nmd, a novel gene differentially expressed in human melanoma cell lines, encodes a new atypical member of the enzyme family of lipases

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Abstract *nmd*, a novel gene, was isolated by applying the differential mRNA display method to human melanoma cell lines with different metastatic capacity. In a panel of 17 other human tumor cell lines, *nmd* RNA expression could only be detected at low levels in T24 (bladder carcinoma) and Caco-2 (colon adenocarcinoma). Furthermore, it was found in placenta and liver, but not in skin, colon, spleen, lung, muscle, prostate and kidney. Sequence analysis classified the *nmd* gene product as a new member of the enzyme family of lipases (almost 30% identity in amino acid sequence with other human lipases). Active site residues of lipases were conserved in NMD, but NMD lacks the regulatory lid domain, which controls entry to the active site in classical lipases. A similar deletion was earlier reported by others in the guinea pig pancreatic (phospho)lipase GPLRP2 and the phospholipase A1 from hornet venom (DolmI).

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Key words: Human cutaneous melanoma; Differential display; Lipase; Guinea pig (phospho)lipase GPLRP2; 'Lid' domain

1. Introduction

The availability of excised lesions as well as cultured cells from different stages has made human cutaneous melanoma particularly suitable for studies of tumor progression [1]. Many changes in gene expression during progression of melanoma have been characterized (for a review see Weterman et al. [2]). The use of molecular markers in tumor diagnosis has become common practice. Moreover, identification of the function of various differentially expressed genes enhances our insight in tumor biology. Recently, we have applied the differential mRNA display technique on a panel of well-defined [3] human melanoma cell lines with different metastatic capacity when xenografted into nude mice. Thus, we isolated nine differentially expressed cDNAs, five of which represented novel gene products [4]. In this report we describe the characterization of one of them, *nmd*, encoding an unknown member of the enzyme family of lipases. The predicted NMD polypeptide lacks a so-called 'lid' domain, as was earlier described for guinea pig pancreatic (phospho)lipase GPLRP2 and the phospholipase A1 from hornet venom (DolmI) [5,6].

2. Materials and methods

2.1. Biological materials

Human melanoma cell lines MV3, BLM, 530 and 1F6 [3,7], T24 bladder carcinoma, PC-3 prostate adenocarcinoma, HeLa cervix carcinoma, MCF7 breast adenocarcinoma, Caco-2 colon adenocarcinoma, A-431 epidermoid carcinoma, HT-1080 fibrosarcoma, 143B PML BK TK and MG-63 osteosarcoma were grown as monolayers, whereas U-937 histiocytic lymphoma, K-562 chronic myelogenous leukemia, KG-1 acute myelogenous leukemia, JEG-3 choriocarcinoma, JAR placenta choriocarcinoma, MOLT-4 acute lymphoblastic leukemia, Raij Burkitt lymphoma, and Jurkat lymphoma were grown in suspension. All cell lines were grown in Dulbecco's modified Eagle's medium as described before [8]. Within the panel of melanoma cell lines, 1F6 and 530 represent poorly metastasizing cell lines, with a metastasis frequency of less than 10% 3 months after subcutaneous inoculation into nude mice. The cell lines BLM and MV3 represent the highly metastatic phenotype, with over 50% metastasis frequency [3]. Excision and processing of the human tissues was performed as described before [9].

2.2. RNA isolation and Northern blot analysis

Total RNA from human tissues and xenografts was isolated using the lithium-urea procedure as described by Auffray and Rougeon [10], whereas total RNA from cell lines was isolated using RNAzol solution (Tel-Test, Friendswood, TX). RNA oligo-deoxythymidine selections were performed using oligo-deoxythymidine columns (type II, Coll. Research, Bedford, MA). 10 μg of total RNA was glyoxylated [11], size fractionated on 1% agarose gels, and blotted onto Hybond N+ (Amersham, Aylesbury, UK). To confirm that equal amounts were loaded in each lane, the blots were afterwards hybridized with an 18S ribosomal RNA probe.

2.3. Construction of cDNA libraries

A cDNA library was constructed from 5 μg of oligo-deoxythymidine selected RNA from the poorly metastatic human melanoma cell line 530, using a λZap cDNA synthesis kit (Stratagene, La Jolla, CA) as described before [4].

2.4. DNA probes and hybridization

DNA probes were radiolabeled by the random prime labelling method by Feinberg and Vogelstein [12]. Hybridization of cDNA library was performed according to standard protocols [13].

