A new human *nm23* homologue (*nm23*-H5) specifically expressed in testis germinal cells

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Abstract We have identified a cDNA encoding a 212 amino acid protein (Nm23-H5) with 27–31% identity to the human members of the *nm23*/nucleoside diphosphate (NDP) kinase gene family. The *nm23*-H5 gene is located on chromosome 5q23–31 and is transcribed as one main transcript of 1.1 kb which is highly and specifically expressed in testis, in the spermatogonia and early spermatocytes. Nm23-H5 possesses most of the residues conserved among NDP kinases plus an additional COOH-terminus end of 55 amino acids unique to this protein. However, under usual assay conditions, Nm23-H5 expressed in *Escherichia coli* did not exhibit NDP kinase activity. These results suggest that Nm23-H5 is specifically involved in early stages of spermatogenesis.

Key words: Nucleoside diphosphate kinase; *nm23*; Testis; Metastasis; Cancer

1. Introduction

The human NDP kinase family to date comprises four members, products of the genes *nm23*-H1 [1], *nm23*-H2 [2], DR-*nm23* [3] and *nm23*-H4 [4]. NDP kinases (EC 2.7.4.6) are ubiquitous and catalyze transfer of γ-phosphates between nucleoside and deoxynucleoside di- and triphosphates [5]. Beside their role in nucleotide synthesis, these enzymes present additional functions, possibly independent of catalysis, in processes such as differentiation, cell growth, tumor progression, metastasis and development (reviewed in [6]). *nm23*-H1 is overexpressed in solid tumors as compared to normal tissues [7] and presents a reduced expression correlated with the metastatic potential of some tumors [6,8]. *nm23*-H2 encodes a protein which is the transcription factor Puf for the c-*myc* proto-oncogene and perhaps other genes [9]. DR-*nm23* was highly expressed in blast crisis transition of chronic myeloid leukemia and was shown, when overexpressed, to suppress granulocyte differentiation and to induce apoptosis of myeloid precursor 32Dc13 cells [3]. Nm23-H4, which was identified in our laboratory [4], possesses an NH_2 extension as compared to the other human NDP kinases and presents characteristics of a mitochondrial NDP kinase.

nm23-H1 and *nm23*-H2 genes are both located on chromosome 17q21.3 [10]. Their products are 88% identical and can form homo- or heterohexamers [11]. DR-*nm23* and *nm23*-H4 genes are localized on different arms of chromosome 16, at 16q13 [12] and 16p13.3 [4], respectively. Their products are respectively 65% and 67%, and 55% and 56% identical to Nm23-H2 and Nm23-H1. It is not known whether they can form polymers with the other *nm23* gene products.

Several expressed sequence tags (ESTs) of the GenBank data base, selected according to their homology to *nm23*-H4 cDNA, reconstituted a complete open reading frame (ORF). We describe here the sequence, mapping and expression of the corresponding cDNA, obtained by RT-PCR using primers deduced from the ESTs. It encodes a novel human homologue of *nm23*, named *nm23*-H5, unique among the *nm23* gene family since its product possesses a COOH extension of 55 amino acids and presents an expression which is testis specific.

2. Materials and methods

2.1. Materials

Restriction enzymes, DNA modifying enzymes and radioactive nucleotides were obtained from New England Biolabs, Gibco-BRL and Amersham Pharmacia Biotech. Ni^{2+}-nitrilotriacetic acid (Ni-NTA)-agarose resin and the His-tagged protein purification protocol were from Qiagen. Human Multiple Tissue Northern (MTN) blots and Human RNA Master Blot with poly(A)$^+$ RNAs from different human tissues were purchased from Clontech. Probes were labeled using the NEBlot kit from New England Biolabs.

2.2. Cloning of nm23-H5 cDNA

Search in the GenBank sequence data base for ESTs homologous to *nm23*-H4, using the Basic Alignment Search Tool, identified several EST-encoded proteins homologous to but different from the sequence of the *nm23* gene products so far identified. A pair of specific primers deduced from EST AA292811 (5′-GCCGCTGAGCCATAATGGAGATATC-3′) and from EST AA133350 (5′-GAAATAAATTTATTTTACTGGTAGTTACTTAAACCCTAG-3′) from human testis and pregnant uterus libraries (WashU-Merck EST project), respectively, were used for first strand cDNA synthesis and PCR amplification using human gallbladder mRNA as template. The single amplification product of 771 bp was subcloned in the pCR 2.1 vector using the TA Cloning kit from Invitrogen and sequenced automatically (Applied Biosystems, ABI) in both directions according to the protocol of Applied Biosystems (Genome Express, Grenoble, France).

