Keratin 9 Is a Component of the Perinuclear Ring of the Manchette of Rat Spermatids

Kazuhiko Mochida, Eugene Rivkin, Mara Gil, and Abraham L. Kierszenbaum

Department of Cell Biology and Anatomical Sciences, The City University of New York Medical School, New York, New York 10031

Previous work in our laboratory has shown that a 62- to 64-kDa protein was a major component of the perinuclear ring of manchettes fractionated from rat spermatids. Mass spectrometry analysis of this protein indicated the presence of a glycine-rich domain homologous to human keratin 9 (K9). Several antibodies to K9, raised against synthetic peptides of human K9, recognized the 64- to 62-kDa protein in the perinuclear ring of the manchette as well as in keratinocytes of the suprabasal layer of the rat and human footpad/sole epidermis in both immunoblotting and immunocytochemical experiments. Based on these data, human-derived K9 primers were used to clone rat K9 cDNA from epidermis by RT-PCR. Rat-specific K9 primers were then used to perform a two-step (nested) PCR to amplify the K9-specific rat testicular RNA and to obtain cDNA to demonstrate K9 gene expression in rat testis. The deduced amino acid sequence of rat K9 cDNA contains 618 amino acids with an estimated molecular mass of 63,020 Da, in agreement with that obtained by electrophoretic fractionation of rat manchette and epidermis footpad proteins. The deduced protein structure correlates with the recognizable pattern of keratins: a rod domain of 304 amino acids with well-conserved initiation and termination sequences (MQNLNSRLASY and EIETYRKLLEG, respectively), flanked by glycine-serine-rich head and tail domains of 141 and 173 amino acids, respectively. A high content of phenylalanine was detected in the head domain and a repetitive motif (SGGSYGGGS) in the tail domain. A comparison with human keratin 9 showed an overall nucleotide and amino acid similarity of 75%. An increased level of K9 transcripts was detected in a cDNA library prepared from fractionated round spermatids. Results of this study show that rat testis expresses K9 and that this protein is a component the perinuclear ring of the manchette of rat spermatids. © 2000 Academic Press

Key Words: spermiogenesis; nuclear shaping; testis; keratins.

INTRODUCTION

One of the intriguing events of mammalian spermiogenesis is the development of the manchette, a transient tubulin-containing structure. The timing of the development of the manchette is very precise: it follows the development of the axoneme and occurs concomitantly with the shaping of the nucleus of the male gamete (Rattner and Brinkley, 1972; Rattner and Olson, 1973; Wolosewick and Bryan, 1977; Clermont et al., 1993). Sperm nuclear shaping, in turn, correlates with the gradual replacement of somatic histones by arginine/lysine-rich protamines, a process that leads gradually to three events (Kierszenbaum and Tres, 1975): (1) general gene inactivation, (2) a change in chromatin pattern, from nucleosome-containing chromatin fibers to a smooth type of chromatin fibers characterized by their side-by-side alignment, and (3) the condensation of the sperm genome. Experimental evidence supporting a role of the manchette in nuclear shaping has been reviewed by Meistrich (1993). Yet, the precise contribution of this structure to nuclear shaping and sperm development remains unknown.

A direct approach to determine whether the manchette is a clutch-like structure modulating nuclear shaping is through the identification and characterization of its com-
ponents. A recently developed procedure has enabled the isolation of intact manchettes from rat and mouse spermatids and the fractionation of two of its major components: the perinuclear ring and its inserted microtubular mantle (rat, Mochida et al., 1998; mouse, Mochida et al., 1999). An immunofluorescence localization analysis of α-tubulin in intact manchette and perinuclear ring fractions, separated from the microtubular mantle by thermocleavage, has shown truncated microtubules still attached to the perinuclear ring (Mochida et al., 1998, 1999). This observation suggests the existence of a strong microtubule-perinuclear ring linkage stabilized by molecules yet to be identified. The possible existence in the perinuclear ring of a complex of macromolecules is indirectly supported by electron microscopy studies showing distinct structural patterns within the ring and at the microtubular mantle insertion site (for example, see Fig. 2 in Mochida et al., 1998). A combined immunoblotting and immunofluorescence analysis has shown that microtubules of the mantle of the manchette contain a substantial number of tubulin isoforms and that a 62- to 64-kDa protein was a major component of the perinuclear ring fraction (Mochida et al., 1998, 1999).