2.5. DNA sequencing and computer analysis

DNA fragments were sequenced according to the dideoxy method as described by Sanger et al. [14] using a Sequenase sequencing kit (USB, Cleveland, OH). Large fragments were sequenced by constructing a set of deletion clones using exonuclease III (Erase-a-base kit, Promega, Madison, WI). Identification of known sequences was performed using the EMBL/Genbank database [15,16]. DNA sequence analysis, including searches for motifs, alignments, and structure predictions, was performed using the CAMMSA programs MOTIFS, PILEUP, CLUSTAL V, BESTFIT, PEPTIDESTRUCTURE, PLOTSTRUCTURE, and MEMBRANE PROPENSITY which are all part of the Wisconsin Package V 7.0 [15].

2.6. DNA isolation and Southern blot analysis

Chromosomal DNA was isolated according to the method of Blin

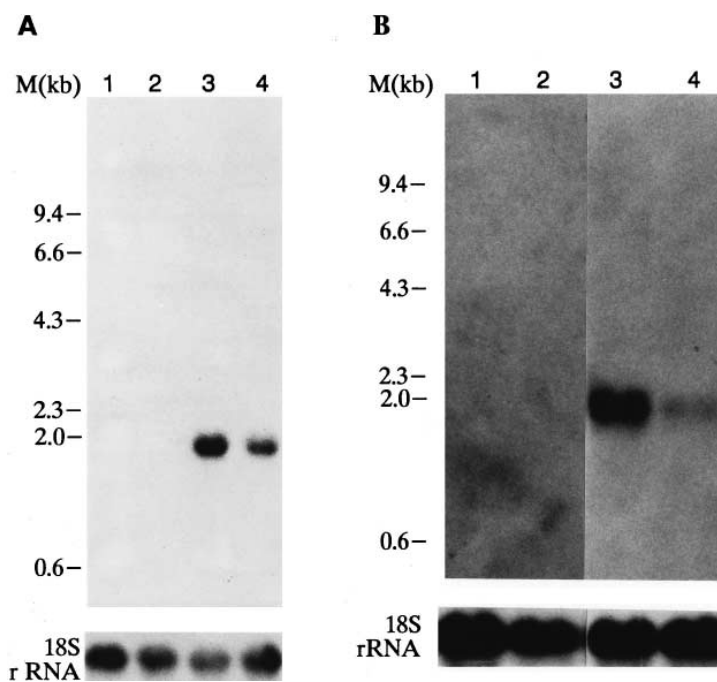*Corresponding author. Fax (31) (24) 3540525.
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Fig. 1. Northern blot analysis of a panel of human melanoma cell lines (A) and xenografts (B). Total RNA samples (10 μg) were loaded in each lane. Lane 1: BLM; lane 2: MV3: lane 3: 1F6; lane 4: 530. The blots were hybridized to radiolabeled 1.8 kb *nmd* cDNA insert of pJG454. The molecular weight marker was λDNA digested with *Hind*III. As a check of the amount of RNA loaded in each lane an 18S ribosomal RNA hybridization is shown.

and Stafford [17]. DNA was digested with *Bam*HI, and 10 μg was size fractionated on 0.6% agarose gels, transferred to Hybond N⁺ and hybridized as described previously for Northern blot analysis. To check for equal loading of DNA, the blot was hybridized afterwards to a chromosome 18-specific centromeric probe. No abnormalities involving chromosome 18 are known in relation with melanoma.

3. Results

3.1. Cloning and sequence analysis of nmd

Using the differential mRNA display technique we isolated nine cDNA clones, which were differentially expressed in human melanoma cell lines. One of these clones, clone 6, representing a gene designated *nmd*, showed a unique expression of a 1.9 kb transcript in the poorly metastatic human melanoma cell lines 530 and 1F6, and derived xenografts, whereas expression was not detected in the highly metastatic human melanoma cell lines MV3 and BLM, and xenografts derived from these cell lines (Fig. 1).

Screening of a cDNA library derived from cell line 530 resulted in the isolation of the *nmd* cDNA clone pJG454. The *nmd* cDNA sequence consists of 1767 nucleotides (U37591 GenBank). The longest open reading frame starting with a methionine codon at position 27 in the nucleotide sequence encodes a 456 amino acids long polypeptide (not shown).