2.3. Expression and purification of the human Nm23-H5 protein

Two primers with *Nde*I and *Bam*HI restriction enzyme sites were designed from the cDNA sequence (5′-GGCGGATCCATATGGAGATATCAATGCC-3′ and 5′-CCGCGGATCCTTATTAATAAGGTTCTTCTACA-3′) and used to amplify the coding region of *nm23*-H5. The PCR-amplification product was inserted into the *Nde*I-*Bam*HI sites of pET-28a(+) plasmid vector (Novagen) to express the Nm23-H5 protein fused to a hexahistidine tag at the NH_2 terminus. The sequence of the construct was verified by sequencing. For production of recombinant protein, the vector was transferred into *Escherichia coli* strain BL21(DE3) (Novagen). A colony was inoculated in LB medium supplemented with 30 μg/ml kanamycin. The

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PII: S0014-5793(98)00996-X

bacteria were grown to an optical density of 0.5 at 600 nm and expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C. The tagged Nm23-H5 protein was purified following the procedure provided by Qiagen. Bacteria were harvested by centrifugation and the bacterial pellets were lysed by sonication in 50 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 8, after a 30 min incubation on ice with 1 mg/ml lysozyme. The extracts were centrifuged at $10\,000 \times g$ for 10 min and the pellets were resuspended in denaturing lysis buffer (8 M urea, 100 mM sodium phosphate, 10 mM Tris-HCl, pH 8). The denatured extract was incubated for 1 h under mild agitation with 5 ml Ni-NTA-agarose resin in lysis buffer. The slurry was transferred to a column, rinsed with wash buffer (8 M urea, 100 mM sodium phosphate, 10 mM Tris-HCl, pH 6.3) and eluted with elution buffer (same composition as wash buffer but pH 4.5). The fractions containing the recombinant protein were pooled, equilibrated in 50 mM HEPES buffer, pH 8, containing 5 mM EDTA, 5 mM DTT and 50% glycerol and stored at −80°C.

2.4. Nucleoside diphosphate kinase assay and autophosphorylation

NDP kinase activity was measured using a coupled pyruvate kinase-lactate dehydrogenase assay as described by Agarwal et al. [5] with minor modifications. The assays were performed at 25°C in a

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                                  <--β1---><α0->   <---α1---->   <--β2-->   <--αA-->
                    1        10        20        30          40        50
                    +--------+---------+---------+------    ---+---------+-------
Nm23-H5             MEISMPPPQIYVEKTLAIIKPDIVDKEEEIQDIILR---SGFTIVQRRKLRLSPEQCSNF  57
Nm23-H1                     MANC.R.FIA....G.QRG-LVGE..K.FEQK..RL.GLKFMQA.EDLLKEH  51
Nm23-H2                     MANL.R.FIA....G.QRG-LVGE..K.FEQK..RL.AMKF..A.E.HLKQH  51
DR-Nm23             -NLFPAACTGAH.R.FLAV...G.QRR-LVGE.VR.FERK..KL.ALKLVQS.E.LLREH  59
Nm23-H4             -RHGSGG.SWTR.R..VAV...G.QRR-LVG.V.Q.FERR...L.GMKM.QAPESVLAEH  59
Rat β                       MANS.R.FIA....G.QRG-LVGE..K.FEQK..RL.GLKF.QA.EDLLKEH  51
Pigeon                     MRANC.R.FIA....G.QRG-LVGE..K.FEQK..RL.GMKFVHA.E.LLKQH  52
Droso. (Awd)               MAANK.R.FIMV...G.QRG-LVGK..E.FEQK..KL.ALKFTWA.K.LLEKH  52
Pea                           MA.Q.FIM....G.QRG-LVGE..S.FEKK..YLKGLKFVNVERAFAEKH  49
D. discoid.             .STNKVNK.R.FLAV...G.ARG-LVGE..A.YEKK..VL.GLKQ.VPTKDLAESH  55
Yeast                      MSSQT.R.FIAV...G.QRG-LVSQ.LS.FEKK.YKL.AIKLVKADDKLLEQH  52
E. coli                       AI.R.FS....NA.A.N-V.GN.FA.FEAA..K..GTKM.H.TV..ARG.  49
M. xanthus                    AI.R..S.....GLE.G-V.GK..S.FEEK.LKP.AI.LQH..QA.AEG.  49
B. subtilis                   MM...FIMV...G.QRQ-L.G..LS.FERK.LQLAGAKLM.VTEQMAEKH  49
P. aerugin.                  MALQR..S.....A.S.N-V.GE.LT.FEKA.LRV.AAKMVQ..EREAGG.  50
  Consensus                       * T   *KP* *     *  * R    G      *          *