In this paper, we have examined the biochemical and molecular nature of the 62- to 64-kDa protein using mass spectroscopic analysis of amino acid composition, immunocytochemistry, immunoblotting, and cDNA cloning. We have made the unexpected finding that the 62- to 64-kDa protein is a rat homologue of human keratin 9 (K9), a glycine-rich keratin previously reported to be exclusively present in the human plantar and palmar epidermis (Fuchs and Green, 1980; Knapp et al., 1986; Langbein et al., 1993), bovine heel pad (Knapp et al., 1986), and mouse footpad sole (Schweizer et al., 1989). Several antibodies, generated against peptides encoded by the human K9 gene cross-reacted with the 62- to 64-kDa protein present in the perinuclear ring of the rat spermatid manchette as well as with keratinocytes of the suprabasal cell layer of the rat and human epidermis as detected by immunoblotting and immunohistochemistry.

MATERIALS AND METHODS

Isolation of Rat Spermatid Manchettes

Manchettes were fractionated by sucrose gradient centrifugation from seminiferous tubules harvested from adult rats (Sprague-Dawley, Taconic Farm, Germantown, NY; 250–300 g) as described (Mochida et al., 1998). Briefly, cellular components of isolated seminiferous tubules (Stages VIII–XIV of spermatogenesis, recognized by their transillumination pattern under a dissection stereo microscope) were collected in a microtubule-stabilizing buffer. Following sequential filtration through nylon mesh membranes of decreasing pore sizes to exclude cells larger than 30 μm in diameter, the resulting sample was dissociated in a detergent-containing buffer and placed in a 1.0–2.05 M sucrose gradient to separate the manchettes by ultracentrifugation.

Mass Spectrometry of the 64- to 62-kDa Protein and Polyclonal Antibody Production

Coomassie blue-stained proteins of the perinuclear ring resolved by two-dimensional polyacrylamide gel electrophoresis (PAGE) were transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). Protein samples were subjected to an enzymatic digestion by endoproteinase (Lys-C) followed by HPLC separation of the peptides and mass spectrometry that weighs each fragment, forming a mass fingerprint of the protein fragments. UCSF Mass-Fit program was utilized to compare the mass spectroscopy profile of the digest with the calculated digest patterns of all proteins in the database. One of the analyzed proteins featured a close relationship to a human K9. Based on a human sequence, an antibody was raised against a glycine-rich peptide located at the carboxyl-terminus of human K9 (wwGGLGRRSGGGSYGRGSRww) (Langbein et al., 1993). An antigen with four attached glycine-rich peptides was synthesized by direct solid-phase synthesis using Fmoc chemistry (Tam, 1978; Mertz et al., 1995) and used for immunization. Polyclonal anti-peptide antibody (designated pcaK9) was raised in rabbits by Biodesign International (Kennebunk, ME) and affinity-purified by coupling cysteine sulphydryls to SulfoLink gel (Pierce Chemical Co., Rockford, IL) using the glycine-rich peptide with a terminal cysteine residue to facilitate coupling as reported (Mertz et al., 1995). In addition, two K9 monoclonal antibodies (mcaK9) raised against synthetic peptides at amino acid positions 450–477 and 4–28 of human K9 (Langbein et al., 1993), commercially available as a mixture (Multi-epitope Cocktail, Research Diagnostics, Flanders, NJ), were used for immunocytochemistry and Western blot analysis.

Immunoblot Analysis

Rat and human epidermis (prepared as described below) and testes from adult rats were Dounce-homogenized in 8 M urea, 1% Triton X-100, 10 mm DTT, sonicated, incubated for 10 min at room temperature, and centrifuged at 14,000g for 10 min. Supernatants were collected and stored at −80°C. For one- and two-dimensional PAGE, protein extracts of fractionated manchettes were prepared as described (Mochida et al., 1998). Following electrophoresis, fractionated proteins were electrotransferred to an Immobilon P membrane. The membranes were treated with PBS containing 2% bovine serum albumin, 0.2% Tween 20, incubated for 1 h with preimmune serum (control, not shown), pcaK9 (working dilution, 1:500), or mcaK9 (1:100), washed, and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (working dilution, 1:30,000; Sigma Chemicals Co., St. Louis, MO). Immunoreactive proteins were detected by using NBT/BCIP substrate (Promega, Madison, WI).

