a dog epididymal cDNA library (Ellerbrock *et al.*, 1994). These dog cDNA probes, CE1, CE4, and CE5, represent the canine homologues of the human epididymis-specific gene products HE1, HE4, and HE5, each of which has already been shown to display a distinct regionally specific pattern of expression in the human epididymis (Kirchhoff *et al.*, 1991; Krull *et al.*, 1993).

All three mRNAs were shown to represent major tran-

scripts of the epithelial cells lining the dog epididymal duct. The patterns of the positive hybridization signals obtained were very similar to those observed in the human, emphasizing once more the suitability of the dog epididymis as a model for the human. Thus, in spite of sometimes striking species differences in the anatomy of the male reproductive tract and in the diversity of epididymal gene products between different mammalian species, the data presented here, using specific marker genes, serve to reveal a common underlying pattern, presumably reflecting essential epididymal function.

Materials and methods

Tissue samples

Dog epididymides, kindly provided by Dr K. Ellerbrock, were obtained from local veterinary practices, where dogs were castrated for non-endocrine, generally behavioural disturbances. Preference was given to small- to medium-sized dogs of mixed breeding (see Ellerbrock *et al.*, 1994). Tissues were removed rapidly, the epididymides cleaned of irrelevant tissue and separated by gross morphological criteria into caput, corpus, and cauda regions. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C .

Preparation of cRNA probes

[$\alpha^{35}\text{S}$]rCTP-labelled cRNA hybridization probes were prepared as described previously (Krull *et al.*, 1993), by in-vitro transcription from pBS-SK (Stratagene, La Jolla, C.A., U.S.A.) cDNA subclones containing the entire cDNA sequences in the case of CE4 (554 bp) and CE5 (483 bp), or an 800bp EcoRI/ApaI 5' subfragment of the CE1 cDNA (Ellerbrock *et al.*, 1994).

In situ hybridization

Caput, corpus and cauda fragments of canine epididymides were freeze-mounted separately, cut into 10 μm serial cryosections, post-fixed in 4% paraformaldehyde, and pretreated for in-situ hybridization as described previously (Krull *et al.*, 1993). Prior to cryosectioning, corpus fragments were oriented to obtain sagittal sections through the entire length of the corpus. Hybridization was performed in 25 μl hybridization buffer (20mM Tris-Cl, pH7.5; 0.3M NaCl; 1mM EDTA, pH8.0; 0.1mM DTT; 1 $\times$ Denhardt's; 50% deionized formamide; 10% dextran sulphate; 500 $\mu\text{g/ml}$ yeast tRNA; 100 $\mu\text{g/ml}$ polyadenylate; 500 $\mu\text{g/ml}$ denatured salmon sperm DNA) containing 10^6 cpm ^{35}S -labelled antisense or sense cRNA per slide. Incubation was overnight at 52°C . Washing procedure and RNase A digestion were as described previously (Krull *et al.*, 1993). The optimal stringency of washing was calculated to be 70°C for CE1 and CE5 hybridization probes, and 74°C for the CE4 probes. Slides were dried and exposed overnight to Kodak XAR5 X-ray film as a control for the washing conditions and exposure times before dipping slides in photographic

emulsion. The preliminary autoradiograms provided additionally a low resolution image of the distribution of the specific hybridization signals which allowed direct visual quantitation of signal strength in all epididymal regions simultaneously. These autoradiograms were photographed macroscopically under transmitted light and constant exposure. After dipping in emulsion (Kodak NBT.2), slides were exposed in the dark for 3 days (CE1 and CE5) or 8 days (CE4), developed, counterstained in Mayer's haemalum and photographed in both transmitted light and reflectance darkfield using a Nikon Epiphot microscope.

Results

Gross morphology and subdivisions of the canine epididymis

For the in-situ hybridization analyses the epididymides were divided on the basis of gross morphology into three segments (Fig. 1). In the absence of a more appropriate terminology, the conventional terms of head (caput), body (corpus), and tail (cauda) were used to designate these segments. Within these segments various subdivisions could be made based upon the position of fibrous septa, on the appearance and contours of the epididymal duct cross-sections, and on the in-situ hybridization labelling characteristics of the epithelium arising from steady-state transcript levels of three epididymal marker genes, CE1, CE4, and CE5 (Ellerbrock *et al.*, 1994). The numbering of the subsegments in Fig. 1 corresponds to the longitudinal pattern of the actual adjacent sagittal sections, and is not intended to correspond to any published subdivision based on histomorphological criteria of the epithelium.

