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Expression and Up-Regulation of Alternatively Spliced Transcripts of Melastatin, a Melanoma Metastasis-Related Gene, in Human Melanoma Cells

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Loss of expression of a novel suppressor of metastasis, melastatin (*MLSN1*), has recently been reported to correlate with metastatic potential of melanoma cells. Using differential display analysis, we identified *MLSN1* among genes overexpressed in pigmented metastatic human melanoma cells treated with the differentiation inducer hexamethylene bisacetamide (HMBA). In this study, we show that multiple short transcripts of *MLSN1* are present in melanocytes and pigmented metastatic melanoma cell lines while the full-length 5.4-kb mRNA is detectable only in melanocytes. Treatment of pigmented melanoma cells with the differentiation-inducing agent, HMBA, results in up-regulation of the 5.4-kb *MLSN1* mRNA as well as short RNAs. Analysis of a panel of nonpigmented primary and metastatic melanoma cell lines showed weak expression of a 1.8-kb mRNA in a few melanoma cell lines. Northern blot and RT-PCR analyses with DNA probes and oligonucleotide primers that correspond to distinct regions of full-length *MLSN1* mRNA indicated that the short transcripts contained sequences corresponding primarily to either 5′- or 3′-end of the 5.4-kb mRNA. HMBA appears to up-regulate *MLSN1* transcripts derived mainly from the 5′-end. Modulators of cAMP and protein kinase C pathways had no significant effect on *MLSN1* expression. Our data show that multiple *MLSN1* transcripts, both constitutively expressed and inducible, are present in cultured pigmented melanoma cells, and suggest that *MLSN1* expression can be regulated at the level of both transcription and mRNA processing.

Key Words: **melastatin (MLSN1); alternative splicing; metastasis; melanoma; pigmentation; differential display; HMBA.**

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Several investigators have employed differential display, a technique that allows comparison of gene expression profiles, to identify genes differentially expressed in mouse and human melanoma cells that differ in their metastatic potential (1–4). Using variants of mouse B16 melanoma cells that display differences in their potential to form pulmonary metastasis after intravenous injection into syngeneic mice, Duncan *et al.* recently identified a novel gene, melastatin (*mlsn1*), that is down-regulated in melanoma variants with higher metastatic potential. The amount of *mlsn1* mRNA detected in mouse B16 melanoma cell lines was shown to be inversely correlated with their metastatic potential. *In situ* hybridization studies using *MLSN1*, the human homologue of mouse *melastatin*, suggested that the extent of loss of *MLSN1* expression correlates with the thickness of primary cutaneous melanomas. Metastatic melanomas were shown to be completely devoid of *MLSN1* RNA expression (5).

Analysis of the genomic organization of *mlsn1* showed that the mouse gene consists of 27 exons which share >85% sequence identity with the 5.4 kb human *MLSN1* cDNA (6). The single 2.8-kb mRNA found in mouse melanoma cells appears to be derived by alternative splicing that truncates mRNA after exon 14. Whereas the 2.8-kb mouse *mlsn1* cDNA is predicted to produce a soluble protein of 542 amino acids, the full length human *MLSN1* cDNA encodes a putative 1533 amino acid long type III integral membrane protein with limited sequence homology to the proteins of the TRP (transient receptor potential) calcium channel family (6). However, MLSN1 polypeptides expressed in human melanocytes and melanoma cells have not yet been identified.

We have demonstrated earlier that treatment of pigmented metastatic human melanoma cell lines with differentiation-inducing agent hexamethylene bisacetamide (HMBA) results in the inhibition of growth, induction of multiple long dendritic extensions and the

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MITF-independent repression of the melanocyte-differentiation marker TRP-1/gp75 (7, 8). In attempts to further characterize this phenotype, we performed differential display analysis and identified *MLSN1* among genes over-expressed in human melanoma cells treated with HMBA. In this report, we describe the identification and characterization of multiple *MLSN1* mRNAs present in normal melanocytes and pigmented metastatic melanoma cell lines. Northern blot and RT-PCR analyses showed that the alternative splicing of *MLSN1* mRNA produces short *MLSN1* mRNAs derived from the 5′- or 3′-ends of the full-length *MLSN1* mRNA. Treatment of pigmented melanoma cells with HMBA results in the up-regulation of the full-length *MLSN1* mRNA and the accumulation of short mRNAs derived from the 5′-end but not the 3′-end of full-length *MLSN1* mRNA.

MATERIALS AND METHODS

Cell culture. Primary culture of human melanocytes was initiated from neonatal foreskins. Fresh skin specimens were washed three times with Hanks' balanced salt solution (HBSS) and excess fat was removed. The samples were cut into small pieces and incubated in 0.25% trypsin solution at 4°C overnight. Epidermis was separated from the dermis and epidermal cells were suspended and cultured in Ham's F10 nutrient medium with 10% FBS, 85 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), and 2.5 nM cholera toxin (CT).

Primary (WM75, WM35, and WM98-1) and metastatic (WM451 and WM1205) human melanoma cell lines were kindly provided by Dr. M. Herlyn at the Wistar Institute (Philadelphia, PA). WM35 is derived from an early-stage radial growth phase (RGP) primary lesion and the patient was cured after the surgical removal of the lesion. WM35 is reported to be metastatically incompetent (9). WM75 is derived from a vertical growth phase (VGP) primary melanoma from a patient who also had a subsequent metastatic lesion. WM98-1 is derived from a VGP primary melanoma and the patient had a recurrence of melanoma during 5-year clinical follow-up. WM98-1 is tumorigenic in nude mice (10, 11). WM1205 is the metastatic derivative obtained from nude mouse after injecting an early primary melanoma WM793. No recurrence was found during >10 year follow-up in the patient from whom WM793 was derived (12). WM451 is derived from a metastatic lesion (13). These melanoma cell lines were grown in Ham's F10 medium containing 10% FBS. Metastatic melanoma cell lines SK-MEL-19, -23 clone 22 (c.22) and clone 22a (c.22a) described earlier (14, 15), were grown in MEM medium supplemented with 10% FBS, 1% antibiotics, 1% nonessential amino acids and 1% glutamine. Cells were seeded at a density of 5×10^5 cells/10 ml culture medium in 100-mm dishes. Culture medium, FBS, HBSS, antibiotic–antimycotic mixture, nonessential amino acids and glutamine were purchased from Gibco-BRL (Bethesda, MD). TPA, IBMX and CT were from Sigma Chemical Co. (St. Louis, MO). HMBA was obtained from Aldrich Chemical Co. (Milwaukee, WI).