Indirect Immunofluorescence and Immunoperoxidase Staining

Fractionated rat spermatid manchettes and spermatogenic cells isolated from seminiferous tubules (Stage VIII–X of spermatogenesis) as described (Mochida et al., 1999) were placed on microscope slides coated with Vectabond (Vector Laboratories, Burlingame, California), allowed to air dry, and fixed with acetone. Following sequential filtration through nylon mesh membranes of
Preparations of RNA and Single-Stranded cDNA from Rat and Human Epidermis and Rat Testis

Rat epidermis (from the footpad sole) and human epidermis (from the plantar region) were separated from the dermis following incubation in 0.9% NaCl at 60°C for 1 min. Testes were obtained from adult rats. Total RNA from testis and epidermis was extracted using the SNAP RNA isolation kit (Invitrogen, Carlsbad, CA), allowed to settle for about 2 min, fixed for 15 min at room temperature in 3% paraformaldehyde in cacodylic buffer, pH 7.2, containing 1 mM each of CaCl₂ and MgCl₂, and allowed to dry. Samples were rehydrated in PBS, blocked with 1% goat serum in PBS for 5 min, and immunoreacted with anti-α-tubulin (monoclonal antibody; Sigma; working dilution, 1:100) and/or pcaK9 (working dilution, 1:50–1:100), or mcaK9 (working dilution, 1:10–1:20).

After incubation with primary antibody, the samples were rinsed and incubated with secondary antibody (anti-mouse or anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRICT; 1:200–300 working dilutions). All incubations were at room temperature. Controls included normal sera, antisera against irrelevant antigens, omission and increasing dilutions of first antibody (results not shown), and preimmune serum. Specimens were mounted with Vectashield (Vector Laboratories) and examined with a Zeiss Universal fluorescence microscope equipped with episcopic illumination. Footpad skin was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2) for 1 h, embedded in paraffin, and sectioned. Sections were immuno-reacted with pcaK9 (working dilution 1:1000) and processed for indirect immunofluorescence and immunoperoxidase staining using standard procedures.

Cloning of Rat K9 cDNA

Human-derived K9 primers were used to clone rat K9 cDNA from epidermis by RT-PCR (see below). To clone K9 cDNA from testicular RNA, rat-specific K9 primers were used and two-step (nested) PCR was performed (Fig. 4). In the first step, single-stranded cDNA reverse transcribed from total rat testis RNA using oligo(dT) primers was used for PCR amplification (40 cycles with annealing temperature 60°C). In the second step, 0.5% of this PCR product was used as a template for a second round of PCR amplification, using nested primers and annealing temperature 64°C. The obtained DNA bands were gel-purified and sequenced. The following primers were used (numbering for human K9-specific primers (h) from Langbein et al., 1993; for rat K9-specific primers (r) as in GenBank database, accession number pending): hF1, 5′-AAT-TCTCGGTCGGCTCTTCTATG-3′; hF2, 5′-GGAAGACAC-GAAAGACCGCTACTTG-3′; hF6, 5′-TGCACTTGGGACGCGGCG-3′; hF9, 5′-GGTTGACTGCCTCTTACTTG-3′; hF10, 5′-TCTCGGCTGGCCTCTTACTTG-3′; rF4, 5′-CTGCCCACTGCTTCTC5′; rF5, 5′-CTGGCATGACTCTCTTCTCTTCTC5′; rF9, 5′-CTTGCCACTTGGGAGAACGAGAAG-3′; rF10, 5′-CTTGGCCGCTGGGCTTTTCTTCGAC-3′; rF5, 5′-GGTTGACTGCCTCTTACTTG-3′; rF10, 5′-CTTGCCACTTGGGAGAACGAGAAG-3′;

