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Localization of Kex2-like processing endoproteases, furin and PC4, within mouse testis by in situ hybridization

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By in situ hybridization analysis, we show here the localization of furin and PC4, which are both members of a growing family of endoproteases structurally related to the yeast precursor processing protease Kex2, within mouse testis. Furin transcript was detected in both germ and somatic cells, while PC4 transcript was found only in round spermatids. Proenkephalin transcript was also localized in round spermatids. These observations suggest that, within testis, PC4 is involved in processing of peptide precursors such as proenkephalin and may play a role in regulation of sperm maturation, while furin may serve as a more general processing endoprotease.

Proenkephalin; Prohormone processing; Spermatogenic cell; Yeast Kex2 protease

1. INTRODUCTION

Many bioactive peptides and proteins are produced from larger precursors through limited endoproteolysis, which occurs at sites marked usually by paired basic amino acids or occasionally by oligo-basic residues [1,2]. Research on processing endoproteases was advanced by the investigation of the yeast Kex2 protease which is involved in processing of pro-α-factor at dibasic sites [3]. Recently, five mammalian Kex2 homologues, furin, PC2, PC1/3, PC4, and PACE4, have been identified by cDNA cloning [4–8]. Among them, PC2 and PC1/3 are expressed in neuroendocrine tissues and are involved in processing of precursors for peptide hormones and neuropeptides at dibasic sites [4,5,9–11], and furin is expressed in all examined tissues and is involved in processing of a wide variety of precursor proteins with the Arg-X-Lys/Arg-Arg motif at the cleavage sites [4,5,12–15].

By contrast, the function of PC4 appears to be somewhat different from those of other endoproteases for the following reasons: first, PC4 mRNA is detectable only in the testis and its expression is developmentally regulated, while furin mRNA is detected in all examined developmental stages [7]; second, coexpression of PC4 with prorenin, which is cleaved by PC1/3 [9,13,16,17], or with a prorenin mutant with an Arg-X-Lys-Arg sequence, which is cleaved by furin [13,15], in cultured cell lines does not result in conversion of the precursor [7].

Peptides and proteins produced locally within the testis have been implicated as potential autocrine and paracrine factors mediating interactions between testicular cells [18]. Therefore, PC4 and furin are thought to be involved in processing of precursors for these peptides and proteins. To gain a further insight into the physiological roles of PC4 and furin, we analyzed the localization of their transcripts within mouse testis by in situ hybridization.

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2. MATERIALS AND METHODS

The following mouse cDNAs were separately subcloned into the following vectors: furin, a 924-bp *Bam*HI-*Bam*HI fragment (nucleotide residues 1,718–2,641) [12] into pBluescript-II KS(+); PC4, a 236-bp *Pst*I-*Xho*I fragment (residues 1,250–1485) [7] into pBluescript-II SK(+); preproenkephalin, a 307-bp *Rsa*I-*Pst*I fragment (residues 320–626) [19] into pGEM-2. Antisense or sense riboprobes labeled with digoxigenin (DIG)-UTP were generated from the linearized vectors using T3, T7, or SP6 RNA polymerase by the aid of a DIG RNA Labeling kit (Boehringer Mannheim).

Testes from sexually mature (60-day-old) or prepuberal (16-day-old) mice were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline at 4°C, dehydrated successively with cold 70%, 80%, 90% and 100% ethanol, embedded in paraffin, and cut into 7-μm sections. The sections were deparaffinized with xylene, washed successively with 100%, 90%, 80%, and 70% ethanol, and phosphate-buffered saline. After refixation with 4% paraformaldehyde, the sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, dehydrated, and air-dried. Hybridization was performed overnight at 50°C in the presence of the DIG-labeled antisense or sense riboprobe. The hybridization solution was composed of 50% formamide, 10 mM Tris-HCl (pH 7.6), 0.2 mg/ml yeast tRNA, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10% dextran sulfate, 0.6 M NaCl, and 0.25% SDS. After the hybridization, the sections were treated successively with 50% formamide in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate) for 30 min at 50°C, with RNase A (50 μg/ml in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.5 M NaCl) for 30 min at 37°C, and with 2 × then again 0.2 × SSC for 20 min at 50°C. Detection of the hybridized probe was