Segment I. Within the tissue sections through the caput segment three regions could be distinguished as follows (Fig. 1).

(Ia) The efferent ducts connecting the rete testis with the epididymal duct proper were identified by their relatively narrow lumina, and the squat epithelium with short epithelial cells.

(Ib) The transition between efferent ducts and epididymal duct was marked by a distinct increase in epithelial height. The epithelial cells of the caput epididymis have been described as containing a large number of cytoplasmic and nuclear inclusions (Chandler *et al.*, 1981). However, these inclusions were not visible on our micrographs (Figs 2–5).

(Ic) Within a third caput region, the appearance of ductular cross-sections as well as the labelling characteristics of the ductular epithelium resembled very much the most proximal region of the corpus segment.

Segment II. Ductular cross-sections within the longitudinal slices through the corpus segment were characterized by relatively broad lumina and were often filled densely with spermatozoa. Maximum sperm density was observed in the middle corpus region. This segment could be subdivided

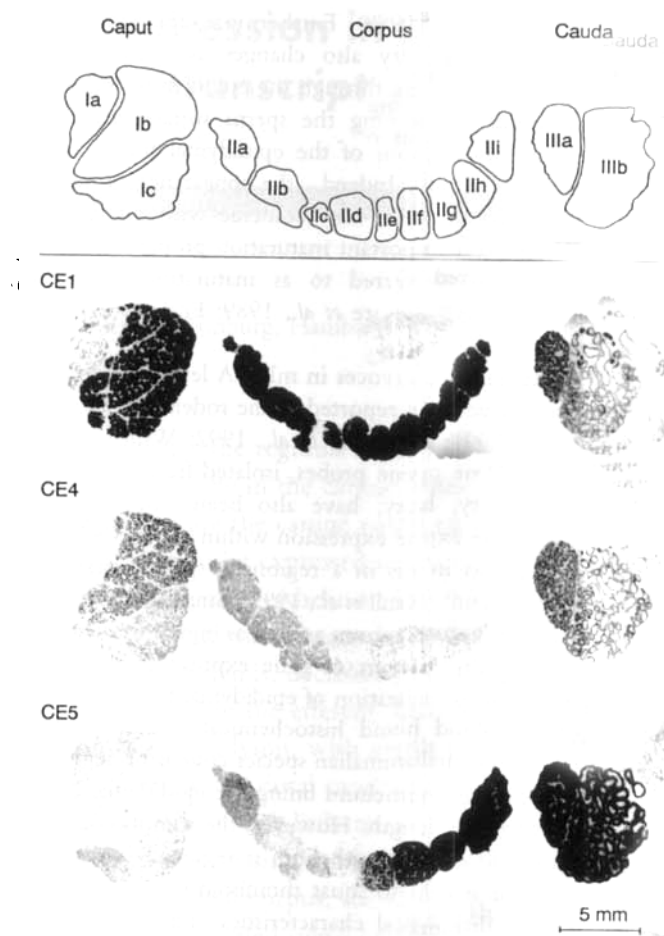


Figure 1. Low-resolution autoradiograms of three entire sagittal sections of an epididymis obtained from a small mongrel dog, visualized by in-situ transcript hybridization to the tissue-specific gene probes CE1, CE4 and CE5, as indicated. All sections were exposed simultaneously to autoradiographic film for the same time period. The roman annotation in the upper outline diagram refers to the segment descriptions given in the text. Scale bar = 5 mm.

into nine discrete subregions (IIa–IIi) delineated by the positions of fibrous septa intercalating along the length of the corpus region (Fig. 1). Significantly, transcript levels also appeared to conform to the same subdivisions, levels within a subregion being relatively homogeneous, but often differing markedly from the transcript levels in the immediately adjacent subregions (Fig. 1).

Segment III. Within tissue sections through the cauda segment two regions were distinguished based upon the height of the epithelium and the diameter of the ductular cross-sections as follows.

(IIIa) A smaller part essentially showed the morphological and labelling characteristics of the most distal cross-sections of the corpus epididymis.

(IIIb) The majority of luminal cross-sections in the cauda region showed an increased luminal diameter and a flattened

epithelium. The vas deferens was not a constituent of the dissected material.

