A novel testis-specific RAG2-like protein, Peas: its expression in pachytene spermatocyte cytoplasm and meiotic chromatin

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Abstract **We report a novel gene *Peas* that constitutes an overlapping gene complex in mammalian genome. We have cloned human and mouse *Peas* cDNAs (h*PEAS*/m*Peas*) and analyzed their tissue and stage-specific expressions. Peas protein contains six repeated kelch motifs, structurally similar to RAG2, a V(D)J recombination activator, and is evolutionarily conserved among mammals, birds, insects, and nematodes. Northern, RNA in situ hybridization and immunohistochemical analyses showed that m*Peas* is specifically transcribed in testis, particularly in pachytene spermatocytes in which it is localized to the cytoplasm and meiotic chromatin. It is suggested that Peas may be involved in meiotic recombination process.**
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Key words: Peas; RAG2; Meiosis; DNA double strand break; Homologous recombination

1. Introduction

We have earlier reported that the human and mouse male-enhanced antigen-1 gene (*Mea1*) are flanked by two overlapping genes, *Peas* and *Ppp2r5d*, forming a *Peas–Mea1–Ppp2r5d* gene complex on human chromosome 6p21.1 and mouse chromosome 17 24.30-cM. Whereas *Peas–Mea1* occurs in a head-to-head orientation, *Mea1–Ppp2r5d* in a tail-to-tail orientation [1]. *Mea1*, a candidate gene for a male-specific antigen, is expressed in the late stage of spermatogenesis [1,2]. *Ppp2r5d* encodes δ isoform of PP2A regulatory subunit B56 family [3,4].

In the sexual reproduction system, four haploid gametes are formed from a single diploid cell at meiosis. This process shuffles genetic material by random segregation of paternal and maternal chromosomes, and in most cases, by physical recombination between the homologous chromosomes. Molecular mechanisms of homologous recombination in eukaryotes have been extensively studied using budding yeasts. It has been shown that the entry to the meiotic stage is marked by expression of many proteins that are involved in the duplication, recombination, and segregation of chromosomes, most of these are conserved amongst eukaryotes [5].

Double strand breaks (DSBs) can be induced physically or chemically, e.g. by ionizing radiations or treatments with mutagens. DSBs, if not repaired, lead to cell death. On the other hand, cells harbor mechanisms to create DSBs during the initiation of meiotic recombination in yeasts [6]. During lymphocyte development, T cell receptor (TCR) and immunoglobulin (Ig) genes are assembled from separate coding segments by a process called V(D)J recombination. The lymphoid-specific recombination activators, RAG1 and RAG2, initiate V(D)J recombination by introducing DSBs adjacent to the specific recombination signal sequences at the border of the coding DNA, producing a blunt-cut signal end and a hairpin coding end [7–9].

Genes involved in meiotic DSBs-introduction of higher eukaryotes have not been identified. We have found a novel testis-specific gene, *Peas*, that encodes a RAG2-like protein. In this study, we report its primary structure, expression, and localization in spermatocytes. Its possible role in meiotic recombination is suggested.

2. Materials and methods

2.1. Cloning of human and mouse PEAS/Peas cDNA

Human and mouse (BALB/c) testis mRNA were purchased from Clontech (Palo Alto, CA, USA). The first-strand cDNAs were prepared by reverse transcription (RT) of 200 ng of the mRNAs using Superscript II (Lifetech, Gaithersburg, MD, USA). Polymerase chain reactions (PCRs) for the coding sequences of h*PEAS*/m*Peas* cDNA were performed by using h*PEAS*-specific primers hPEAS-NF, 5′-GGATGTTACGGTGGACAGTGC-3′, and hPEAS-CR, 5′-CTACCCATGGGAGGAGACGAT-3′, and m*Peas*-specific primers mPeas-NF, the same to hPEAS-NF, and mPeas-CR, 5′-CTAGCCATGGGAGGAGACAAT-3′, using Pyrobest polymerase system (Takara, Japan). The cycling conditions were 1 min at 94°C, 1 min at 58°C, 2 min at 74°C for 30 cycles. Amplified products were separated by 1% (w/v) agarose electrophoresis, purified and subcloned into the pBluntII-TOPO vector (Invitrogen).