RNA Isolation. Cells grown as monolayers were washed twice with HBSS, harvested by trypsinization, washed once with ice-cold PBS. Poly(A)$^+$ RNA and total RNA were isolated from cell pellets using Micro-FastTrack mRNA (Invitrogen Corp., Carlsbad, CA) and Ultraspec-II RNA isolation system (Biotecx Laboratories, Inc., Houston, TX), respectively. Total RNAs were treated with DNase I (Clontech Laboratories Inc., Palo Alto, CA) for RT-PCR to remove remaining genomic DNA.

RT-PCR. Two micrograms of total RNA was used for the first-strand cDNA synthesis (GeneAmp RNA PCR kit, Perkin–Elmer Corp.). RNA was reverse transcribed in 20 μl of final volume of 1× PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP, 20 units of RNase inhibitor, 2.5 μM oligo$(dT)_{16}$ and/or random primers, and 2.5 units of MuLV reserve transcriptase. The reaction mixture was incubated at 42°C for 1 h and then 75°C for 10 min. PCR was routinely performed using 1 μl of cDNA in 50 μl containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM each primer, and 1.25 units of AmpliTaq DNA polymerase. Higher concentration of $MgCl_2$ (4 mM) was used for probe A and C. The following cycling conditions were used unless specified otherwise: 94°C for 5 min for denaturation; 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 30 cycles; followed by 72°C for 7 min. For probe A and C, 45 cycles of 94°C for 30 s, 52°C for 1 min, and 72°C for 1 min were performed. For probe D amplification, 45 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1:30 min were used. The primers were designed based on the human *MLSN1* sequence (AF071787) and are listed in Table I.

Northern analysis. The PCR product of 5′-probe was sequenced directly after purification using QIAquick Gel Extraction kit (Qiagen, Chatsworth, CA). The PCR amplified cDNA fragments for probe A-D were cloned into the pCR2.1 vector using a TOPO TA cloning kit (Invitrogen) and the sequences of probe templates were confirmed by DNA sequencing. Human GAPDH probe template was from Ambion (Austin, TX). Northern analysis was performed as described previously using Northern Max kit and Strip-EZ DNA probe synthesis and removal kit (Ambion; 8). The blots were washed at room temperature for 20 min with 2× SSC, 0.5% SDS, followed by washes at 55–60°C for 20 min with 0.5× SSC, 0.5% SDS and then 0.1× SSC, 0.5% SDS. Quantitative analysis was performed with an Image-QuaNT software and background correction was made using an area next to bands. Relative intensities for each probe were obtained by normalizing to GAPDH.

RESULTS

Identification of MLSN1 by Differential Display Analysis

In earlier studies we showed that treatment of pigmented human melanoma cells with the differentiation inducing agent HMBA results in selective down-regulation of the melanocyte differentiation marker TRP-1/gp75, induction of polydendritic morphology and expression of neuronal marker MAP-2 (microtubule associated protein-2; Refs. 7, 8 and manuscript in preparation). Flow cytometric analyses showed that treatment of human melanoma cells with 5mM HMBA for 48 h caused an accumulation of cells in G_0/G_1 phase and a significant decrease in the population of cells in G_2/M phase (data not shown).

To characterize changes in gene expression associated with the HMBA-induced phenotype, we performed differential display analysis (Delta differential display kit from Clontech) using total RNA obtained from control and HMBA-treated (for 48 h) SK-MEL-19 melanoma cells. The arbitrary primer, ATTAACCCTCACTAAATGTGGCAGG, and oligo(dT) primer CATTATGCTGAGTGATATCTTTTTTTTTCC amplified a cDNA band that showed increased expression in the growth-arrested SK-MEL-19 melanoma cells. Nucleotide sequence analysis of the ~170-bp fragment obtained by PCR re-amplification of DNA excised from

TABLE I

Probes and Primers Used in This Study

Primer name	Exon[a] location	nt position[b]	Primer sequence[c]	cDNA size (bp)	Probe name
Ex2 5′	2	121–140	CTAACAGGTGTTGCTGTGGC	700	5′-probe
Ex6 3′	6	801–820	TGGAGGGAGATGTGCTTTTC		
Ex4 5′	4	318–335	CGGGGTACCATGTATATCCGTGTA	1292	Probe A
Ex12 3′	12	1595–1614	GTTATAGAGCTCCTCCAG		
Ex19 5′	19	2568–2585	ATCTGTGAATTCTATAAC	868	Probe C
Ex25 3′	25	3436–3455	TTAAAATGATCATCGGTG		
Ex25 5′	25	3436–3455	CACCGATGATCATTTTAA	1480	Probe D
Ex27 3′	27	4898–4919	CGGGGTACCGCATTCAGTTTCTGTGGAAGC		
Ex23 5′	23	3167–3186	AGACCTCTACGCCATGGAAA		
Ex15 3′	15	1815–1838	TTCCATTCCCAGAAGTTTAAGAGC		
Ex16 3′	16	2072–2091	GATCACTCTCGGAGGACTCG		
Ex17 3′	17	2301–2320	CACATATCGGTCAGCAGCAT		

[a] Locations correspond to mouse exons 1–27 (Ref. 6).
[b] Nucleotide positions corresponding to AF071787.
[c] Primer sequences shown 5′–3′.

the gel band showed 98% identity to human melastatin gene (*MLSN1;* GenBank Accession No. AF071787) near the 5′-end of the 5.4-kb cDNA (between nt 88–205; Ref. 6).

