KIN17 encodes an RNA-binding protein and is expressed during mouse spermatogenesis

Ghislaine Pinon-Lataillade[1,*], Christel Masson[1,‡], Jacqueline Bernardino-Sgherri[2], Véronique Henriot[1,§], Philippe Mauffrey[1], Yveline Frobert[3], Silvia Araneda[4] and Jaime F. Angulo[1,*]

[1]Laboratoire de Génétique de la Radiosensibilité, CEA, Direction des Sciences du Vivant (DSV), Département de Radiobiologie et de Radiopathologie (DRR), B.P. 6, 92265 Fontenay aux Roses CEDEX, France
[2]Laboratoire de la Radiosensibilité des Cellules Germinales, CEA, DSV, DRR, U 566 CEA-INSERM-Université ParisVII, B.P. 6, 92265 Fontenay aux Roses CEDEX, France
[3]Service de Pharmacologie et d'Immunologie, Département de Recherche Médicale, DSV, CEA-Saclay, 91191 Gif-sur Yvette, France
[4]Physiologie Integrative, Cellulaire et Moléculaire, UMR 5123 CNRS/UCB Lyon 1, 43 Bd. 11 novembre 1918, 69622 Villeurbanne CEDEX, France
*Authors for correspondence (e-mail: latailla@dsvidf.cea.fr; angulo@dsvidf.cea.fr)
‡Present address: Département de Médecine Nucléaire et de Radiobiologie, Université de Sherbrooke, (QC), J1H 5N4, Canada
§Present address: Laboratoire populations, génétique et évolution. CNRS, 91190 Gif-sur-Yvette, France

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Summary

Genotoxic agents deform DNA structure thus eliciting a complex genetic response allowing recovery and cell survival. The *Kin17* gene is up-regulated during this response. This gene encodes a conserved nuclear protein that shares a DNA-binding domain with the bacterial RecA protein. The KIN17 protein binds DNA and displays enhanced expression levels in proliferating cultured cells, suggesting a role in nuclear metabolism. We investigated this by studying the expression profile of KIN17 protein during mouse spermatogenesis. As expected, the expression level of *Kin17* is higher in proliferating than in differentiated cells. KIN17 is selectively extracted from this tissue by detergents and a fraction was tightly associated with the nuclear matrix. Germinal cells ubiquitously express *Kin17* and the protein is located mainly in the nucleus except in elongated spermatids where cytoplasmic staining is also observed. Sertoli and germ cells that are no longer mitotically active express KIN17, suggesting a general role in all testicular cell types. In adult testis a significant proportion of KIN17 co-purifies with polyadenylated RNA. KIN17 directly binds RNA, preferentially poly(G) and poly(U) homopolymers. These results together with the identification of KIN17 as a component of the human spliceosome indicate that this protein may participate in RNA processing.

Key words: KIN17 protein, Sertoli cell, Spermatogenesis, DNA, RNA binding, Nuclear matrix

Introduction

Genotoxic agents deform DNA structure thereby stimulating a complex response in mammalian cells. When there are a high number of DNA lesions, many cannot be processed and cells undergo apoptosis. When there are few lesions, several metabolic pathways are activated to remove these lesions and survival is more likely. Several genes playing key roles in this response have already been identified and characterised (Fornace et al., 1999; Friedberg et al., 1995). We identified a mammalian gene called *Kin17* because it codes for a protein immunologically related to RecA protein. This gene is located on band A of chromosome 2 in mouse (Angulo et al., 1991) and on human chromosome 10 at p15-14. In adult mouse tissues this gene is ubiquitously expressed at a low level (Tissier et al., 1996). KIN17 is a nuclear protein of 45 kDa conserved from yeast to human (Kannouche et al., 2000) which is up-regulated after UV and γ irradiation (Biard et al., 2002; Blattner et al., 2000; Kannouche et al., 1998; Masson et al., 2001; Masson et al., 2003). The structures of *E. coli* RecA and KIN17 proteins are different, but nevertheless share an epitope of 40 residues belonging to a DNA-binding domain located in the C-terminal end of RecA protein and in the core of KIN17 protein (Kurumizaka et al., 1996; Mazin et al., 1994a). KIN17 protein possesses a modular structure comprising four motifs: a zinc finger (amino acids 27-50), a bipartite nuclear localization signal (239-256), the core domain homologous to RecA protein (161-201) and a KOW motif (335-373) (Ponting, 2002; Tissier et al., 1995). The mouse KIN17 protein produced in *E. coli* binds to DNA in vitro (Angulo et al., 1991; Mazin et al., 1994b) particularly to curved DNA and can substitute for H-NS, a bacterial transcriptional regulator. The binding of KIN17 near promoters induces a topological change in DNA that leads to gene activation (Timchenko et al., 1996).

In mammals, the KIN17 protein forms intranuclear foci in proliferating cells and is redistributed in the nucleoplasm after UV or γ irradiation (Biard et al., 2002; Kannouche et al., 2000). A fraction of KIN17 is directly associated with chromosomal DNA (Biard et al., 2002). The ectopic overexpression of human KIN17 protein indicates a colocalisation with SV40 large T antigen in intranuclear foci (Kannouche and Angulo, 1999). The overproduction of KIN17 protein in vivo and the introduction of increased amounts of human KIN17 protein in an in vitro assay reduced SV40 T-dependent DNA replication, leading to the conclusion that it might be involved in DNA replication (Miccoli et al., 2002). However, the expression of KIN17 protein in mouse and rat nervous cells unable to

undergo DNA replication (Araneda et al., 1997; Araneda et al., 2001) suggests it participates in other aspects of nuclear metabolism. We sought to analyse this point further by studying the expression profile of KIN17 protein during mouse spermatogenesis. Germ cell development is intimately supported by Sertoli cells, the somatic component of the seminiferous epithelium. In postnatal mice, Sertoli cells are mitotically active concomitant with development of the initial wave of spermatogenesis. This proliferation almost stops at 12 days post partum (p.p.) (Kluin et al., 1984). Postmitotic Sertoli cells are not replaced during adult life. In adult testis, spermatogenesis consists of three main phases: mitotic proliferation of spermatogonia, meiosis and spermiogenesis. Cells at different stages within these phases can be identified by histochemical methods (Oakberg, 1956). Therefore we followed up the occurrence of *Kin17* RNA and immunoreactive protein in developmental and adult testis. We show that in the mouse testis, KIN17 protein is present in a fraction extractable by detergent and also in a fraction tightly associated with a nuclear structure resistant to detergent. Immunohistochemical detection of KIN17 locates it in the nucleus of both Sertoli and germ cells but it was detected in the cytoplasm of the elongating spermatids. Biochemical analyses indicate that fractions of KIN17 protein are distributed between the nucleoplasm, chromatin and the nuclear matrix. This protein directly binds to RNA. Our results suggest that KIN17 protein may participate in the complex stepwise process of regulation of eukaryotic gene expression that begins with transcription initiation, elongation and termination.

Materials and Methods

Tissue collection

Whole testes were collected from 5-, 12-, 17-, 22-, 28-, 48- and 53-day-old NMRI mice, immediately detunicated, stored at –70°C and then processed for RNA or protein extraction.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA purification and semi-quantitative RT-PCR was performed as described (Masson et al., 2001). *β Actin* was used as positive control. Primers for mouse *Kin17* and *β Actin* were 5′-AGCCCCAAGGC-CATCGCCAA-3′ (forward) and 5′-ATACCTTCAACTCTGCGTC-CTT-3′ (reverse), 5′-TGACCCAGATCATGTTTGAGA-3′ (forward) and 5′-ACGCAGCTCAGTAACAGTC-3′ (reverse), respectively. Expected products were 1125 and 794 base pairs (bp) long, respectively. For negative controls, reverse transcriptase or cDNA was omitted.

Protein gel electrophoresis and western blotting

Total protein lysates were prepared by homogenising testes (0.1 g /ml of buffer) on ice in a Potter homogeniser with RIPA buffer (PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate) including 1 mM PMSF and 1% protease inhibitor cocktail (Roche). The cells were lysed for 20 minutes on ice and centrifuged (20,000 ***g*** for 30 minutes at 4°C). The supernatant fraction contains cytoplasmic and nuclear extractable proteins and the pellet fraction contains proteins unextractable with detergent associated with nuclear and cytoplasmic structures. These fractions were diluted in 2×Laemmli buffer (Laemmli, 1970) and protein levels were measured using DCprotein assay kit (BioRad). 20 μg of protein of each fraction were subjected to 10% SDS-PAGE for western blot analysis as described (Masson et al., 2001). Primary antibodies against PCNA (mAb PC10, Roche) diluted 1:2000 in TBT, and KIN17 protein (mAb k58, diluted 1:200 in TBT) were incubated with the blots overnight at 4°C. The antibodies used to detect KIN17 protein were the supernatants of the hybridomas k58 (IgG2b) or purified IgG from ascites fluid (Igk58, 1:20,000). These monoclonal antibodies were directed against human KIN17 protein (Miccoli et al., 2002). Since the human and mouse KIN17 proteins are 92.4% identical (Kannouche et al., 2000), we were able to use these monoclonal antibodies to study the distribution of mouse KIN17 protein in spermatogenesis. Their specificity was tested by immunoblotting mouse testes and HeLa cell extracts. These mAbs produced similar immunocytochemical staining patterns for nuclear detection but a better cytoplasmic detection was observed with purified Igk58. Protein level analysis was performed as described (Masson et al., 2001). In order to verify further the quality of protein detection the membranes were reprobed with a mAb anti-β ACTIN diluted 1:200 in TBT (Santa Cruz Biotechnology).

