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Identification of a novel MAGE D2 antisense RNA transcript in human tissues

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Abstract

Using cDNA microarray analysis, we identified a cDNA clone, DD74, from primary human bronchial epithelial cells, which exhibits increased expression in vitro after treatment with all-*trans* retinoic acid. This clone corresponded to MAGE D2 mRNA, a gene previously identified to be upregulated in several cancer tissues. Surprisingly, in situ hybridization of lung tissue demonstrated positive hybridization signals with sense, but not antisense, MAGE D2-specific cRNA probes. Examination of several cell lines by Northern blot hybridization confirmed significant expression of two RNA bands. With strand-specific riboprobes, we identified a 2.0 kb RNA transcript with the antisense probe as expected and identified a 4.1 kb transcript by the sense probe. Further sequence analysis of the 4.1 kb transcript revealed at least a 509 nucleotide sequence exactly complementary to the 2.0 kb MAGE D2 mRNA sequence. This MAGE D2i sequence contains unique structural features not shared with those of previously described antisense transcripts. Identification of this transcript potentially has important implications for future studies examining MAGE D2 expression patterns in cancer and normal tissues.

Keywords: MAGE; MAGE D2; Non-coding RNA; Gene expression; Antisense RNA; Airway epithelium

To better understand the biology of airway epithelium and lung cancer development, we recently used a cDNA library obtained from well-differentiated primary human bronchial epithelial cells. The goal was to discover novel genes that are differentially regulated by all-*trans* retinoic acid [1,2]. Using this technique, we isolated a novel cDNA clone, DD74, whose corresponding message is also elevated by all-*trans* retinoic acid. Nucleotide sequence analysis demonstrated that the DD74 cDNA clone encodes MAGE D2 sequence.

MAGE family proteins were first recognized as tumor-specific antigens in melanoma cells [3]. Several investigators have since demonstrated that MAGE proteins have functional importance in cell cycle progression, gametogenesis, neuronal development, and cell death [4]. MAGE family members are recognized to contain two different expression patterns, a cancer- and testis-specific expression pattern (e.g., MAGE family A, B, and C), and a ubiquitous expression pattern (e.g., MAGE family D, E1, and F1) [5,6].

Along with its ubiquitous expression in multiple tissue types, MAGE D2 mRNA sequence has been identified in fetal lung tissue (AF128528), and several cancer tissues [7], suggesting a role of this gene in cell differentiation and/or tumorigenesis. Because the cellular distribution and function of this 65 kilodalton (kDa) putative protein remains unknown, we carefully examined MAGE D2 expression in airway epithelium.

Surprisingly, we identified a novel, 4.1 kb RNA transcript in multiple human tissues that is complementary to MAGE D2 mRNA at the 5′ end. This 4.1 kb transcript

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is unique in that its 5′ end precisely complements a 509 nucleotide region of MAGE D2 message across an intron–exon boundary. Furthermore, our data suggest this RNA transcript functions to inhibit MAGE D2 protein expression. We have therefore named this transcript MAGE D2i.

Experimental procedures

Cell sources and culture. HBE1 cells, a papilloma virus-immortalized human bronchial epithelial cell line kindly provided by Dr. J. Yankaskas from the University of North Carolina [8], were maintained in a serum-free, hormone-supplemented Ham's F12 medium as described previously [9,10]. Other cell lines, including a melanoma cancer cell line, G361 (ATCC# CRL1424), and a colorectal adenocarcinoma cell line, SW480 (ATCC# CCL228), were maintained in appropriate media as per the supplier (ATCC). All cell lines were grown to confluence in 6-well tissue culture plates without additional substratum.

RNA isolation and Northern blot hybridization. Cells were harvested for total RNA isolation by a single-step acid guanidium thiocyanate extraction method [11]. Total RNA was further fractionated in an oligo(dT) column to obtain poly(A)-enriched and poly(A)-poor fractions. For Northern blot hybridization, equal amounts of total RNA (50 μg/lane) or poly(A)-enriched RNA (1 μg/lane) were subjected to electrophoresis, transferred overnight to a Nytran N+ nylon membranes, and underwent UV cross-linking using UV Stratalinker 2400 (Stratagene, La Jolla, CA). cDNA probes were labeled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA) to a specific activity of $\sim 2 \times 10^9$ dpm/μg with a Ready-To-Go random primer labeling kit (Amersham–Pharmacia Biotech, Life Sciences, Arlington Heights, IL). cRNA probes were generated from a 368 base pair region of the MAGE D2 sequence using T7-and T3-tailed PCR primers:

T7: 5′-TAATACGACTCACTATAGGGAGAGTCTAGCAGCTGACACCAA-3′
T3: 5′-GAAATTAACCCTCACTAAAGGGAGCTATGGGACCCCTTGAA-3′

PCR products were in vitro transcribed using T7 and T3 polymerase, and labeled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA) to produce sense and antisense cRNA probes, respectively. Membranes were pre-hybridized in hybridization solution (50% formamide, 5× SSC, 0.02% SDS, 0.01 M EDTA, 1% *N*-lauroylsarcosine, and DEPC water) at 68 °C for a minimum of 1 h followed by hybridization in the same solution plus specific probe at 68 °C overnight. Standard washing procedures were utilized. PhosphorImager screens (Molecular Dynamics, Amersham–Pharmacia Biotech, Life Sciences) and/or X-Omat film (Kodak, Rochester, NY) were exposed to hybridized membranes for various times.

In situ hybridization. The same cRNA probes generated for Northern blots were used for in situ hybridization. PCR product templates were in vitro transcribed with T7 and T3 RNA polymerases using a Dig RNA labeling kit (Roche Diagnostics, Indianapolis, IN) to produce Dig-11-labeled sense and antisense cRNA probes, respectively. In situ hybridization was carried out as described previously [12–14].

5′-Rapid amplification of cDNA end. A modified procedure using the Clontech Smart Race cDNA Amplification Kit (Clontech Laboratories) was utilized to obtain the 509 nucleotide novel RNA sequence. Total RNA was isolated from HBE1 cells grown to confluence in tissue culture plates. Due to the lack of a polyadenylated tail in our transcript, we performed first-strand cDNA synthesis using an 18-nucleotide gene-specific primer (GSP-1). Second strand cDNA synthesis and adaptor ligation to terminal ends were performed according to the company protocol. Polymerase chain reaction (PCR) with a second GSP (GSP-2) and the long and short universal primers provided in the 5′-rapid amplification of cDNA end (5′-RACE) kit was performed for product amplification.

GSP-1: 5′-TCTAGCAGCTGACACCAA-3′
GSP-2: 5′-GCAGCTGACACCAAGAAACAGAATGCTGACC-3′

The PCR products were subcloned into the pCR II TOPO TA vector (Invitrogen, San Diego, CA) and sequenced. DNA sequencing was carried out at the Institutional DNA Core Facility (Department of Plant Genetics, UC Davis) using the fluorescence-labeled automatic sequencing approach and separated by the ABI Prism Model 377 Automated DNA sequencer (Applied Biosystems, Foster City, CA).

Construction and transfection of a MAGE D2 Flag-tagged fusion protein. To prepare the FLAG-MAGE D2 expression vector, the ORF of MAGE D2 was amplified by PCR using appropriate primers containing *Eco*RI and *Xha*I sites. The PCR products were inserted into *Eco*RI/*Xha*I sites of the p3XFLAG-CMV-14 expression vector (Sigma–Aldrich, St. Louis, MO). For transient transfection studies, 0.5 μg of FLAG-MAGE D2 plasmid was transfected into 5×10^4 HBE1 cells in a 35 mM Petri dish with Fugene 6 (Gibco). Protein isolation for Western blot analysis was performed 72 h post-transfection.

Polyclonal antibody production and Western blot analysis. A 19-mer oligopeptide antigen was synthesized, using the deduced amino acid sequence from Gln^{166} to Asp^{184} of MAGE D2, and conjugated to multiple antigen peptide to increase its antigenicity (Bio-Synthesis,

Lewisville, TX). From this peptide, mono-specific rabbit polyclonal antibodies were generated. This region of the MAGE D2 protein was chosen both for its antigenic properties and the lack of homology with other MAGE proteins. The specificity of the polyclonal antiserum was determined by Western blot analysis.