In mouse melanoma cell lines, *mlsn1* (GenBank Accession No. AF 047714) is expressed as a 2.8-kb mRNA and is predicted to encode a protein of 542 amino acids with neither signal sequence nor hydrophobic transmembrane domain sequences. The predicted protein has no significant similarity to any protein of known function. Expression of *mlsn1* mRNA is restricted to melanocytic lesions and the eye (5). The human homologue of *MLSN1* cDNA is 5.4 kb with a 4.6-kb open reading frame predicted to encode 1533 amino acid long protein containing six putative transmembrane domains. Sequence similarity analysis revealed that human MLSN1 protein has limited homology to transient receptor potential (TRP) family of calcium channel proteins, and also ~45% identity within the first 1200 amino acids to a *C. elegans* predicted protein of 1753 amino acids (6, 16). Sequence alignment of predicted amino acid sequences of human and mouse melastatin proteins is shown in Fig. 1.

Expression of Short MLSN1 Transcripts in Melanocytes and Pigmented Melanomas Cells

To study the expression of *MLSN1* in human melanocytic cells, we performed Northern hybridization analysis of melanocyte and melanoma cell RNA using the 5′-probe (Table I). In cultured neonatal melanocytes, three prominent bands of 1.8, 4.0, and 5.4 kb size could be readily detected (Fig. 2A). In a panel of nonpigmented cell lines derived from primary (WM35, WM75, and WM98-1) and metastatic melanomas (WM451 and WM1205), a weak 1.8-kb band was detectable only in WM35 and WM451. In pigmented metastatic melanoma cell lines SK-MEL-19, -23 (c.22) and nonpigmented (c.22a) clones by injecting SK-MEL-23 c.22 into nude mice, expression of 1.8- and 4.0-kb *MLSN1* mRNA was readily detectable (Fig. 2A). A 1.3-kb band could also be seen in SK-MEL-19 and -23 c.22 melanoma cells. The melanocytic origin of all cell lines used in this study was confirmed by the expression of tyrosinase and MITF (data not shown). These data show that, while a few nonpigmented melanoma cell lines express only 1.8-kb *MLSN1* mRNA, both pigmented and nonpigmented clones derived from pigmented metastatic melanomas express significant amounts of short *MLSN1* transcripts.

Up-Regulation of Full-Length MLSN1

Treatment of pigmented SK-MEL-19 and -23 c.22 cells with HMBA for 48 h resulted in the appearance of the 5.4-kb *MLSN1* mRNA corresponding to the full-length cDNA and also an increase in the intensity of 1.3,, 1.8-, and 4.0-kb bands (Fig. 2B, lane 1: control and lane 2: treated). The up-regulation of *MLSN1* RNA by HMBA confirmed the basis for the identification of this gene by differential display in our study. However, treatment of non-pigmented melanomas, including those which showed a weak expression of the 1.8-kb mRNA (WM35, WM451), with HMBA did not result in induction of *MLSN1* expression (data not shown). These data suggest that the induction of full-length *MLSN1* expression is associated with pigmented phenotype of melanoma cells.