Sequence analysis showed no identities with known genes or proteins, except for the 3′ and 5′ ends of a partially sequenced human cDNA clone 120969 with accession numbers T96131 and T96213 respectively. Similarities at the protein level could be detected with human and several other lipases. This is illustrated by the alignment of NMD with five different human lipases (Fig. 2). The overall identity of NMD with different human lipases is almost 30%.

Hydrophobicity plot analysis of the predicted amino acid sequence of NMD revealed a hydrophobic leader peptide with a putative signal cleavage site located 25 amino acids downstream of the translation start (Fig. 2). Potential N-glycosylation sites are located at positions 54 and 340, protein kinase C phosphorylation sites at positions 259, 344 and 379, and casein kinase II phosphorylation sites at positions −3, 74, 162, 175, 193, 244, 316 and 330. Furthermore a serine active site for lipase is located at position 141.

3.2. Northern blot analysis of nmd in human melanocytic lesions

Total RNA isolated from human melanocytic lesions was hybridized with the 1.8 kb *nmd* cDNA insert of pJG454 as a probe (Fig. 3). The expression of *nmd* mRNA could not be detected in normal skin (lane A) and three metastatic lesions (lanes K, L and M). A weak *nmd* expression was detected in cultured melanocytes (lane B), xenograft derived from radial growth phase (lane C) and vertical growth phase (lane D), and three metastatic lesions (lanes G, J and O), whereas expression was moderate to high in seven out of 13 metastatic lesions (lanes E, F, H, I, N, P and Q).

3.3. Expression of nmd is restricted to a few human organs and tumor cell lines

A Northern blot containing total RNA from gut, spleen, placenta, liver, muscle, lung, prostate, kidney cortex and kidney medulla was hybridized with the 1.8 kb *nmd* cDNA insert of pJG454 as a probe (Fig. 4). Expression of *nmd* was only detected in placenta (lane 3) and liver (lane 4). For further characterization a Northern blot containing total RNA from 17 different human tumor cell lines was screened with the *nmd* cDNA insert. No expression was

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nmd     MPPGPWESCF WVGGLILWLS VGSSG   / /     DAPPT PQPKCADFQS ANLFEGTDLK V---QFLLFV PSNPSCGQLV   42
hPL      L LWTL LL LGAVAG-   / /   KEV CYERLGCFSD DS WSGITER PLHILPWSP DVNTR    YT NE  NNF E      53
hPLRP1  LIFWTITLE LL AAKG    / /   KEV CYEDLGCFSD TE WGGTAIR PLKILPWSPE KIGTR   YT NE  NNF IL     53
hPLRP2   L PWTLGLL LLATVRG   / /   KEV CYGQLGCFSD EKPWAGTLQR PVKLLPWSPE DIDTR   YT NE  NNF I      53
hHL     DTS LCFSI LLVLC FIQS SAL   / /   GQSLK E  FGRR QAVE T KTLHEMK-  ---TR    G ET -QGC IR     42
hLPL    ESKALLVLT LAVY QSLTA SRGGVAA  / /   ADQ RRDFIDIES- ---------- ----K A RT PEDTAEDTCH       28
                                                                      *****     *      *

            *      *   ****** *        *    *    **       *   ***        *   *   * **** *
nmd     EG--SSDLQN -SGFNATLGT KLIIHGFRVL GTKPSWIDTF IRTLLRA--T NANVIAVDWI YGSTGVYFSA VKNVIKLSLE  117
hPL     AADS  ISGS --N KTNRK RF      -ID KGEEN LANV CKN FKV--E SV C C   K G  RTG TQ SQ IRIVGA    128
hPLRP1  LLSDP TIEA - N QMDRK RF      -ID KGDE  VTDM CKK FEV--E EV C C   K K  QAT TQ AN  RVVGAQ    129
hPLRP2  TGTEPDTIEA - N QLDRK RF      -LD KAED  PSDM CKKMFEV--E KV C C   R H  RAM TQ Q  IRVVGA     129
hHL     INHPDTLQEC --   SS PL VM  WS D  VLEN  WQM VAA KSQPAQ PV  GL      TLAHDH TI   R TRLVGK     120
hLPL    LIPGVAESVA TCH  HSSK FMV  WT T  MYE  VPKL VAA YKREPD S-   V    L SRAQEH PVS AGYTKLVGQD   107
            *       *  ****** *        *   *       **      *   ***        *    *     ** *