For Western blot analysis, cultured cells were harvested as described [15,16]. Supernatant protein concentrations were determined by the method of Lowry using the Bio-Rad D_c assay (Bio-Rad, Hercules, CA). Twenty micrograms/lane of protein was subjected to discontinuous SDS–polyacrylamide gel electrophoresis according to Laemmli [17]. Proteins were blotted onto nitrocellulose membranes overnight according to the manufacturer's recommendations with a semi-dry blotting apparatus (Schleicher and Schuell) at 20 V/45 min/10 cm^2 gel surface area. Western blot hybridization was done using Western Blotting Luminol Reagent (Santa Cruz Biotechnologies, Santa Cruz, CA) and the appropriate primary and secondary antibodies.

Results

Identification of a novel RNA transcript complementary to MAGE D2 mRNA

Based on previous reports that MAGE D2 is upregulated in fetal lung (AF128528) and several cancer tissues [7], we initially examined MAGE D2 localization in normal lung tissues by in situ hybridization. Due to the presence of multiple MAGE gene family members, a computer-based search program was used to identify a nucleotide region specific for MAGE D2. A 368-base region, specific for and corresponding to the 5′-end of MAGE D2, was selected to generate sense and antisense cRNA probes. To our surprise, we observed strong hybridization signal from the sense probe and weak to absent signal from the antisense probe (Fig. 1). Importantly, the sense probe demonstrated hybridization only in the airway epithelium. There was no sense or antisense signal in the remainder of the lung parenchyma (data not shown).

To explain the discrepancy between our in situ hybridization results and previous reports, we performed Northern blot hybridization with MAGE D2-specific cRNA probes. We isolated RNA from an immortalized normal human bronchial epithelial cell line, HBE1, and two human cancer cell lines (G361, a malignant melanoma cancer cell line (ATCC #CRL-1424), and SW480, a colorectal adenocarcinoma cell line (ATCC #CCL-228)). Northern blot analysis demonstrated the presence of two MAGE D2-specific transcripts; a 2.1 kb message corresponding to MAGE D2 mRNA, and a 4.1 kb message (Fig. 2). Using poly(A)-enriched RNA, we observed that the 2.0 kb transcript,

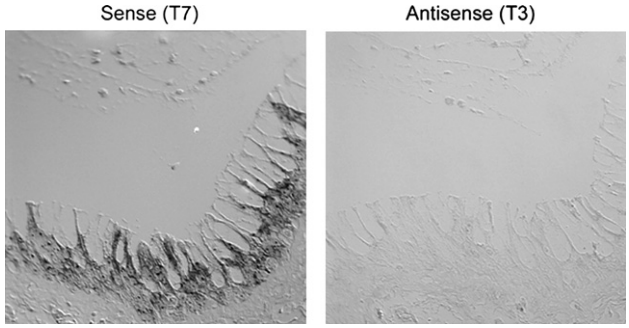

Fig. 1. An endogenous MAGE D2 antisense transcript is present in airway epithelia by in situ hybridization. Dig-labeled riboprobes were prepared and hybridized to tracheal tissues as described. There was no significant signal obtained using the T3 polymerase-generated antisense probe (T3), but strong signal limited to the airway epithelium was observed with the T7 polymerase-generated sense probe (T7).

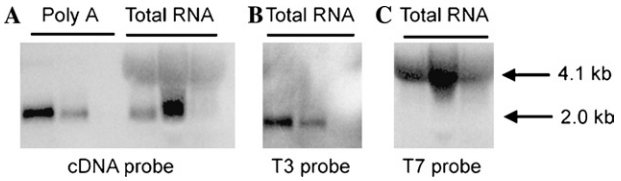

Fig. 2. Identification of a novel 4.1 kb transcript, which lacks a poly(A) tail. Northern blot hybridization using (A) cDNA probes to identify MAGE D2-specific bands in poly(A)-enriched and total RNA from G361 (lanes 1 and 4), SW480 (lanes 2 and 5), and HBE1 (lanes 3 and 6) cell lines, (B) MAGE D2 mRNA-specific T3 cRNA riboprobes to identify MAGE D2 mRNA in G361 (lane 1), SW480 (lane 2), and HBE1 (lane 3) cell lines, and (C) antisense-specific T7 cRNA riboprobes to identify the 4.1 kb transcript in G361 cells, SW480 cells, and HBE1 cells (lanes 1, 2, and 3, respectively). Equal loading of all lanes was confirmed by stripping the membrane and reprobing with a radiolabeled 18S probe (data not shown).

corresponding to MAGE D2 mRNA, hybridized with our cDNA and antisense cRNA probes in the two cancer cell lines, but not in HBE1 cells. The 4.1 kb transcript, observed in all three cell lines, hybridized with the cDNA and sense cRNA probes. However, hybridization with the 4.1 kb transcript only occurred when we utilized total RNA.