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        10         20         30         40         50         60
MYIRVSYDTKPDSLLHLMVKDWQLELPKLLISVHGGLQNFEMQPKLKQVFGKGLIKAAMT
:::::::::::::::::::::::::::::::::::::.:::: ::::::::::::::::::
MYIRVSYDTKPDSLLHLMVKDWQLELPKLLISVHGGLQSFEMQSKLKQVFGKGLIKAAMT
        70         80         90        100        110        120
TGAWIFTGGVSTGVISHVGDALKDHSSKSRGRVCAIGIAPWGIVENKEDLVGKDVTRVYQ
:::::::::::::.:::::::::::::::.:::::::::.:::::::.:::::::::::
TGAWIFTGGVSTGVVSHVGDALKDHSSKSRGRLCAIGIAPWGMVENKEDLIGKDVTRVYQ
       130        140        150        160        170        180
TMSNPLSKLSVLNNSHTHFILADNGTLGKYGAEVKLRRLLEKHISLQKINTRLGQGVPLV
:::::::::::::::::::::::::::::::::::: :::::::::::::::::::.:
TMSNPLSKLSVLNNSHTHFILADNGTLGKYGAEVKLRRQLEKHISLQKINTRLGQGVPVV
       190        200        210        220        230        240
GLVVEGGPNVVSIVLEYLQEEPPIPVVICDGSGRASDILSFAHKYCEEGGIINESLREQL
:::::::::::::::::::.:.::.:::.:::::::::::::::::::.:::.:::::::.::
GLVVEGGPNVVSIVLEYLKEDPPVPVVVCDGSGRASDILSFAHKYCDEGGVINESLRDQL
       250        260        270        280        290        300
LVTIQKTFNYNKAQSHQLFAIIMECMKKKELVTVFRMGSEGQQDIEMAILTALLKGTNVS
:::::::::.:.::.:::::::::::::::::::::::::::::::::::::::::.:
LVTIQKTFNYSKSQSYQLFAIIMECMKKKELVTVFRMGSEGQQDVEMAILTALLKGTNAS
       310        320        330        340        350        360
APDQLSLALAWNRVDIARSQIFVFGPHWTPLGSLAPPTDSKATEKEKKPPMATTKGGRGK
:::::::::::::::::::::::::::: ::::::::.:.::.::::::: ::::: :::
APDQLSLALAWNRVDIARSQIFVFGPHWPPLGSLAPPVDTKAAEKEKKPPTATTKG-RGK
       370        380        390        400        410        420
GKGKKKGKVKEEVEEETDPRKIELLNWVNALEQAMLDALVLDRVDFVKLLIENGVNMQHF
:::::::::::::::::::::.:::::::::::::::::::::::::::::::::::::::
GKGKKKGKVKEEVEEETDPRKLELLNWVNALEQAMLDALVLDRVDFVKLLIENGVNMQHF
       430        440        450        460        470        480
LTIPRLEELYNTRLGPPNTLHLLVRDVKKSNLPPDYHISLIDIGLVLEYLMGGAYRCNYT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LTIPRLEELYNTRLGPPNTLHLLVRDVKKSNLPPDYHISLIDIGLVLEYLMGGAYRCNYT
       490        500        510        520        530        540
RKNFRTLYNNLFGPKRPKALKLLGMEDDEPPAKGKKKKKKKEEEIDIDVDDPAVSRFQY
::.:::::::::::::: .
RKSFRTLYNNLFGPKRVELSRHTVSCASQSNMWFLDVLPQKPTCAECNSSPHLSQTDITP
       550        560        570        580        590        600
PFHELMVWAVLMKRQKMAVFLWQRGEESMAKALVACKLYKAMAHESSESDLVDDISQDLD
:
PLP
       610        620        630        640        650        660
NNSKDFGQLALELLDQSYKHDEQIAMKLLTYELKNWSNSTCLKLAVAAKHRDFIAHTCSQ
       670        680        690        700        710        720
MLLTDMWMGRLRMRKNPGLKVIMGILLPPTILFLEFRTYDDFSYQTSKENEDGKEKEEEN
       730        740        750        760        770        780
TDANADAGSRKGDEENEHKKQRSIPIGTKICEFYNAPIVKFWFYTISYLGYLLLFNYVIL
       790        800        810        820        830        840
VRMDGWPSLQEWIVISYIVSLALEKIREILMSEPGKLSQKIKVWLQEYWNITDLVAISTF
       850        860        870        880        890        900
MIGAILRLQNQPYMGYGRVIYCVDIIFWYIRVLDIFGVNKYLGPYVMMIGKMMIDMLYFV
       910        920        930        940        950        960
VIMLVVLMSFGVARQAILHPEEKPSWKLARNIFYMPYWMIYGEVFADQIDLYAMEINPPC
       970        980        990       1000       1010       1020
GENLYDEEGKRLPPCIPGAWLTPALMACYLLVANILLVNLLIAVFNNTFFEVKSISNQVW
      1030       1040       1050       1060       1070       1080
KFQRYQLIMTFHDRPVLPPPMIILSHIYIIIMRLSGRCRKKREGDQEERDRGLKLFLSDE
      1090       1100       1110       1120       1130       1140
ELKRLHEFEEQCVQEHFREKEDEQQSSSDERIRVTSERVENMSMRLEEINERETFMKTSL
      1150       1160       1170       1180       1190       1200
QTVDLRLAQLEELSNRMVNALENLAGIDRSDLIQARSRASSECEATYLLRQSSINSADGY
      1210       1220       1230       1240       1250       1260
SLYRYHFNGEELLFEDTSLSTSPGTGVRKKTCSFRIKEEKDVKTHLVPECQNSLHLSLGT
      1270       1280       1290       1300       1310       1320
STSATPDGSHLAVDDLKNAEESKLGPDIGISKEDDERQTDSKKEETISPSLNKTDVIHGQ
      1330       1340       1350       1360       1370       1380
DKSDVQNTQLTVETTNIEGTISYPLEETKITRYFPDETINACKTMKSRSFVYSRGRKLVG
      1390       1400       1410       1420       1430       1440
GVNQDVEYSSITDQQLTTEWQCQVQKITRSHSTDIPYIVSEAAVQAEQKEQFADMQDEHH
      1450       1460       1470       1480       1490       1500
VAEAIPRIPRLSLTITDRNGMENLLSVKPDQTLGFPSLRSKSLHGHPRNVKSIQGKLDRS
      1510       1520       1530
GHASSVSSLVIVSGMTAEEKKVKKEKASTETEC
```