        * **  *      *    ****** *****  * **     *     * ********* * *      ** * *** *** ***
nmd     ISLFLNKLLV -LGVSESSIH IIGVSLGAHV GGMVGQLFGG Q--LGQITGL DPAGPEYTRA SVEERLDAGD ALFVEAIHTD  194
hPL     VAY VEF QS AF Y P NV  V H    A A EA RRTN T  I R    E CFQGT PELV   PS   K  DV            206
hPLRP1  VAQM DI  T EYSYPP KV  L H    A EA SKTP -   SR    VEASFEST PE V   PS    D  DV            206
hPLRP2  TAFLIQA ST Q Y LEDV  V H    T AAEA RRL - RV R    CFQDE PE V   PS    V  DV              207
hHL     VAAL RW EE SVQL R HV  L Y    V S FA SSI THKI R    A  LFEGS APSN  SPD   S  D    F        200
hLPL    VAR I WMEE EFNYPLDNV  LL Y    A A IA S --T NKKVNR    NFEY EAPS  SPD   D  DVL  F          185
        * **  *       *    ****** *****  * **    *     * ********* * *      ** * *** *** ***

          *  *   ************   ****        ***       * * ***     *   **  *        **
nmd     TD------NL GIRIPVGHVD YFVNGGQDQP GC-------- ---PTFFYAG YSYLICDHMR AVHLYISALE NSCPLM-AFP  256
hPL     GAPIVPNLGF MSQV   L F P  VEM   KKNILSQI VDIDGIWEGT RDFAA N L  SYKY TDSIV  PDGFA G        285
hPLRP1  AAPLIPFLGF TNQQM  L F P  ESM   KKNALSQI VDLDGIWAGT RDFVA N L  SYKY LESIL  PDGFA  Y       285
hPLRP2  SSPIVPSLGF MSQK   L F P  KEM   KKNVLSTI TDIDGIWEGI GGFVS N L  SFEY S SVL  PDGFL GY       286
hHL     REHMG LSV  KQ I  Y FYP  SF    HFLELYRH IAQHG- N I TQTIK S E  S   F DS L  HAGTQSM Y       278
hLPL    RGSPG RSI  QK    IYP  TF    NIGEAIRV IAERGL-GDV DQLVK S E  SI  F DS L  EENPSK YR        263
          *  *   ************   ****        ***       * * ***     *   **  *        **

        * *  *     *  *        *   *             ** *       *      * *
nmd     CASYKAFLAG RCLDCFNPFL LSCPRIGLVE QGGVKIEPLP KEVKVYLLTT SSAPYCMHHS LVEFHLKELR NKDTNIEVT-  335
hPL      NV T N K FP PSG-- -G  QM HYA -DRYPGKTND VGQ F  D G DASNFARWRY K SVT SGKK VTGHILVSL-     360
hPLRP1   T  S ESD K FP PDQ-- -G  QM HYA -DKFAGRTSE EQQ FF N G EASNFARWRY G SIT SGRT ATGQIKVAL-    360
hPLRP2   DE QES K FP PDE-- -G  KM HYA -DQF GKTSA V QTFF N G ESGNFTSWRY K SVT SGKE KVNGY RIAL     362
hHL     GDMNS SQ L  S KKG-- -R NTL YHV RQEPRSKS-- --KRLF V R AQS FKVY Y QLKIQF-INQ TETPIQTTFT    350
hLPL    S KE  EK L  S RKN-- -R NNL YEI NKVRAKRS-- --S M  K R QM  KVF Y Q KI FSGTE SETHTNQAFE    336
        * *  *     *  *        *   *             ** *       *      * *

              *              *            **** *    *** *
nmd     ---FLSSNIT SSSKITIPKQ QRYGKGII-- -AHATPQCQI NQVKFKFQSS NRV---WKKD ---------R TTII------  391
hPL      GNKGN-          YEIF  TL-- -KPDSTHSNE FDSDVDVGDL QM KFI Y-N NV-------I NPTL------       412
hPLRP1   GNKGN --------H YSIFR L-- -KPGSTHSYE FDA LDVGTI EK KFL -NN NV-------I NPTL------        412
hPLRP2   YG NEN- -------  YEIF SL-- -KPDASHTCA ID D NVGKI QK KFL -NK RG-------I NLSE------       414
hHL     MSLLGTKE-- -------- M KIPITLGKG IASNKTYSFL ITLDVDIGEL IMIKFK ENS AVWANVWDTV Q  PWSTGP    420
hLPL    ISLYGTVA-- --------ES ENIPFTLPE- VSTNKTYSFL IYTEVDIGEL LMLKLK KSD SYFS--WSD- -----WWSSP  397
              *              *            **** *    *** *