                                <---α2--->  <--β3-->   <--α3--->    <-------Kpn-----
                     60        70        80        90       100       110
                    --+---------+---------+---------+---------+---------+-------
Nm23-H5             YVEKYGKMFFPNLTAYMSSGPLVAMILARHKAISYWLELLGPNNSLVAKETHPDSLRAIY  117
Nm23-H1             ..DLKDRP..AG.VK..H...V...VWEGLNVVKTGRVM..ET.PADS.PG---TI.GDF  108
Nm23-H2             .IDLKDRP...G.VK..N...V...VWEGLNVVKTGRVM..ET.PADS.PG---TI.GDF  108
DR-Nm23             .A.LRERP.YGR.VK..A...V...VWQGLDVVRTSRA.I.AT.PAD.PPG---TI.GDF  116
Nm23-H4             .QDLRR.P.Y.A.IR......V...VWEGYNVVRASRAMI.HTD.AE.APG---TI.GDF  116
Rat β               .TDLKDRP..TG.VK..H...V...VWEGLNVVKTGRVM..ET.PADS.PG---TI.GDF  108
Pigeon              .IDLKDRP.Y.G.VK..N...I...VWEGLNVVKTGRVM..ET.PADS.PG---TI.GDF  109
Droso. (Awd)        .ADLSARP...G.VN..N...V.P.VWEGLNVVKTGRQM..AT.PADSLPG---TI.GDF  109
Pea                 .ADLSA.P..SG.VD.II...V....WEGKNVVTTGRKII.AT.PAQSEPG---TI.GDF  106
D. discoid.         .A.HKERP..GG.VSFIT...V...VFEGKGVVASARLMI.VT.P.ASAPG---.I.GDF  112
Yeast               .A.HV..P...KMVSF.K...IL.TVWEGKDVVRQGRTI..AT.P.GSAPG---TI.GDF  109
E. coli             .A.HD..P..DG.VEF.T...I.VSV.EGEN.VQRHRD...AT.PAN.LAG---T...D.  106
M. xanthus          .AVHKARP..KD.VQF.I...V.L.V.EGEN.VLANRDIM.AT.PAQ.A.G---TI.KDF  106
B. subtilis         .A.HQ..P..GE.VEFIT...VF..VWEGENV.EVTRQ.I.KT.PKE.LPG---TI.GD.  106
P. aerugin.         .A.HKARP..KD.VSF.T...V.VQV.EGED..AKNR..M.ATDPKK.DAG---TI..DF  107
  Consensus         Y      * F*  *   ** SGP*    *       *      **G  **   *  *    **R  *

