Int. J. Cancer: **65,** 460–465 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

EXPRESSION OF *nma,* A NOVEL GENE, INVERSELY CORRELATES WITH THE METASTATIC POTENTIAL OF HUMAN MELANOMA CELL LINES AND XENOGRAFTS

Winfried G.J. DEGEN[1], Marian A.J. WETERMAN[1,2], Jan J.M. VAN GRONINGEN[1], Ine M.A.H. CORNELISSEN[2], Jolanda P.W.M. LEMMERS[1], Marloes A. AGTERBOS[1], Ad GEURTS VAN KESSEL[3], Guido W.M. SWART[1] and Henri P.J. BLOEMERS[1,4]

Departments of [1]Biochemistry, [2]Pathology and [3]Human Genetics, University of Nijmegen, Nijmegen, The Netherlands.

***nma,* a novel gene, was isolated by using a subtractive hybridization technique in which the gene expression was compared in a panel of human melanoma cell lines with different metastatic potential. *nma* mRNA expression (1.5 kb) is high in poorly metastatic human melanoma cell lines and xenografts and completely absent in highly metastatic human melanoma cell lines. Fluorescence *in situ* hybridization combined with the analysis of a panel of human–rodent somatic cell hybrids indicated that the *nma* gene is located on human chromosome 10, in the region p11.2–p12.3. Sequence analysis of *nma* showed no homologies with other known genes or proteins, except for several partially sequenced cDNAs. The predicted amino acid sequence suggests that the protein encoded by *nma* contains a transmembrane domain. Expression of *nma* is high in human kidney medulla, placenta and spleen, low in kidney cortex, liver, prostate and gut and absent in lung and muscle. Whereas *nma* is not expressed in normal skin tissue, expression is high in melanocytes and in 3 out of 11 melanoma metastases tested.**

Metastatic melanoma is one of the most invasive cancers, the incidence of which has rapidly increased in the past decade. The process of metastasis is thought to be characterized by subsequent distinct stages as defined by Clark *et al.* (1984) and Herlyn *et al.* (1987), and eventually enables a primary tumor to spread and colonize at secondary sites. A set of biological markers defining unequivocally each stage of tumor development is lacking. New markers are thus sought for better diagnosis and therapeutic strategies. They may also lead to a better understanding of the tumor biology of melanoma.

In cultured human melanocytic cells, several tumor markers have been defined that are possibly indicative for metastatic potential (Weterman *et al.,* 1994). Some of these markers may be very useful in clinical practice as well as in studies of tumor biology. Several strategies have been used to isolate tumor progression markers. The use of monoclonal antibodies (MAbs), in particular, was successful in the isolation of membrane associated proteins, such as membrane receptors and adhesion molecules (Real *et al.,* 1985). A number of melanoma markers represent (potential) tumor suppressor activities, *e.g., nm*23 (Leone *et al.,* 1991) and *p16* (Kamb *et al.,* 1994).

In the last few years, we have identified several potential progression markers, *e.g.,* calcyclin (Weterman *et al.,* 1992), thymosin-β10 (Weterman *et al.,* 1993), *nmb* (Weterman *et al.,* 1995) and *nma* (this report), by using differential and subtractive hybridization techniques. Calcyclin and thymosin-β10 are expressed in highly metastatic human melanoma cell lines, whereas *nma* and *nmb* are expressed in poorly metastatic human melanoma cell lines.

The absence of expression of *nma* and *nmb* in highly metastatic cell lines does not imply a role in tumor progression. It merely indicates that these markers are part of the phenotype of a non-metastasizing cell line. Because this phenotype may be relevant for tumor biology, we now present a characterization of the *nma* gene.

MATERIAL AND METHODS

Biological materials

Human melanoma cell lines 1F6, 530, BLM, MV3 and MV1 (van Muijen *et al.,* 1991*a, b, c*) were cultured as described by Weterman *et al.* (1992). In this panel of cell lines, BLM and MV3 represent the highly metastatic cell lines, *i.e.,* those in which metastases occur in more than 50% of the tumor-bearing mice. 1F6, 530 and MV1 produced metastases in less than 10% of the tumor-bearing mice, representing the poorly metastatic cell lines (van Muijen *et al.,* 1991*a*); 3×10^6 cells were used for s.c. inoculation into nude mice (*nu/mu* BALB/c; Bomholtgaard, Ry, Denmark).

Excision and processing of the human tissues was performed as described by Weterman *et al.* (1995).

RNA isolation and Northern blot analysis

Total RNA was isolated using the lithium-urea procedure described by Auffray and Rougeon (1980). RNA oligo-deoxythymidine selections were performed using oligo-deoxythymidine columns (type II; Collaborative Research, Bedford, MA). Total RNA (10 μg) was glyoxylated (McMaster and Carmichael, 1977), size fractionated on 1% agarose gels and blotted onto Hybond N-plus filters (Amersham, Aylesbury, UK). Northern blots were hybridized according to Church and Gilbert (1984), with the addition of 0.1 mg denatured herring sperm DNA/ml of hybridization mixture. To confirm that equal amounts of RNA were loaded in each lane, the blots were hybridized afterwards to an 18S ribosomal probe.