          * *  *        * ***    *    *
nmd     --GKFCTALL PVNDREKMVC LPEPVNLQAS VTVSCD-L                                           426
hPL      --PRVGASKI I ETNVG--- -K-QF FCSP E  REEV LT LTPC                                   449
hPLRP1   --P VGATKI T QKG E--- -KTVY FCSE D  RE T LT LTPC                                   450
hPLRP2   --P LGASQI T QSG D--- -GTEY FCS D  EENV QS LYPC                                    452
hHL     RHSGLVLKTI R KAG T--- -QQRMTFCSE N --D L LR PTQEKIFVKC EIKSKTSKRK IR                476
hLPL    ---GFAIQKI R KAG T--- -QKK IFCSR EK --SH QK GKAPAVFVKC HDKSLNKKSG                   448
          * *  *        * ***    *    *
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Fig. 2. Alignment of the predicted protein NMD with the other human lipases (hPL: human pancreatic lipase; hPLRP1: human pancreatic lipase-related protein 1; hPLRP2: human pancreatic lipase related protein 2; hHL: human hepatic lipase; hLPL: human lipoprotein lipase). Published sequences of other human lipases [28–32] are compared with our sequence. The alignment starts with the leader peptide and the cleavage sites are indicated by slashes (/ /). Numbering starts from the first amino acids of the mature products. Identical amino acids are indicated by empty spaces, amino acid deletions by dashes (-). Conservation of amino acids is indicated by an asterisk (*) above and below the sequences; bold in the case of a conservation in at least five out of six sequences. See text for further details.

found in cell lines derived from carcinomas of the prostate, cervix and breast or from choriocarcinoma, epidermoid carcinoma, lymphoma and myeloid leukemia. In T24 cells (bladder carcinoma) and Caco-2 cells (colon carcinoma), the 1.8 kb *nmd* transcript was only detected at low levels (results not shown).

4. Discussion

This paper describes the characterization of *nmd*, a novel gene, expressed in two melanoma cell lines with a low metastatic potential and in xenografts derived from these cell lines. Expression was absent in two highly metastatic cell lines and
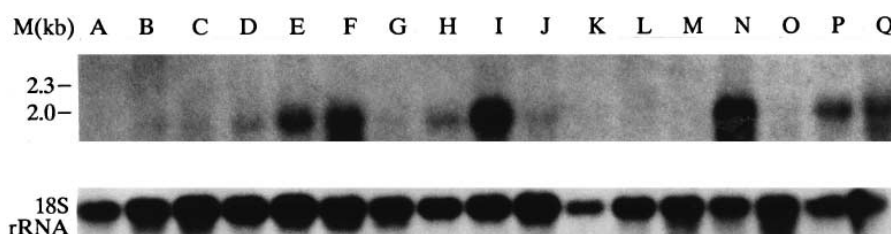
M(kb) A   B   C   D   E   F   G   H   I   J   K   L   M   N   O   P   Q

2.3–
2.0–

18S
rRNA

Fig. 3. Northern blot analysis of human cutaneous lesions. Total RNA samples (10 μg) were loaded in each lane. Lane A: normal skin (13 biopsies from 12 patients); lane B: melanocytes; lane C: xenograft derived from radial growth phase; lane D: xenograft derived from vertical growth phase; lanes E–Q: melanoma metastatic lesions. The blot was hybridized to radiolabeled 1.8 kb *nmd* cDNA insert of pJG454. The molecular weight marker was λDNA digested with *Hin*dIII. As a check of the amount of RNA loaded in each lane an 18S ribosomal RNA hybridization is shown. Densitometric scanning showed that the maximum variation in 18S rRNA loading (between lanes K and C) was not more than 3-fold, whereas this was 50-fold for *nmd* mRNA (between lanes N and G, lanes A, K, L and M being negative).

derived xenografts (Fig. 1). The differences in *nmd* mRNA expression between poorly and highly metastasizing melanoma cell lines cannot be ascribed to gene amplification or other major chromosomal rearrangements, as demonstrated by Southern blot analysis (results not shown).