                    --->   <-β4->  <----α4--->
                     120       130       140       150       160       170
                    --+---------+---------+---------+---------+---------+-------
Nm23-H5             GTDDLRNALHGSNDFAAAEREIRFMFPEVIVEPIPIGQAAKDYLNLHIMPTLLEGLTELC  177
Nm23-H1             CIQVG..II...DSVES..K..GLW.HPEELVDYTSCAQNWI.E°                152
Nm23-H2             CIQVG..II...DSVKS..K..SLW.KPEELVDYKSCAHDWV.E°                152
DR-Nm23             CIEVGK.LI...DSVES.R...ALW.RADELLCWEDSAGHWL.E°                160
Nm23-H4             SVHIS..VI.A.DSVEG.Q...QLW.QSSELVSWAD.GQHSSIHPA°              162
Rat β               CIQVG..II...DSVKS..K..SLW.QPEELVEYKSCAQNWI.E°                152
Pigeon              CIQVG..II...DSVES.QK..NLW.KPAELIDFKSCAHDWI.E°                153
Droso. (Awd)        CIQVG..II...DAVES..K..ALW.N.KELVTWTPAAKDWI.E°                153
Pea                 AI.IG..VI...DAVES.NK..ALW...GA-ANWESSLHSWI.E°                149
D. discoid.         .V.VG..II...DSVES.N...ALW.KPEELLTEVKPNPN-L.E°                155
Yeast               .I.LG..VC...DSVDS.....NLW.KKEELVDWESN..KWI.E°                153
E. coli             ADSLTE.GT...DSVES.A...AYF.G.GE.C.RTR°                        142
M. xanthus          A.SIDK.TV...DSLEN.KI..AYF.R.TEIHSY.YQK°                      144
B. subtilis         .MFVGK.II...DSLES.....NIF.KNEELVSYQQLM.GWI.°                 149
P. aerugin.         AVSIDE..V...DSE.S.A...VYF.ADTE.CERIR°                        143
  Consensus               N  H*S*    A  EI   F    *

                     180       190       200       210
                    --+---------+---------+---------+---
Nm23-H5             KQKPADPLIWLADWLLKNNPNKPKLCHHPIVEEPY°                         212
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Fig. 1. Alignment of the amino acid sequence of the human Nm23-H5/NDP kinase with other NDP kinases of either human (Nm23-H1, Nm23-H2, DR-Nm23 and Nm23-H4), eukaryotic (rat, pigeon, *Drosophila*, pea, slime mold and yeast) or prokaryotic (*E. coli*, *M. xanthus*, *B. subtilis* and *P. aeruginosa*) origin. The numbering at the top refers to the Nm23-H5 sequence. A dot indicates that the amino acid residues are identical to the Nm23-H5 sequence. The consensus sequence represents identical (one-letter code) and conserved (*) residues within all sequences. Identical residues in bold typeface are those involved in nucleotide binding and catalysis [19–21]. Gaps are indicated by dashes and the COOH terminal residue by °. The sequences are either referenced in [4] or available through their SwissProt database accession number: Q90380 for pigeon, P47922 for garden pea, P32917 for yeast, P31103 for *B. subtilis*, and Q59636 for *P. aeruginosa*. Secondary structural elements, named and defined as in [20], are also indicated.

1 ml reaction mixture containing 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.1 mM NADH, 1 mM ATP, 0.2 mM dTDP, 0.1 mg/ml bovine serum albumin and 2 units of pyruvate kinase and lactate dehydrogenase. The reaction was started by the addition of 50 μl (about 50 μg) of the purified protein. To detect the autophosphorylated intermediate, the reactions were performed with purified Nm23-H5 (1 μg) for 10 min at 4°C with 1 μCi [γ-^{32}P]ATP in 50 mM HEPES buffer pH 8 containing 1 mM EDTA and 1 mM $MgCl_2$. As a control, 0.5 ng of the purified *E. coli* NDP kinase (a kind gift from Dr. M. Konrad) was autophosphorylated in parallel. The samples were diluted in Laemmli buffer without boiling and run on a 4–20% SDS-PAGE. The gel was dried and the radioactivity was detected by autoradiography.

2.5. Northern blot analysis

MTN blots and Human RNA Master Blot with poly(A)$^+$ RNAs from different human tissues were probed according to the protocol provided by Clontech using the 771 bp PCR amplification product radiolabeled with [α-^{32}P]dCTP (6000 Ci/mmol) by random priming.