Construction of cDNA and subtraction libraries

cDNA libraries were constructed from 5 μg of oligo-deoxythymidine selected RNA using a cDNA cloning kit (Invitrogen, San Diego, CA) or a λZAP cDNA synthesis kit (Stratagene, La Jolla, CA). Construction of the MV3-MV1 subtraction library with a subtractor kit (Invitrogen) was performed as described before (Weterman *et al.,* 1993). The poorly metastatic human melanoma cell line 530 was used for the construction of the 530 λZAP cDNA library.

DNA probes and hybridization of cDNA and genomic libraries

DNA probes were radiolabeled using the random prime method as recommended by Boehringer Mannheim (Germany). Hybridization of cDNA libraries and the human fetal brain genomic library (kindly provided by Mr. A. van Bokhoven, Department of Urology, Nijmegen) was performed for

[4]To whom correspondence and reprint requests should be sent, at Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: 31 243540525.

Received: June 29, 1995 and in revised form September 19, 1995.

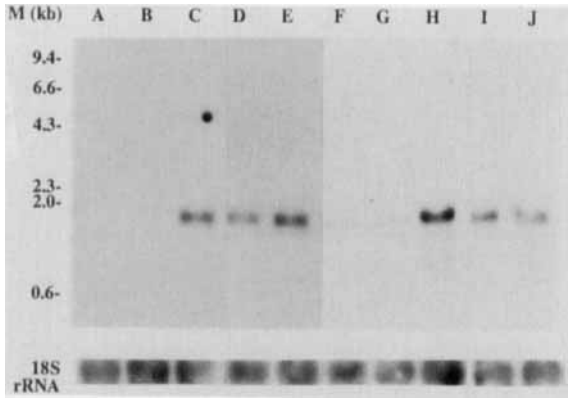

FIGURE 1 – Northern blot analysis of human melanoma cell lines and xenografts; 10 μg of total RNA were loaded in each lane. Lanes A–E, cell lines; Lanes F–J, xenografts. Lanes A and F, BLM; B and G, MV3; C and H, MV1; D and I, 1F6; E and J, 530. The blot was hybridized to radiolabeled 1.5 kb *nma* cDNA. The m.w. marker was HindIII digested λDNA. As a control, an 18S ribosomal hybridization is shown.

16 hr in 5× SSPE, 5× Denhardt's and 0.5% SDS in the presence of 0.1 mg/ml denatured herring sperm DNA at 65°C. Filters were washed in a stringent manner, using decreasing salt concentrations (2× and 1× SSPE/0.1% SDS) at room temperature and 65°C, respectively.

Chromosomal DNA isolation and Southern blot analysis

Chromosomal DNA was isolated using the method of Blin and Stafford (1976). DNA was digested with EcoRI, and 10 μg was size fractionated on 1% agarose gels. DNA was transferred to Hybond N-plus filters and hybridized as described previously for Northern blot analysis. To confirm that equal amounts of DNA were loaded in each lane, blots were hybridized afterwards to a chromosome 18-specific centromeric probe. Abnormalities involving chromosome 18 have not been described in relation to melanoma.

DNA sequencing and computer analysis

DNA fragments were sequenced according to the dideoxy method described by Sanger *et al.* (1980) 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). Searches for known DNA sequences were performed using the GenBank and EMBO databases (Devereux *et al.*, 1984; Pearson and Lipman, 1988). 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 (Devereux *et al.*, 1984).