RESULTS

Biochemical Characterization of the 62- to 64-kDa Protein of the Perinuclear Ring of the Manchette

A protein with a molecular mass of about 62- to 64-kDa and pl 5.75–5.30 was detected by two-dimensional PAGE in the perinuclear ring fraction of rat spermablast manchettes isolated by sucrose gradient centrifugation and thermolysis of manchettes (Mochida et al., 1998). This protein, regarded as a major component of the perinuclear ring, was harvested from intact isolated manchettes, fractionated by two-dimensional PAGE, blotted onto Immobilon P, digested (Lys-C), and digested-separated by HPLC. A small amount (2%) of the total digest and 10% of the selected HPLC fractions were taken for mass spectrometry analysis. The obtained peptide fingerprint was compatible with that of a protein homologous to human K9.

Construction of a CDNA Expression Library of Round Spermatids

A cDNA library was prepared using fractionated round spermatids from testes of 35-day-old rats as described previously (Rivkin et al., 1997). Briefly, a round spermatid fraction was collected following centrifugal elutriation (Möstrich, 1972). This fraction was further purified by density gradient centrifugation (25–37% Percoll). Cell purity (~98.5%) was monitored by both phase-contrast microscopy of unfixed cells and microscopic examination of oscein-stained preparations to visualize nuclear structure and acrosome. Poly(A)⁺ mRNA from purified round spermatids was isolated and used for directed CDNA synthesis (Riboclone CDNA synthesis system; Promega, Madison, WI). First-strand synthesis was primed with oligo(dT)-NotI primer adaptor. After the second strand was synthesized, the CDNA As were blunt-ended and fractionated on Sephacryl S-400 spin columns. EcoRI adapters were then ligated to both ends. The NotI site in the 3′ adapter was then exposed by NotI digestion. Ligation into SfiI-NotI arms of agt11 and packaging into λ particles (Packagene Lambda DNA packaging system, Promega) provided the initial library. The CDNA library with a titer of 6 × 10⁶ independent clones was amplified once and stored at −70°C.

Institutional Review of Animal Use and Sample of Human Skin

Human epidermis samples were from autopsy specimens. Its use was approved by the IRB/Human Subjects Committee (Assurance Number M-1111-XM-4XM; Protocol Number H-0021). The use of animals in this research has also been approved by the IACUC according to guidelines approved by the National Institutes of Health.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.
ated polypeptides from rat manchette and epidermis, respectively, migrating within the 62- to 64-kDa range. Each row consisted of at least three polypeptides with an isoelectric point ranging from 5.75 to 5.30 (refer to Fig. 5 in Mochida et al., 1998, for the position of the 62- to 64-kDa

protein relative to other protein components of the fractionated whole manchettes and perinuclear rings). A comparison of the immunoblot patterns of these proteins in the manchette and footpad epidermis samples showed slightly different isoelectric points in the polypeptide components of the upper row (compare panels B and D in Fig. 1). Confirming the antigenic homology between rat and human epidermis, one-dimensional immunoblotting experiments demonstrated that the anti-K9 antibody recognized similar immunoreactive proteins in both specimens (Fig. 1E). The immunoreactive proteins from either rat footpad or human sole epidermis displayed a multilayered profile within the expected molecular mass range, although the human K9 protein was more closely spaced. Thus, protein extracts of human epidermis, used as positive control, yielded a protein migrating at the same electrophoretic range as that of rat manchette, and rat proteins were recognized by pcaK9 serum raised against a human glycine-rich antigen. Subsequent cloning and sequencing of rat K9 cDNA (see below) showed that the rat K9 sequence contains a homologous region (GGSGGSYGGS) essentially identical (10 out of 11 amino acids) to the part of the human K9 peptide used to raise the pcaK9 serum. These results help to explain why the pcaK9 serum showed strong cross-reactivity for both human and rat K9 protein in the manchette and epidermis. We have not determined whether the anti-K9 serum recognized the manchette in human spermatids. The specific reactivity of this antibody with the rat K9 protein was confirmed by its recognition of rat K9-fusion protein produced in Escherichia coli using 3' end of rat K9 cDNA cloned into the pET-29b+ expression vector (Novagen, data not shown).