5′- and 3′-RACE (rapid amplification of cDNA ends) analyses of h*PEAS*/m*Peas* cDNA were performed on testis cDNA using h*PEAS*-specific anti-sense primers hPEAS-GSP1, 5′-TGCACTGTCCACCGTAACATC-3′, and hPEAS-GSP2, 5′-GTCCTCGAGCTGCCTGCAGCC-3′, h*PEAS*-specific sense primers hPEAS-323(L)F, 5′-CTTATAGATCATTCTGACTTA-3′, and hPEAS-338(L)F, 5′-CTGAAGACTCTGTGCAAACTG-3′, m*Peas*-specific anti-sense primers mPeas-GSP1, the same to hPEAS-GSP1, and mPeas-GSP2, 5′-CACAGGTTACAGGAACATGCC-3′, and m*Peas*-specific sense primers mPeas-323(L)F, 5′-CTCATAGATCATTCTGACTTA-3′, and mPeas-338(L)F, 5′-CTGAAGACTCTGTGCAAGCTG-3′, following the manufacturer's instruction (human: 5′- and 3′-RACE kit, Stratagene, mouse: Cap Site cDNA®dT kit, Nippon Gene, Japan), respectively. Amplified products were separated by 2% (w/v) agarose electrophoresis and subcloned into the pCR2.1-TOPO vector (Invitrogen). All DNA sequencing was performed with ABI PRISM™ 310 genetic analyzer (PE Biosystems, Foster City, CA, USA).

2.2. Northern analysis

Mouse multiple tissue Northern blot (2 μg of poly(A)$^+$ RNA from

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a variety of tissues) was purchased from Clontech. The membrane was hybridized with a random-primed ^{32}P-labeled DNA fragment of the full-length coding region of m*Peas* cDNA for 16 h at 45°C in a hybridization solution containing 50% (v/v) formamide and was washed under stringent conditions.

2.3. *In situ hybridization*

Paraffin-sections (3 μm thick) of the mouse (BALB/c; age, 8 weeks) testis were hybridized with anti-sense and sense digoxigenin (DIG)-11-UTP-labeled RNA probes that were prepared by in vitro transcription of DNA fragments (~500 bases) of m*Peas* cDNA using a DIG RNA labeling kit (T7/SP6) (Roche, Germany). To add T7 promoter sequence, the DNA fragments were amplified using m*Peas*-specific primers T7-mPeas/sense, 5′-TAATACGACTCACTATAGGGAGACCACATGTTACGGTGGACAGTGCAT-3′, mPeas/anti-sense, 5′-CATGTCATGGTGCTGGTATCC-3′, mPeas-NF (see Section 2.1), and T7-mPeas/anti-sense, 5′-TAATACGACTCACTATAGGGAGACCACCATGTCATGGTGCTGGTATCC-3′, with Pyrobest polymerase system (Takara). Immunohistochemical detection of DIG-labeled RNA was carried out with alkaline phosphatase-conjugated anti-DIG antibody (Roche) and an alkaline phosphatase substrate kit VI (BCIP/NBT) (Vector, Burlingame, CA, USA). Counter-staining was performed with 0.1% safranin O.

2.4. *Anti-Peas antibody*

A KLH-conjugated synthetic oligopeptide corresponding to amino acids 4–18 of hPEAS/mPeas/*Ce*Peas (WTVHLEGGPRRVNHAC) was used as an antigen. Rabbit polyclonal antisera against the peptide were raised. The antisera were affinity-purified by column chromatography using a HiTrap NHS-activated column (Amersham Pharmacia Biotech) linked with the antigen peptide.

