

# Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, *mda-5*: a novel type I interferon-responsive apoptosis-inducing gene

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Melanoma differentiation associated gene-5 (*mda-5*) was identified by subtraction hybridization as a novel upregulated gene in HO-1 human melanoma cells induced to terminally differentiate by treatment with IFN- $\beta$  + MEZ. Considering its unique structure, consisting of a caspase recruitment domain (CARD) and an RNA helicase domain, it was hypothesized that *mda-5* contributes to apoptosis occurring during terminal differentiation. We have currently examined the expression pattern of *mda-5* in normal tissues, during induction of terminal differentiation and after treatment with type I IFNs. In addition, we have defined its genomic structure and chromosomal location. IFN- $\beta$ , a type I IFN, induces *mda-5* expression in a biphasic and dose-dependent manner. Based on its temporal kinetics of induction and lack of requirement for prior protein synthesis *mda-5* is an early type I IFN-responsive gene. The level of *mda-5* mRNA is in low abundance in normal tissues, whereas expression is induced in a spectrum of normal and cancer cells by IFN- $\beta$ . Expression of *mda-5* by means of a replication incompetent adenovirus, Ad.*mda-5*, induces apoptosis in HO-1 cells as confirmed by morphologic, biochemical and molecular assays. Additionally, the combination of Ad.*mda-5* + MEZ further augments apoptosis as observed in Ad.*null* or uninfected HO-1 cells induced to terminally differentiate by treatment with IFN- $\beta$  + MEZ. The *mda-5* gene is located on human chromosome 2q24 and consists of 16 exons, without pseudogenes, and is conserved in the mouse genome. Present data documents that *mda-5* is a novel type I IFN-inducible gene, which may contribute to apoptosis induction during terminal differentiation and during IFN treatment. The conserved genomic and protein structure of *mda-5* in human and mouse will permit

analysis of the evolution and developmental aspects of this gene.

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## Introduction

Differentiation therapy of cancer is based on the hypothesis that transformed tumor cells are derived from normal cells displaying defects in their ability to terminally differentiate thereby resulting in a loss of normal growth control mechanisms (Jiang *et al.*, 1994; Fusenig *et al.*, 1995; Scott, 1997; Garattini and Terao, 2001; Lotem and Sachs, 2002). A logical extension of this concept is that cancer cells can be induced to differentiate by suitable treatment to reactivate endogenous growth control mechanisms, which in a mutated and genetically damaged cancer cell context can culminate in apoptosis (Wang and Chen, 2000; Leszczyniecka *et al.*, 2001). This possibility has been addressed experimentally utilizing various model systems with the capacity to undergo *in vitro* differentiation (Lovat *et al.*, 1997; Petersen *et al.*, 1998; Wang and Chen, 2000; Leszczyniecka *et al.*, 2001). Analysis of gene expression changes in differentiation therapy models provides a powerful experimental tool for delineating and understanding biochemical and molecular events mediating growth arrest of transformed cells (Jiang and Fisher, 1993; Huang *et al.*, 1999a, b; Jiang *et al.*, 2000; Zhang *et al.*, 2000). In addition, studies in such systems can lead to identification of specific molecules, which are important activators of the terminal differentiation process. Such molecules, in addition to being important analytical tools, also have therapeutic potential in anticancer gene therapy or drug design (Petersen *et al.*, 1998; Zhang *et al.*, 2000; Su *et al.*, 2001; Fisher *et al.*, 2003).