Our next approach was to use immunocytochemistry to determine in whole spermatids whether pcaK9 recognized the perinuclear ring of the manchette as well as keratinocytes of the suprabasal layer of the rat epidermis (footpad) as previously reported in the mouse and rat footpads (Schweitzer et al., 1989) and human palm and sole epidermis (Langbein et al., 1993). Whole spermatids collected from spermatogenic stage-specific seminiferous tubular segments were considered more suitable than paraffin or frozen sections of whole testis for our immunocytochemical studies because of limitations in the microscopic resolution of sectioned manchettes. Figures 2A–C illustrate the immunostaining of the ring of the manchette in a step 9 spermatid. Figures 2D–F show a fractionated rat spermatid manchette stained with pcaK9 (Fig. 2D) and anti-α-tubulin antibody (Sigma Chemicals) to visualize the mantle of the manchette (Fig. 2E) and the merged images to indicate the relative position of the ring with respect to the microtubular mantle (Fig. 2F). Immunofluorescence (Fig. 2G) and immunoperoxidase techniques (Figs. 2H and I) were used to demonstrate K9 immunoreactive sites in the rat footpad epidermis. Both procedures demonstrated an immunoreactive product in keratinocytes of the suprabasal layers of the epidermis. Preimmune serum, used as a negative control, did not display cross-reactivity in the epidermis of the

FIG. 1. Western blot analysis of rat tissues using anti-K9 antibody (pcaK9). Two-dimensional PAGE fractionation of proteins of intact manchette (A and B) and rat footpad epidermis (C and D). (A) Immunoblot of purified rat manchettes, showing a 62- to 64-kDa protein (K9, dotted box) immunoreacted with pcaK9. (B) An enlarged view of K9 shown in A. (C) Similar to A, except that the sample was from rat footpad epidermis. (D) Enlarged view of K9 shown in B. Note a shift toward the basic end of the upper row polypeptides in the manchette sample (A and B) when compared to the epidermis sample (C and D). (E) One-dimensional PAGE immunoblot showing cross-reactivity between urea-extracted K9 from rat (footpad) and human (sole) epidermis.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.
We concluded that a major protein component of the perinuclear ring of the manchette displayed antigenic homology with K9 and that the pcaK9 was antigen specific.

To further confirm that K9 was a protein component of the perinuclear ring of the manchette, a mixture of monoclonal antibodies to K9 was used (see Materials and Methods). One of the antibodies in the mixture was raised footpad (Fig. 2I). We concluded that a major protein component of the perinuclear ring of the manchette displayed antigenic homology with K9 and that the pcaK9 was antigen specific.

FIG. 2. Indirect immunofluorescent localization of K9 in the perinuclear ring of a whole spermatid (A–C) and a fractionated manchette (D–F). (A–C) The perinuclear ring was detected by pcaK9 (red staining) and the microtubular mantle of the manchette was recognized using an α-tubulin antibody (green staining). The location of the acrosomal region and the components of the manchette are indicated in the phase-contrast microscopy view of the spermatid (B). (C) Merged images shown in A and B. (D and E) Fractionated manchette with a pcaK9-decorated, partially collapsed perinuclear ring (red in D) and microtubular mantle (α-tubulin antibody, green in E). (F) Merged images of D and E. (G) Immunofluorescent staining of rat footpad epidermis. The bracket indicates the predominantly immunoreactive suprabasal layer of the epidermis (pcaK9, working dilution 1:100). (H) Immunoperoxidase staining of the same preparation as in G. The bracket indicates the immunoreacted suprabasal layer of the epidermis (pcaK9; working dilution 1:1000). (I) Same preparation as in H except that preimmune serum was used as the primary antibody (working dilution 1:1000). The bracket indicates the nonimmunoreactive suprabasal layer of the epidermis. The arrow in G, H, and I indicates the position of the basal lamina. Scale bar: A–C, 4 μm; D–F, 1 μm; G, 50 μm; H, 30 μm; I, 15 μm.
against a glycine-serine-rich peptide (amino acids 4–28) with poor homology with rat K9, and therefore would not cross-react with the rat antigen. In contrast, the second antibody was raised against a peptide (amino acids 450–477) displaying 100% homology with rat K9 in a region with the highest antigenicity (amino acids 8–25). Therefore, cross-reactivity of this mcaK9 for the rat K9 protein was expected. In fact, Figs. 3D and E demonstrate that mcaK9 recognized a single 64-kDa protein band in immunoblots of purified rat manchette extracts (Fig. 3A). Work is in progress to determine the time course expression of K9 mRNA and protein during spermatogenesis as well as the immunogold electron microscopic localization of K9 immunoreactivity during the development of the manchette.