FIG. 1. Sequence alignment of human and mouse MLSN1 polypeptides. Alignment of predicted amino acid sequences of human and mouse melastatin. Upper: Human MLSN1; Lower: Mouse mlsn1. Sequence identity is indicated by (:) and similarity is indicated (.). Six putative transmembrane (TM) domains in human *MLSN1* are underscored. Note sequence identity between human and mouse proteins at the amino terminal end (between amino acids 1–496) and divergence of mouse sequence at the carboxyl end (5, 6).

Characterization of Alternatively Spliced MLSN1 Transcripts

Reproducible appearance of similar shorter (<5.4 kb) RNA species hybridizing with the 5′-probe in both melanocytes and melanoma cells raised the possibility that the short forms of *MLSN1* RNA are generated by alternative splicing of the primary transcript. Studies on the genomic organization found that mouse *mlsn1* gene consists of 27 exons which show >85% sequence identity with the 5.4-kb human cDNA (6). The single 2.8-kb mRNA found in mouse B16 melanoma cells consists of sequence derived from exons 1–14, followed by sequences of intron 14, exon 15 and intron 15. This is evident by the presence of exons 14 and 15 splice donor/acceptor site sequences in the 2.8-kb cDNA. Nucleotide sequences corresponding to exons 16–27 are not found in the mouse *mlsn1* cDNA (6).

We therefore investigated the relationship of the short mRNA species to the 5.4-kb full-length *MLSN1* mRNA in the pigmented melanoma cell line SK-MEL-19. Oligonucleotide primers for RT-PCR were designed to generate DNA probes that span the full-length open reading frame of *MLSN1* and to amplify cDNA fragments from 5′- or 3′-end of *MLSN1*. Sequences of these primers, length of the probes, nucleotide positions and mouse exon numbers to which they correspond are listed in Table I. Sequences of all PCR-amplified cDNA probes were confirmed directly after PCR or after cloning in TOPO vector. The specific hybridization of probes A, C and D with *MLSN1* was confirmed using a panel of RNAs shown in Fig. 2A (data not shown).

Analysis with 5′-End Probes

In poly(A)$^+$ enriched RNA isolated from untreated SK-MEL-19 cells, the 5′-probe (exons 2–6) detected 1.8-kb transcript (Fig. 3B, panel 1, lane 1). Weak bands corresponding to the 1.3- and 4.0-kb transcript could be detected upon prolonged exposure (>72 h) of the blot to the X-ray film. Treatment of SK-MEL-19 melanoma cells with HMBA resulted in the up-regulation of the 5.4-kb mRNA and the accumulation of 1.3, 1.8, and 4.0 kb bands (lane 2). Probe A, corresponding to mouse exons 4–12, gave a weak signal of 1.8-kb band in control cells. The up-regulation of this 1.8-kb band and 4.0- and 5.4-kb transcripts by HMBA could be seen in lane 2 of the second panel in Fig. 3B. Probe A, however, did not detect the 1.3-kb mRNA. Since all blots were hybridized, washed and exposed to X-ray film under the identical conditions, weaker signals obtained for probe A (compared to the 5′-probe) suggests a weak hybridization of *MLSN1* to nucleotide sequences within this probe. These data show that the 1.8-kb band hybridizing with the overlapping 5′-probe and probe A (spanning exons 2–12) represents a major poly(A)$^+$ transcript of *MLSN1*.

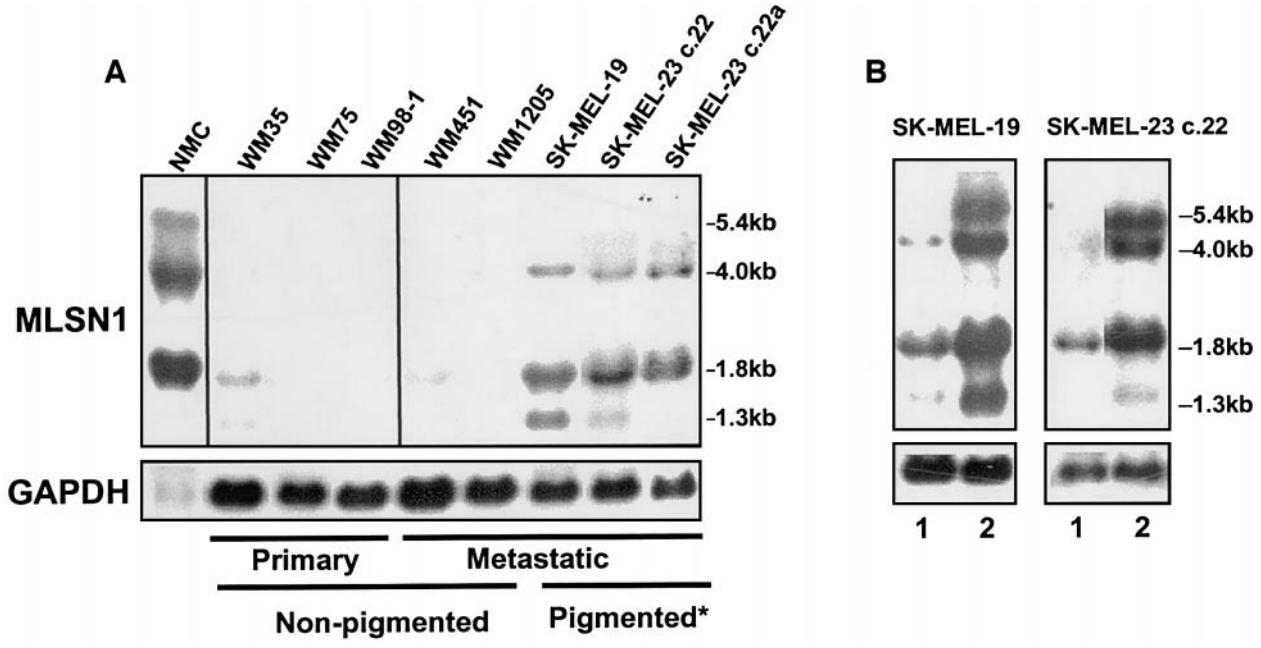

FIG. 2. Expression of *MLSN1* mRNA in human melanocytes and melanoma. (A) Northern blot analysis of total RNA isolated from pigmented neonatal melanocytes (NMC), nonpigmented primary (WM35, WM75, and WM98-1) and metastatic (WM451, WM 1205) melanoma cell lines, and pigmented metastatic melanoma cell lines SK-MEL-19, -23 (c.22) and nonpigmented (c.22a*) clones derived from SK-MEL-23 c.22. 10 μg of RNA was electrophoresed on denaturing agarose gel, transferred to nitrocellulose membrane, and hybridized with ^{32}P-dATP labeled 700 bp *MLSN1* (nt 121–820) 5′-probe. The numbers on the right are estimated sizes of autoradiographic bands. (B) Northern blot analysis of up-regulation of the 1.3-, 1.8-, 4.0-, and 5.4-kb *MLSN1* mRNA in pigmented melanoma cells. SK-MEL-19 and -23 c.22 cells were treated with 5 mM HMBA for 2 days. Total RNA from control (lane 1) and treated cells (lane 2) was isolated and analyzed as in A.