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Combined treatment of HO-1 human melanoma cells with IFN- $\beta$  and mezerein (MEZ), an antileukemic PKC activating compound, induces terminal differentiation, a process involving irreversible changes in growth kinetics and morphology and ultimately apoptosis (Fisher *et al.*, 1985; Jiang *et al.*, 1993, 1994). It should be noted that treatment of HO-1 cells with IFN- $\beta$  alone inhibits cell proliferation and increases melanogenesis, while combined treatment with MEZ induces changes in cell growth characteristics and morphology resembling a terminally differentiated state (Fisher *et al.*, 1985; Jiang *et al.*, 1993, 1994). To define the molecular aspects of the terminal differentiation process, changes in gene expression patterns were analysed between undifferentiated and IFN- $\beta$  + MEZ differentiated cells by subtracted cDNA library screening approaches (Jiang and Fisher, 1993; Jiang *et al.*, 1994), including rapid subtraction hybridization (RaSH) (Jiang *et al.*, 2000) and differentiation induction subtraction hybridization (DISH) (Huang *et al.*, 1999b). Melanoma differentiation associated gene-5 (*mda-5*) was identified as an upregulated sequence in a cDNA subtraction library screening of terminally differentiating HO-1 cells by combined treatment with IFN- $\beta$  and MEZ, although treatment with IFN- $\beta$  is also sufficient to induce significant gene expression over basal levels (Jiang and Fisher, 1993; Kang *et al.*, 2002).

Protein motif analysis through the PROSITE database indicated that *mda-5* has unique structural attributes (Kang *et al.*, 2002). MDA-5 consists of an amino-terminal caspase recruitment domain (CARD) and a carboxyl-terminal putative RNA helicase domain, representing a novel combination of functional domains that is unprecedented in any previously identified helicase family protein (Kang *et al.*, 2002; Kovacovics *et al.*, 2002). The closest type of 'dual-function heterologous domain fusion' currently recognized in helicase molecules is the fusion of functionally interconnected helicase and RNase motifs found in several putative RNA helicases (Jiang *et al.*, 2000; Bernstein *et al.*, 2001). The CARD motif is found in diverse apoptotic molecules, such as Apaf-1, caspase-2, -4 and -9 and inhibitors of apoptosis, and is involved in apoptotic signaling by homotypic protein-protein interactions (Hofmann *et al.*, 1997; Bouchier-Hayes and Martin, 2002).

The carboxyl-terminus two-thirds of MDA-5 contains putative RNA helicase domains (Kang *et al.*, 2002). RNA helicases function primarily in destabilizing and unwinding dsRNA using energy generated by ATP hydrolysis (Gorbalenya and Koonin, 1993; Luking *et al.*, 1998). They belong to helicase superfamily II and are grouped into three subfamilies based on the structure of their ATPase B motifs. The three currently recognized families include the DEAD, DEAH and DExH box containing helicases (Gorbalenya and Koonin, 1993; Luking *et al.*, 1998; Jankowsky and Jankowsky, 2000). RNA helicases are involved in every step of RNA metabolism including transcription, translation, RNA editing, splicing and degradation (Luking *et al.*, 1998; Jankowsky and Jankowsky, 2000). RNA helicases