Cloning of Rat K9 cDNA from Rat Epidermis and Testis

The cloning of K9 cDNA from rat testis was undertaken to determine whether the immunocytochemical data of the perinuclear ring of the manchette correlated with the expression of a specific gene. Our preliminary PCR studies indicated that testicular K9 mRNA was not abundant and that human-derived K9 primers were not effective in our attempts to clone rat testis K9 cDNA. Since the sequence of
rat K9 was not known, we first cloned by RT-PCR and sequenced the considerably more abundant rat epidermal K9 cDNA using total RNA extracted from the footpads and K9 primers (Fig. 4). Based on obtained rat K9 cDNA nucleotide sequence information, rat-specific primers were synthesized and used to demonstrate the presence of K9 RNA in rat testis by a nested PCR procedure. The cloned and sequenced segments of rat testicular K9 were identical to the corresponding portions of epidermal K9 and clearly different from human K9 (see below).

Two approaches were used to exclude the possibility that K9-specific PCR bands might have derived from contaminating genomic DNA. First, the same primers were used to amplify testicular K9 cDNA or genomic DNA (Fig. 5A). Second, a single-stranded cDNA was synthesized from total testis RNA in the presence (RT+) or absence (RT–) of reverse transcriptase followed by PCR reactions (Fig. 5B). Both the size difference of the PCR products (0.8 kb cDNA versus 2.4 kb genomic fragment) and the absence of actin (control) and K9-specific bands in PCR from RT– reactions confirm that K9 RNA is indeed expressed in testis and is not the result of genomic DNA contamination of RNA, which, in fact, was pretreated with DNase I (see Materials and Methods).

The deduced amino acid sequence of rat K9 is shown in Fig. 6. It contains 618 amino acids with estimated molecular mass of 63,020 Da. This value matches well with that obtained by electrophoretic fractionation of rat manchette and epidermis proteins (Fig. 2). The predicted protein structure correlates with the recognizable pattern of keratins: a rod domain of 304 amino acids with well conserved start and end sequences (MQNLNSRLASY and E1ETYRKLLEG, respectively), flanked by the glycine/serine-rich head and tail domains of 141 and 173 amino acids, respectively. Of interest is the high content of phenylalanine (F) in the head domain and a repetitive motif SGGSYGGGS in the tail domain. A comparison with the human K9 protein (Fig. 6) shows a close relationship with an overall amino acid similarity of 75%. Although the identity is relatively high in all domains, gaps in the alignment of the head and tail domains were introduced to maximize the existing homology.
Expression of the K9 Gene in Round Spermatids

A round spermatid cDNA expression library was used to screen for the presence of K9 cDNA by PCR. An example of such cloning is shown in Fig. 5B (lane 2). In this case, PCR amplification was efficient, eliminating the need for nested reactions. In contrast, the same primers could not produce a detectable signal upon amplification from testicular RNA (not shown) but did so when nested primers were used (Fig. 5B, lane 1). Thus, K9 protein is expressed in round spermatids in comparison with total testis. Studies are in progress to determine K9 gene expression in rat spermatogonia and spermatocytes.