In order to clarify that the 4.1 kb transcript is in fact RNA, and not a transcript that resulted from DNA contamination during RNA preparation, we digested total RNA samples with RNAses and DNases (Fig. 3). With these samples we performed Northern blot hybridization using T7 RNA polymerase-generated sense cRNA probes. The 4.1 kb hybridization signal was sensitive to RNase digestion, but resistant to DNase.

Characterization of the 5′ end of the 4.1 kb transcript

Further characterization of the 4.1 kb transcript included rapid amplification of the 5′ end (5′ race). RACE products were cloned and underwent DNA sequencing. All clones produced nucleotide sequences precisely

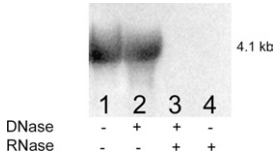

Fig. 3. The 4.1 kb MAGE D2 antisense transcript is an RNA molecule. Northern blot hybridization using antisense-specific T7 cRNA riboprobes to identify the 4.1 kb transcript in total RNA from HBE1 cells without digestion (lane 1) with DNase digestion alone (lane 2), digestion with DNase and RNase (lane 3), or RNase digestion alone (lane 4). Equal loading of all lanes was confirmed by stripping the membrane and reprobing with a radiolabeled 18S probe (data not shown).

complementary to a region within exons 3 and 4 of the MAGE D2 mRNA transcript (Fig. 4A). The longest sequence obtained was 509 nucleotides. Of importance, the start of the 4.1 kb transcript is just prior to the region where the MAGE D2 mRNA codes amino acids within the MAGE homology domain. Also, the 4.1 kb transcript does not contain any sequence contained within the intron between exons 3 and 4 (Fig. 4B). The start site of this transcript relative to MAGE D2 mRNA sequence strongly suggested to us that the new transcript may be an endogenous RNAi molecule.

The ratio of the 4.1 kb transcript and MAGE D2 mRNA affects protein expression

To elucidate a potential regulatory role of the 4.1 kb MAGE D2 antisense transcript, we examined protein expression in four different cell lines utilizing an anti-MAGE D2 rabbit polyclonal antibody (Fig. 5). We were unable to identify the 65 kDa MAGE D2 protein, or identified MAGE D2 protein at very low levels, in untransfected HBE1 cells (Fig. 5A, lane 1). As predicted, HBE1 cells transfected with a FLAG-MAGE D2 construct expressed the 68 kDa MAGE D2-FLAG fusion protein that was recognized by both anti-FLAG and anti-MAGE D2-specific antibodies (Fig. 5A, lane 2). We were able to identify high levels of endogenous MAGE D2 protein in the two cancer cell lines that expressed high levels of the endogenous 2.0 kb MAGE D2 mRNA transcript (Fig. 5A, lanes 3 and 4). Impor-

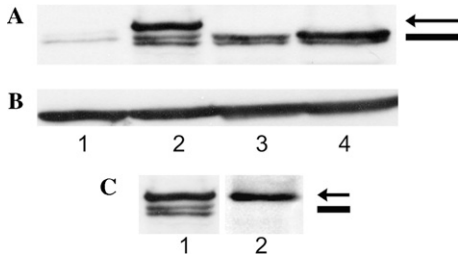

Fig. 5. Differential MAGE D2 protein expression in three different cell lines. (A) Western blot using MAGE D2-specific rabbit polyclonal antibodies against cell lysates from untransfected HBE1 cells (lane 1), HBE1 cells 24 h after transfection with a MAGE D2-FLAG chimeric construct (lane 2), and two cancer cell lines, SW480 (lane 3), and G361 (lane 4). The 68 kDa (arrow) and 65 kDa (bold line) positions were estimated from molecular weight markers. (B) Western blot membrane reprobed using anti-β-actin to ensure equal protein loading of all samples. (C) Western blot of cell lysates from HBE1 cells transfected with MAGE D2-FLAG chimeric construct using MAGE D2 specific antibodies (lane 1) and anti-FLAG antibodies (lane 2).