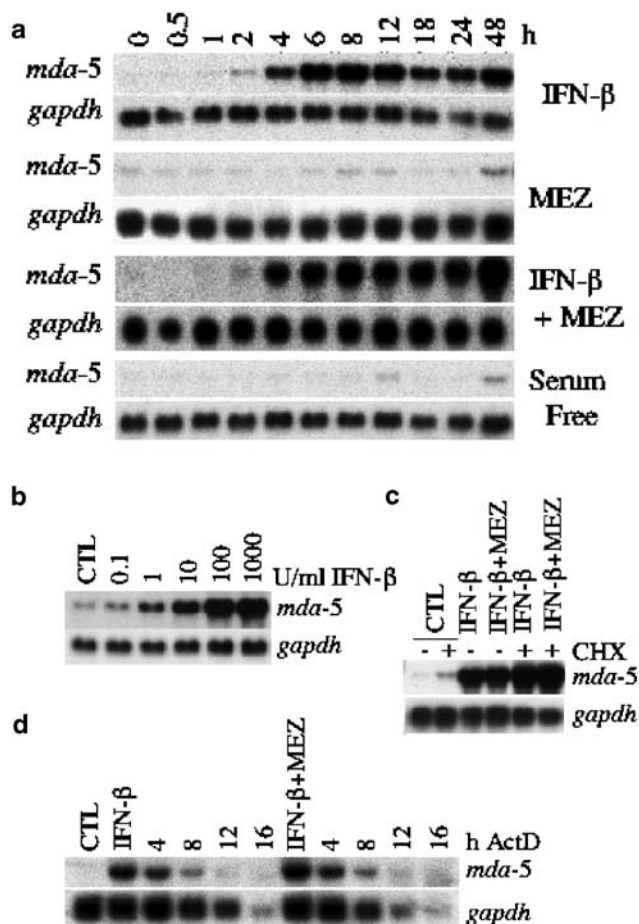
consist primarily of motifs for ATPase, binding and unwinding RNA (Gorbalenya and Koonin, 1993; Luking *et al.*, 1998; Jankowsky and Jankowsky, 2000). MDA-5 contains eight of the nine conserved signatures identifying the DExH subfamily of RNA helicases (Luking *et al.*, 1998; Jankowsky and Jankowsky, 2000). Among the RNA helicases, a subset where the helicase domain is mostly fused with an RNase III motif has been shown to share further homology with the helicase motif of MDA-5 (Kang *et al.*, 2002), the implications of which are presently unclear. Both purified full-length GST-MDA-5 and the recombinant helicase domain of murine MDA-5/Helicard manifest significant dsRNA-dependent ATPase activity (Kang *et al.*, 2002; Kovacovics *et al.*, 2002). DNA-dependent ATPase activity of MDA-5 is much weaker than dsRNA-dependent ATPase activity, supporting its RNA specificity (Kang *et al.*, 2002).

Based on its novel structure and distinctive properties, the IFN- $\beta$ -inducible *mda-5* gene is uniquely poised to address relevant questions relating to cellular differentiation, apoptosis and oncogenesis. Analyzing *mda-5*'s molecular properties offers potential to obtain insights into the processes of growth control and apoptosis associated with induction of terminal differentiation and irreversible growth arrest in cancer cells. In this report we begin to address several aspects of *mda-5* regulation and perform detailed genomic characterization and expression profile studies. Our results document that *mda-5* is an early type I IFN-inducible gene whose expression is elevated in a wide-spectrum of normal and cancer cell types following treatment predominantly with IFN- $\beta$  and forced expression of *mda-5* in HO-1 human melanoma cells induces apoptosis.

## Results

### *Expression pattern of mda-5*

The expression pattern of *mda-5* was analysed in detail by Northern blot hybridization to determine whether induction of *mda-5* was directly responsive to IFN- $\beta$  or expression was indirectly induced as a result of secondary cellular changes (Figure 1). Treatment of HO-1 cells with IFN- $\beta$  or IFN- $\beta$  + MEZ resulted in a biphasic induction of *mda-5* (Figure 1a). A threefold increase in steady-state *mda-5* message level was evident 2 h after treatment with IFN- $\beta$  or IFN- $\beta$  + MEZ. The maximum level of *mda-5* mRNA was apparent by 8 h after treatment and then decreased by a small extent before a subsequent increase was observed at 24 h postinduction. Treatment with IFN- $\beta$  plus MEZ further increased both the amount and the duration of the *mda-5* mRNA increase in comparison with treatment with IFN- $\beta$  alone. Both MEZ and serum starvation, which cause growth inhibition in HO-1 cells induced *mda-5* expression in the absence of IFN- $\beta$  treatment. This induction was significantly delayed (requiring 48 h) as compared to the IFN- $\beta$  response and the overall levels of *mda-5* mRNA produced were considerably lower