Determination of the transcription start site of the nma *gene by S1 mapping*

Nuclease S1 mapping was performed using the method of van Leen *et al.* (1986). Briefly, 50 μg of total 530 RNA or yeast tRNA was hybridized with 1×10^6 cpm of a ^{32}P-labeled antisense ssDNA probe derived from the human genomic *nma* subclone pWD117. Subclone pWD117 consists of a 418 bp AvaI DNA restriction fragment subcloned in pGEM-3zf(−). Nucleotide positions 304 to 418 of this AvaI fragment correspond to positions 1 to 115 of the human *nma* cDNA sequence. This fragment also contains a putative TATA and CAAT box. The insert was labeled using the method of Sanger *et al.* (1980),

```
                                                                    25
                                                CTGGCGCGGGCGGGAGCTGCGGCGG
                                        60                          96
ATACCCTTGCGTGCTGTGGAGACCCTACTCTCTTCGCTGAGAACGGCCGCTAGCGGGGACTGAAGGCCGGG
                                       131                         167
AGCCCACTCCCGACCCGGGGCTAGCGTGCGTCCCTAGAGTCGAGCGGGGCAAGGGAGCCAGTGGCCGCCGA
                                       202                         238
CGGGGGACCGGGAAACTTTTCTGGGCTCCTGGAGAGCCCTGTAGCCGCGCTCCATGCTCCGGCAGCGGCCC
                                       273                         309
GAAACCCAGCCCCGCCGCTGACGGAGCCCGCCGCTCCGGGCAGGGCCCATGCCCTGCGCGCTCCGGGGGTC
                                       344                         378
GTAGCTGCCGCCGAGCCGGGGCTCCGGAAGCCGGCGGGGGCGCCGCGGCCGTGCGGGGCGTCA ATG GAT
                                                                   Met Asp
                                       405                         432
CGC CAC TCC AGC TAC ATC TTC ATC TGG CTG CAG CTG GAG CTC TGC GCC ATG GCC
Arg His Ser Ser Tyr Ile Phe Ile Trp Leu Gln Leu Glu Leu Cys Ala Met Ala
                  I | II
                                       459                         486
GTG CTG CTC ACC AAA GGT GAA ATT CGA TGC TAC TGT GAT GCT GCC CAC TGT GTA
Val Leu Leu Thr Lys Gly Glu Ile Arg Cys Tyr Cys Asp Ala Ala His Cys Val
                                       513                         540
GCC ACT GGT TAT ATG TGT AAA TCT GAG CTC AGC GCC TGC TTC TCT AGA CTT CTT
Ala Thr Gly Tyr Met Cys Lys Ser Glu Leu Ser Ala Cys Phe Ser Arg Leu Leu
                                       567                         594
GAT CCT CAG AAC TCA AAT TCC CCA CTC ACC CAT GGC TGC CTG GAC TCT CTT GCA
Asp Pro Gln Asn Ser Asn Ser Pro Leu Thr His Gly Cys Leu Asp Ser Leu Ala
                                       621                         648
AGC ACG ACA GAC ATC TGC CAA GCC AAA CAG GCC CGA AAC CAC TCT GGC ACC ACC
Ser Thr Thr Asp Ile Cys Gln Ala Lys Gln Ala Arg Asn His Ser Gly Thr Thr
                                       675                         702
ATA CCC ACA TTG GAA TGC TGT CAT GAA GAC ATG TGC AAT TAC AGA GGG CTG CAC
Ile Pro Thr Leu Glu Cys Cys His Glu Asp Met Cys Asn Tyr Arg Gly Leu His
                                       729          II | III        756
GAT GTT CTC TCT CCT CCC AGG GGT GAG GCC TCA GGA CAA GGA AAC AGG TAT CAG
Asp Val Leu Ser Pro Pro Arg Gly Glu Ala Ser Gly Gln Gly Asn Arg Tyr Gln
                                       783                         810
CAT GAT GGT AGC AGA AAC CTT ATC ACC AAG GTG CAG GAG CTG ACT TCT TCC AAA
His Asp Gly Ser Arg Asn Leu Ile Thr Lys Val Gln Glu Leu Thr Ser Ser Lys
                                       837                         864
GAG TTG TGG TTC CGG GCA GCG GTC ATT GCC GTG CCC ATT GCT GGA GGG CTG ATT
Glu Leu Trp Phe Arg Ala Ala Val Ile Ala Val Pro Ile Ala Gly Gly Leu Ile
                                       891                         918
TTA GTG TTG CTT ATT ATG TTG GCC CTG AGG ATG CTT CGA AGT GAA AAT AAG AGG
Leu Val Leu Leu Ile Met Leu Ala Leu Arg Met Leu Arg Ser Glu Asn Lys Arg
                                       945                         972
CTG CAG GAT CAG CGG CAA CAG ATG CTC TCC CGT TTG CAC TAC AGC TTT CAC GGA
Leu Gln Asp Gln Arg Gln Gln MET Leu Ser Arg Leu His Tyr Ser Phe His Gly
                                       999                        1026
CAC CAT TCC AAA AAG GGG CAG GTT GCA AAG TTA GAC TTG GAA TGC ATG GTG CCG
His His Ser Lys Lys Gly Gln Val Ala Lys Leu Asp Leu Glu Cys MET Val Pro
                                      1053                        1080
GTC AGT GGG CAC GAG AAC TGC TGT CTG ACC TGT GAT AAA ATG AGA CAA GCA GAC
Val Ser Gly His Glu Asn Cys Cys Leu Thr Cys Asp Lys MET Arg Gln Ala Asp
                                      1107                        1134
CTC AGC AAC GAT AAG ATC CTC TCG CTT GTT CAC TGG GGC ATG TAC AGT GGG CAC
Leu Ser Asn Asp Lys Ile Leu Ser Leu Val His Trp Gly MET Tyr Ser Gly His
                                      1162                        1198
GGG AAG CTG GAA TTC GTA TGA CGGAGTCTTATCTGAACTACACTTACTGAACAGCTTGAAGGCC
Gly Lys Leu Glu Phe Val
                                      1233                        1269
TTTTGAGTTCTGCTGGACAGGAGCACTTTATCTGAAGACAAACTCATTTAATCATCTTTGAGAGACAAAAT
                                      1304                        1340
GACCTCTGCAAACAGAATCTTGGATATTTCTTCTGAAGGATTATTTGCACAGACTTAAATACAGTTAAATG
                                      1375                        1411
TGTTATTTGCTTTTAAAATTATAAAAAGCAAAGAGAAGACTTTGTACACACTGTCACCAGGGTTATTTGCA
                                      1446                        1482
TCCAAGGGAGCTGGAATTGAGTACCTAAATAAACAAAAATGTGCCCTATGTAAGCTTCTACATCTTGATTT
                                      1517
ATTGTAAAGATTTAAAAGAAATATATATATTTTGTCTG(A)n
      ******
```

FIGURE 2 – Complete nucleotide sequence of *nma* (GenBank accession number U23070) and translated predicted protein. A putative polyadenylation signal is marked by asterisks. Starting points of exons II and III (see also Fig. 4 and the Results section) are also indicated.

and a single stranded DNA probe was isolated following SacI digestion and electrophoresis on a 4% polyacrylamide/7 M urea gel. Annealing was performed at 40°C overnight. Nuclease S1 (Boehringer Mannheim) was used at 1000 units/ml for 1 hr at 37°C. The remaining product was electrophoresed on a 6% polyacrylamide/7 M urea gel.