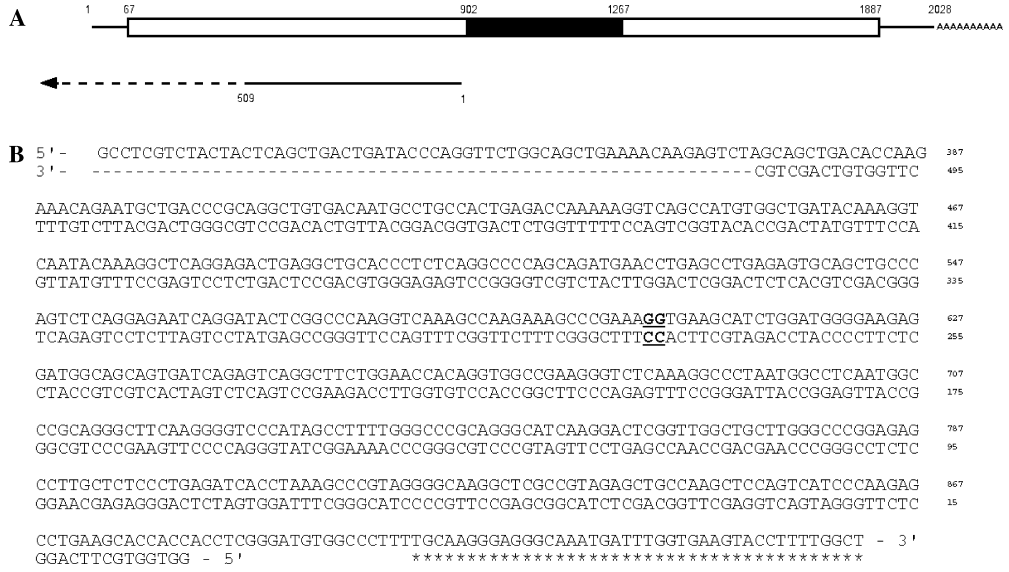

Fig. 4. Relative position of the 4.1 kb antisense transcript to MAGE D2 mRNA. (A) Representation of MAGE D2 mRNA sequence (top) in comparison to the 4.1 kb antisense sequence (bottom), open box, open reading frame; filled box, MAGE homology domain; dashed line, unsequenced 3′ end of the antisense transcript. (B) Portion of the MAGE D2 mRNA nucleotide sequence (top, nucleotides 307–942) compared with the known nucleotide sequence of the antisense transcript (bottom, nucleotides 1–509). The exon–intron splice site between exons 3 and 4 of the MAGE D2 mRNA is identically matched by the antisense transcript (bold underline). *, start of the MAGE homology domain.

tantly, the level of endogenous MAGE D2 protein was significantly elevated in transfected HBE1 cells compared with untransfected HBE1 cells (Fig. 5C). These observations suggest a potential regulatory mechanism between the 4.0 kb antisense transcript and MAGE D2 protein expression.

Discussion

At the time of this report, there are four published papers specifically examining the expression of MAGE D2 [6,7,18,19]. The 13-exon genomic structure, location on the X chromosome, and ubiquitous tissue distribution of this gene are well characterized. In our study of MAGE D2, we identified a novel, 4.1 kb RNA transcript in multiple tissue types that contains, minimally, a 509-nucleotide region exactly complementary to spliced MAGE D2 mRNA. To date, no investigators have identified this transcript, which we have preliminarily named MAGE D2i.

Because MAGE D2i shares at least 509 nucleotides of complementary sequence to MAGE D2, we performed several database searches, including GenBank databases and the Celera database, to identify transcripts or genomic sequences that shared this 509-base homology. Up until the time of this report, we were unsuccessful in finding any sequence other than the MAGE D2 gene.

There are several reasons why previous reports of MAGE D2 have not identified both transcripts. For one, two out of the four reports use RT-PCR alone to characterize gene expression [6,18]. This technique will selectively identify only one transcript. Two, the types of blots previously used were not able to determine the presence of two unique transcripts. For example, Langnaese et al. [19] utilized poly(A)-enriched RNA for some of their Northern blot data. As we have shown, our MAGE D2i RNA lacks a poly(A) tail and therefore our transcript will not be identified using this technique. Similarly, Kurt et al. [7] utilized dot blots and cDNA probes. Neither the blot itself nor the probes will be able to discriminate between two transcripts that are complementary to each other.