Immunohistochemical staining of paraffin-embedded sections

Antibody detection was performed by mounting 5-μm-thick paraffin sections of testis treated with Carnoy's fixative on poly-L-lysine-coated slides. De-waxed and hydrated sections were boiled in a microwave oven twice for 6 minutes in 0.01 M sodium citrate pH 6.0 for antigen retrieval (Shi et al., 1991). After 20 minutes' incubation at room temperature, slides were washed in PBS and endogenous peroxidase was blocked by incubation with 3% H_2O_2 in PBS for 10 minutes. Non-specific binding sites of the antibodies were blocked by incubating the slides in PBS included 5% normal horse serum (NHS) for 30 minutes. The primary antibody was purified mAb Igk58 diluted to 1:500 or PC10, 1:1000 in PBS including 3% NHS and was applied to the slides and incubated overnight at 4°C.

Protein detection was performed as described (Masson et al., 2001). The slides were then counterstained with Mayer's haematoxylin. Staining specificity was tested. Serial dilution of the antibody resulted in a decreased signal as expected. Preincubation of the primary antibody with a 15-fold excess of recombinant KIN17 protein or replacing mAb Igk58 with normal mouse IgG resulted in no detectable staining.

BrdU incorporation and confocal microscopy

Adult mice were injected i.p. with 5-bromodeoxyuridine (BrdU, Sigma) at a dose of 50 mg/kg of body weight, killed 3 hours later and their testes were fixed in Carnoy's fluid as above. Double immunostaining was performed for detection of incorporated BrdU and KIN17 protein. mAb Igk58 (1:500 dilution in PBS with 3% NHS) was used to detect KIN17 protein followed by Cy3™-conjugated affini-Pure goat anti-mouse IgG secondary antibody (1:500 dilution in PBS including 3% NHS). After washing in PBS, slides were incubated for 60 minutes at 37°C with FITC-conjugated anti-BrdU monoclonal antibody (Roche; 1:2 dilution in PBS with 3% NHS). Slides were then counterstained with DAPI and mounted. Control negative sections were treated as described above. For cellular colocalisation studies, the preparations were observed with a Leica confocal system TCS-SP2 microscope (Leica, Heidelberg, Germany). Source images were excited at 488 nm and captured with filters set at 498-520 nm for FITC and 594-692 nm for Cy3™. Images were analysed and merged using Adobe Photoshop and JASC Paint Shop Pro (JASC software Inc., USA).

Chromosome preparations, cell suspensions and spreading

Freshly removed testes were dissociated as described (Bernardino et al., 2000). Large tubular fragments were removed by sedimentation. The turbid supernatant was collected, pre-fixed in methanol-acetic acid (3:1) fixative and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in the same fixative and kept at 4°C until

spreading. The suspension was dropped on pre-chilled glass slides and air-dried. Slides were stored at –20°C until further use. To detect KIN17 protein the immunofluorescence procedure was performed on these cells as described for paraffin-embedded sections, with some modifications. After postfixation in Carnoy's fluid for 15 minutes, cell preparations were boiled as described above and permeabilised with 0.3% Triton in PBS for 15 minutes. Then cell preparations were incubated with PBS including 5% NHS before incubation with anti-KIN17 mAb k58 (1:20 dilution in PBS including 3% NHS). Bound antibodies were visualised using Cy3™-conjugated secondary antibody as described above. Cells were counterstained with DAPI and the slides were mounted in Vectashield. Microscopy was performed using a ×100 objective on an Olympus fluorescence microscope equipped with a CCD camera and standard filters for DAPI and Cy3™. Pictures were recorded with IPLabSpectrum software.

DNase I and RNase A treatment

Dewaxed and hydrated testes sections of adult mice were incubated in either RNase-free DNaseI (400 U/ml, Roche), in RNase A (200 μg/ml, Roche) or in a combination of the two as described (Larsson et al., 1995). Incubations were performed in PBS with 3 mM $MgCl_2$, 0.4% Tween 20 for 2 hours at room temperature for DNase I and in PBS for 1 hour at 37°C for RNase A. The efficiency of the respective treatments was verified by: loss of DAPI staining after DNase I treatment and the unaffected detection of the splicing factor SC35 (mouse mAb, Sigma) (Spector, 1993); a preserved DAPI stain after RNase A treatment and the unaffected detection of Rad51 (a protein involved in homologous recombination and DNA repair) (Haaf et al., 1995) and the loss of propidium iodide (PI) staining after DNase I plus RNase A treatment.

In vivo DNA and RNA cross-linking assays

The interaction of KIN17 protein with polynucleotides was assessed as described (Kuroda et al., 2000; Zinszner et al., 1997). Briefly, cells were isolated by treating testes of adult mice with 1 mg/ml type IA collagenase (Sigma) for 10 minutes at 37°C. The recovered cells were suspended in ice-cold PBS and irradiated on ice with 900 mJ/cm^2 of UVC light using a spectrolinker™ cross-linking oven (Stratagene). The cells were then lysed in ice-cold RIPA buffer as above. The lysate was treated with DNase I (400 U/ml) for 50 minutes at 32°C, or with RNase A (10 μg/300 μl) for 30 minutes at 20°C and centrifuged at 100,000 ***g*** for 30 minutes at 4°C using the TLA-100-3 rotor in a tabletop ultracentrifuge (Optima™ TL100 Beckman). The supernatant was pre-cleared by incubation with 5 μl rabbit pre-immune serum and protein A Sepharose CL-4B (Amersham).

Rabbit polyclonal anti-KIN17 serum (Miccoli et al., 2002) or rabbit pre-immune serum (PIS) for control was added to the resulting supernatant and incubated on ice for at least 2 hours. Then protein A Sepharose was added to the reaction medium. Immunoprecipitation reactions were incubated at 4°C overnight. The immune complexes were washed three times in RIPA, once in RIPA containing 0.5 M NaCl, and 3 times in kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT containing 1% protease inhibitor cocktail). The beads containing immune complexes were resuspended in 30 μl kinase buffer and the attached polynucleotides were end-labelled with T4 polynucleotide kinase for 30 minutes at 37°C (5 units, USB) using 90 μCi [γ-^{32}P]ATP (3000 Ci/mmole; Amersham). The beads were washed three times with RIPA buffer and resuspended in 20 μl Laemmli buffer. The labelled species were resolved on two 10% SDS-PAGE gels. One gel was dried and autoradiographed. The proteins in the other gel were transferred to a nitrocellulose filter for western blotting and autoradiography. Anti-KIN17 mAb k58 (1:200 dilution in TBT) was used for immunoblotting detection of [^{32}P]KIN17 protein.

The labelled RNA or DNA molecules cross-linked to KIN17 were recovered as described (Zinszner et al., 1997) and resolved on a 10% acrylamide gel containing 8 M urea.

Oligo(dT) chromatography

Adult mouse testes were disrupted with collagenase. Cells were irradiated with UVC at 10^{-1} joules/cm^2, dounced and further treated as described (Lalli et al., 2000). Poly(A^+) RNA was purified using the rapid purification kit of polyadenylated RNA from eukaryotic total RNA (Amersham). Flowthrough (FT), high-salt wash (HS) (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5 M NaCl), low-salt wash (LS: 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 M NaCl) and eluate fractions (10 mM Tris-HCl pH 7.4, 1 mM EDTA) were collected from the columns. Eluate fractions were concentrated to 50 μl, FT to 1 ml and HS, LS to 100 μl using Vivaspin concentrator (Vivascience). 15-25 μl of the supernatant, 25 μl of the FT, HS, LS and the eluate were subjected to 10% SDS-PAGE and analysed by western blot using mAb Igk58, anti-RAD51 (Haaf et al., 1995), anti-HUR (Gallouzi et al., 2000) and anti DAX-1 2F4 (Tamai et al., 1996).