Chromosomal localization of the nma *gene*

The initial chromosomal assignment of the *nma* gene was carried out by using a panel of monochromosomal human–

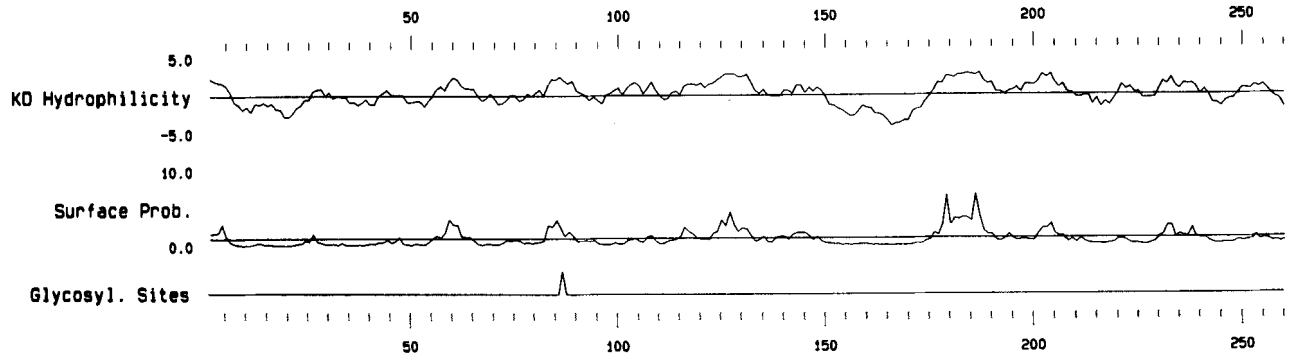

FIGURE 3 – Predicted characteristics of the protein encoded by *nma*. The upper panel shows a Kyte and Doolittle (1982) prediction of hydrophilicity. Hydrophobic regions are indicated by negative values.

rodent somatic cell hybrids (Coriell Repository, Camden, NJ) and the complete *nma* cDNA clone as a probe for Southern blot analysis. Further refinement of the localization was obtained through fluorescence *in situ* hybridization (FISH) on normal human metaphase spreads using the complete *nma*-1 genomic clone as a probe. Briefly, probe DNA was nick translated with digoxigenin-11-dUTP (Boehringer Mannheim). For efficient hybridizations, 10 ng of labeled probe were applied under a 20 × 20 mm carrier slip onto normal lymphocyte-derived metaphase spreads. Visualization of the hybridization signals was accomplished using fluorescein-conjugated sheep anti-digoxigenin and fluorescein-conjugated donkey anti-sheep antibodies (Jackson Immunoresearch, West Grove, PA). Slides were immersed in antifade solution supplemented with DAPI (Sigma, St. Louis, MO) for counterstaining of the chromosomes. Digital images were captured using a cooled CCD-camera (Photometrics, Tucson, AZ) coupled to a Macintosh computer.

RESULTS

Cloning and characterization of a differentially expressed nma *cDNA clone*

During the isolation of cDNA clones differentially expressed in a panel of poorly metastatic *versus* highly metastatic human melanoma cell lines, 2 novel genes, designated *nmb* (Weterman *et al.*, 1995) and *nma*, were identified that showed expression in the poorly metastatic human melanoma cell lines 1F6, 530 and MV1. The expression of *nma* is shown in Figure 1. *nma* mRNA expression is high in the poorly metastatic human melanoma cell lines 1F6, 530 and MV1 and derived xenografts. Expression was not detected in the highly metastatic cell lines BLM and MV3 and xenografts derived from these cell lines.

Sequencing revealed that *nma* represents a novel gene. Screening of a cDNA library derived from the poorly metastatic human melanoma cell line 530 resulted in the isolation of the *nma* cDNA clone pWD58. An S1 mapping experiment described below indicates that clone pWD58 contains the complete cDNA. The *nma* cDNA sequence with a putative polyadenylation signal, translation start site and open reading frame (260 amino acids) is shown in Figure 2. A consensus signal cleavage site located downstream of amino acid 27 (Glu) indicates that the predicted nma protein contains a signal sequence. A glycosylation site and a Kyte and Doolittle (1982) prediction of hydrophilicity are schematically depicted in Figure 3. The predicted amino acid sequence contains a transmembrane domain indicating, together with the other data, that *nma* codes for a transmembrane protein.

Sequence comparison showed no similarities with known genes or proteins, except for several partially sequenced cDNAs (150 to 500 bp) with accession numbers D25985, T73919, T85484, T90372, Z39158 and Z41678.