In-situ localization of CE1, CE4, and CE5 transcripts in the dog epididymis

CE1, CE4, and CE5 mRNAs were localized in tissue sections through the dog epididymis employing ^{35}S -rCTP-labelled antisense cRNA probes obtained by in-vitro transcription from plasmid subclones (Ellerbrock *et al.*, 1994) as described in Materials and methods. Transcript hybridization was visualized as dark silver grains on brightfield transmission micrographs (Fig. 2a) or as white grains on darkfield reflectance micrographs (Figs 2c, 3, 4 and 5).

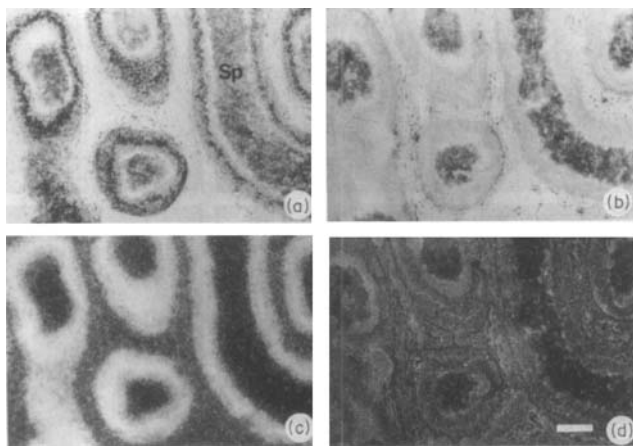


Figure 2. High-resolution in-situ transcript hybridization of segment IIa using the gene probe for CE1. (a) transmitted light, anti-sense cRNA. (b) transmitted light, control sense cRNA. (c) dark-field reflectance, anti-sense cRNA. (d) darkfield reflectance, control sense cRNA. Scale bar = 70 μm . Note that although the lumen of tubules are filled with spermatozoa (Sp), these do not hybridize to the cRNA probes. Only the principal cells of the epithelium gave a positive hybridization signal.

The micrographs shown (Fig. 2) represent the hybridization patterns observed for the proximal dog corpus epididymis using adjacent tissue sections in three independent in-situ hybridization experiments. Hybridization was found exclusively in the epithelial cell layers of the epididymal duct. Few or no silver grains were observed within the duct lumen (i.e. the location of spermatozoa) nor in the inter-tubular tissue surrounding the duct cross-sections. The specific localization of the transcripts within the ductular epithelium was confirmed by the absence of silver grains beyond a background level of signal when adjacent tissue sections were hybridized with ^{35}S -rCTP-labelled sense cRNAs as negative controls (Fig. 2b and d).

CE1. The CE1 gene transcript is the most frequent of the tissue-specific mRNAs identified by us both in the canine