RNA homopolymer binding assay

The fixation of different proteins was performed essentially as described (Lalli et al., 2000) using ^{35}S-labelled $_{MMU}$KIN17 protein and mutants $_{MMU}$KIN17$_{335}$ΔKOW and $_{MMU}$KIN17$_{160}$ proteins carrying respectively the first 335 and 160 residues of the wild-type $_{MMU}$KIN17 protein. These labelled proteins were synthesised using the TNT rabbit reticulocyte lysate (Promega) and *Kin17* cDNA fragments carrying the T7 RNA polymerase promoter upstream of the translation initiation ATG. These fragments were amplified by PCR using the following pairs of primers: (a) $_{MMU}$*Kin17*DIR (5′-GGATCCTAATACGACACTATAG-GGAGACCACCATGATGATGATGATGGGCAAGTCGGATTTTCT-GAGCCCCAAG-3′) and REV1 (5′-CAATATGAAGACATATC-TAAACTTGCTATGGCCATGTGA-3′) for $_{MMU}$KIN17 protein; (b) $_{MMU}$*Kin17*DIR and REV2 (5′-CTGGACCAGACTCATTTAG-AGACAGTCATGGCCATGTGA-3′) for $_{MMU}$KIN17$_{335}$ ΔKOW; (c) $_{MMU}$*Kin17*DIR and REV3 (5′-TTAGAAAAAAAGAAGAAGCAAG-ATATGGCCATGTGA-3′) for $_{MMU}$KIN17$_{160}$ protein.

The matrix used for PCR amplification was the plasmid pLex$_{MMU}$*Kin17* carrying the whole *Kin17* cDNA. RNA homopolymer-conjugated agarose beads were obtained from Amersham [poly(A) and poly(U)] and from Sigma [poly(C) and poly(G)]. [^{35}S]-labelled $_{MMU}$KIN17 proteins or $_{HSA}$KIN17 protein produced in a baculovirus system were eluted from beads under the indicated conditions and loaded on a 10% SDS-PAGE. After electrophoretic separation, the proteins were transferred onto a nitrocellulose filter for autoradiography and western blotting using the mAb Igk58 antibody. Radioactivity was quantified using Storm phosphoimager (Amersham).

Northwestern analysis

This assay was performed as described (Bertrandy et al., 1999) using $_{HSA}$KIN17 protein produced in baculovirus (Miccoli et al., 2002), $_{MMU}$KIN17 protein produced in *E. coli* (Mazin et al., 1994a), bovine serum albumin (Sigma) and BioLabs Prestained molecular weight marker, broad range, P7708S (New England). Each protein blot was incubated with 5×10^5 cpm of either [^{32}P]-labelled poly(G) or RNA for 2 hours at room temperature and washed twice for 15 minutes as described (Siomi et al., 1993). [^{32}P]-labelled probes: poly(G) (Sigma) was end-labelled using T4 polynucleotide kinase as described (Siomi et al., 1993). RNA probe was synthesised from a $_{MMU}$*Kin17* cDNA obtained using primers $_{MMU}$*Kin17*DIR and REV1 described above. An RNA fragment of 1200 bp of *Kin17 ORF* was synthesised for 14 hours at 37°C using the Message Amp™ aRNA kit (Ambion) supplemented with [^{32}P]UTP (Amersham). The 1200 bp RNA displayed a specific radioactivity of 1.5×10^6 cpm/μg.

Chromatin and nuclear matrix preparation

Testes of adult mice were homogenised in cytoskeletal buffer (CSK) (10 mM pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.5% Triton-X-100, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 25 mM NaF and 1% protease inhibitor cocktail). Following incubation at 4°C for 3 minutes, samples were centrifuged at 5000 ***g*** for 3 minutes. Chromatin was released by digestion with 1000 units/ml RNase-free DNase I (Roche) in CSK buffer for 15 minutes at 37°C. Ammonium sulphate in CSK buffer was added to a final concentration of 0.25 M, samples were incubated at 4°C for 5 minutes and centrifuged. The pellet was extracted with 2 M NaCl in CSK buffer for 5 minutes at 4°C and then centrifuged. The resulting pellet containing the nuclear matrix fraction was solubilised in urea buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.) as described (Reyes et al., 1997; Wu and Means, 2000).

Results

Expression of mouse *Kin17* gene during testicular development

We used the male germinal tissue to check whether *Kin17* is upregulated in vivo as described in actively proliferating cultured mouse cells (Kannouche et al., 1998). The temporal development of spermatogenesis in prepubertal mice is well established, so it is possible to correlate cellular patterns of gene expression with specific stages of male germ cells (Nebel et al., 1961). The expression of *Kin17* RNA was first determined in testes at days 5, 12, 17, 22, 28, 48, 53 p.p. by RT-PCR. A DNA fragment of the expected size (1125 bp for *Kin17* and 794 bp for *β Actin*) was obtained with every RNA sample (Fig. 1A). The ratio *Kin17/β Actin* mRNA in whole testis almost doubled from day 5 to days 17-22 and then decreased by day 28 p.p. to reach a stable level in adult testis (Fig. 1A). No signal was observed when reverse transcriptase or cDNA was omitted (not shown).

In parallel we detected KIN17 protein by western blotting in detergent-extractable and non-extractable fractions from testes at the same stage of development. KIN17 was detected in both fractions (Fig. 1B,C). The highest levels of KIN17 were observed from day 5 to 22 p.p. in both fractions. Afterwards the levels decreased which correlates well with *Kin17* RNA expression (Fig. 1A). PCNA expression, detected as control, showed a similar pattern in agreement with its mRNA expression in rat testis (Aguilar-Mahecha et al., 2001). The highest PCNA level was found in the detergent-extractable fraction in agreement with reported data (Balajee et al., 1999) (Fig. 1B,C).

Localization of KIN17 and PCNA in seminiferous tubules of developmental and adult mouse testes

Using the same antibodies, we determined the expression of KIN17 and PCNA proteins in different germ cell types by immunohistochemical (IHC) analysis of testes during mouse development. In immature testis these proteins were found only in the nucleus. In newborn mice at day 1 p.p. the seminiferous tubules contained only Sertoli cells and gonocytes. All Sertoli cell nuclei were clearly labelled for KIN17 (Fig. 2A) and PCNA (not shown) but no staining could be observed in gonocyte nuclei. At day 5 p.p., in addition to Sertoli cells, tubules contained spermatogonia which were also stained for KIN17 (Fig. 2B) and PCNA (not shown). Sertoli cell nuclei

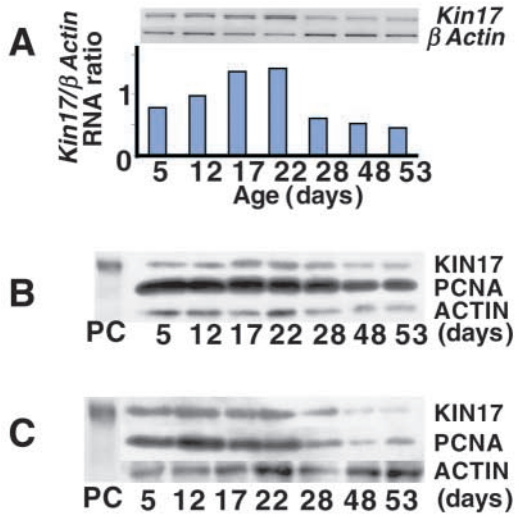

Fig. 1. Expression of *Kin17* mRNA and protein in developmental and adult mouse testes. (A) Total RNA from testes of mice at days 5, 12, 17, 22, 28, 48 and 53 p.p. were subjected to RT-PCR. PCR products were analysed in a 2% agarose gel. The results shown are from two independent experiments done in triplicate. The numerical interpretation of a representative gel is shown. *β Actin* RNA was determined as reference. The intensity of the bands was quantified using NIH image software. The ratios *Kin17*:*β Actin* RNA were calculated and plotted. (B,C) Western analysis of KIN17, PCNA and β ACTIN proteins from extracts of mouse testes. (B) 20 μg of the detergent-extractable protein fraction and (C) 20 μg of the protein remaining in the pellet were loaded per lane. The positive control (PC) includes purified mouse KIN17 protein produced in bacteria.

were stained for KIN17 but not for PCNA. By day 12 p.p. leptotene and zygotene spermatocytes appeared and were labelled for KIN17 and PCNA (not shown). In pachytene spermatocytes seen at day 17 p.p., KIN17 staining was detected throughout development of these spermatocyte cells (Fig. 2E) whereas PCNA was only detected in the earliest stages of the spermatocyte cells (Fig. 2D). By day 22 p.p. young round spermatids (Rs) appeared which were slightly labelled for KIN17 as confirmed by observation of 28-day-old testes. The labelling was associated with chromatin (Fig. 2F).