Isolation and partial sequencing of a genomic clone containing the nma *gene*

Screening of a human fetal brain genomic library with *nma* cDNA clone pWD58 yielded 1 positive clone with an insert of approximately 30 kb. Characterization of this genomic clone *nma*-1 was performed by producing a set of subclones using several restriction endonucleases. A 9 kb BamHI fragment was isolated that hybridized with both a 5′ and a 3′ *nma* cDNA probe and, thus, contained the complete coding sequence. Subclones of this 9 kb BamHI fragment were partially sequenced in order to locate exon-intron junctions. The gene consists of 3 exons, 448, 288 and 784 bp, respectively, separated by 2 introns of 3 kb and 435 bp (Fig. 4; GenBank accession numbers for exon-containing sequences I, U29188; II and III, U29189).

The 9 kb BamHI *nma* genomic fragment is flanked by 2 EcoRI restriction sites and also contains 1 EcoRI restriction site between exons II and III, and 1 EcoRI site in exon III (Fig. 4). When hybridizing a Southern blot containing EcoRI digested genomic DNA from human melanoma cell lines to a complete *nma* cDNA probe, 3 bands are expected to hybridize. In Figure 5, the 3 EcoRI bands are indicated by arrows. An additional band of approximately 4 kb is visible in melanoma cell line BLM that may be the result of cross hybridization.

S1 mapping was performed with a genomic *nma* antisense ssDNA probe overlapping the putative transcription start site and total RNA from the poorly metastatic melanoma cell line 530, to define precisely the 5′ transcription start site of the *nma* gene. Besides the first 115 nucleotides of the cDNA sequence, this genomic *nma* DNA fragment also contains a putative TATA and CAAT box. A transcription start region corresponding to protected transcripts of 113 to 115 nucleotides long is visible in Figure 6, indicating that 2 or 3 alternative transcription start sites are used.

Chromosomal localization of the nma *gene*

The initial chromosomal assignment of the *nma* gene was carried out via Southern blot analysis of a well-defined panel of monochromosomal human–rodent somatic cell hybrids with *nma* cDNA pWD58 as a probe. A single hybrid revealed a human-specific hybridizing fragment (not shown). This hybrid contains chromosome 10 as its only human constituent, thus mapping the gene to this human chromosome. Further refinement of the localization was obtained by using FISH with the genomic *nma*-1 clone. In this case, hybridization signals were observed in several (telomeric) chromosomal regions (not

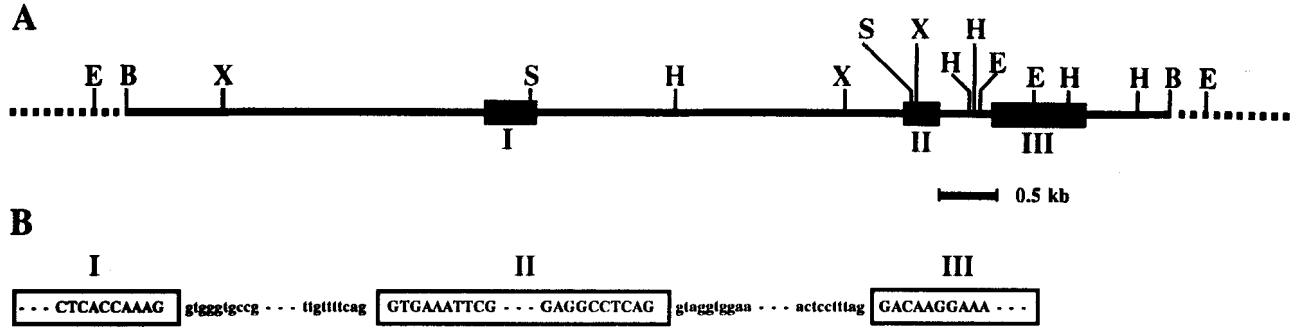

FIGURE 4 – Schematic map of the *nma* gene. *(a)* The schematic map of *nma* with the major restriction sites. The black boxes in the gene correspond to the 3 exons. B, BamHI; E, EcoRI; H, HindIII; S, SacI; X, XbaI. *(b)* Nucleotide sequences of exon-intron junctions of exons I, II and III. Lowercase letters indicate intron sequences, whereas exon sequences are indicated by capitals (GenBank accession numbers for exon-containing sequences I, U29188; II and III, U29189).

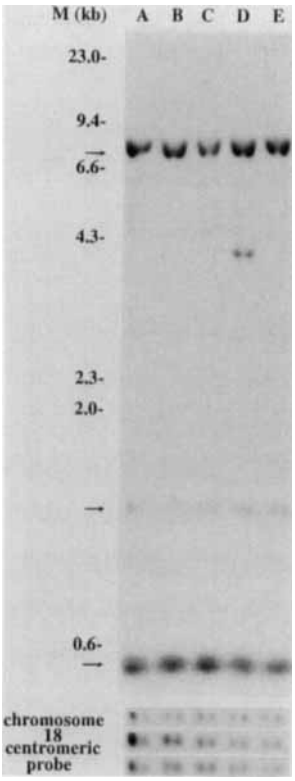

FIGURE 5 – Southern blot analysis of several human melanoma cell lines; 10 μg of EcoRI digested chromosomal DNA were loaded in each lane. Lane A, MV1; Lane B, 530; Lane C, 1F6; Lane D, BLM; Lane E, MV3 DNA digested with EcoRI. The blot was hybridized to radiolabeled 1.5 kb *nma* cDNA. Hybridizing bands are indicated with arrows. The m.w. marker was HindIII digested λDNA. As a control, a chromosome 18-specific centromeric hybridization is shown (3 bands of approximately 20, 1.3 and 0.6 kb). Abnormalities involving chromosome 18 have not been described in relation to melanoma.