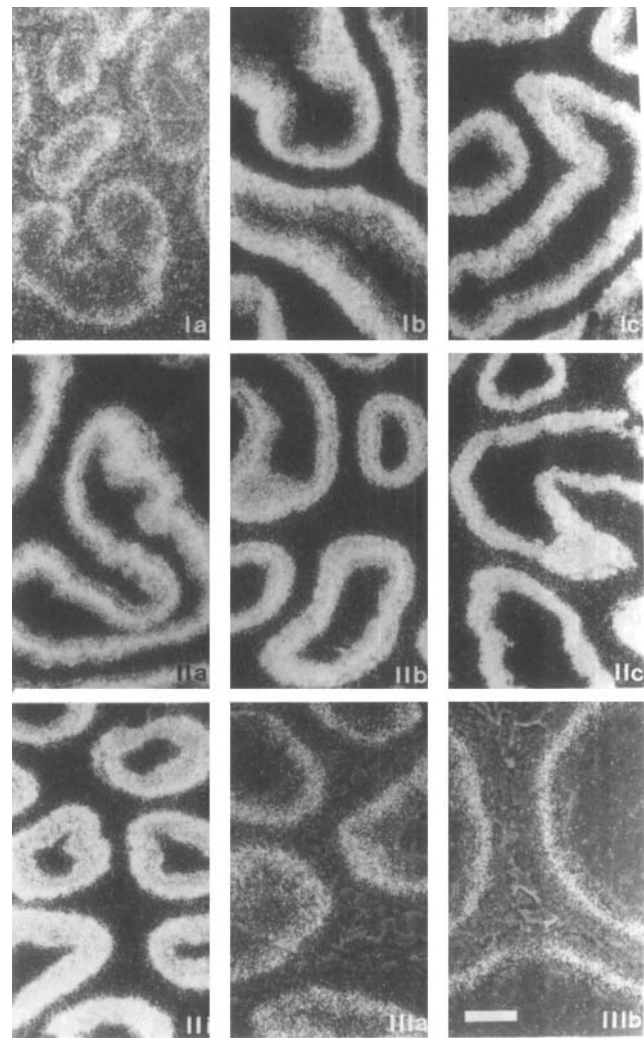


Figure 3. In-situ transcript hybridization using the anti-sense cRNA probe for CE1. Sections are selected from the epididymal regions indicated (see Fig. 1), with the panel upper left being most proximal and the panel lower right most distal. For details see text. Scale bar = 90 μm . Autoradiographic exposure, 3 days.

epididymis (Ellerbrock *et al.*, 1994) and as its homologue, HE1, in the human epididymis (Kirchhoff *et al.*, 1990). This was also true for the in-situ transcript hybridization pattern (Fig. 3), where there was a consistently strong hybridization signal in nearly all regions of the epididymis excluding the efferent ducts, segment Ia, where there was only a very weak positive signal, and the proximal cauda (IIIa) where the signal was reduced when compared to the more proximal corpus segments. The signal remained reduced also in the distal cauda (IIIb).

While for the CE1 transcript there appeared to be little variation in overall expression levels from one segment to the next, there was an interesting variation in silver grain localization within the epithelium (Fig. 3). Within the high epithelia of the proximal corpus epididymis, silver grains

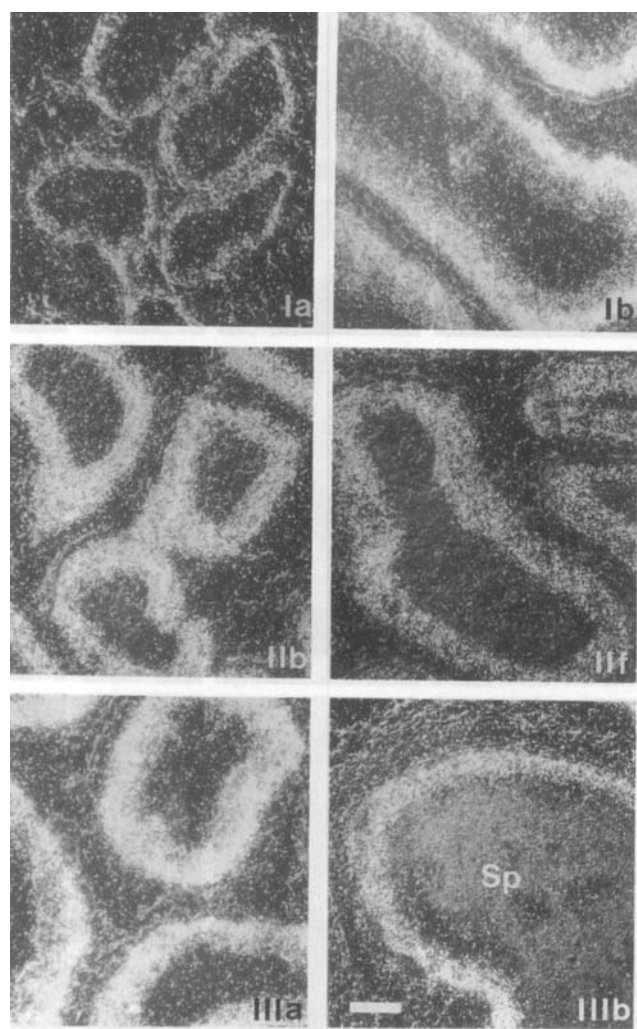


Figure 4. In-situ transcript hybridization using the anti-sense cRNA probe for CE4. Sections are selected from the epididymal regions indicated (see Fig.1), with the panel upper left being most proximal and the panel lower right most distal. For details see text. Scale bar = 90 μ m. Autoradiographic exposure, 8 days.

were concentrated in a central position within the epithelial cells (Fig.3, *Ib–IIb*). In more distal corpus segments of the same longitudinal sections, silver grains were distributed evenly throughout the cytoplasm of the cells. This shift in the intracellular transcript distribution possibly reflects a structural change within the epithelial layer between the proximal and distal regions of the corpus; the proximal corpus only is reported to be furnished with marked cytoplasmic inclusions (Chandler *et al.*, 1981), which may sterically hinder an even distribution of the protein synthetic machinery and mRNA on the rough endoplasmic reticulum.