In adult testis, as in immature testis, KIN17 staining was clearly observed in the nuclei of all spermatogonia and spermatocyte cells mainly associated with chromatin (Fig. 2G-K). This staining remained during meiotic divisions and was always present but at low level in early Rs nuclei (Fig. 2G). After stage IV (Oakberg, 1956) the staining disappeared in spermatid nuclei (Fig. 2H-J). This failure to detect KIN17 protein may be due to a transient decrease in its expression. Biochemical analysis showed that from day 28 the *Kin17* RNA and protein levels decrease (Fig. 1). At this time round spermatids were present and the elongating spermatids started to appear in the seminiferous epithelium. This may account for a decrease in the average concentration of KIN17 protein. From stage XII the cytoplasm of elongating spermatids (Es) was slightly stained (Fig. 2K) and staining progressively increased until complete spermiogenesis. This cytoplasmic staining was rather homogeneous with a higher intensity in a spherical component that remains to be identified. (Fig. 2H; see also Fig. 5D,G,J,M). In residual bodies KIN17 staining was

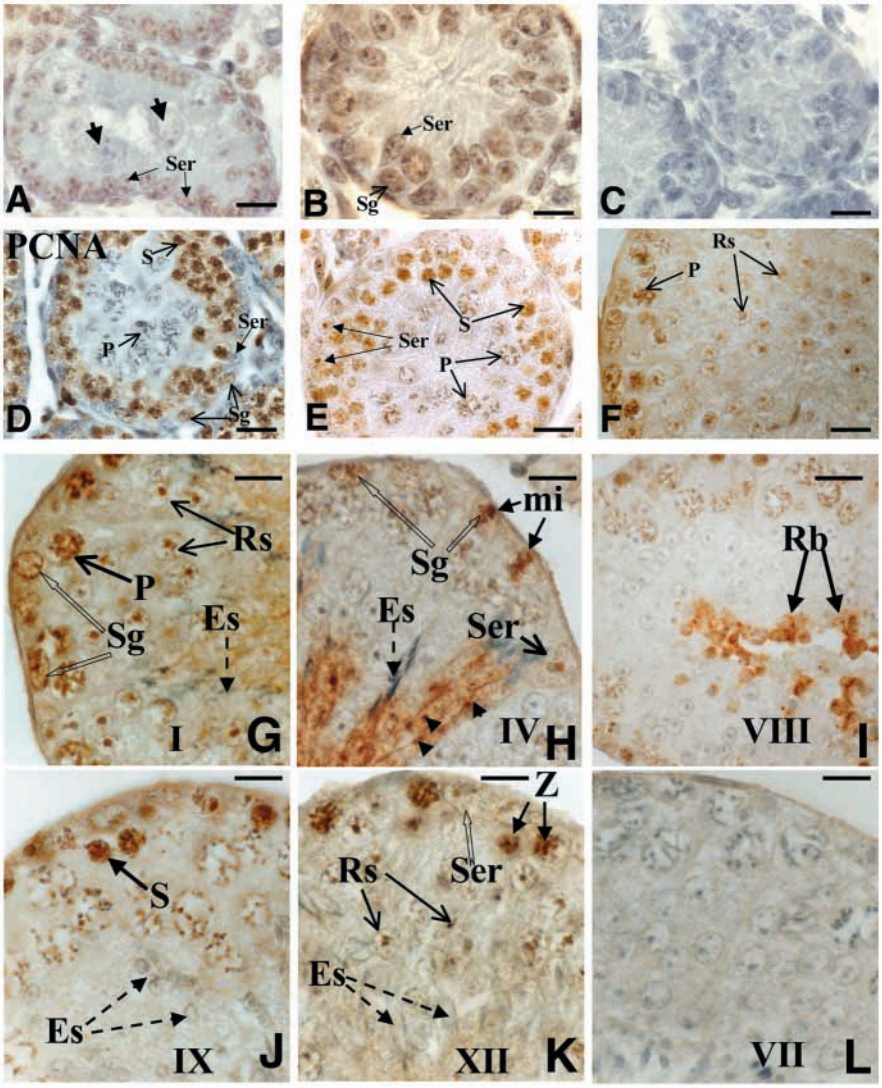

Fig. 2. Immunohistochemical detection of KIN17 (panels A,B,E,F) and PCNA (panel D) proteins during development of mouse testis. Nuclear staining of Sertoli cells (Ser) for KIN17 was observed in testis sections of 1-day-old (panel A), 5-day-old (panel B), 17-day-old (panel E) and 28-day-old (panel F) mice. Nuclei of spermatogonia (Sg), leptotene (S), pachytene (P) and round spermatid (Rs) cells also labelled for KIN17 (panels B,E,F). No staining was detected in gonocytes (unlabelled bold arrows, panel A). Note the nuclear staining of spermatogonia and leptotene spermatocytes for PCNA in 17-day-old testis whereas pachytene and Sertoli cells are unstained (panel D). A control section of a 5-day-old mouse testis is also shown (panel C). Bars, panels A, B and C: 12 μm, panel D: 20 μm, panel E: 18 μm, panel F: 15 μm. In adult mouse testis, staining for KIN17 protein was observed in the nuclei of Sertoli cells (Ser), spermatogonia (Sg) and on chromosomes of cells undergoing mitosis (mi); spermatocyte cells: leptotene (S) zygotene (Z), pachytene (P), early round spermatid (Rs), in the cytoplasm of elongating and late spermatids (Es) and in residual bodies (Rb) (panels G-K). Arrowheads in panel H show nonidentified Es cytoplasmic spherical components. A control testicular section where anti-KIN17 antibody was replaced by normal mouse IgG remained unstained (panel L). Stages of the seminiferous epithelium are indicated with Roman numerals. Bars: panels G, H and J, 12 μm; panel I, 19 μm; panel K, 10 μm and panel L, 11 μm.

clearly detected (Fig. 2I). In Sertoli cells, KIN17 staining was concentrated in the two perinucleolar satellites in which the centromeric region of the chromosomes and the telomeric sequences of the short arms are clustered (Guttenbach et al., 1996) (Fig. 2H,K).

The staining pattern of PCNA in adult testis was very similar to that observed in immature testis; it disappeared in pachytene spermatocytes at stage VIII-IX when they become associated with mid-leptotene. Haploid cells lacked detectable PCNA reactivity as well as Sertoli cells (not shown) in agreement with published observations (Kamel et al., 1997). Negative control sections where primary antibody was replaced by mouse IgG remained unstained (Fig. 2C; Fig. 2L).

Co-localisation of KIN17 and BrdU in the nucleus of S-phase preleptotene spermatocytes and spermatogonia cells

Considering the possible implication of KIN17 protein in DNA replication, we determined BrdU incorporation and KIN17 protein expression patterns in the testis using confocal laser microscopy. The individual expression patterns of BrdU incorporation (Fig. 3A,D) and KIN17 (Fig. 3B,E) appeared to be the same as those found using immunohistochemistry. BrdU was incorporated in spermatogonia and preleptotene spermatocytes in agreement with Monesi's observations on DNA synthesis using tritiated thymidine (Monesi, 1962). In all the cells that incorporated BrdU, namely preleptotene spermatocytes (Fig. 3A) and spermatogonia (Fig. 3D), the green fluorescence colocalised with the red KIN17 fluorescence as shown in Fig. 3C,F. We concluded that in S-phase germ cells, a fraction of KIN17 protein resides near the site of BrdU incorporation as has been previously reported in cultured cells (Biard et al., 2003).

Expression of KIN17 in isolated testicular cells

To examine further the association of KIN17 protein with chromosomes during mitosis, we performed an immunocytochemical (ICC) analysis of isolated testicular cells of seminiferous tubule from testes of 2- or 8-day-old mice or adult mice. In cells from immature testes, Sertoli cells (Fig. 4A), gonocytes which enter mitosis at day 2 p.p. (Fig. 4B), and spermatogonia mitoses (Fig. 4C) were observed. They showed

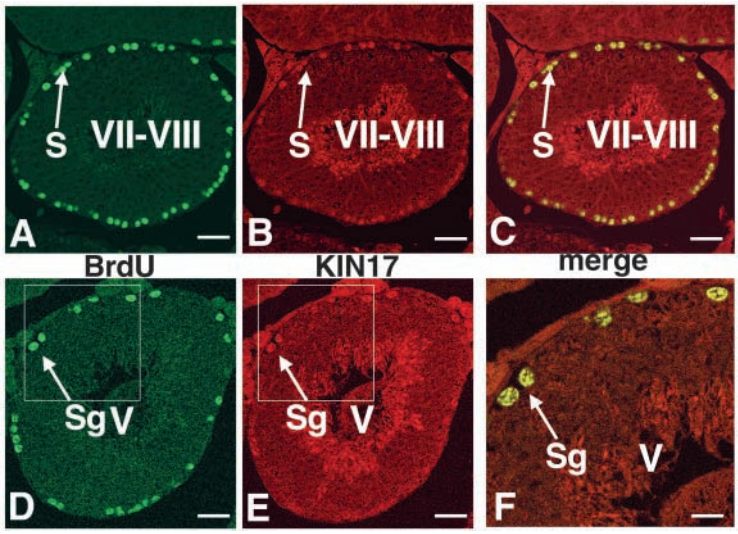

Fig. 3. In vivo colocalisation of incorporated BrdU and KIN17 protein in adult testis. (A,D) BrdU staining appears green. (B,E) KIN17 staining appears red. (C,F) merged images where the colocalisation of KIN17 protein and BrdU appears yellow. (F) shows a magnification of the spermatogonia in merged images of the indicated area in D and E. Stages of the seminiferous epithelium are indicated with Roman numerals. Preleptotene spermatocytes: S, spermatogonia: Sg. Bars: A, B, C, D and E, 32 μm; F, 13 μm.