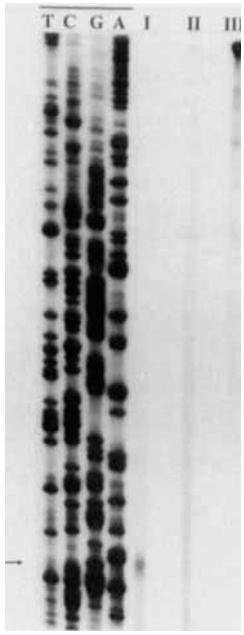

FIGURE 6 – Determination of the 5′ transcription start site of the *nma* gene by S1 mapping. An antisense *nma* ssDNA genomic subclone containing the 5′ untranscribed region with putative TATA and CAAT boxes and flanking the first 115 nucleotides of the cDNA sequence was hybridized with 50 μg of 530 total RNA or yeast tRNA. Upon treatment of the hybrid with nuclease S1, the product was analyzed under denaturing conditions (indicated with an arrow). Lane I, 530 total RNA; lane II, yeast tRNA; lane III, intact undigested ssDNA probe. A portion of the corresponding dideoxy sequencing reactions is shown at the left.

shown). A specific signal was observed on the short arm of chromosome 10, in the region p11.2–p12.3. We conclude that the *nma* gene maps to this region. A number of other signals may be due to cross hybridization and/or the presence of other *nma*-like sequences in the human genome.

Expression of nma *in human melanocytic lesions*

A Northern blot containing several human melanocytic lesions was screened for *nma* expression (Fig. 7). Whereas *nma* (indicated with an arrow) is highly expressed in melanocytes and in 3 out of 11 melanoma metastases, expression in the other metastases is low or absent. Remarkably, 3 out of 4 metastases with strong pigmentation (Lanes I, J, M) show high *nma* expression levels. *nma* does not seem to be expressed in normal skin tissue (Lane B). The upper 1.9 kb band may be the result of cross-hybridization of *nma* with the 1.9 kb 18S rRNA (compare with control hybridization).

Expression of nma *in human organs*

The expression of *nma* in several human organs was tested using a Northern blot containing kidney cortex and kidney medulla, lung, muscle, liver, placenta, spleen, prostate and gut total RNA (Fig. 8). *nma* is expressed at high levels in kidney

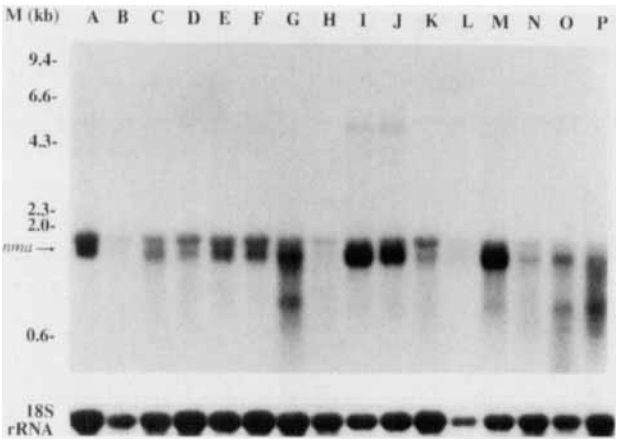

FIGURE 7 – Northern blot analysis of human cutaneous melanocytic lesions; 10 μg of total RNA were loaded in each lane. Lane A, melanocytes; Lane B, normal skin tissue (obtained from melanoma patients); Lane C, pooled dysplastic naevi (6 lesions from 6 patients); Lane D, xenograft derived from radial growth phase (RGP); Lane E, xenograft derived from vertical growth phase (VGP); Lanes F–P, melanoma metastases. Pigmentation was strong in the metastases in lanes I, J, L and M, weak in lanes F and N and absent in lanes G, H, K, O and P. The blot was hybridized to radiolabeled 1.5 kb *nma* cDNA. Expression of *nma* is indicated with an arrow. The m.w. marker was HindIII digested λDNA. As a control, an 18S ribosomal hybridization is shown.

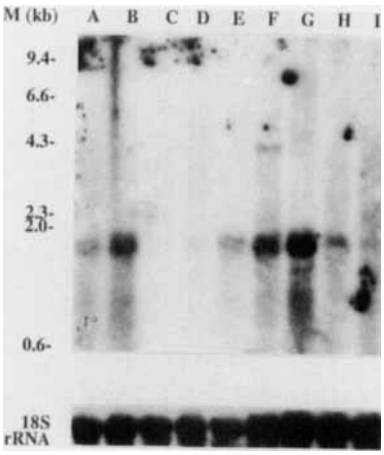

FIGURE 8 – Northern blot analysis of several human organs; 10 μg of total RNA were loaded in each lane. Lane A, kidney cortex; Lane B, kidney medulla; Lane C, lung; Lane D, smooth muscle; Lane E, liver; Lane F, placenta; Lane G, spleen; Lane H, prostate; Lane I, colon. The blot was hybridized to radiolabeled 1.5 kb *nma* cDNA. The m.w. marker was HindIII digested λDNA. As a control, an 18S ribosomal hybridization is shown.

medulla, placenta and spleen, and at moderate levels in kidney cortex, liver, prostate and gut. Expression is absent in lung and muscle. These results indicate that *nma* is not melanocyte-specific.