2.6. In situ hybridization

Human testis samples were taken at surgery and kept frozen at −80°C until use. Sections (10 μm) were cut in a cryostat, mounted on gelatine-coated microscope slides and stored at −20°C. A 24-mer oligonucleotide probe (5′-GATGTGTCTCCTTCGCTACTAAGC-3′) complementary to nucleotides 353–377 of the coding region of the human Nm23-H5 was labeled using the 3′-terminal deoxynucleotidyl transferase (Promega) and digoxigenin-11-deoxyuridine triphosphate (DIG-dUTP, Boehringer Mannheim). The tailing of the probe was performed by incubating 300 pmol of oligonucleotide for 45 min at 37°C in 12 μl tailing buffer 5× (500 mM potassium cacodylate, 5 mM $CoCl_2$, 0.5 mM dithiothreitol) containing 50 mM DIG-dUTP, 0.5 mM deoxy-ATP and terminal transferase (1 U/μl) in a final volume of 60 μl. The tailed probe was purified by spin column chromatography on RNase-free Sephadex G-50. For hybridization, the sections were fixed and treated as reported by Chen et al. [13] with minor modifications. Each section was prehybridized for 2 h at 37°C in 100 μl of hybridization buffer (50% deionized formamide, 0.1 M sodium phosphate (pH 7.0), 4×SSC, 1×Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.01 M DTT, 1% sodium laurylsarkosine and 1 mg/ml denatured salmon sperm DNA) under Nescofilm coverslips in a humid chamber. The sections were briefly rinsed in 2×SSC, dehydrated using ethanol, and air dried. Hybridization was performed for 16 h at 42°C in 100 μl of hybridization buffer containing 10% dextran sulfate and 100 nM DIG-labeled probe under Nescofilm coverslips in a humid chamber. After hybridization, the sections were rinsed at room temperature in 2×SSC (2×10 min), then for 15 min at 40°C in 2×SSC, and finally at 40°C in 1×SSC (2×15 min). Hybridization signals were detected using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim) with alkaline phosphatase-conjugated anti-DIG antibody and nitroblue tetrazolium salt/bromo-chloro-indolyl-phosphate (NBT/BCIP) as chromogen. Sections were then counterstained using nuclear fast red to aid visualization of nuclei.

2.7. Chromosomal localization

The *nm23*-H5 gene was localized by PCR analysis of radiation cell hybrids (90 hamster somatic cell hybrid lines from GeneBridge4 panel) [14] using a pair of specific oligonucleotides (upstream primer: from base 163 to base 182 of the cDNA, 5′-GCCCTGAGCAATGTAGTAAC-3′ and downstream primer: from base 323 to base 342 of the cDNA, 5′-GATGTGTCTCCTTCGCTACT-3′). We verified that there was no amplification of the hamster genomic DNA and that the amplified band of human genomic DNA was of the expected size (179 bp, not shown).

3. Results

3.1. Isolation of human cDNA related to the nm23 gene family

The cDNA sequence (data not shown, GenBank accession number Y14992), obtained from two independently isolated clones, starts 14 bp before two in frame ATG. Only the upstream one fits the Kozak consensus sequence for a eukaryotic initiation codon [15]. It is preceded by an in frame TGA stop codon suggesting that the protein is indeed translated from this ATG. The 639 nucleotide ORF ends with a TAA stop codon and predicts a 212 amino acid protein with a calculated M_r of 24 200 and a deduced p*I* of 6.6, indicating an acidic protein. When compared to NDP kinase sequences from different organisms (Fig. 1), it possesses a COOH extension of 55 amino acids which is unique to this enzyme. Considering the rest of the primary sequence, homology with NDP kinases was seen to extend throughout their common sequence but Nm23-H5 is more divergent than the other NDP kinases which all share at least 42% identity. Indeed, this sequence is 29.5, 30.8, 26.9, and 30.6% identical to Nm23-H1, Nm23-H2, DR-nm23 and Nm23-H4, respectively. In addition, it presents two insertions, of one (aa 28) and of three residues (aa 109–111) and one deletion of three residues (position 36) not found in any of the other NDP kinases so far sequenced. Nm23-H5 possesses at position 127, the specific histidine residue which is phosphorylated during catalysis in the other NDP kinases. Around this histidine, the NDP kinase consensus motif (NXXHG/ASD) [16] presents one change, of an Asp (D) to an Asn (N), which is not found in any of the others.