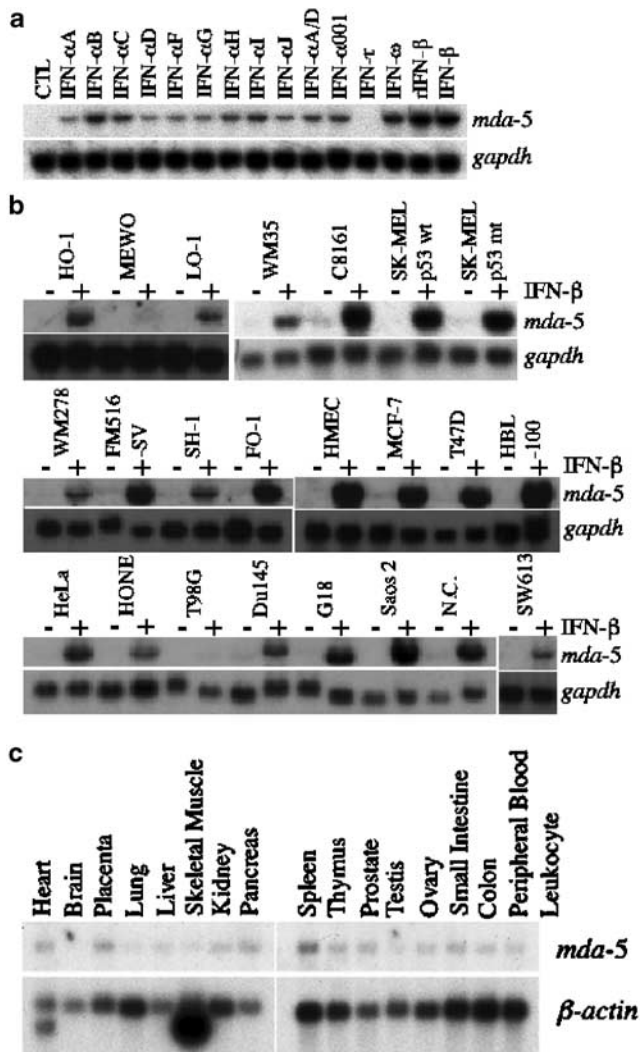
**Figure 1** Northern blot analysis of *mda-5* expression I. (a) Temporal kinetics of *mda-5* expression. RNA samples were extracted from HO-1 cells treated with 2000 U/ml IFN- $\beta$ , 10 ng/ml MEZ or 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ or serum free media, and harvested at the indicated times. Northern blots were prepared as in Materials and methods and were probed with a  $^{32}$ P-labeled *mda-5* cDNA, the blots were stripped and reprobed with a  $^{32}$ P-labeled *gapdh* cDNA. (b) Dose-response of *mda-5* expression. RNA samples were extracted from HO-1 cells treated with the indicated amount of IFN- $\beta$  for 16 h. Northern blot hybridizations were performed as above. (c) The effect of cycloheximide (CHX) treatment on *mda-5* message level. RNA samples were extracted from HO-1 melanoma cells pretreated with 50  $\mu$ g/ml CHX for 30 min and treated with the indicated reagents for 8 h. Northern blot hybridizations were performed as above. (d) The effect of ActD treatment on induced *mda-5* message level. HO-1 melanoma cells were treated with 5  $\mu$ g/ml ActD after incubation with 2000 U/ml IFN- $\beta$  or 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ for 24 h. Cells were harvested at the indicated time after ActD treatment. Northern blot hybridizations were performed as above

(only 2–4-fold over basal activity) suggesting a secondary mediator of this response, for example, growth inhibition (Figure 1a). The induction of *mda-5* was apparent with as little as 0.1 U/ml IFN- $\beta$ , a dose of IFN which does not alter HO-1 cell growth, suggesting a specific response to this cytokine rather than a secondary effect resulting from growth suppression (Figure 1b). The induction of *mda-5* was gradually increased up to 1000 U/ml of IFN- $\beta$  in HO-1 cells.