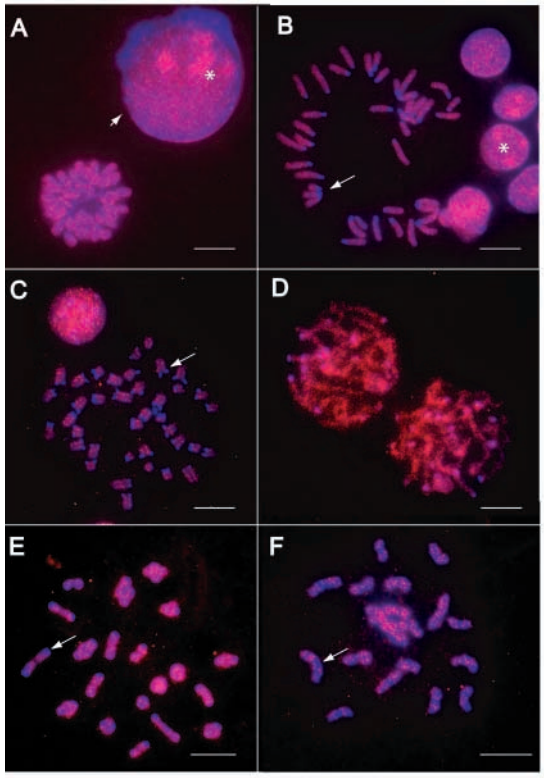

Fig. 4. Detection of KIN17 protein by immunofluorescence on testicular cell spreads. (A,B) 2-day-old (C) 8-day-old and (D-F) adult mice cells. In neonatal testes, germ cell interphase nuclei are much larger (arrow in A) than somatic nuclei (B). Both germ and somatic interphase nuclei are stained positive (red) for KIN17 all over the nucleoplasm with stronger staining of large intranuclear structures (* in A and B). Germ and somatic metaphase chromosomes also exhibit different shapes and, in the same preparation, germ cell metaphases are well spread (B) compared to Sertoli cell metaphases (A). In both cell types staining is not detected at pericentromeric heterochromatic regions as shown after counterstaining with DAPI (blue, arrows in B and C). (C) In 8-day-old mice spermatogonia metaphase chromosomes (with premature centromeric region separation as previously described) (Bernardino-Sgherri et al., 2002) exhibit the same staining pattern. At different stages of adult germ cell, chromatin of pachytene (D), metaphase I (E) and metaphase II (F) spermatocytes is stained by anti-KIN17 antibodies, whereas reduced staining is observed at pericentromeric regions (arrows in E and F). Bars, 10 μm.

homogenous KIN17 staining on chromosomes in a granular/punctate pattern except in pericentromeric heterochromatin regions where the labelling was strongly reduced. Interphase somatic cells and germ cells from immature testes (Fig. 4A,B) showed bright staining for KIN17 that was distributed in discrete foci throughout the nucleoplasm but also in large intranuclear structures (LIS). Variable levels of KIN17 labelling in cells from adult testes were observed compared to the high level seen in cells from immature testes. High levels of KIN17 are associated with the S phase of the cell cycle as shown for 83% of the 142 counted cells incorporating BrdU. The other 17% showed a lower level of this protein. Nevertheless, in some negative BrdU cells strong KIN17 staining was observed as in pachytene spermatocytes (Fig. 4D). KIN17 foci remained at metaphase as in immature testes and were also observed on chromosomes of the two meiotic divisions (Fig. 4E,F). In Sertoli cells perinucleolar satellites presented a staining similar to that observed in paraffin-embedded sections together with a slight staining in discrete nucleoplasmic foci (not shown).

KIN17 protein detection in adult mouse testicular sections after nuclease treatment

To test whether the detection of KIN17 protein depends on the integrity of nucleic acids in vivo, testicular sections were treated with either DNase I, RNase A or both. Thereafter KIN17 was detected with purified mAb Igk58. Sections were stained with DAPI to check the removal of DNA before (Fig. 5A) and after (Fig. 5E) DNase I treatment. After DNase I treatment we only observed DAPI staining corresponding to the very condensed DNA of elongated spermatid heads. The integrity of RNA was checked with propidium iodide, a dye that detects the two nucleic acids (not shown) and by the unchanged detection of the splicing factor SC35 (Fig. 5B,F). KIN17 staining after DNase I treatment was slightly decreased but always detectable in Sertoli and germ cells; the spermatid cytoplasmic signal remained clearly detectable compared to controls (Fig. 5D,G). RNase treatment did not affect DNA detection using DAPI staining (Fig. 5H) and RAD51 protein detection (Fig. 5C,I) but slightly decreased the level of KIN17 protein except in spermatid cytoplasm (Fig. 5J). RAD51 staining completely disappeared following DNase I treatment. After RNaseA digestion the SC35 staining was reduced although a residual staining in pachytene spermatocyte cells was systematically observed owing to its nuclear matrix localisation (data not shown) (Spector, 1993). Treatment with RNase A and DNase I reduced but did not

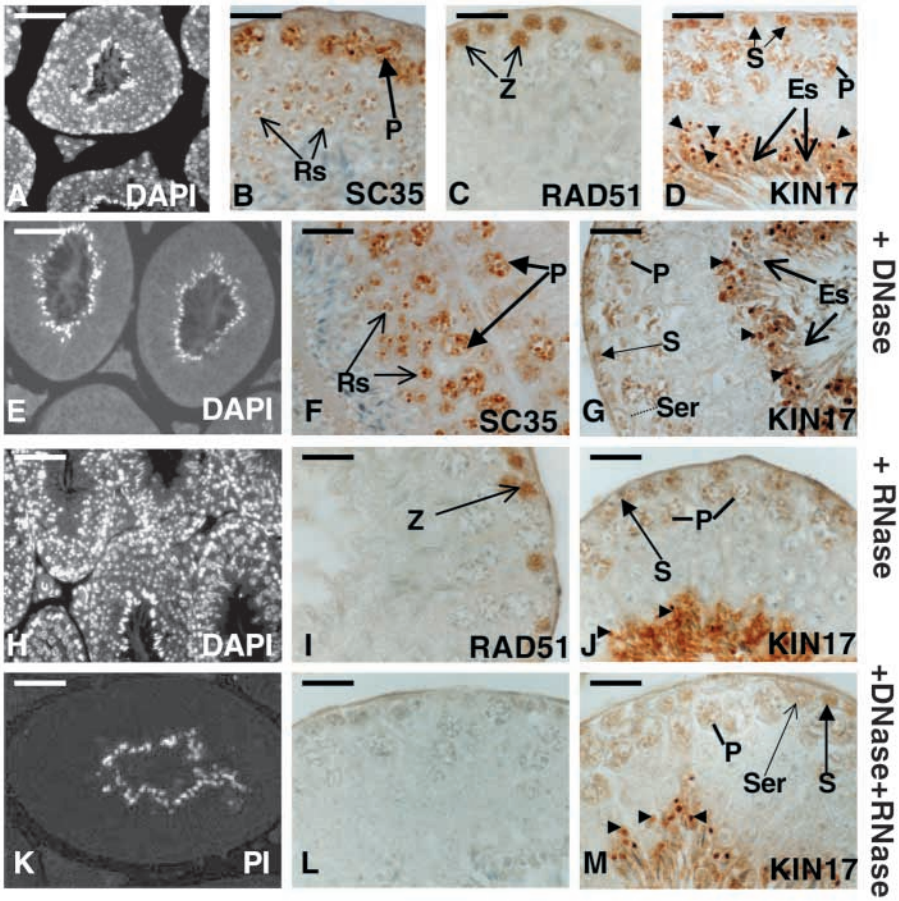

Fig. 5. Detection of KIN17 protein in adult mouse testis after nuclease digestion. Immunohistochemical detection of KIN17 protein (D,G,J,M) in testicular sections digested either with DNase I (E,F,G), or with RNase A (H,I,J), or in a combination of the two (K,L,M). DAPI staining (A, control) shows that DNase I digestion released DNA from nuclei of testicular cells, except elongated spermatid heads (E) and that DNA is preserved by RNase A digestion (H). PI staining shows that DNA and RNA are released from nuclei by DNase and RNase digestion (K). Whatever the treatment, a residual staining for KIN17 is observed in cell nuclei; cytoplasmic staining of elongating spermatids is well preserved (arrowheads in D, G, J and M) compared to control (D). SC35 detection is not affected by DNase I digestion (F) compared to control (B) and RAD 51 is not affected by RNase digestion (I) compared to control (C). A negative control where anti-KIN17 mAb was preadsorbed with pure KIN17 protein is shown (L). Ser, Sertoli cells; S, preleptotene, leptotene; Z, zygotene; P, pachytene spermatocytes; Rs, round spermatides; Es, elongated spermatids. Bars: A,E,H, 80 μm; B,C,D,F,G,I, 22 μm; J,L,M, 23 μm; K, 55 μm.