CE4. The CE4 in-situ hybridization signal was weak in all regions of the dog epididymis (note that the in-situ hybridization for CE4 required 8 days autoradiographic

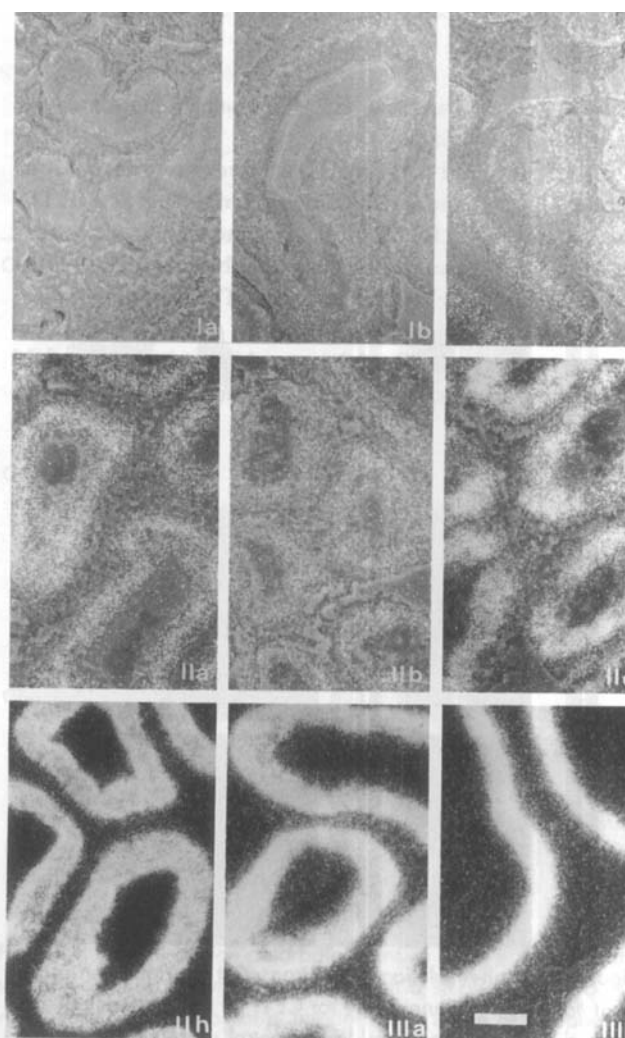


Figure 5. In-situ transcript hybridization using the anti-sense cRNA probe for CE5. Sections are selected from the epididymal regions indicated (see Fig.1), with the panel upper left being most proximal and the panel lower right most distal. For details see text. Scale bar = 90 μ m. Autoradiographic exposure, 3 days.

exposure as opposed to 3 days for the other gene probes), reflecting the relatively low frequency of CE4 mRNA as already revealed by screening a dog epididymal cDNA library and Northern blot hybridization (Ellerbrock *et al.*, 1994). In this respect, it very much resembles its human homologue HE4 (Kirchhoff *et al.*, 1991).

There was, however, a mild variation in levels of CE4 steady state mRNA, with lowest levels in the middle corpus region, and relatively higher levels in both caput (*Ib*), and proximal cauda segments (*IIIa*, Fig. 4). The efferent ducts and segment *Ia*, were devoid of a specific CE4 hybridization signal (Fig.4). The CE4 gene transcript illustrates one curious feature, namely a distinct reduction in mRNA level within one subsegment in the central corpus region (Fig.1,

IId). This feature was also detectable using the CE5 marker gene probe (see below), although it was not as pronounced as with the CE4 antisense cRNA.

CE5. Of the three marker gene probes employed, the antisense CE5 cRNA provided the most pronounced pattern of longitudinal variation in steady state transcript levels (Fig. 5). Virtually no CE5 hybridization signal could be detected in cross-sections of the efferent ducts, segment *Ia*. Positively hybridizing transcripts were first observed in the epithelial cells of the distal caput epididymis, segment *Ic*. In the following corpus regions (*IIa–IIIi*) CE5 transcript levels increased progressively. However, the silver grains were not distributed evenly in the epithelium, but appeared as a 'mottled' pattern, with positive hybridization signals often restricted to 'islands' of cells, within an otherwise negative epithelial layer (Figs 1 and 5, *Ile*). Maximum CE5 hybridization levels were reached in the distal corpus and cauda epididymis.