Treatment with the protein synthesis inhibitor cycloheximide (CHX) prior to IFN- $\beta$  was used to determine whether new protein synthesis was needed for *de novo* *mda-5* gene expression. Pretreatment with CHX increased *mda-5* mRNA levels in uninduced and in IFN- $\beta$  or IFN- $\beta$  + MEZ treated HO-1 cells by ~1.5–2-fold (Figure 1c). This enhanced induction of *mda-5* in the presence of CHX supports the existence of a message degradation mechanism targeting *mda-5* mRNA for destruction. Treatment with actinomycin D (ActD) to inhibit transcription indicated that the *mda-5* message has a half-life of ~7 h in both IFN- $\beta$ - and IFN- $\beta$  + MEZ-treated cells (Figure 1d). Since the degradation kinetics of *mda-5* message was not affected by the presence of MEZ, prolonged maintenance of *mda-5* message following combined treatment with IFN- $\beta$  + MEZ compared to IFN- $\beta$  alone may reflect cooperative induction by these agents. It is possible that the delayed induction kinetics of *mda-5* following MEZ treatment might contribute to the longer and higher induction level seen with the combination. The rapid induction kinetics, sensitivity to IFN- $\beta$  treatment and the ineffectiveness of CHX in blocking *mda-5* induction by IFN- $\beta$  suggest that *mda-5* is an IFN- $\beta$  early-response gene, not requiring prior protein synthesis for induction.

The induction of *mda-5* expression in HO-1 cells by additional type I IFNs was analysed by Northern blot hybridization (Figure 2a). Both natural and recombinant IFN- $\beta$ , which induced *mda-5* at similar levels, elicited the strongest response among the IFNs tested (Figure 2a). The relative extent of induction compared with IFN- $\beta$  was ~75% by IFN- $\omega$ , ~50% by IFN- $\alpha$ B, - $\alpha$ C, - $\alpha$ H, - $\alpha$ I, - $\alpha$ A/D and - $\alpha$ 001, and ~25% by IFN- $\alpha$ A, - $\alpha$ D, - $\alpha$ F, - $\alpha$ G and - $\alpha$ J, respectively. Since a similar pattern of induction of *HuUBP43* mRNA was also evident in HO-1 cells treated with the same IFN subtypes (Kang *et al.*, 2001), it appears that the differential response of *mda-5* reflects different potencies of the various IFNs as inducers of the specific IFN-inducible genes. Although the extent of message level varied, *mda-5* expression induced by IFN- $\beta$  was observed in most cell types including primary normal human cells (HMEC), immortalized normal human cell lines (FM516-SV, HBL-100 and normal cerebellum) and human cancer cell lines including melanoma, breast cancer, glioblastoma multiforme, cervical cancer, nasopharyngeal cancer, prostate cancer, osteosarcoma and colon cancer (Figure 2b). While *mda-5* expression was increased by ~15-fold on average, less than threefold induction was observed in MeWo and T98G cells (Figure 2b), although the signaling pathways for type I IFNs are reportedly functional in both these cell lines (Friedman *et al.*, 1984; Jackson *et al.*, 2003). The reduced induction of *mda-5* mRNA correlates with relative resistance to IFN- $\beta$ -induced inhibition of proliferation in MeWo melanoma cells and T98G glioblastoma cells (Tanabe *et al.*, 2000; Jackson *et al.*, 2003).

The basal level of *mda-5* expression in different organs was examined by Northern hybridization of multiple tissue Northern blots (BD Bioscience, Palo Alto, CA,



**Figure 2** Northern blot analysis of *mda-5* expression II. (a) Induced expression of *mda-5* by various IFNs. RNA samples were extracted from HO-1 cells treated with 100 U/ml of the indicated IFN subtypes for 24 h. Northern blot hybridizations were performed as described in Figure 1. (b) IFN-β-induced *mda-5* expression in various human normal and tumor cell lines. RNA samples were extracted from the indicated cells treated with 2000 U/ml IFN-β for 24 h. Northern blot hybridizations were performed as described in Figure 1. (c) Organ-specific expression of *mda-5*. Multiple tissue Northern blots containing 2 μg of poly A<sup>+</sup> RNA from various organs were probed with a <sup>32</sup>P-labeled *mda-5* cDNA, the blots were stripped and re-probed with a <sup>32</sup>P-labeled β-actin cDNA