3.2. Expression and purification of the recombinant Nm23-H5 protein and assay of its NDP kinase activity

Purification of Nm23-H5 was carried out as described in Section 2. As shown in Fig. 2A, most of the protein was expressed in an aggregated form (inclusion bodies) and obtained in the pellet. Nm23-H5 was purified by chromatography

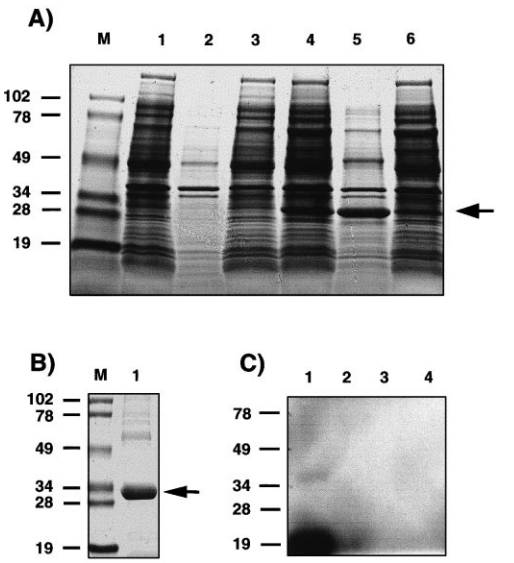

Fig. 2. Expression and purification of Nm23-H5 and assay of autophosphorylation. Nm23-H5 was purified from recombinant bacteria as described in Section 2. A: SDS-PAGE was performed, with bacteria transformed with the pET-28a(+) vector, with no insert (lanes 1–3) or containing the *nm23*-H5 insert (lanes 4–6), using total bacterial extract (lanes 1 and 4) and the soluble (lanes 2 and 5) and pellet (lanes 3 and 6) fractions obtained after centrifugation. B: The 6×His-tagged protein was purified from the pellet after denaturation with 8 M urea (lane 1) by chromatography through a nickel column as described in Section 2. C: Autoradiogram of a dried gel: lanes 1 and 2, *E. coli* NDP kinase; lanes 3 and 4, Nm23-H5. Lanes 2 and 4, reactions were further incubated for 10 min at 4°C in the presence of 100 μM GDP. Positions of molecular mass markers (in kDa) and of the Nm23-H5 protein are indicated on the left and on the right, respectively.

through a Ni-NTA-agarose column, after solubilization of the inclusion bodies with 8 M urea. The protein was approximately 80% pure, as judged by Coomassie blue staining of an SDS-PAGE gel (Fig. 2B). This purified Nm23-H5 protein did not exhibit any NDP kinase activity following renaturation by dilution under conditions which allowed full renaturation and restoration of activity of the Nm23-H4 NDP kinase (data not shown). In addition, although NDP kinases rapidly form a phosphoenzyme intermediate at 0°C in the presence of EDTA when incubated with ATP, Nm23-H5 failed to autophosphorylate under these experimental conditions. A control was run in parallel, demonstrating autophosphorylation of the *E. coli* enzyme (Fig. 2C) under similar conditions. As expected for a NDP kinase, the incorporated radioactivity was readily displaced by further incubation with unlabeled GDP. We also observed a lack of NDP kinase activity and autophosphorylation for the small amount of Nm23-H5 protein present in the bacterial supernatant and purified under native conditions (data not shown).

3.3. Northern blot analysis of nm23-H5 mRNA

The expression pattern and size of the *nm23*-H5 mRNA were analyzed using Northern blots of poly(A)$^+$ RNAs from 16 different human tissues (Fig. 3). A prominent 1.1 kb band was detected in testis together with a less abundant mRNA at 1.4 kb. Trace amounts of the 1.1 kb mRNA was detected in kidney together with the larger mRNA which was also detected in brain. A dot blot containing 50 human tissue poly(A)$^+$ mRNAs (Human RNA Master Blot) was also probed and showed expression only in testis and kidney and trace amounts in trachea and in some brain structures such as caudate nucleus and medulla oblongata (not shown).