Sequence analysis revealed that *nmd* is a novel gene encoding a 50 kDa protein, which probably represents a new member of the enzyme family of lipases [5,18,19]. The similarity with other lipases is sufficiently high to classify NMD as an α/β-hydrolase-fold enzyme [20,21]. Amino acid residues 135–145 (IHIIGVSLGAH), centered around Ser-141, are completely homologous to the consensus for the active site of lipases (Fig. 2). Beside Ser-141, NMD also contains two other active site residues (Asp-165 and His-235) and the oxyanion residue Phe-66. When compared to other human lipases, NMD is characterized by a deletion of 11 amino acids between Cys-220 and Pro-221. The deleted sequence constitutes a large part of the so-called lid domain of the other lipases (Fig. 2). The lid domain or 'flap' regulates access to the active site of the classical pancreatic lipase [19]. A similar deletion is present in guinea pig pancreatic (phospho)lipase GPLRP2 and in phospholipase A1 from hornet venom (DolmI), two other member of the lipase family [6]. In the absence of an aggregated lipid substrate, the lid domain prevents access to the active site. In the presence of water-insoluble substances, the lid domain as well as another surface loop (the so-called β5 loop) undergo large conformational changes thus opening access to the active site and creating the oxyanion hole [5,19,21]. In GPLRP2, containing a 'mini-lid', the catalytic site is freely accessible, and it displays a high phospholipase activity [5,6]. In the pancreatic (phospho)lipase of the coypu (coypu pancreatic lipase-

related protein 2 or CoPLRP2), the lid domain is not deleted, but the stabilizing interactions observed in classical pancreatic lipase between the lid domain, the protein core and the β5 loop, are missing. The observed similarities suggest that NMD represents a new branch in the gene family of lipases and possesses an inherent phospholipase activity.

During evolution guinea pig and coypu, both South American rodents, have lost the metabolic enzyme phospholipase A$_2$ [22,23]. It is speculated that the atypical substrate specificity of GPLRP2 and CoPLRP2, caused by a deletion in the lid domain, compensates for this loss [6,24]. Our discovery of *nmd* in the human genome indicates that the use of a 'lidless' lipase is not confined to a few exceptional species.

Among the cell lines and tissues tested expression of *nmd* was restricted to some human melanoma cell lines and lesions, as well as to human liver and placenta and, only at low levels, to the tumor cell lines T24 and Caco-2. The expression profiles of *nmd* mRNA in human melanoma cell lines and derived xenografts suggest it to be a potential marker for early stages of melanoma progression. Its presence, however, in a considerable number of human melanoma metastatic lesions seems to be in contrast with this suggestion. It is remarkable that other potential early progression markers, notably *nm23* [25], *nma* [26] and *nmb* [27] display a similar expression distribution. Genes like *nm23*, *nma*, *nmb* and also *nmd* may be involved in attenuating metastatic properties of melanoma cells, possibly as a consequence of tumor-host interactions. Tumor progression may require that genes are (temporarily) switched off in the course of metastasis, but sometimes turned on again later in a secondary, distantly growing tumor [4,26].

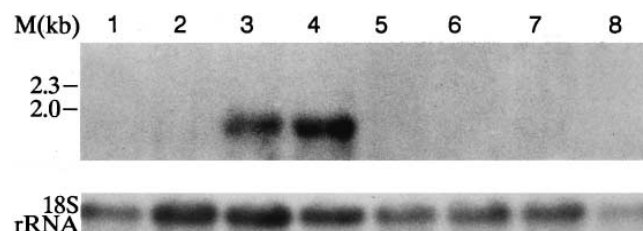M(kb)   1   2   3   4   5   6   7   8

2.3–
2.0–

18S
rRNA

Fig. 4. Northern blot analysis of several human organs. Total RNA samples (10 μg) were loaded in each lane. Lane 1: colon; lane 2: spleen; lane 3: placenta; lane 4: liver; lane 5: smooth muscle; lane 6: lung; lane 7: prostate; lane 8: kidney. The blot was hybridized to radiolabeled 1.8 kb *nmd* cDNA insert of pJG454. The molecular weight marker was λDNA digested with *Hin*dIII. As a check of the amount of RNA loaded in each lane an 18S ribosomal RNA hybridization is shown.

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