Discussion

The in-situ hybridization of specific epididymal transcripts using the three marker gene probes, CE1, CE4 and CE5, identified in the previous study (Ellerbrock *et al.*, 1994), has verified two important postulates. First, the relative levels of the positive signals obtained using probes radiolabelled using standard procedures, are very similar to the relative frequencies estimated for the whole organ from Northern hybridization and from cDNA cloning. Thus the quantitative aspects of the study can be relied upon to reflect true differences in transcript level within different regions of the epididymis. Second, the patterns of transcript distribution conform closely with the mRNA patterns obtained using the same technique in sections cut serially through the human epididymis (Kirchhoff *et al.*, 1991; Krull *et al.*, 1993), using as probes the human homologues of the marker gene probes used in the study described here. This finding is especially relevant to demonstrate the close similarity in pattern of gene expression and hence physiology between the canine and human epididymides, and hence the suitability of the former to model human function.

An additional advantage of this methodology is that it provides further discriminatory characteristics to those provided by conventional morphological techniques. So, for example, it could be shown that within the corpus region there are subsegments which, on morphological criteria, may appear homogeneous but which, for example, using the CE4 marker probe, exhibit a lower level of these specific gene transcripts. This discriminatory ability of the technique was shown more dramatically using the CE5 probe. Not only were the distal and proximal corpus regions clearly different from one another in terms of the level of CE5 gene transcription, but even within a single tubule cross-section, apparently identical cell types showed substantial differences in CE5 mRNA level compared to a neighbouring cell (Fig. 5, *Ile*).

Figure 1 illustrates another feature which may be of considerable functional relevance, namely that the fibrous septa appear to separate the corpus region into subregions with discrete but homogeneous levels of gene transcription. This was most marked for CE5 mRNA and was observed also for CE4 mRNA. This may indicate that each anatomically defined subdivision is subject to an independent microenvironment of hormones, substrates or metabolites. Since the epididymal arteries ramify along the surface of the epididymis and enter the organ within these connective tissue septa (Setchell *et al.*, 1994), with the veins following the arteries, the septally delimited subregions could represent effective functional units, each with a defined milieu determined by the vascularization. Because expression of the CE1 mRNA was homogeneous within these segments, a focal pathological explanation for these observations is unlikely.

The CE5 mRNA encodes a peptide with an unusual structure (Ellerbrock *et al.*, 1994). As in its human and rodent homologues, which have been shown to represent also major lymphocyte surface antigens (Kubota *et al.*, 1990; Xia *et al.*, 1991; Hale *et al.*, 1993), there appears to be substantial post-translational modification with cleavage of signal peptide sequences at both the N- and C-termini of the peptide, attachment of a glycosyl-phosphatidyl-inositol (GPI)- anchor at the new C-terminus, as well as probable N- and O-glycosylation along the remaining length of the mature peptide. It has been hypothesized that the resulting mature glycopeptide assumes a mucin-like structure, intercalated into the sperm plasma membrane via the GPI-anchor (Kirchhoff *et al.*, 1993; Kirchhoff, 1994). Given the high level of this protein in seminal fluid, it seems likely that this structure is a major coating protein of ejaculated spermatozoa, as has been postulated recently for the rodent homologues of CE5, rB7 and mB7 (Kirchhoff, 1994), and possibly has immunosuppressive properties. It is thus easier to understand why CE5 gene expression should first occur in the distal corpus region (Figs 1 and 5). This would allow other maturation events, involving protein-protein interactions, to occur at the sperm surface in the more proximal epididymal segments without the steric hindrance of the fully developed glycocalyx.

This basic study thus suggests that future use of the gene products both at the protein and mRNA level, represented by the tissue-specifically expressed cDNAs for CE1, CE4 and CE5, or their human counterparts, should offer a considerable refinement in diagnostic potential to detect subtle pathologies of epididymal function. Future studies will involve developing this canine model, both *in vitro* and *in vivo* to provide a laboratory substitute of clear relevance to human epididymal function and sperm maturation.

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