completely abolish KIN17 labelling in Sertoli and germ cells, whereas the signal in Es cytoplasm (Fig. 5M) and residual bodies remained clearly detectable (not shown). The removal of nucleic acids was confirmed by PI staining (Fig. 5K). These observations suggest that KIN17 protein might interact with DNA and RNA in vivo but also with other cellular substructures.

Association of KIN17 with nucleic acid

To confirm the molecular interaction of KIN17 with nucleic acid in vivo we analysed cells released from collagenase-treated testes. Cells were irradiated with UVC light, ex vivo, to covalently cross-link proteins to associated polynucleotides (Wagenmakers et al., 1980). Whole cell extracts were treated with DNase I or RNase and KIN17 was immunoprecipitated from the soluble phase using the indicated anti-KIN17 antibody. The polynucleotide covalently attached to KIN17 protein was revealed by labelling the free 5′-OH end of the polynucleotide with [^{32}P] using T4 polynucleotide kinase. The adduct was resolved by SDS-PAGE after boiling the sample in denaturing buffer (Fig. 6A,C). A radioactive adduct was detected only in UV-irradiated cells and migrated at the apparent molecular weight of KIN17. The labelled species were excised and digested with proteinase K. They were sensitive to subsequent digestion with DNase I or RNase A, respectively, thus identifying them as a DNA (Fig. 6B) or an RNA (Fig. 6D). These results agree with the above observations following nuclease treatments of mouse testicular sections. We concluded that in testes KIN17 protein may bind to both DNA and RNA.

Copurification of KIN17 with poly(A$^+$) RNA

To test the possibility that KIN17 may be directly associated with poly(A$^+$) RNA in testis we performed oligo(dT)-cellulose chromatography on lysates of testis cells after in vivo UV cross-linking under native conditions. While most of the protein was found in the flowthrough (FT) and wash fractions, a subset of KIN17 coeluted with poly(A$^+$) RNA (Fig. 7). It may be possible that the amount of KIN17 protein retained on this column is underestimated because during purification under our experimental conditions poly(A$^+$) RNAs could be complexed to cellular proteins and therefore may be unable to bind to the oligo(dT). However, the efficiency of the column was confirmed by the retention of HUR and DAX-1 proteins known to bind to nuclear and cytoplasmic mRNA (Gallouzi et al., 2000; Lalli et al., 2000) (Fig. 7). Conversely, RAD51, which interacts with DNA, was not retained on the oligo (dT) column (Fig. 7) and nor was the DNA-binding component Ku70 (Hamer et al., 2003) (not shown). RNase treatment of the extracts abrogated binding of KIN17 to the oligo (dT) column, showing that the binding is mediated by poly(A$^+$) RNA (Fig. 7).

As KIN17 protein is associated with mRNA within the cell we asked whether it may bind directly to RNA. We tested the binding of [^{35}S]-labelled mouse KIN17 and truncated forms translated in vitro using rabbit reticulocyte lysate, to the

ribonucleotide homopolymers poly(A), poly(C), poly(G) and poly(U) immobilised on agarose beads. Full-length KIN17 (a.a. 1-391) bound to RNA homopolymers with different affinities in the order poly(G)>poly(U)>poly(C)>poly(A) (Fig. 8A). Mutant C-terminal truncated, KINΔKOW (a.a. 1 to 335) showed a similar selectivity but reduced binding to poly(G), poly(U) and poly(C) RNA homopolymers compared with the full-length protein. A protein containing the sequence corresponding to the first N-terminal 160 amino acids showed a very low level of binding to poly(G) and poly(U) and did not bind to poly(C) or poly(A). Thus another region important for RNA binding seems to be localised out of the KOW domain between residues 160 and 335. Mouse KIN17 protein binding to poly(G) decreases in the presence of 0.5 M NaCl while binding to poly(U) is more salt sensitive. Binding to poly(A) and poly(C) was dissociated at 0.2 M.

To rule out the possibility that RNA binding by KIN17 was mediated by association with other proteins present in the reticulocyte lysate, we determined the RNA homopolymer binding properties of purified human KIN17 expressed in the baculovirus system under the same conditions. The protein bound to beads was revealed by western blot. This protein shows the same specificity of binding to RNA homopolymers as the mouse protein translated in vitro (Fig. 8A). Finally, human and mouse KIN17 proteins produced and purified from baculoviral and bacterial systems, respectively, were separated by SDS-PAGE, blotted onto a membrane and probed with [^{32}P]-labelled poly(G) or [^{32}P]-labelled RNA. Protein staining showed an apparent molecular weight of 45 kDa for KIN17 proteins and about 65 kDa for BSA. In the case of the human protein, a band heavier than 83 kDa was also observed. The radioactive detection revealed the presence of bands of 45 kDa corresponding to the human and mouse KIN17 proteins. The heavier band in the human KIN17 protein slot also bound both radioactive probes. The high molecular weight of this band might correspond to a dimer, and indeed mAb k58 clearly recognized it (data not shown). Surprisingly, this mAb also revealed a similar high molecular weight band in the mouse KIN17 protein. The amount was much lower than in the case of the human preparation, perhaps because it did not bind the radioactive probe at detectable levels or because of the differential maturation of the proteins (baculoviral versus bacterial system). The specificity of the detected interaction is further demonstrated by the fact that bovine serum albumin (BSA) and all the proteins used as molecular weight markers did not bind to poly(G) or RNA (Fig. 8B,C). Notably, chicken egg white lysosyme has a pI of 9.3 like KIN17 protein. We conclude that mouse and human kin17 protein have an intrinsic RNA-binding capacity.

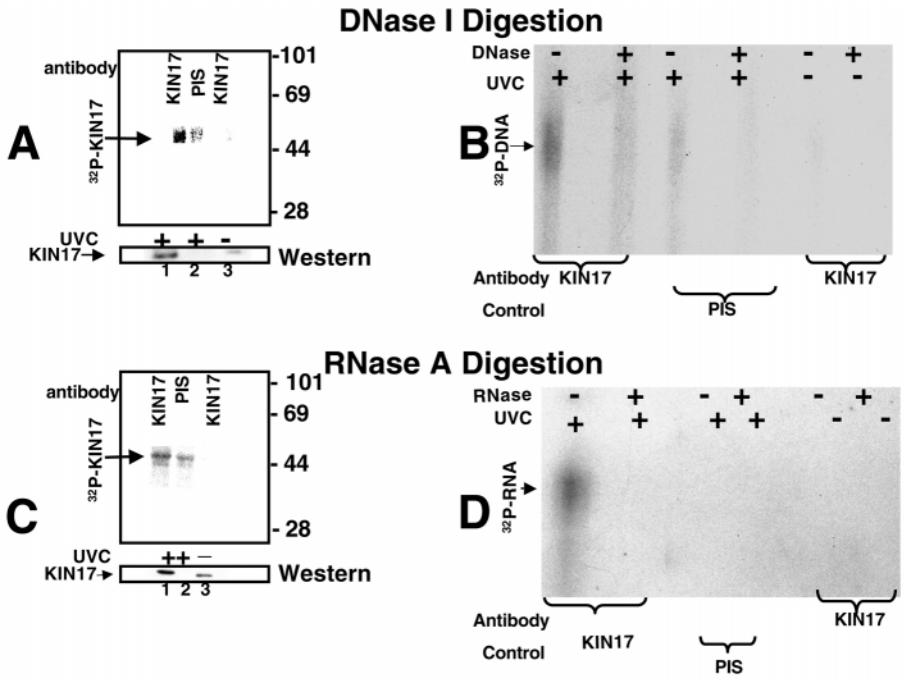

Fig. 6. Mouse KIN17 protein associates with DNA and RNA in testes. (A) In vivo UVC cross-linking of DNA to KIN17 in testicular cells. Suspensions of freshly isolated testicular cells were irradiated with UVC. Cells were then lysed and treated with DNase I. KIN17 protein was immunoprecipitated with rabbit anti-KIN17 antibody (lanes 1 and 3) or pre-immune serum (PIS) (lane 2, control) (lower panel). The immune complex was end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and resolved on a 10% SDS-PAGE gel. The autoradiogram is shown in the upper panel. (B) The labelled material migrating at the position of KIN17 protein was excised from the gel, digested with proteinase K, subsequently re-digested with DNaseI and resolved by 10% PAGE in 8 M urea; the autoradiogram of this gel is shown. A labelled smear is observed only in lysates from UVC cross-linked cells and mainly in the lane containing the immunoprecipitation with anti-KIN17 polyclonal antibody. The deproteinated labelled polynucleotide that had been cross-linked to KIN17 protein is sensitive to degradation by DNase 1. (C) In vivo UVC cross-linking of RNA species to KIN17 in testicular cells. The cells were treated as above excepting that RNase A was used instead of DNase 1. (D) Note that the deproteinated labelled polynucleotide that had been cross-linked to KIN17 is sensitive to degradation by RNaseA.