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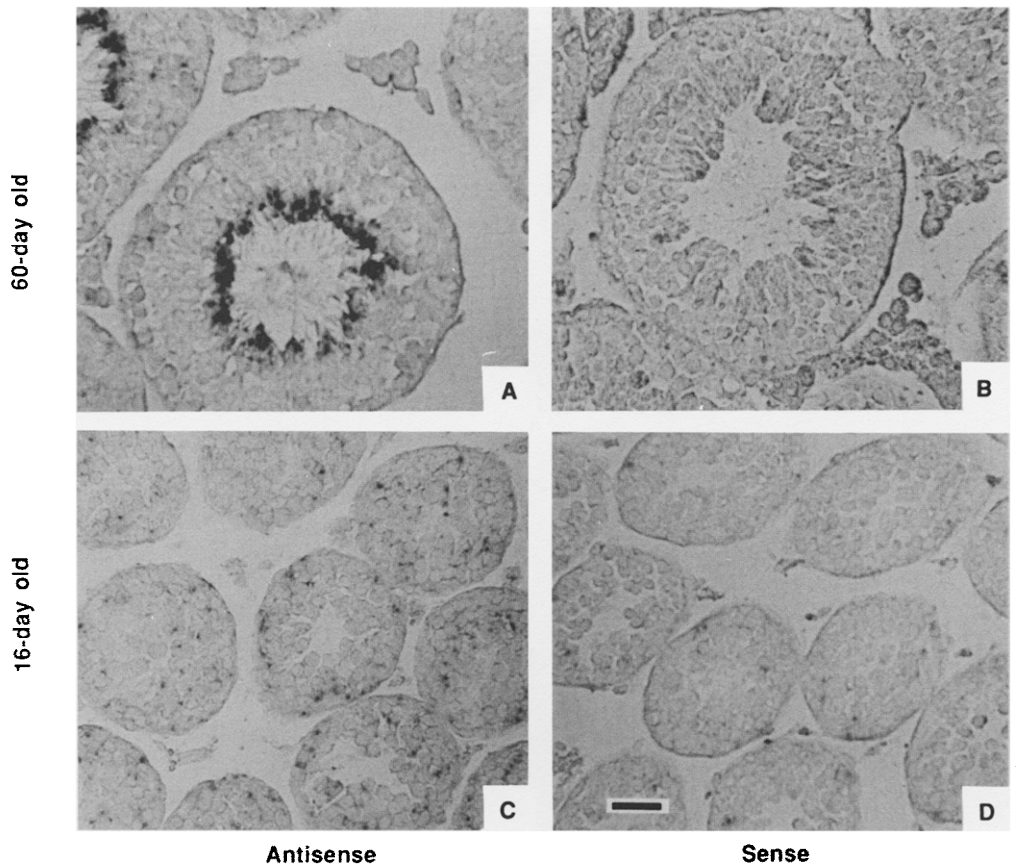

Fig. 1. Localization of PC4 transcript. Sections from 60-day- (A,B) and 16-day- (C,D) old mouse testes were hybridized with the antisense (A,C) or sense (B,D) riboprobe for PC4 under the conditions described in Materials and Methods. Bar = 2 mm.

performed using a DIG Nucleic Acid Detection kit (Boehringer Mannheim) under the conditions described by the manufacturer.

3. RESULTS

As shown in Fig. 1, localization of PC4 transcript in the testis of sexually mature (60-day-old) mouse using the PC4-specific antisense riboprobe revealed strong signals in cells adjacent to the lumen of the seminiferous tubule (Fig. 1A). These cells are round spermatids [18]. By contrast, no signal was detected in spermatozoa, spermatocytes, nor spermatogonia. When the sense probe was used as a negative control, no specific signal was observed in cells throughout the testis (Fig. 1B). It is known that in mice the spermatocytes begin to undergo meiosis and differentiate into the haploid round spermatids around the 18-day stage after birth [20]. Therefore, we then examined the expression of PC4 transcript in the testis from a prepuberal (16-day-old) mouse. No specific signal was observed using the antisense (Fig. 1C) nor sense (Fig. 1D) riboprobe. These observations indicate that PC4 transcript is expressed in the early stages of spermiogenesis, and support our previous Northern blot data using RNAs from fractionated spermatogenic cell populations [7].

The distribution pattern of the furin transcript was quite different from that of the PC4 transcript. As shown in Fig. 2, specific signals on sections of 60-day-old mouse testis were observed in virtually all cell types (Fig. 2A). Within the seminiferous tubules, the intensity of staining appeared to be higher in outer cells, such as spermatogonia, than in inner cells, such as spermatids. A similar pattern of staining was obtained for the testis from a 16-day-old mouse (Fig. 2C). By contrast, no specific signal was apparent with the sense probe (Fig. 2B,D). These observations are compatible with our previous data that furin mRNA is detectable in all examined developmental stages of mouse testis [7].