3.4. In situ hybridization of nm23-H5 to human testis

The cellular localization of *nm23*-H5 mRNA expression was determined by in situ hybridization on human testis sections. The specific signal exhibited a crescent-shape distribution inside the tubules (Fig. 4B–D), compatible with the helical organization and morphogenesis of the human seminiferous tubule [17]. Such a distribution is characteristic of stage specific expression of *nm23*-H5 during human spermatogenesis. The positive cells correspond to spermatogonia located along the basement membrane as well as early spermatocytes distant from the boundary of the tubules. A sparse and weak labeling of extratubular cells was occasionally observed. Under identical conditions the control probe did not give any signal (Fig. 4A).

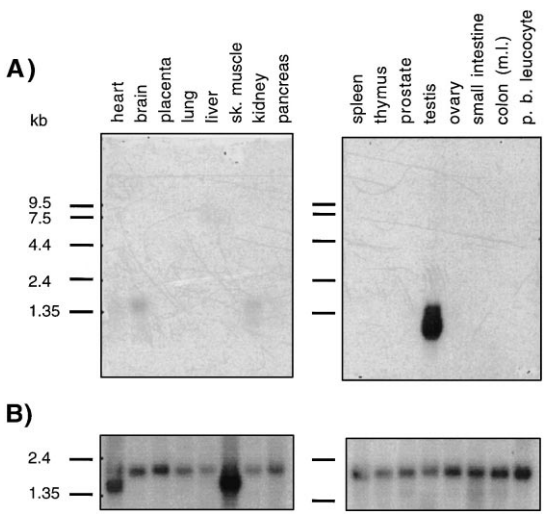

Fig. 3. Expression analysis of *nm23*-H5. Blots, containing 2 μg of poly(A)$^+$ RNAs of the indicated human tissues, were hybridized with the *nm23*-H5 cDNA probe (A) and the β-actin probe to compare relative loadings of each lane (B). The size of RNA standards is indicated on the left. Shown here is an overnight exposure. Abbreviations: sk., skeletal; m.l., mucosal lining; p.b., peripheral blood.

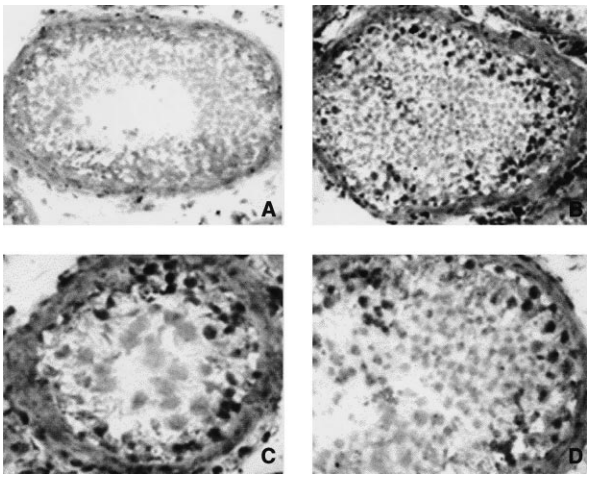

Fig. 4. Germinal cell localization of *nm23*-H5 in human testis detected by in situ hybridization. In situ hybridization to human testis sections of sense (A) or antisense (B, C, D) DIG-labeled oligonucleotide probes specific for *nm23*-H5. Signals indicating the presence of *nm23*-H5 transcript were confined to the seminiferous tubules in association with germinal cells (B, C, D). Sections were counterstained with nuclear fast red. Original magnifications: 100× (A, B) and 250× (C, D).

3.5. Chromosomal localization of the nm23-H5 gene

The PCR analysis of radiation cell hybrids revealed that the *nm23*-H5 gene closely mapped to the marker AFM240xg3 (D5S500) on chromosome 5q23–31 at a distance of 3 $cRay_{3000}$, as indicated by a pairwise lod score of 17.8, according to the method previously described [14]. No genetic disorder has been found associated with this region.

4. Discussion

The data presented herein identify another human gene homologous to *nm23*, located on chromosome 5 and specifically expressed in testis. Unique to Nm23-H5 is a 55 amino acid extension at the COOH terminal end. Curiously, this extension contains a 35 residue stretch 42% identical to a part of the *Caenorhabditis elegans* dpy-30 protein which is nuclear and essential at early stages of embryogenesis for dosage compensation of the X chromosome [18]. This particular domain does not possess any known function.