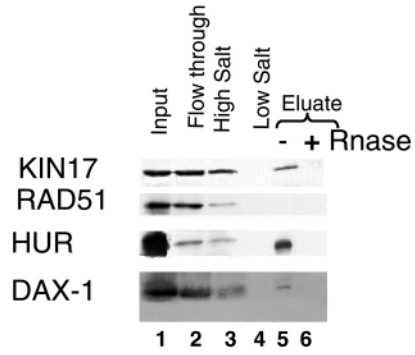

Fig. 7. KIN17 protein co-purifies with poly(A$^+$) RNA. Extracts from mouse testicular cells were subjected to oligo (dT) chromatography under native conditions. Aliquots from total cell extract (lane 1), flow-through (lane 2), high-salt (lane 3) and low-salt (lane 4) washes and eluate (lane 5) fractions were subjected to SDS-PAGE and analysed by western blot. Previous RNase treatment of cell extracts abolishes KIN17, HUR and DAX-1 fractionation in the eluate (lanes 5 and 6).

Association of KIN17 with chromatin and the nuclear matrix.

Since KIN17 staining is always detectable in histological testis sections after nuclease digestion, we reasoned that a fraction might be associated with the nuclear skeleton or nuclear matrix. To test this, we prepared nuclear matrix as reported (Wu and Means, 2000). In the first fractionation step, soluble proteins were extracted with Triton X-100. The detergent-insoluble pellet was treated with DNase I followed by 0.25 M ammonium sulphate to release chromatin-bound proteins. After washing the pellet with 2 M NaCl, the last fraction was resuspended in urea buffer. This fraction was composed of structural nuclear matrix proteins and nuclear matrix-associated proteins. Western blotting of supernatants of each extraction step and the final nuclear matrix pellet demonstrated that KIN17 is mainly present in the detergent-soluble fraction and the chromatin-associated proteins as also shown by western blot analysis (Fig. 1C). A small fraction was also associated with the nuclear matrix (Fig. 9). To rule out the possibility that nuclear matrix preparation induced the precipitation of some nuclear proteins, we detected PCNA which is predominantly found in the soluble nuclear fraction (Balajee et al., 1999). As expected PCNA was almost completely released in the first two fractions. To verify that the cytoplasmic and chromatin fractions were not contaminated with nuclear matrix protein we detected LAMIN B, one of the major components of the nuclear matrix. As expected LAMIN B was solely found in the nuclear matrix fraction. This result shows that our preparation retained specifically true nuclear matrix-associated proteins. This was further confirmed by the detection of the SR protein SF_2/ASF which is predominantly associated with the nucleus at steady state (Caceres et al., 1998) (Fig. 9).

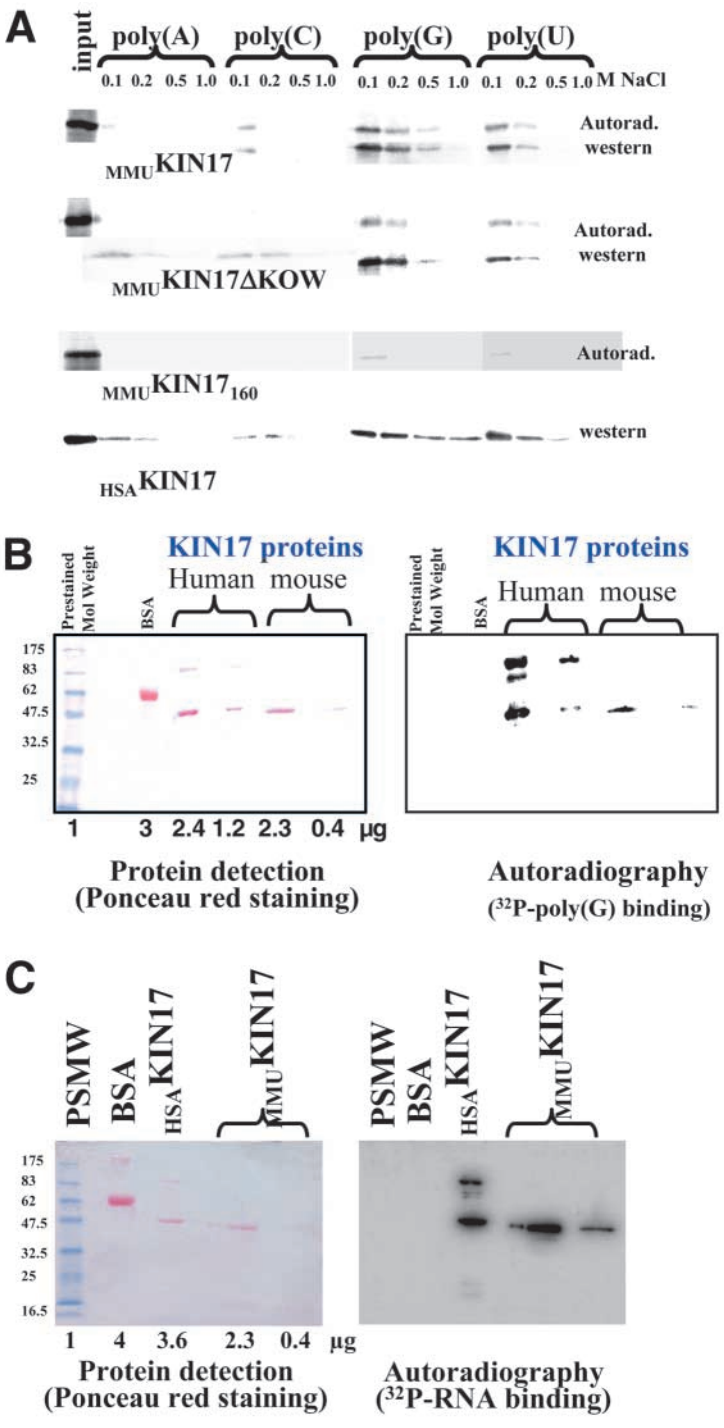

Fig. 8. KIN17 protein binds to RNA. (A) RNA homopolymer binding assay. Binding to agarose beads coupled to poly(A), poly(C), poly(G) and poly(U) is shown for wild-type mouse KIN17 protein, modified proteins $KIN17_{335}$ΔKOW and $KIN17_{160}$ translated in a rabbit reticulocyte lysate. The binding of human KIN17 protein expressed in insect cells to RNA homopolymers and detected by western blot is also shown. A 1:3 ratio of the input protein is shown in each case. The binding of protein to homopolymers was evaluated by radioactivity detection using STORM phosphoimager and immunodetection with the exception of $KIN17_{160}$ protein that was not recognized by the mAb. Salt sensitivity of the binding was tested in buffers containing 0.1, 0.2, 0.5 and 1 M NaCl. (B) Northwestern binding assay using a [^{32}P]-labelled poly(G) or (C) [^{32}P]-labelled RNA probed against the proteins blotted on a nitrocellulose membrane. PSMW, prestained molecular weight markers, 1 µg of each protein was loaded.

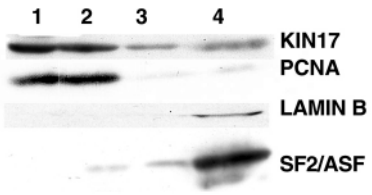

Fig. 9. A fraction of KIN17 protein is associated with chromatin and nuclear matrix. Testicular nuclear matrix was prepared as described in the Materials and Methods. Proteins were sequentially extracted with 0.5% Triton X-100 (lane1), DNase I and 0.25 M $(NH_4)_2SO_4$ (chromatin, lane 2) and 2 M NaCl (lane 3); the remaining pellet was solubilised in 8 M urea buffer (nuclear matrix, lane 4). 50 µg of protein from each step of the extraction protocol were subjected to SDS-PAGE and immunoblotted with antibodies against KIN17 protein, PCNA or LAMIN B (Santa Cruz Biotechnology) or SF_2/ASF (gift from A. Krainer).