USA). The steady-state *mda-5* message level was extremely low (requiring extended exposure for ~3 days for visualization) (Figure 2c). Among the various tissues, *mda-5* message level was highest in spleen and placenta, and almost undetectable in brain, lung, liver, testis and smooth muscle. Higher expression of *mda-5* in spleen, but not in thymus, suggests a possible association of *mda-5* in humoral immune response and the low basal level of *mda-5* message in most tissues highlights the inducible rather than constitutive nature of expression of this gene. Taken together, the Northern blot

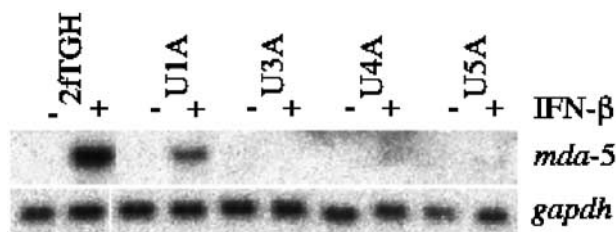
analysis of *mda-5* expression clearly demonstrates that *mda-5* is an early and highly sensitive IFN-inducible gene displaying rapid temporal induction kinetics with limited basal expression in normal tissue. In addition, the induction of *mda-5* by various subtypes of IFNs in a wide variety of cells implicates *mda-5* as a general type I IFN-responsive gene.

*Signaling pathways involved in IFN-induced mda-5 expression*

The signal transduction pathways involved in IFN-stimulated gene (ISG) expression includes activation of JAK family protein tyrosine kinases and STAT proteins (Stark *et al.*, 1998; Samuel, 2001). Specifically, Jak1, Tyk2, STAT1 and STAT2 play a major role in type I IFN receptor signaling. Based on these considerations, studies of *mda-5* induction by IFN were performed using a series of fibrosarcoma cell lines defective in IFN signaling. Derivatives of the 2fTGH fibrosarcoma, wild type in IFN signaling, were compared with U1A, U3A, U4A and U5A cells whose signaling defects could be complemented by expression of tyk2, STAT1, jak1 or IFNAR2, respectively (Pellegrini *et al.*, 1989; Darnell *et al.*, 1994; Lutfalla *et al.*, 1995). While IFN-β treatment induced *mda-5* expression in 2fTGH cells, inducible expression of *mda-5* was severely impaired in all of the mutant cell lines (Figure 3). A partial induction of *mda-5* was observed in U1A cells, which was anticipated due to the lack of tight signaling regulation in this clone (Darnell *et al.*, 1994). This data confirms that the well-established JAK/STAT signaling pathway is directly involved in IFN-inducible *mda-5* expression. The fact that *mda-5* is an early-response gene whose expression requires JAK/STAT signaling suggests that *mda-5* may be a direct target of transcriptional regulation by the ISGF3 complex (Stark *et al.*, 1998; Samuel, 2001).

*Overexpression of mda-5 induces apoptosis*

Ectopic expression of *mda-5* reduces colony forming ability by ~70% in HO-1 melanoma cells (Kang *et al.*, 2002). This effect could be the consequence of *mda-5*-induced inhibition of cell proliferation or induction of cell death. In order to distinguish between these two