2.5. *Western blotting analysis*

Total testis protein (10 μg) from a variety of species (human, bovine, swine, rat, mouse, and chicken) were separated in 12% SDS–PAGE, and transferred to Immobillon™ PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with the blocking solution (3.7% skim milk (Difco), 1% normal goat serum (Vector) in TBS-T (20 mM Tris–HCl, pH 7.4; 150 mM NaCl; 0.2% (w/w) Tween-20)) for 1 h and reacted to the anti-Peas antibody (1:6000 dilution with the blocking solution) for 16 h at 4°C. The detection was performed using goat anti-rabbit IgG antibody peroxidase conjugate (1:8000 dilution with the blocking solution, Sigma) and ECL™ detection kit (Amersham Pharmacia Biotech).

2.6. *Immunostaining analyses*

For indirect immunohistochemistry, paraffin sections (3 μm thick) of the mouse (BALB/c; age, 8 weeks) testis were incubated with a blocking solution (5% (w/v) skim milk, 1% (v/v) normal goat serum (Vector) in PBS-T) and probed with an anti-Peas rabbit polyclonal antibody (1:1000 dilution with the blocking solution). The signal was revealed using a biotin-conjugated goat anti-rabbit IgG (1:1000, Sigma), an ABC kit (VECTASTAIN, Vector), and a peroxidase substrate kit (DAB) (Vector) and a Cy3-conjugated goat anti-rabbit IgG (1:500, Amersham Pharmacia Biotech). Sections were counter-stained with hematoxilin (Wako, Japan) and DAPI (100 ng/ml in PBS), respectively.

3. Results

3.1. *Human and mouse* PEAS/Peas *gene*

We had previously analyzed human and mouse male-enhanced antigen-1 gene (*MEA1*/*Mea1*) genomic sequences and found that *MEA1*/*Mea1* was flanked by two overlapping genes on the chromosome 6p21.1 (human) and 17 24.30-cM (mouse) in the vicinity of HLA/H-2 complex loci. One of them is *PPP2R5D*/*Ppp2r5d*, encoding a protein serine/threonine phosphatase 2A regulatory subunit B56δ isoform. The other is a novel gene named *PEAS*/*Peas* [1].

We designed several PCR primers for cDNA on the basis of genomic DNA sequences and cloned human and mouse *PEAS*/*Peas* cDNA by combining RT-PCRs and the RACE technique. Complete cDNAs (human: accession no. AB055925 and mouse: no. AB053465) have sizes of 1875/1994 bases and both encode polypeptides consisting of 382 amino acid residues (aa) with a calculated molecular weight of ~43 kDa.

The predicted protein sequences (Fig. 1A) can be organized into two distinguishable parts. The amino-terminal region (human and mouse: 13–338) contains six tandem-repeated kelch motifs of ~50 aa (Fig. 1B). The carboxy-terminal region consists of a domain with relatively hydrophobic amino acids. The kelch motif was earlier identified as six-fold tandem elements in the sequence of the *Drosophila* kelch ORF1 protein [10]. The repeated kelch motifs predict a conserved tertiary structure, a β-propeller. This module appears in many different polypeptide contexts and contains multiple potential protein–protein contact sites. Members of this growing superfamily are present both intra- and extra-cellularly and have diverse activities [11]. The sequence alignment of hPEAS/mPeas, *Dm*Peas (*Drosophila melanogaster* CG12081 gene product: AAF46395), and *Ce*Peas (*Caenorhabditis elegans* hypothetical protein F53E4.1: CAB03122) shows that *Peas* proteins are evolutionarily conserved and much more similar to each other than to the other kelch-repeat-containing proteins (Fig. 1A). The most conserved motifs consist of an Arg residue between the fourth and the first β-strand, a Gly doublet and hydrophobic amino acids that seem to be crucial for the transition from the second to the third β-strand, and a Trp residue in the fourth β-strand (Fig. 1A,B). A putative nuclear localization signal is predicted at amino acid residues 279–296 KKIEPKGKGPCPRRRQCC in both human and mouse proteins. The hPEAS/mPeas are similar to RAG2 almost throughout the whole length including six repeated kelch motifs to form a six-bladed β-propeller structure excluding the β-strand–β-strand regions (Fig. 1C).