Discussion

We report here the expression profile of mouse *Kin17* gene during mouse spermatogenesis. This gene participates in the cellular response to several genotoxic agents in mouse and human cells and encodes a nuclear zinc-finger protein of 45 kDa which seems to be involved in several DNA transactions (Kannouche et al., 1998; Mazin et al., 1994a; Miccoli et al., 2003; Timchenko et al., 1996). The hypothesis of a role in nucleic acid metabolism is further confirmed here by the analysis of a tissue presenting rapidly dividing cells together with arrested differentiated cells. In the male germinal tissue, the expression of the *Kin17* gene increases during the first 17 days p.p. when spermatogonia cells actively proliferate into spermatocytes. Furthermore, in the seminiferous tubule, the bulk of KIN17 is localised in the nuclei of Sertoli and germ cells until early round spermatids form indicating that the bipartite nuclear localisation signal present in its amino acid sequence (Angulo et al., 1991) is functional in vivo. This ubiquitous expression of KIN17 suggests its participation in a common regulatory mechanism for all cell types. In germ cells, KIN17 protein is essentially nuclear and associated with condensed chromatin during the mitotic metaphase and the two meiotic divisions. The nuclease treatment of testicular histological sections suggests that KIN17 might be associated with chromatin DNA and other nuclear components. Biochemical approaches indicated that this is the case, giving further support to the previously reported data. Indeed, mouse KIN17 protein binds to DNA in vitro (Angulo et al., 1991; Biard et al., 2002; Mazin et al., 1994b) and in HeLa cells (Biard et al., 2002) and is associated with the nuclear matrix in cultured human cells (Miccoli et al., 2003). Most germ cells express KIN17. In the particular case of the S-phase spermatogonia and spermatocytes, we observed a colocalisation of the sites with BrdU incorporation with KIN17 protein (Fig. 3) which supports the involvement in DNA replication in vivo. Furthermore, the identification of KIN17 in the adducts formed between deoxypolynucleotides and testicular protein after UVC cross-linking most likely confirms that the interaction previously reported in cultured cells also takes place in testicular tissue (Fig. 6, panels A and B).

Interestingly, postmitotic cells such as Sertoli cells, as shown here in IHC analysis, and neurons can express KIN17 protein (Fig. 2H,K) (Araneda et al., 1997). Moreover, under our experimental conditions PCNA, a nuclear protein involved in DNA replication, recombination and repair (Kelman, 1997) is undetectable in adult mouse Sertoli cells and many germ cells are labelled for KIN17 and not for BrdU. These data reveal that KIN17 protein may be involved not only in DNA replication but also in other biological functions in vivo.

The identification of KIN17 as a component of the human spliceosome (Rappsilber et al., 2002) together with the presence of a KOW motif, known to mediate the protein-RNA interaction (Kyrpides et al., 1996) in the C-terminal end of the protein, and the fact that RNase treatment decreases the KIN17 staining in testicular sections, are all suggestive of a possible role in RNA metabolism. Biochemical analysis confirmed that a fraction of the total KIN17 protein copurified with poly(A^+) RNA after UV cross-linking in vivo (Fig. 7). This copurification may be explained by direct binding to poly(A^+) RNA. Indeed, the mouse and human KIN17 proteins interact with RNA and share a similar affinity profile for the RNA homopolymers (Fig. 8A). These data indicate that the 92.4% structural identity shared by these two proteins determines similar functional properties. The deletion of the KOW domain decreases but does not negate the fixation to RNA homopolymers, indicating that other regions may also be involved in this interaction (Fig. 8A). The fact that a mutant containing only the first 160 N-terminal residues of KIN17 protein is unable to bind to poly(G) and poly(U) make a direct involvement of the zinc finger in RNA recognition unlikely (Lu et al., 2003). Nevertheless, an indirect role cannot be excluded since we detect dimers of KIN17 that bind to RNA (Fig. 8C,D). Therefore a possible polymerisation mediated by the zinc finger cannot for the moment be completely excluded. Further investigations should help to determine the domain(s) directly interacting with RNA.

These results are consistent with preliminary data showing that KIN17 interacts with DDX1 in a two-hybrid analysis and a significant fraction co-immunoprecipitates with DDX1 protein in nuclear extracts of HeLa cells (our unpublished results). DDX1 is a DEAD box protein that resides in both the nucleoplasm and within cleavage bodies. It is a component of the spliceosome which interacts with CstF-64, a protein involved in mRNA 3′ cleavage and polyadenylation (Bleoo et al., 2001). This observation suggests that KIN17 may be part of a complex composed of DDX1 and CstF-64 proteins that may participate in pre-mRNA processing. It should be mentioned that CstF-64 mRNA is highly expressed in mouse testicular cells (Dass et al., 2001; Wallace et al., 1999). Further studies are required to define the possible involvement of KIN17 in RNA transactions such as pre-mRNA processing which is tightly coupled to transcription (Bentley, 2002; Maniatis and Reed, 2002).

The RNA-binding activity of KIN17 protein may help to explain its detection in the cytoplasm of elongating spermatids (Fig. 2G-K). As global transcription ceases several days before the completion of spermiogenesis, in the late stages of germ cell maturation, protein synthesis is achieved from stored mRNA located in cytoplasmic ribonucleoprotein particles. Translation takes place after the mRNAs undergo a deadenylation that shortens their poly(A) tail (Sassone-Corsi, 1997). Our results suggest that KIN17 may be involved in mRNA processing and in this way it may indirectly regulate gene expression. Moreover, the release of the cytoplasmic KIN17 protein in residual bodies probably indicates that this fraction of KIN17 is no longer useful for spermatozoa functions. Nevertheless, the high isoelectric point of KIN17 (9.3) suggests a possible structural role in spermatid maturation.

However, it is likely that only a fraction of the total nuclear KIN17 detected in the germinal tissue is involved in these types of RNA transaction as judged by the direct staining of testis sections and nuclease treatments (Figs 2, 5) and confirmed by biochemical analysis such as detergent extraction fractionation or nuclear matrix preparation (Figs 1, 9) or oligo(dT) affinity purification (Fig. 7). This is consistent with previous reports that in human cells KIN17 is differentially extracted by detergents (Biard et al., 2002; Miccoli et al., 2002) and that only a fraction of the total cellular KIN17 protein binds tightly to the non-chromatin sub-structure of the nucleus called the nuclear matrix (Miccoli et al., 2003). The latter is a fundamentally important nuclear component upon which the

processes of DNA replication, recombination, repair and transcription take place (for a review, see Berezney et al., 1995; Nickerson, 2001).

Our results point to a differential association of KIN17 protein with nuclear structures in testicular cells. This raises the possibility that KIN17 may be implicated in several functional protein complexes of reduced half-lives, as nuclear components are in perpetual flux (Misteli, 2000). It has been previously proposed that the binding to the nuclear matrix occurs through the C-terminal domain of KIN17 (Kannouche and Angulo, 1999). Nevertheless, it must be considered that in chromosomal DNA there are matrix or scaffold associating regions (called MAR or SAR respectively) that usually comprise AT-rich sequences which bind specifically to the nuclear matrix and have a high unwinding propensity (Bode et al., 1992). Among AT-rich sequence chromatin-binding proteins, SAF-B protein couples transcription and pre-mRNA splicing to S/MAR elements (Nayler et al., 1998). This would also be the case for the KIN17 protein as it is shown, in vitro, that a core domain of KIN17 protein allows preferential binding to curved DNA containing AT-rich regions (Mazin et al., 1994a; Mazin et al., 1994b). Independent of the identity of the domains governing the interaction with the nuclear matrix, our results indicate that in vivo this interaction may point to a possible participation of KIN17 protein in chromatin remodelling and subsequent gene expression (Dickinson et al., 1992). The fact that KIN17 protein can substitute for the bacterial nucleoid protein H-NS, which is a chromatin-associated protein interacting with curved DNA, to influence topology and gene expression (Owen-Hughes et al., 1992; Timchenko et al., 1996) lends further support to this idea.

In conclusion, our in vivo data confirm the observations reported in cultured cells and illustrate for the first time a direct interaction of KIN17 with RNA. To elaborate on our findings we now aim to identify proteins interacting with the KIN17 protein to define its participation in mRNA processing or editing, in order to gain insight into its biological function(s) in the mouse testis.

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