X-ray structures of NDP kinases [19,20] and site-directed mutagenesis experiments [21] identified nine residues, conserved among NDP kinases and important for quaternary structure, nucleotide binding and catalysis. Seven (Lys-20, Tyr-58, Phe-66, Arg-114, Asn-124, His-127 and Glu-138) of these nine residues are present in Nm23-H5. The two divergent ones (Leu and Asn at positions 94 and 100 in place of

Arg and Thr) are located in the *Kpn* loop which lines the catalytic site and maintains the quaternary structure. They are involved in interaction with the β-phosphate [22]. Their single mutation in the *Dictyostelium discoideum* NDP kinase greatly reduced but did not suppress the catalytic activity [21]. The *Kpn* loop is named after the presence of the *Killer of prune* mutation (proline to serine) of the *Drosophila* Awd NDP kinase [23]. This mutation is lethal in conjunction with the *prune* eye color mutation, but the mechanism involved is still unknown. Interestingly, the Nm23-H5 protein also contains a serine at this critical position (102), which is only found in Nm23-H4 [4] and in the pigeon mitochondrial NDP kinase [24].

Although the structure of the monomer is essentially identical, NDP kinases form either tetramers (dimers of dimers perpendicularly associated), in most prokaryotes [25,26] or hexamers (trimers of parallel dimers) in eukaryotes [19,20]. The polymeric structure is required for activity since dissociated subunits of NDP kinases do not possess catalytic activity, although there is one catalytic site per monomer. The purified recombinant Nm23-H5 protein is devoid of detectable NDP kinase activity under the usual assay conditions and it is unable to autophosphorylate. Some features, deduced from the primary sequence, are in favor of a lack of stability of the polymeric structure which could explain the defective catalytic activity. The three amino acid deletion and insertion observed in the Nm23-H5 sequence are located in the turn, between the α_1 helix and the β_2 strand, and in the *Kpn* loop which are both involved in the contacts between trimers to form hexamers. Moreover, the COOH terminus which stabilizes the hexamer by forming an interaction between two monomers of different trimers is much longer and does not present the Tyr-Glu motif found in hexameric enzymes. The residues involved in the dimer interface, mostly in the α_1 helix and the β_2 strand, are conserved. Interestingly, the *D. discoideum* hexameric cytosolic NDP kinase is dimeric and looses its catalytic activity with a combination of the *Kpn* Pro-Ser substitution and a C-terminal deletion, probably due to a defect in the binding of the nucleotide substrate [27].

Taken together, these data suggest that the trimeric association might be greatly altered, and are strongly in favor of a dimeric structure for Nm23-H5, not observed and/or not predicted nowadays for the NDP kinase family, with a very low or no NDP kinase activity. It is possible that, in vivo, a specific interaction with a co-factor or a maturation step removing the COOH extension could be necessary for catalytic activity. One can also hypothesize that this protein possesses another function or other functions still to be discovered. Indeed, the DNA binding property of NDP kinase B/Nm23-H2 is independent of its catalytic activity [28].

Nm23-H5 is specifically expressed in testis, which is in contrast to Nm23-H1, Nm23-H2, DR-Nm23 and Nm23-H4 which are ubiquitously expressed, although at different levels depending on the tissue. However, it seems that a low level of *nm23*-H5 mRNA can be observed in other tissues. From the Northern blots, it appears that there are two mRNA, a 1.1 kb transcript which is very abundant in testis and a 1.4 kb transcript mainly observed in testis, but also in brain and kidney. In addition, using PCR amplification, a low level of *nm23*-H5 mRNA can be detected in gallbladder (data not shown), which is absent from the commercially available tissue mRNA panel. It is interesting to note that ESTs corresponding to *nm23*-H5 have been characterized from pregnant uterus as well as from tumors of the ovary and parathyroid. In situ hybridization demonstrates the presence of the *nm23*-H5 mRNA in germinal cells at early stages of spermatogenesis. Since *nm23*-H5 mRNA was not present in mature ovary, lacking the first steps of prophase I of the first meiotic division, one can postulate that this protein is important for the initial stages of spermatogenesis occurring before the first meiotic division. Future work is needed to investigate whether Nm23-H5 participates in nucleotide metabolism of germ cells or if it contributes to more subtle regulation occurring at earlier stages.

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