To further characterize this 1.8-kb mRNA, we performed RT-PCR analysis using primers corresponding to exon 4 (sense primer) and exons 15 or 16 or 17 (antisense primers; see Table I for nt position of the primers on human *MLSN1*). Exon 4 and exon 15 primer pair readily amplified the expected 1.52-kb fragment (Fig. 3C, left panel) from untreated cells. An increase in the yield of the PCR product from HMBA treated cells was apparent. The relative yield of these PCR fragments from control and treated cells was consistent with the relative abundance of 1.8-kb *MLSN1* transcript (Fig. 3B, panels 1 and 2). DNA sequence analysis showed that this PCR fragment contained uninterrupted sequence spanning exon 4-15 (data not shown). PCR amplifications with primers for exons 16 and 17, however, produced weak 1.7- and 2.0-kb bands from control cells and bands of higher yield from HMBA treated cells (*lanes 1 and 2*). Since, primers for exons 4–15 readily amplify a PCR fragment of expected size and sequence (presumably from the most abundant 1.8 kb mRNA), weak amplification by antisense primers from exons 16 and 17 suggest that mRNAs containing exons 16 and 17 are relatively of low abundance in control cells. These data also suggest that the major 1.8 kb *MLSN1* mRNA consisting of 5′ ~1800 nucleotides is generated by alternative splicing at or near the position corresponding to mouse exon 15 and does not contain sequences derived from exons 16 and 17. Attempts to determine nucleotide sequence downstream of exon 15 by 3′ RACE using oligo $(dT)_{16}$ primer were unsuccessful due to the presence of several stretches of A residues, including a stretch of 14 A residues between nucleotide positions 1866–1879 in exon 16, that produced multiple short cDNA fragments (data not shown).

Analysis with 3′-End Probes

Hybridization with probe C (exons 19–25) showed two major bands of 0.8 and 1.7 kb, and a weak 2.3 kb band. Exposure of the X-ray film for prolonged periods showed weak signals corresponding to 4.0 and 5.4 kb (Fig. 3B, panel 3, lane 1). Treatment with HMBA did not affect the intensity of bands hybridizing with probe C (lane 2). Probe D (exons 25–27) revealed two major bands of 1.2 and 1.8 kb size and a weak >3.0 kb band. There was no difference in the intensity of 1.2 and 1.8 kb bands between control and HMBA-treated cells (Fig. 3B, panel 4, lanes 1 & 2). These 0.8, 1.7, 1.8, and 2.3 kb RNAs did not hybridize with the 5′-probe and probe A (compare panels 3 and 4 with panels 1 and 2 in Fig. 3B). Since treatment with HMBA also did not affect their expression, these data suggest that the short RNAs detected with probes C and D (excepting

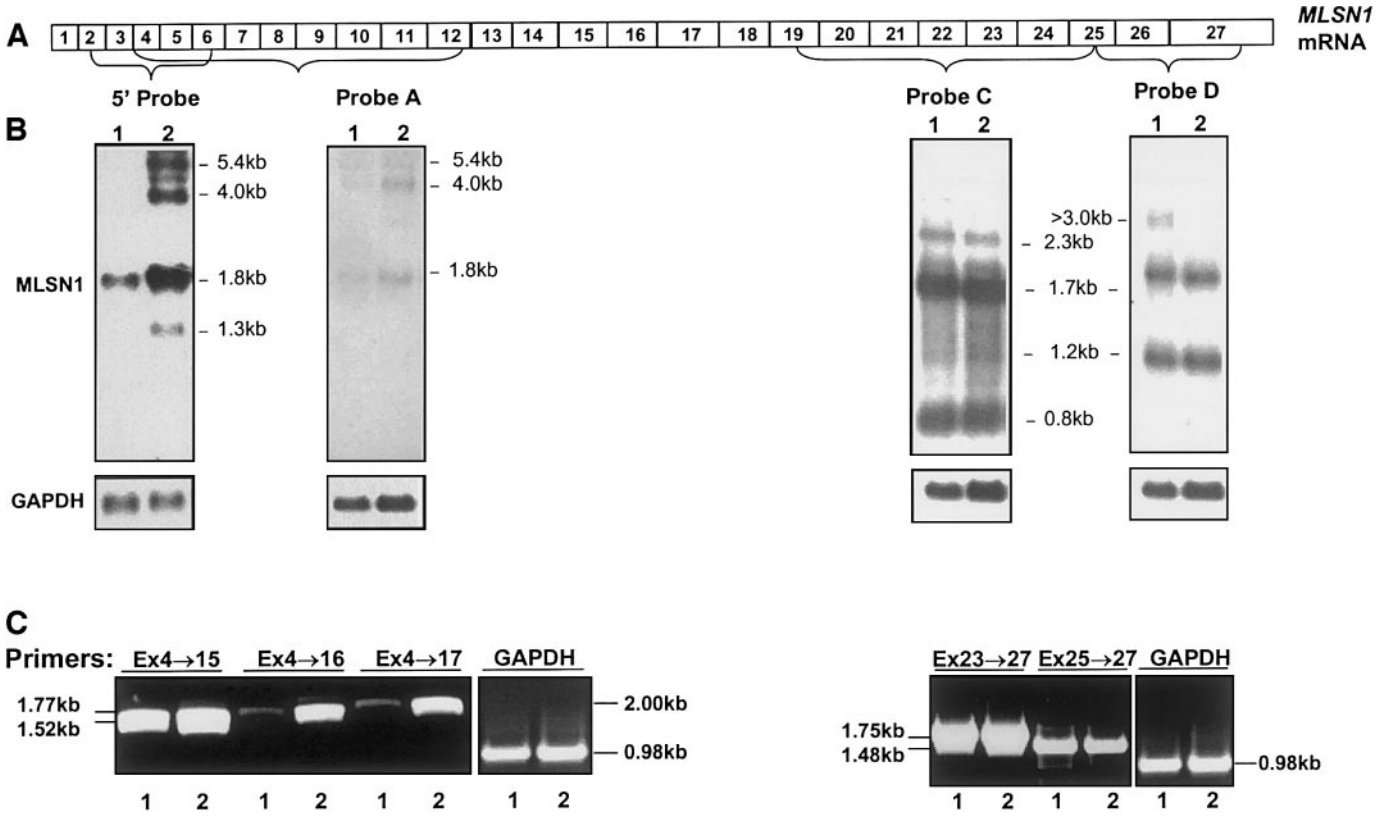

FIG. 3. Alternative splicing of *MLSN1* mRNA. The 5.4-kb full-length mRNA of human *MLSN1* and boundaries of exons corresponding to the 27 exons of mouse *melastatin* are shown in *A*. The exon sizes range from 21 to 1200 bp (6) and are not drawn to scale. cDNA probes used for Northern blot analysis are indicated below bracketed regions. (B) Northern blot analysis of poly(A)$^+$ RNA from control (lanes 1) and 5 mM HMBA-treated (48 h; lanes 2) SK-MEL-19 melanoma cells with probes indicated above each panel. The estimated sizes of autoradiographic bands are indicated. Poly(A)$^+$ RNA (3 μg/lane) was electrophoresed, blotted simultaneously, hybridized under the identical conditions with the indicated probes labeled simultaneously, washed under the identical conditions, and exposed to the film for same amount time. (C) RT-PCR analysis of *MLSN1* mRNA. Total RNA from control (lanes 1) and 5 mM HMBA-treated cells (48 h; lanes 2) was reverse transcribed with random hexamers and oligo(dT)$_{16}$ primers, and 1 μl of cDNA was used for PCR. Primer pairs Ex4/Ex15, Ex4/Ex16, and Ex4/Ex17 amplified 1.52-, 1.77-, and 2.00-kb fragments, respectively (left panel). The 1.75- and 1.48-kb fragments amplified by primer pairs Ex23/Ex27 and Ex25/Ex27 (right panel). All PCRs were performed using identical cycling parameters. Amplification of GAPDH is shown as control.

the >3.0 kb mRNA detected by probe D) are generated constitutively by alternative splicing that excludes *MLSN1* sequences corresponding to mouse exons upstream of exon 20.

RT-PCR analysis using primers that amplify sequences between mouse exons 23 and 27 and between exons 25 and 27 showed amplification of abundant cDNAs of expected size, i.e., 1.75 and 1.48 kb (Fig. 3C, right panel). There was no difference in the yield of these PCR fragments between control (lane 1) and HMBA-treated cells (lanes 2). Direct sequencing of PCR products showed that these DNA fragments contained uninterrupted sequences between the primers used for amplification. Taken together with the Northern blot analyses, these data suggest that alternative splicing of *MLSN1* generates several *MLSN1* transcripts containing mostly 3′-end sequences corresponding to mouse exons 20–27.

Regulation of MLSN1 Expression