It is of particular interest what precursor protein(s) is the physiological substrate for PC4. Although many peptides which are synthesized from precursors through cleavage at basic sites have been reported to be present in the testis, most of them are produced in the somatic cells, such as Leydig and Sertoli cells [18]. One of the candidate substrates is proenkephalin, since its mRNA is detected in fractionated spermatogenic cell populations [21,22], and since one of its processed products, Met-enkephalin-Arg-Phe, is detectable in the sperm acrosome [22]. However, the distribution of proenkephalin transcript in individual cells within the intact testis

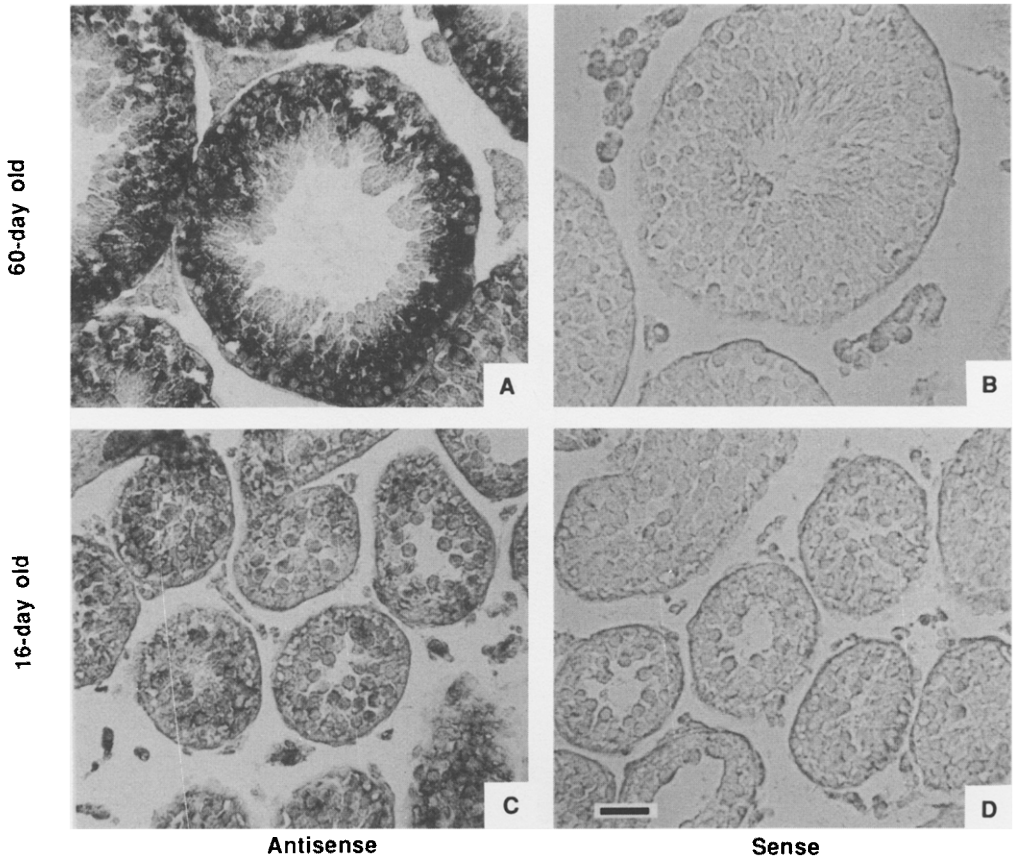

Fig. 2. Localization of furin transcript. Sections from 60-day- (A,B) and 16-day- (C,D) old mouse testes were hybridized with the antisense (A,C) or sense (B,D) riboprobe for furin under the conditions described in Materials and Methods. Bar = 2 mm.

has not been examined. Therefore, we analyzed the distribution of proenkephalin transcript by in situ hybridization. As shown in Fig. 3, specific signals were detected mainly in the round spermatids; the distribution pattern of proenkephalin transcript within 60-day-old mouse testis (Fig. 3A,B) resembled that of PC4 transcript (see Fig. 1). Specific signals were barely detectable in 16-day-old mouse testis (Fig. 3C,D). These data suggest that proenkephalin can be serve as a substrate for PC4.

4. DISCUSSION

Many bioactive peptides have been found to be produced within the testis, and appear to play the role of mediators among different testicular cells [18]. Most of them are produced from precursors through cleavage at basic residues catalyzed by specific endoproteases. We have recently shown that Kex2-like proteases, PC4 and furin, are expressed in the testis, and proposed that they are involved in the precursor processing [7]. To gain a further insight into their roles, we analyzed the localization of their transcripts within mouse testis by in situ hybridization.