There are two observations, however, in conflict with our data and difficult to explain. The in situ data from the Langnaese paper clearly demonstrate antisense probe tissue hybridization in rat brain. This conflicts with our in situ hybridization data in human lung tissue, where only the sense probe was able to generate a signal. This discrepancy may be due to a species-specific difference or a tissue-specific difference in gene expression. Further investigation of human brain tissue using strand-specific riboprobes will be able to resolve this issue.

Another set of observations that are in conflict with our data are the Northern blots using total RNA by both Langnaese et al. and Kurt et al. Their cDNA-probed Northern blots show only one RNA species in contrast to the two we observed. Again, species-specific difference or a tissue-specific difference may explain these data. Alternatively, the less stringent hybridization conditions we used may have allowed identification of the 4.1 kb transcript. It is possible that the cDNA probes did not have sufficient overlap with the 5′ end of the MAGE D2i transcript to allow stable hybridization in the more stringent hybridization conditions.

Due to technical limitations, we have been unable to fully sequence the MAGE D2i transcript. The lack of a polyadenylated tail has made it exceptionally difficult for our laboratory to obtain further sequence information. We have attempted several methodologies to resolve the 3′ end of the molecule without success. However, we have identified important elements of the transcript.

We have unambiguously established that the MAGE D2i RNA transcript is a RNA molecule of approximately 4.1 kb (Fig. 3). Importantly, we have determined that this new RNA species lacks a poly(A) tail. This is in direct contrast to MAGE D2 mRNA, which we were able to identify in both total RNA and poly(A)-enriched RNA. Furthermore, we have characterized the 5′ end of the molecule and established two important features. One, the 5′ end of the molecule will hybridize to MAGE D2 mRNA prior to the start of the MAGE homology domain (Fig. 4A). This strongly suggests that the MAGE D2i molecule is specifically regulating MAGE D2 mRNA and protein expression. Two, based on the X chromosome genomic sequence, the MAGE D2i RNA molecule contains splice sites that are identical to the MAGE D2 mRNA (Fig. 4B). This is a unique feature not previously identified with other antisense transcripts. It will be important to determine how this MAGE D2i molecule is made, as the splice junctions for the MAGE D2i molecule do not contain the well-established splicing consensus sequences identified for processing mRNA.

The MAGE D, E, and F family members have been characterized as having ubiquitous expression in several tissues in contrast to the cancer- and testis-specific expression of other MAGE family members [5,6]. Importantly, our data suggest this categorization may not fully describe the expression pattern of MAGE D2. We demonstrated significant expression of MAGE D2i RNA, but not MAGE D2 mRNA, in airway epithelia. In comparison, both transcripts were highly expressed in the two cancer lines we examined. Previous reports demonstrating ubiquitous MAGE D2 mRNA expression utilized techniques that are unable to determine which transcript is present. It is possible, based on our data, that MAGE D2i expression is ubiquitous and MAGE D2 expression remains cancer-specific. Future studies examining MAGE D2 expression will need to consider our newly identified transcript.

Our MAGE D2 protein data suggest MAGE D2i regulates MAGE D2 mRNA expression. As shown in Fig. 5, untransfected HBE1 cells express little to no endogenous MAGE D2 protein. In parallel with this, RNA data from Fig. 2 demonstrate minimal to no MAGE D2 mRNA expression but high MAGE D2i RNA expression in HBE1 cells. However, in HBE1 cells transfected with a MAGE D2-FLAG construct, we observed increased expression of the 65 kDa endogenous MAGE D2 protein in addition to the expected expression of the 68 kDa MAGE D2-FLAG fusion protein (Fig. 5). We suggest that overexpression of the MAGE D2-FLAG construct generates sufficient MAGE D2-specific mRNA nucleotide sequence to temporarily overwhelm the mechanisms responsible for endogenous MAGE D2 mRNA suppression; this results in transient expression of endogenous MAGE D2 protein.

It follows from this argument that the relative levels of both MAGE D2i RNA and MAGE D2 mRNA determine whether or not MAGE D2 protein expression occurs. This concept is supported when we closely compare the relative amounts of MAGE D2i RNA and MAGE D2 mRNA (Fig. 2) to the amount of endogenous protein expressed (Fig. 5) in the three examined cell lines. However, we recognize establishing any functional nature for this 4.1 kb transcript is beyond the scope of this report.