Since activators of cAMP-dependent and protein kinase C pathways are known to affect the expression of melanocyte differentiation markers, we tested the effect of cholera toxin (CT) and TPA on expression of *MLSN1* in SK-MEL-19 cells. As shown in Fig. 4, addition of CT or TPA to the growth medium had no significant effect on *MLSN1* expression, whereas HMBA up-regulated expression of all transcripts including the full-length 5.4-kb mRNA (Fig. 4A). Densitometric analysis (all bands combined) showed that treatment with HMBA caused a time-dependent, 5-fold accumulation of *MLSN1* mRNA (Fig. 4B).

In summary, our data show that human *MLSN1*, unlike its mouse counterpart, produces multiple alternatively spliced mRNAs in human melanocytes. In pigmented melanoma cells, which express only the short RNAs, the full-length *MLSN1* mRNA can be induced

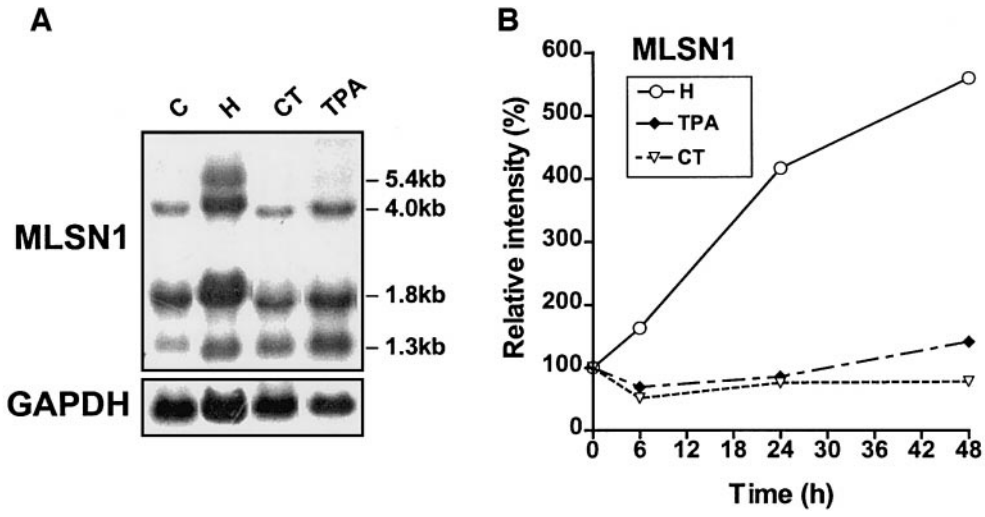

FIG. 4. Up-regulation of *MLSN1* was independent of cAMP or protein kinase C cellular signaling pathways. (A) SK-MEL-19 melanoma cells were treated with 5 mM HMBA (H), or 2.5 nM CT, or 10 ng/ml TPA for 2 days. Untreated cells are shown as control (C). 10 μg of total RNA/lane was loaded for Northern analysis using 5′-probe of *MLSN1*. (B) Time course of HMBA-induced up-regulation of *MLSN1* compared to CT and TPA treatment. The relative intensity (of all *MLSN1* transcripts combined) was plotted against time. The data shown are means from two independent experiments.

by treatment with pharmacological compounds such as HMBA through mechanisms that appear to be independent of protein kinase C and cAMP signaling pathways.

DISCUSSION

We identified *MLSN1* among genes over-expressed in human metastatic melanoma cells treated with the differentiation-inducing agent HMBA. Our data show that both cultured normal human melanocytes and pigmented metastatic melanoma cells express short *MLSN1* mRNAs, in contrast to the single 2.8 kb mRNA observed in mouse melanoma cells. In pigmented melanoma cells which constitutively express short *MLSN1* mRNAs, the 5.4 kb mRNA representing full-length *MLSN1* and selected short forms of mRNA derived from its 5′-end are up-regulated by the differentiation inducer. Identification of an abundant 1.8 kb mRNA, which corresponds to the 5′-end sequences of the full length *MLSN1*, predicted to produce an intracellular 48 kDa polypeptide lacking transmembrane domains has implications for biological functions of this short MLSN1 polypeptide in both melanocytes and melanoma cells. Interestingly, this polypeptide appears to be the human counterpart of the single melastatin polypeptide predicted to be produced by mouse melanoma cells (5).

Patterns of MLSN1 Expression in Melanocytes and Melanoma

A role for melastatin in suppression of mouse melanoma metastasis was proposed based on an inverse relationship of its expression to the metastatic potential. *In situ* hybridization analysis of human melanocytic neoplasms also suggested that a focal loss of melastatin expression correlates with the thickness of the primary cutaneous melanomas. Melastatin expression is absent in metastatic melanoma tumor specimens and cell lines (5). In the present study, we utilized a panel of human melanoma cell lines that represent different well-defined clinical and pathological stages of disease progression to investigate *MLSN1* expression (10, 17–20). In human melanocytes, in addition to the 5.4 kb full-length *MLSN1* mRNA, we found abundant expression of 1.8 and 4.0 kb mRNA (Fig. 2A). This is particularly significant since varying amounts of these RNAs, specifically the 1.8 kb mRNA, are found in both primary and metastatic melanoma cell lines. For example, we found abundant expression of the short mRNAs, but not the full-length 5.4 kb mRNA, in three cell lines derived from pigmented metastatic melanomas. But, only a small amount of 1.8 kb mRNA was detectable in a primary RGP melanoma cell line WM35 (Fig. 2A). This cell line, derived from a primary melanoma ($<$0.76 mm thick), was shown to be metastatically incompetent and nontumorigenic in nude mice (9). WM451, a cell line derived from a metastatic lesion also had detectable amounts 1.8 kb *MLSN1* mRNA. There are several possible explanations for these discordant observations. First, continuous passage *in vitro* alters the expression of *MLSN1* in cell lines and may not represent the expression pattern *in vivo*. This appears to be unlikely, since all cell lines used in this study showing either abundant expression of *MLSN1* or no expression have