PC4 transcript was detected only in round spermatids. The localization was consistent with that of proenkephalin transcript. In view of the presence of a processed product of proenkephalin, Met-enkephalin-Arg-Phe, in the sperm acrosome [22], PC4 may be responsible for processing of this precursor. In this context, the data of Kew et al. [22] is noteworthy; Met-enkephalin, which is the major product of proenkephalin in other tissues (e.g. hypothalamus and adrenals), is undetectable in the testis. We speculate that PC4 may have a dibasic site-preference different from those of other Kex2-like proteases for the following reasons: first, PC2 and PC1/3 have been shown to be expressed in hypothalamus and adrenals but not in the testis [9,23–25]; second, PC2 and PC1/3 have been shown to have different dibasic site-preferences for the same precursor, proopiomelanocortin [10,11]; third, prorenin, which is processed at the dibasic site by PC1/3 [9,13,16,17], is not processed by PC4 [7].

On the other hand, furin transcript was detected in all cell types within the testis. In view of the fact that furin cleaves precursors at sites marked by the Arg-X-Lys/Arg-Arg motif but not at dibasic sites [13,15,26], furin may be involved in maturation of some growth factors,

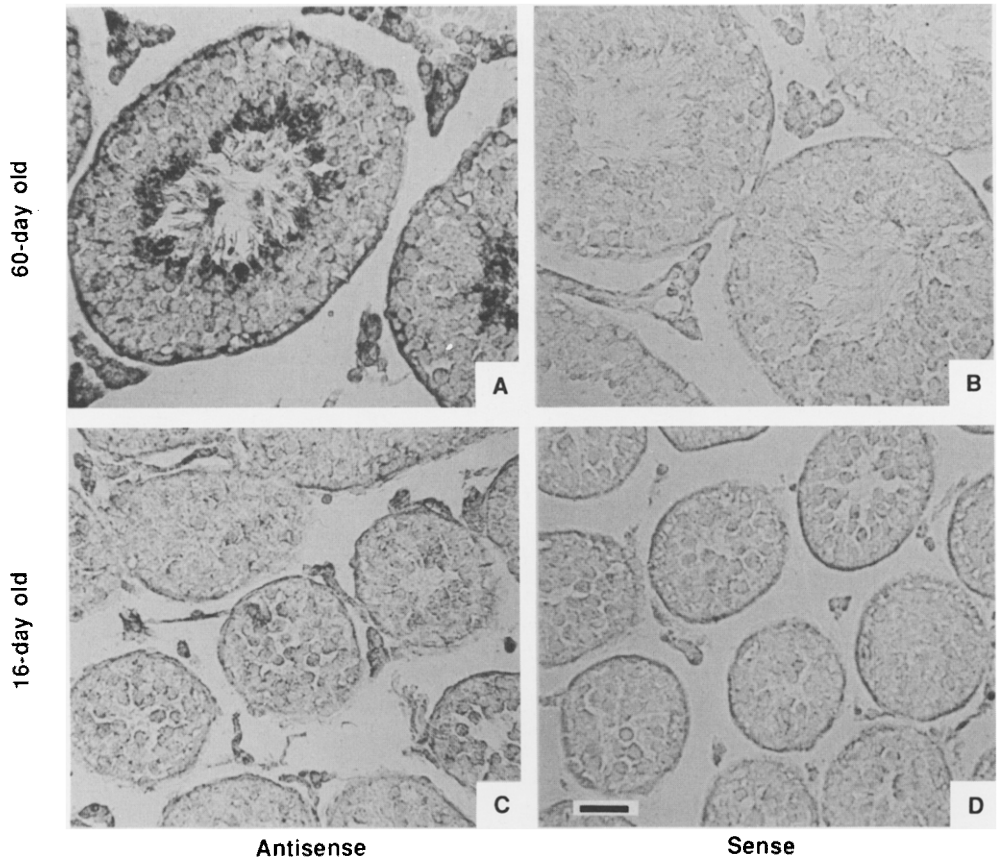

Fig. 3. Localization of proenkephalin transcript. Sections from 60-day- (A,B) and 16-day- (C,D) old mouse testes were hybridized with the antisense (A,C) or sense (B,D) riboprobe for proenkephalin under the conditions described in Materials and Methods. Bar = 2 mm.

such as nerve growth factor, transforming growth factor-β, and inhibin, for the following reasons: first, they are present in testicular cells [18,27]; second, they are produced from precursors through cleavage at Arg-X-Lys-Arg sites [13]; third, pro-nerve growth factor have been shown to be cleaved by furin [28].

In summary, this study determined the localization of Kex2-like endoproteases, PC4 and furin, within the testes. However, it must await experimental proof what precursors are the actual substrates for these proteases. Studies are under way to settle this problem.

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