3.2. *Tissue-specific Peas expression*

Northern blot analyses on various mouse tissues revealed that m*Peas* was specifically expressed in the testis (Fig. 2). In mouse testis, in situ hybridization with the sense probe did not show any signals (Fig. 3a,c). When antisense probe was used, *Peas* transcripts were abundantly detected in pachytene spermatocytes and moderately in spermatids (Fig. 3b,d).

Anti-Peas antibodies were raised against KLH-conjugated synthetic oligopeptide corresponding to amino acids 4–18 of hPEAS/mPeas/*Ce*Peas in rabbits. The cross-reactivity and specificity of the antibody was examined by Western blot analysis for endogenous testicular proteins from various species (Fig. 4). Endogenous proteins were detected as major 43-kDa bands supposedly corresponding to products of cloned Peas cDNA. A few, high-molecular proteins also reacted weakly to the antibody. The data indicated that Peas protein is conserved in human, bovine, swine, rat, mouse, and chicken. The weakly reacting proteins may either represent cross-reactivity to related proteins or may be the products of alternative splicing. In fact, database search hit several expressed sequence tags that could present splice-variants in the C-terminal regions (data not shown).

3.3. *Localization of Peas*

Immunohistochemical examination of mouse testis revealed that Peas is expressed in a stage-specific manner and localized mainly in pachytene spermatocytes (Fig. 5a). High-magnifica-