DISCUSSION

Our report describes the characterization of *nma,* a novel gene, whose expression is inversely correlated with the metastatic potential of a panel of human melanoma cell lines and xenografts. *nma* is highly expressed in the poorly metastatic human melanoma cell lines 1F6, 530 and MV1 and derived xenografts, and completely absent in the highly metastatic melanoma cell lines BLM and MV3 (Fig. 1). The differences in RNA expression of *nma* between poorly and highly metastatic melanoma cell lines cannot be explained by major chromosomal rearrangements, as demonstrated by Southern blot analysis (Fig. 5). The observed differences in *nma* mRNA expression may, therefore, reflect differences in promoter activity of the *nma* gene.

Sequence analysis revealed that *nma* is a new gene. The presence of a signal sequence and a hydrophobic area of 25 amino acid residues (positions 151 to 175) bordered by charged residues indicates that the gene encodes a transmembrane protein.

The *nma* gene is located on human chromosome 10 in the region p11.2–p12.3 (results not shown). Loss of heterozygosity (LOH) of chromosome 10 in relation to malignant melanoma has already been described. Isshiki *et al.* (1993) concluded from their results that a tumor suppressor gene may be located on 10q, although allelic losses on 10p were also found.

Although its expression pattern in cell lines and derived xenografts offers the perspective that *nma* could be a potential marker for early stages of melanoma progression (Fig. 1), its presence in a considerable number of human melanoma metastasis lesions is not in line with this suggestion (Fig. 7). Other (potential) early progression markers, notably *nm*23 and also *nmb,* display expression profiles similar to that of *nma.* Experimental evidence is available in support of a metastasis suppressor function for the *nm*23 gene (Leone *et al.,* 1991) and an *nmb* clone encoding an N-terminally truncated product (Weterman *et al.,* 1995). One could speculate that genes like *nm*23, *nmb* and also *nma* may be involved in attenuating metastatic properties of melanoma cells. Tumor progression may require that these genes are (temporarily) switched off in the course of metastasis but turned on again later in a secondary, distantly growing tumor. Alternatively, their expression may only partially inhibit the metastasis. Evidence for a transient role of a presumed suppressor of metastasis is given by the experiments of Florenes *et al.* (1992). They demonstrated that the level of *nm*23 mRNA was significantly lower in tumors from patients with a short interval between primary diagnosis and the appearance of metastases than in those with more prolonged relapse-free intervals. This observation was explained by assuming that the expression of *nm*23 in metastases is inversely correlated with aggressive behavior. This conclusion was strengthened by Xerri *et al.* (1994) and Caligo *et al.* (1994), who found a correlation between high expression of *nm*23 in patients with a high overall survival following metastasis resection and in cell lines derived from these metastases. It is possible that *nma* and *nmb* follow the same pattern as *nm*23 does and, therefore, are found in some but not all metastases. Although clinical data were available on the melanoma metastases used in this study, no final conclusions can be drawn because of their small number.

Expression of *nma* in several human organs indicates that the expression is not melanocyte-specific: *nma* is expressed at variable levels in most organs tested, except in lung and muscle (Fig. 8).

Further characterization of *nma,* especially of its biological functions, should eventually clarify its role in melanoma metastasis.

ACKNOWLEDGEMENTS

The authors thank Ms. D. Olde Weghuis and Dr. D. de Bruijn (Department of Human Genetics, Nijmegen) for their help in the chromosomal localization of the gene. This work was supported by the Dutch Cancer Society.

REFERENCES

AUFFRAY, C. and ROUGEON, F., Purification of mouse immunoglobulin heavy chain mRNAs from total myeloma tumor RNA. *Europ. J. Biochem.*, **107**, 303–314 (1980).

BLIN, N. and STAFFORD, D.W., A general method for isolation of high molecular weight DNA from eukaryotes. *Proc. nat. Acad. Sci. (Wash.)*, **3**, 2303–2308 (1976).

CALIGO, M.A., GRAMMATICO, P., CIPOLLINI, G., VARESCO, L., DEL PORTO, G. and BEVILACQUA, G., A low NM23.H1 gene expression identifying high malignancy human melanomas. *Melanoma Res.*, **4**, 179–184 (1994).

CHURCH, G.M. and GILBERT, W., Genomic sequencing. *Proc. nat. Acad. Sci. (Wash.)*, **81**, 1991–1995 (1984).

CLARK, W.H., JR., ELDER, D.E., GUERRY, D., EPSTEIN, M.N., GREENE, M.H. and VAN HORN, M., A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Hum. Pathol.*, **15**, 1147–1165 (1984).

DEVEREUX, J., HAEBERLI, P. and SMITHIES, O., A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395 (1984).