DISCUSSION

In this paper, we report that one of the components of the perinuclear ring of the manchette is an acidic polypeptide with a molecular mass of 62- to 64-kDa, particularly rich in glycine and serine/glycine in the head and tail region of the molecule with 75% amino acid similarity to human keratin 9. Keratin 9 is a relatively large acidic (type I) protein abundant in human foot soles and palms as well as in the rodent and bovine equivalents (footpad and heel pad of the hoof, respectively). K9 has not been found in the epidermis of other body sites (cf. Knapp et al., 1986). The acidic polypeptide consists of two closely associated strings of polypeptide components when resolved in two-dimensional electrophoretic fractionation maps. The finding that the rat equivalent of human K9 was expressed in spermatids was surprising since previous studies have shown that spermatogenic cells in the developing gonads of rodents (rat, Fridmacher et al., 1992, 1995; mouse, Appert et al., 1998), and adult testis (Franke et al., 1979; Appert et al., 1998) do not express keratins. However, none of the reported attempts to identify keratins in the seminiferous epithelium have used immunological probes directed to K9.

The finding of a keratin particularly abundant in the palm region in human epidermis was of concern since it could have been attributed to a laboratory contamination of rat specimens by human K9 protein during purification and preparation procedures, resulting in false positive results during protein sequencing, and in immunoblot studies using pcaK9. We have confirmed that K9 in the manchette is not a contaminant by several approaches. First, our immunocytochemical and immunohistochemical observations indicate that K9 antibodies recognize the perinuclear ring in whole rat spermatids (Figs. 2A–C) and purified manchettes (Figs. 2D–F and Figs. 3B and C), as well as in the suprabasal layer of the footpad rat epidermis and human (sole) epidermis. The latter observations are in agreement with previously published results of other laboratories (Knapp et al., 1986; Moll et al., 1987; Langbein et al., 1993). Second, K9 cDNA cloned from rat testis RNA has a sequence distinct from that of human K9. The specific-ity of the antibody was confirmed by its cross-reactivity with a rat K9 fusion protein. Fourth, a commercial K9 monoclonal antibody gave identical results in immunoblot analysis of purified manchettes (Fig. 3A) and in immuno-

---

**FIG. 6.** Comparison of the deduced amino acid sequences of rat and human K9. Numbering is indicated on the right margin. Identical amino acids are indicated between sequences. The symbol + designates conservative amino acid exchanges. The arrows demarcate the beginning and the end of the rod domain. N note the abundant aromatic residue (F) in the head domain and the glycine-rich SGGSYGGGS repeat in the tail domain. Note the high homology in all domains of human and rat K9. Gaps were introduced in the head and tail domains to maximize the existing homology.

---

**Expression of the K9 Gene in Round Spermatids**

A round spermatid cDNA expression library was used to screen for the presence of K9 cDNA by PCR. An example of such cloning is shown in Fig. 5B (lane 2). In this case, PCR amplification was efficient, eliminating the need for nested reactions. In contrast, the same primers could not produce a detectable signal upon amplification from testicular RNA directly (not shown) but did so when nested primers were used (Fig. 5B, lane 1). Thus, K9 transcripts are more abundant in round spermatid cDNA than in whole testis cDNA. This conclusion is confirmed by the immunoblotting results shown in Fig. 3A. Both pcaK9 and mcaK9 detected the antigen in protein extracts of the manchettes, but produced no detectable signal on blots of extracts from total testis (not shown). Thus, K9 protein is enriched in manchette preparations in comparison with total testis. Studies are in progress to determine K9 gene expression in rat spermatogonia and spermatocytes.

---

**Discussion**

In this paper, we report that one of the components of the perinuclear ring of the manchette is an acidic polypeptide with a molecular mass of 62- to 64-kDa, particularly rich in glycine and serine/glycine in the head and tail region of the molecule with 75% amino acid similarity to human keratin 9. Keratin 9 is a relatively large acidic (type I) protein abundant in human foot soles and palms as well as in the rodent and bovine equivalents (footpad and heel pad of the hoof, respectively). K9 has not been found in the epidermis of other body sites (cf. Knapp et al., 1986). The acidic polypeptide consists of two closely associated strings of polypeptide components when resolved in two-dimensional electrophoretic fractionation maps. The finding that the rat equivalent of human K9 was expressed in spermatids was surprising since previous studies have shown that spermatogenic cells in the developing gonads of rodents (rat, Fridmacher et al., 1992, 1995; mouse, Appert et al., 1998), and adult testis (Franke et al., 1979; Appert et al., 1998) do not express keratins. However, none of the reported attempts to identify keratins in the seminiferous epithelium have used immunological probes directed to K9.