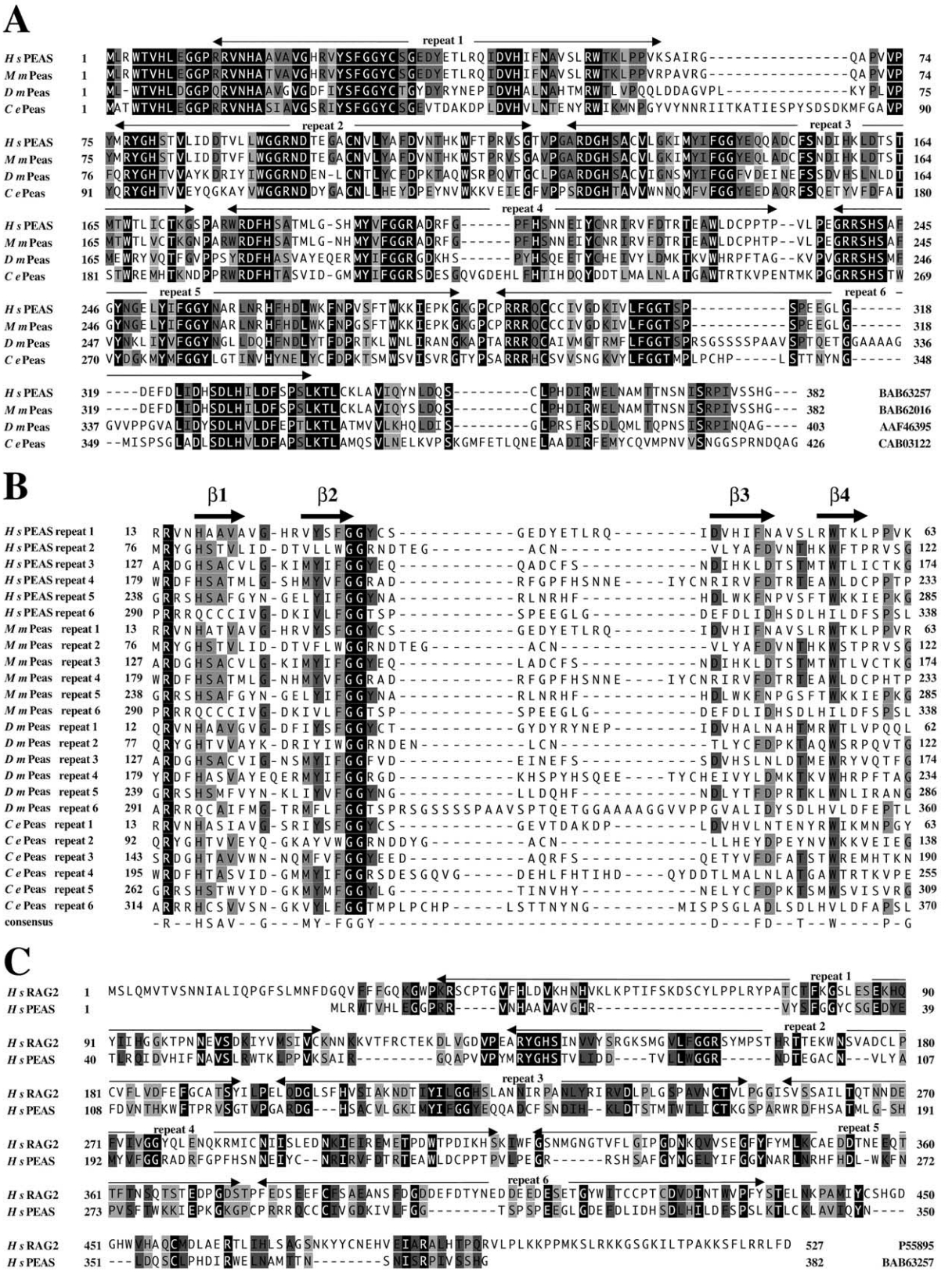

Fig. 1. A: Multiple alignment of Peas sequence from human (*Hs*PEAS, BAB63257), mouse (*Mm*Peas, BAB62016), *D. melanogaster* (*Dm*Peas, CG12081 gene product, AAF46395), and *C. elegans* (*Ce*Peas, hypothetical protein F53E4.1, CAB03122). The six tandem-repeated kelch motifs are indicated by arrows. B: Multiple alignment of kelch motifs in Peas. The predicted regions to form β-strands are indicated by arrows. C: Alignment of human RAG2 and human PEAS. The six tandem-repeated kelch motifs are indicated by arrows.

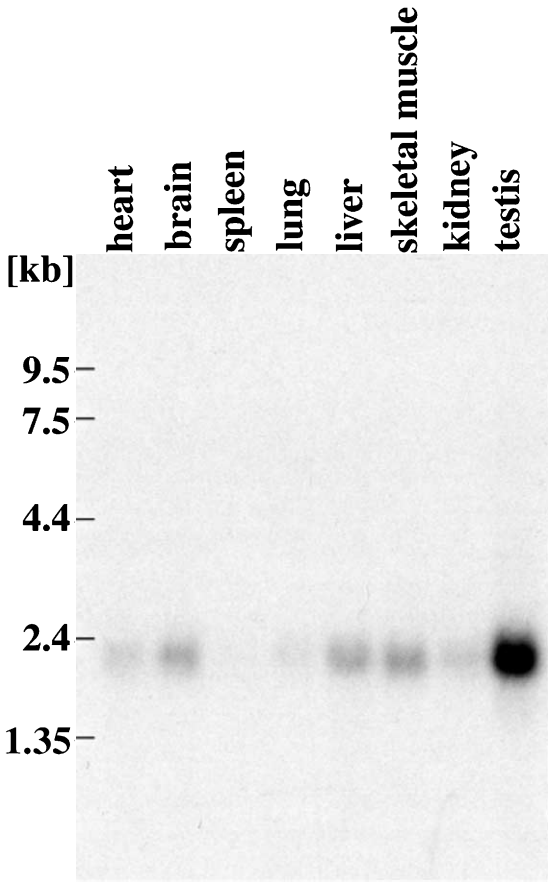

Fig. 2. Comparison of expression of *Peas* in various tissues. Northern blot (2 μg of poly(A)$^+$ RNA per lane) analysis with random-primed ^{32}P-labeled DNA fragments of the full-length coding region of m*Peas* cDNA.