FLORENES, V.A., AAMDAL, S., MYKLEBOST, O., MAELANDSMO, G.M., BRULAND, O.S. and FODSTAD, O., Levels of *nm*23 messenger RNA in metastatic malignant melanomas: inverse correlation to disease progression. *Cancer Res.*, **52**, 6088–6091 (1992).

HERLYN, M., CLARK, W.H., RODECK, U., MANCIANTI, M.L., JAMBROSIC, J. and KOPROWSKI, H., Biology of tumor progression in human melanocytes. *Lab. Invest.*, **56**, 461–474 (1987).

ISSHIKI, K., ELDER, D.E., GUERRY, D. and LINNENBACH, A.J., Chromosome 10 allelic loss in malignant melanoma. *Gene Chromosomes Cancer*, **8**, 178–184 (1993).

KAMB, A., GRUIS, N.A., WEAVER-FELDHAUS, J., LIU, Q., HARSHMAN, K., TAVTIGIAN, S.V., STOCKERT, E., DAY, R.S., JOHNSON, B.E. and SKOLNICK, M.H., A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436–440 (1994).

KYTE, J. and DOOLITTLE, R.F., A simple method for displaying the hydropathic character of a protein. *J. mol. Biol.*, **157**, 105–132 (1982).

LEONE, A., FLATOW, U., KING, C.R., SANDEEN, M.A., MARGULIES, I.M.K., LIOTTA, L.A. and STEEG, P.S., Reduced tumor incidence, metastatic potential, and cytokine responsiveness of *nm*23-transfected melanoma cells. *Cell*, **65**, 25–35 (1991).

MCMASTER, G.K. and CARMICHAEL, G.G., Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. *Proc. nat. Acad. Sci. (Wash.)*, **74**, 4835–4838 (1977).

PEARSON, W.R. and LIPMAN, D.J., Improved tools for biological sequence comparison. *Proc. nat. Acad. Sci. (Wash.)*, **85**, 2444–2448 (1988).

REAL, F.X., HOUGHTON, A.N., ALBINO, A.P., CORDON-CARDO, C., MELAMED, M.R., OETTGEN, H.F. and OLD, L.J., Surface antigens of melanomas and melanocytes defined by mouse monoclonal antibodies: specificity analysis and comparison of antigen expression in cultured cells and tissues. *Cancer Res.*, **45**, 4401–4411 (1985).

SANGER, F., COULSON, A.R., BARRELL, B.G., SMITH, A.J.H. and ROE, B.A., Cloning in single-stranded bacteriophage as an aid to rapid sequencing. *J. mol. Biol.*, **143**, 161–178 (1980).

VAN LEEN, R.W., KASTROP, P.M., VAN ROOZENDAAL, K.E. and SCHOENMAKERS, J.G., Sequence divergence and selection of cap sites in the rat gamma-crystallin gene family. *Europ. J. Biochem.*, **157**, 203–208 (1986).

VAN MUIJEN, G.N., CORNELISSEN, L.M., JANSEN, C.F., FIGDOR, C.G., JOHNSON, J.P., BROCKER, E.B. and RUITER, D.J., Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. *Clin. exp. Metastasis*, **9**, 259–272 (1991*a*).

VAN MUIJEN, G.N.P., JANSEN, C.P.J., CORNELISSEN, L.M.A.H., SMEETS, D.F.C.M., BECK, J.L.M. and RUITER, D.J., Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. *Int. J. Cancer*, **48**, 85–91 (1991*b*).

VAN MUIJEN, G.N.P., WETERMAN, M.A.J., QUAX, P.H.A., VERHEIJEN, J.H. and RUITER, D.J., Phenotypic profile of two human melanoma cell lines isolated from the same patient with different metastatic behavior in nude mice. *Proc. Amer. Ass. Cancer Res.*, **32**, 62 (1991*c*).

WETERMAN, M.A.J., AJUBI, N., VAN DINTER, I.M.R., DEGEN, W.G.J., VAN MUIJEN, G.N.P., RUITER, D.J. and BLOEMERS, H.P.J., *nmb*, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. *Int. J. Cancer*, **60**, 73–81 (1995).

WETERMAN, M.A.J., STOOPEN, G.M., VAN MUIJEN, G.N.P., KUZNICKI, J., RUITER, D.J. and BLOEMERS, H.P.J., Expression of calcyclin in human melanoma cell lines correlates with metastatic behavior in nude mice. *Cancer Res.*, **52**, 1291–1296 (1992).

WETERMAN, M.A.J., VAN MUIJEN, G.N.P., BLOEMERS, H.P.J. and RUITER, D.J., Molecular markers of melanocytic tumor progression. *Lab Invest.*, **70**, 593–608 (1994).

WETERMAN, M.A.J., VAN MUIJEN, G.N.P., RUITER, D.J. and BLOEMERS, H.P.J., Thymosin β10 expression in melanoma cell lines and melanocytic lesions: a new progression marker for human cutaneous melanoma. *Int. J. Cancer*, **53**, 278–284 (1993).

XERRI, L., GROB, J.J., BATTYANI, Z., GOUVERNET, J., HASSOUN, J. and BONERANDI, J.J., *nm*23 expression in metastasis of malignant melanoma is a predictive prognostic parameter correlated with survival. *Brit. J. Cancer*, **70**, 1224–1228 (1994).