The finding of a keratin particularly abundant in the palm region in human epidermis was of concern since it could have been attributed to a laboratory contamination of rat specimens by human K9 protein during purification and preparation procedures, resulting in false positive results during protein sequencing, and in immunoblot studies using pcaK9. We have confirmed that K9 in the manchette is not a contaminant by several approaches. First, our immunocytochemical and immunohistochemical observations indicate that K9 antibodies recognize the perinuclear ring in whole rat spermatids (Figs. 2A–C) and purified manchettes (Figs. 2D–F and Figs. 3B and C), as well as in the suprabasal layer of the footpad rat epidermis and human (sole) epidermis. The latter observations are in agreement with previously published results of other laboratories (Knapp et al., 1986; Moll et al., 1987; Langbein et al., 1993). Second, K9 cDNA cloned from rat testis RNA has a sequence distinct from that of human K9. The specific-ity of the antibody was confirmed by its cross-reactivity with a rat K9 fusion protein. Fourth, a commercial K9 monoclonal antibody gave identical results in immunoblot analysis of purified manchettes (Fig. 3A) and in immuno-
staining of perinuclear rings of fractionated manchettes (Fig. 3, compare B and C with D and E). Fifth, we demonstrated that K9 transcripts are higher in the round spermatid cDNA library than that in whole testis cDNA (Fig. 5B, compare lanes 2 and 1).

Our initial studies indicated that the overall expression of K9 RNA in rat testis was low, a finding that alerted us against using in situ hybridization procedures to determine the localization of specific transcripts in sections of rat testis. An RT-PCR procedure using rat-specific nested primers was used to overcome the obstacle of a limited amount of K9 mRNA in rat testis. The possibility of contamination of rat testis RNA with rat genomic DNA in the RT-PCR cloning experiments was excluded by conducting parallel PCR experiments using genomic DNA as a template. Figure 5A demonstrates that the same primers produced bands of different size using genomic DNA or cDNA as templates. Subsequent sequencing of the 2.4-kb genomic fragment revealed the presence of introns (not shown). In addition, RT (minus) reactions were used as controls to demonstrate the absence of genomic DNA contamination (Fig. 5B).

The sequencing of rat K9 cDNA demonstrated high homology (75%) with human K9 cDNA (Langbein et al., 1993). Although molecular variances in domains were detected, the general organization and content of the head, rod, and tail domains of rat K9 correlate with that of other keratins, and the presence of glycine and glycine/serine-rich domains in the head and tail regions is characteristic of K9. It has been proposed that these variable domains have evolved through a series of tandem duplications of distinct sequence motifs (Steinert and Roop, 1990). Although the distribution of glycine and glycine/serine residues in human and rat K9 is different, these residues are particularly abundant, probably reflecting species-specific requirements for the assembly of this protein into filaments at distinct sites to fulfill a particular function. In this respect, the presence of K9 in the footpad and perinuclear ring of the manchette could suggest that this K9 is expressed at sites subject to pronounced mechanical stress (footpad) and where a transient clumping force is required, for example, during spermatid nuclear shaping events as proposed (Rivkin et al., 1997).

Because K9 is one of the components of the perinuclear ring of the manchette and most keratins appear to be expressed in pairs, the intriguing question is whether other keratin(s) coexists with K9 within the manchette. In this respect, we have found that another rat keratin (Sak57), a protein with structural homology to human keratin 5 (manuscript in preparation), is associated with microtubules of the manchette just before being observed in the outer dense fibers of the sperm tail (Kierszenbaum et al., 1996; Tres et al., 1996). Although K9 and Sak57 have different distribution and fate during spermiogenesis, they represent examples of keratins expressed in pairs. Further mutational studies should determine the specific role of these two keratins during nuclear shaping and sperm development.

REFERENCES


Received for publication February 14, 2000
Revised August 15, 2000
Accepted August 15, 2000
Published online October 20, 2000