Conclusion

We have identified a novel RNA transcript that is present in multiple tissue types and shares complementary homology with the MAGE D2 gene. This finding has important implications for future studies that aim to characterize expression and function of the MAGE D2 gene. The unique structural features of the MAGE D2i transcript, complementary overlap with MAGE D2 mRNA and absence of a poly(A) tail, has implications for the origination and regulation of MAGE proteins as a whole.

Acknowledgments

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References

[1] Y.P. Di, R. Harper, Y. Zhao, N. Pahlavan, W. Finkbeiner, R. Wu, Molecular cloning and characterization of spurt, a human novel gene that is retinoic acid-inducible and encodes a secretory protein specific in upper respiratory tracts, J. Biol. Chem. 278 (2003) 1165–1173.

[2] C.M. Soref, Y.P. Di, L. Hayden, Y.H. Zhao, M.A. Satre, R. Wu, Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol, J. Biol. Chem. 276 (2001) 24194–24202.

[3] P. vander Bruggen, C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Vanden Eynde, A. Knuth, T. Boon, A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma, Science 254 (1991) 1643–1647.

[4] P.A. Barker, A. Salehi, The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease, J. Neurosci. Res. 67 (2002) 705–712.

[5] A.F. Kirkin, K.N. Dzhandzhugazyan, J. Zeuthen, Cancer/testis antigens: structural and immunobiological properties, Cancer Invest. 20 (2002) 222–236.

[6] P. Chomez, O. De Backer, M. Bertrand, E. De Plaen, T. Boon, S. Lucas, An overview of the MAGE gene family with the identification of all human members of the family, Cancer Res. 61 (2001) 5544–5551.

[7] R.A. Kurt, W.J. Urba, D.D. Schoof, Isolation of genes overexpressed in freshly isolated breast cancer specimens, Breast Cancer Res. Treat. 59 (2000) 41–48.

[8] J.R. Yankaskas, J.E. Haizlip, M. Conrad, D. Koval, E. Lazarowski, A.M. Paradiso, C.A. Rinehart Jr., B. Sarkadi, R. Schlegel, R.C. Boucher, Papilloma virus immortalized tracheal epithelial cells retain a well-differentiated phenotype, Am. J. Physiol. 264 (1993) C1219–C1230.

[9] R. Wu, W.R. Martin, C.B. Robinson, J.A. St George, C.G. Plopper, G. Kurland, J.A. Last, C.E. Cross, R.J. McDonald, R. Boucher, Expression of mucin synthesis and secretion in human tracheobronchial epithelial cells grown in culture, Am. J. Respir. Cell Mol. Biol. 3 (1990) 467–478.

[10] R. Harper, K. Wu, M.M. Chang, K. Yoneda, R. Pan, S.P. Reddy, R. Wu, Activation of nuclear factor-kappa b transcriptional activity in airway epithelial cells by thioredoxin but not by *N*-acetyl-cysteine and glutathione, Am. J. Respir. Cell Mol. Biol. 25 (2001) 178–185.

[11] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[12] H. Cui, R. Ohlsson, An improved protocol for purification of 35S-labelled oligonucleotide DNA probes for in situ hybridization applications, Trends Genet. 12 (1996) 506–507.

[13] P. Komminoth, Digoxigenin as an alternative probe labeling for in situ hybridization, Diagn. Mol. Pathol. 1 (1992) 142–150.

[14] P. Komminoth, J. Roth, S. Schroder, P. Saremaslani, P.U. Heitz, Overlapping expression of immunohistochemical markers and synaptophysin mRNA in pheochromocytomas and adrenocortical carcinomas. Implications for the differential diagnosis of adrenal gland tumors, Lab. Invest. 72 (1995) 424–431.

[15] Y.J. Wu, L.M. Parker, N.E. Binder, M.A. Beckett, J.H. Sinard, C.T. Griffiths, J.G. Rheinwald, The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia, Cell 31 (1982) 693–703.

[16] T.H. Huang, J.A. St George, C.G. Plopper, R. Wu, Keratin protein expression during the development of conducting airway epithelium in nonhuman primates, Differentiation 41 (1989) 78–86.

[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[18] S. Lucas, F. Brasseur, T. Boon, A new MAGE gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells, Cancer Res. 59 (1999) 4100–4103.

[19] K. Langnaese, D.U. Kloos, M. Wehnert, B. Seidel, P. Wieacker, Expression pattern and further characterization of human MAGED2 and identification of rodent orthologues, Cytogenet. Cell Genet. 94 (2001) 233–240.