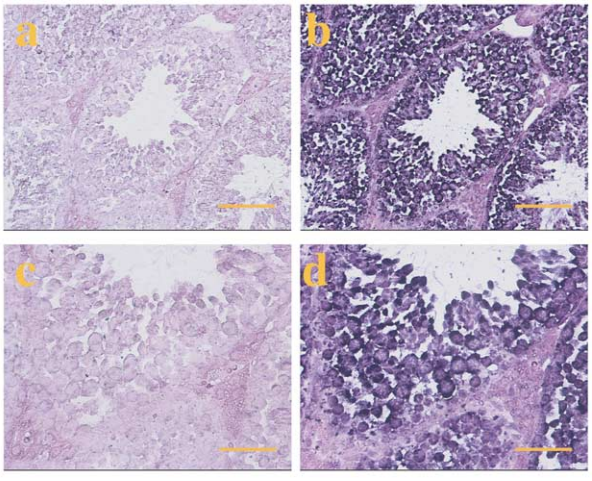

Fig. 3. Cell type- and stage-specific expression of *Peas* transcripts. In situ hybridization of mouse testis with *Peas* sense probe (a, c) and antisense probe (b, d). The bars indicate 100 (a, b) and 50 μm (c, d).

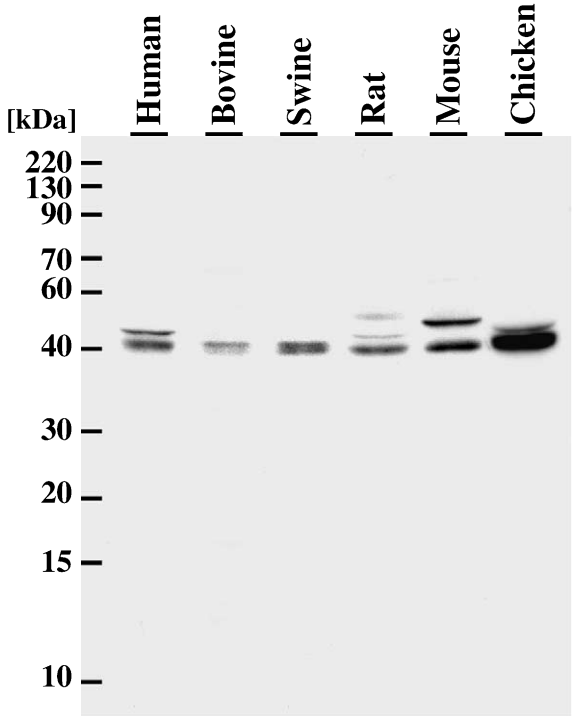

Fig. 4. Western blot analysis. Western blot analysis was performed for endogenous testes protein from various species with a rabbit anti-Peas polyclonal antibody. Peas protein was detected as a major 43-kDa band and a few minor, weakly reacting high-molecular bands.