been cultured for a long period (14, 21). Second, stable expression of small amounts of the 5.4 kb mRNA in non-metastatic primary melanomas, not detectable by Northern blot technique, may be sufficient for the production of a suppressor of metastasis. Thus, the abundant short RNAs in some metastatic melanomas arise from a regulatory mechanism that produces alternatively spliced and partially degraded non-productive RNAs. However, the abundant expression of these short RNAs is also found in normal melanocytes, suggesting that the generation of these short RNAs is not a property unique to the malignant cells. It is of interest, however, all cell lines with abundant expression of 1.8 and 4.0 kb mRNA are pigmented or clonal derivatives of pigmented melanoma, suggesting that *MLSN1* expression is related to melanocytic differentiation and pigmentation.

Alternative Splicing of MLSN1

In both normal melanocytes and melanoma cells, alternative splicing of *MLSN1* appears to produce several short mRNAs. Northern blot analysis, RT-PCR and DNA sequencing suggested that the 1.8 kb mRNA containing the 5′ ~1800 nucleotides encodes a putative ~500 amino acids-long N- terminal isoform of MLSN1. Similarly, the 1.3 kb mRNA which hybridized primarily with the 5′-probe (exons 2–6) also appears to represent alternative splicing after exon 10. This is supported by the finding that nucleotide sequence of a 367 bp human EST (GenBank Accession No. AI638469) corresponds to mouse exon 10 and is contiguous with the splice donor sequence of intron 10 (exon 10. . .GGCCG/gtgaa. . .intron). This splice variant is predicted to produce a 339 residue amino terminal polypeptide of MLSN1.

Alternative splicing of pre-mRNA is a versatile regulatory mechanism and is known to generate isoforms with diverse functions for many types of proteins (22). For example, mechanisms that alter the ratios of alternative splice variants of Wilm's tumor locus WT1 have been implicated as a cause of abnormal urogenital development in Denys-Drash syndrome (23). Alternative splicing of several genes in melanoma has been reported. Aberrant splicing of *Bin1,* a tumor suppressor has been implicated in melanoma progression (24). Similarly, expression of *CD44* isoforms has also been reported to correlate with melanoma progression (25). Included among prominent examples of protein isoforms generated by alternative splicing are ion channels (26). Melastatin is a distant relative of the TRP (transient receptor potential) calcium channel family. Members of TRP family are known to produce alternative transcripts (27–29). It has been reported, recently, that alternative splicing of the transcript of MTR1, a novel gene with homology to melastatin and TRPC7 also produces two possible proteins of 872 and 1165 amino acids (16). Alternatively spliced transcripts of TRP family Ca^{2+} channels are thought to form multimers and regulate the activity of the full-length proteins (30). Interestingly, in human melanocytic cells, a transcript corresponding to the mouse 2.8 kb *mlsn1* mRNA was not detectable. This suggests that melastatin undergoes species specific alternative splicing similar to alternative splicing of N-type calcium channels that produce distinct variants in different regions of the nervous system (26, 31).

Regulation of MLSN1 in Human Melanoma Cells

RT-PCR analysis of a panel of mouse tissues showed that *mlsn1* expression is restricted to eye, presumably normal melanocytes, and melanocytic lesions (5). Interestingly, *cis*-regulatory elements, M box and E box, have been found in the promoter region of mouse *mlsn1* (6). These regulatory elements which are conserved among tyrosinase family genes are known to bind MITF, the melanocyte-specific transcription factor and activate tissue-specific expression (32). It is, therefore, reasonable to predict that melanocyte-specific expression of mouse *mlsn1* is also regulated by MITF (6). Our analysis of human melanocytes and melanoma cells showed no strict correlation between levels of *MITF* mRNA and expression of *MLSN1* mRNA in melanoma cell lines (data not shown).

HMBA is a hybrid polar compound shown to induce terminal differentiation in mouse erythroleukemia cell and a variety of human tumor cells (33). The mechanism of action of HMBA has been studied extensively (34–37). HMBA-induced changes in expression of *MLSN1*does not appear to involve cAMP or protein kinase C pathways (34, 37). Lack of correlation of *MLSN1* expression to MITF and the inability of the cAMP activator to up-regulate *MLSN1* in human melanoma cells suggests that this gene may be regulated by different mechanisms in mouse and human melanocytes. Characterization of *cis*-regulatory sequences of human *MLSN1* will be important to investigate regulation of human *MLSN1* gene.

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