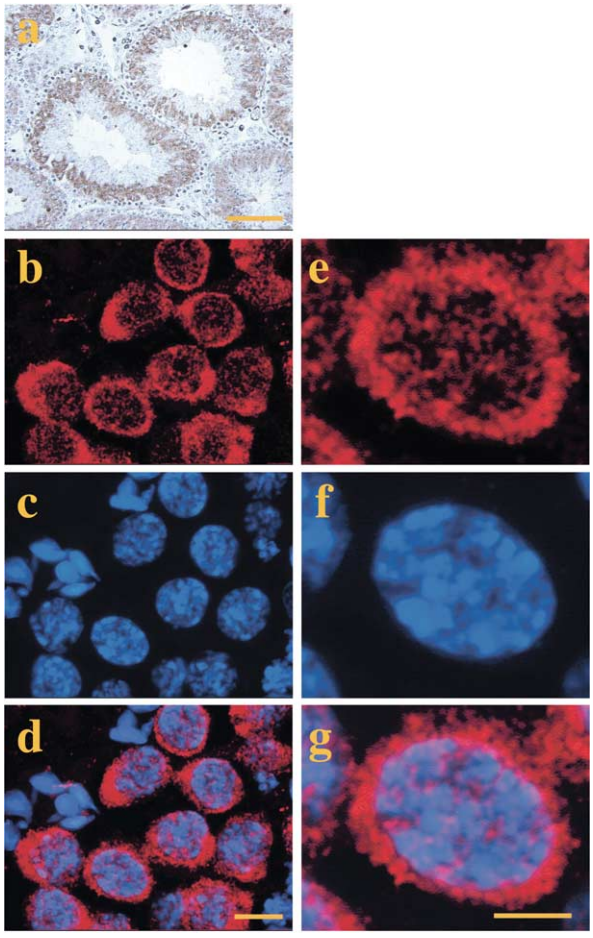

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Fig. 5. Immunostaining of Peas in the mouse testis. For indirect immunostaining, paraffin sections (thickness, 3 μm) of the mouse (BALB/c; age, 8 weeks) testis were used. Immunohistochemical detection of Peas (a). The bar indicates 100 μm. Immunofluorescence detection of Peas (b, e), DAPI staining (c, f), merge of b and c (d), merge of e and f (g). The bars indicate 10 (d) and 5 μm (g).

tion visualization of differential fluorescent staining showed that Peas is localized in both the cytoplasm and nucleus of the spermatocytes (Fig. 5b–d). Peas was distributed granularly in the cytoplasm while in the nucleus, it was localized on the meiotic chromatin (Fig. 5e–g).

4. Discussion

Many genes take part in the meiotic process. *SPO11* encoding topoisomerase II-like protein, *MER2*, *RAD50*, *MEI4*, *MRE2*, *MRE11*, *XRS2*, *REC102*, *REC104* and *REC114* have been characterized in budding yeasts [12]. *RAD50*, *MRE11*, *XRS2* and *SAE2/COM1* are involved in the digestion of the cut-terminals to form recessed ends [13]. The single-stranded DNA was used to search for homologous sequence and for pairing each other. After heteroduplex formation the gap was filled. The recombination was completed by resolving Holliday structures [14].

In mammals, many genes and/or proteins involved in homologous recombination have been identified. *Rad51* is homologous to yeast *RAD51* [15,16]. Dmc1 structurally resembles Rad51 and is specifically expressed at meiosis [17,18]. Rad51B [19], Rad51C [20] and Rad51D [21] show partial homology to Rad51. *Xrcc2* [22] and *Xrcc3* [23] could compensate the mutated state of Chinese hamster cells that were chromosomally unstable and sensitive to DNA cross-linkers. Xrcc2 and Xrcc3 are also slightly homologous to Rad51. There are mammalian homologs to RAD52 [24] and RAD54 [25]. These findings suggest that many proteins commonly take part in homologous recombination processes throughout eukaryotes. However, genes involved in meiotic DSBs-introduction in higher eukaryotes have not yet been identified.

RAG2 is an essential nuclear co-component of RAG1 in the V(D)J recombination of TCR and Ig genes [7–9]. RAG1 inefficiently induces V(D)J recombinase activity when transfected into fibroblasts, but co-transfection with RAG2 resulted in, at least, 1000-fold increase in the frequency of recombination [26]. Amino acid residues 1–355 of RAG2 are sufficient for RAG1 binding and in vitro recombination and thus form a kelch-repeat β-propeller structure [27]. In the present study, hPEAS/mPeas are homologous to RAG2 including six repeated kelch motifs and excluding their β-strand–β-strand regions (Fig. 1C). Multiple mutations within the β-strand–β-strand regions of kelch repeats of RAG2 had either mild or no effects on RAG1–RAG2 interaction and hence on the ability to mediate recombination [28].

In the present study, we report primary structure, expression, and localization of Peas. The findings that Peas is specifically expressed in testis, localized in pachytene spermatocyte cytoplasm and chromatin, and homologous to RAG2 raise a possibility that Peas might be involved in the meiotic recombination in testis. Further studies using knockout and/or transgenic approach are warranted to clarify the exact function of Peas in testis.

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