**Figure 3** IFN-β-induced *mda-5* expression in human fibrosarcoma cell derivatives defective in the interferon signaling pathway. The indicated cells were treated with IFN-β (2000 U/ml) for 16 h and harvested for RNA extraction. Northern blot hybridizations were performed as described in Materials and methods. The complementing genes for the mutant cell lines are as follows: 2fTGH, wild-type; U1A, tyk2; U3A, STAT1; U4A, jak1; U5A, IFNAR2

possibilities, *mda-5* was overexpressed using a replication incompetent adenoviral vector to overcome low transfection efficiency of HO-1 melanoma cells (Figure 4a). In an MTT assay, viable cell counts were reduced in Ad.*mda-5*-infected HO-1 cells by ~75% in comparison with Ad.*null*-infected cells (Figure 4b). Moreover, addition of MEZ to Ad.*mda-5*-infected HO-1 cells essentially eliminated cell viability and this effect actually exceeded what was observed in Ad.*null*-infected cells exposed to the combination of IFN- $\beta$  and

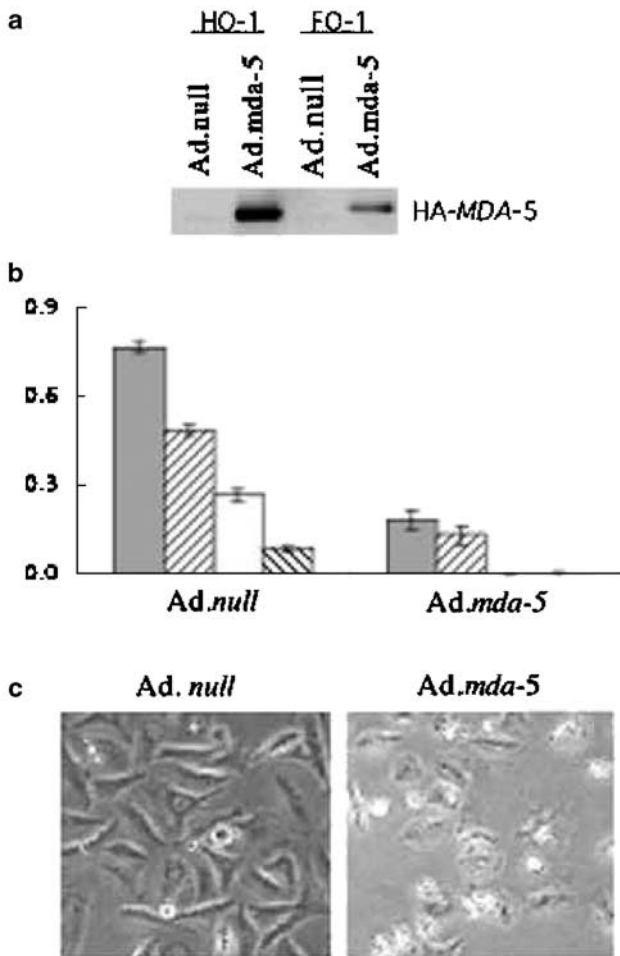
MEZ (Figure 4b). This implies that the endogenous *mda-5* gene product may play a role in cell death observed in uninfected cells treated with IFN- $\beta$  + MEZ.

Ad.*mda-5* infection of HO-1 cells resulted in typical morphological signs of cell death, including membrane irregularity and cell rounding (Figure 4c). The MTT assay results were supported by cell cycle analysis by FACS (Figure 5a). The sub-G<sub>0</sub> cell population significantly increased in Ad.*mda-5*-infected HO-1 melanoma cells (~28%), compared with ~3% in Ad.*null*-infected cells. Moreover, Ad.*mda-5* infection combined with MEZ increased the sub-G<sub>0</sub> population, while only combined treatment with IFN- $\beta$  + MEZ significantly increased the sub-G<sub>0</sub> population in Ad.*null*-infected cells. As observed in the MTT assay, only a minor increase in sub-G<sub>0</sub> population was detected by treatment with IFN- $\beta$  combined with Ad.*mda-5* infection (28% for Ad.*mda-5* alone and 38% for Ad.*mda-5* + IFN- $\beta$ ). These results confirm that overexpression of *mda-5* by itself induces cell death and this effect is augmented by cotreatment with MEZ in a similar manner as observed in terminally differentiating HO-1 cells exposed to the combination of IFN- $\beta$  + MEZ.

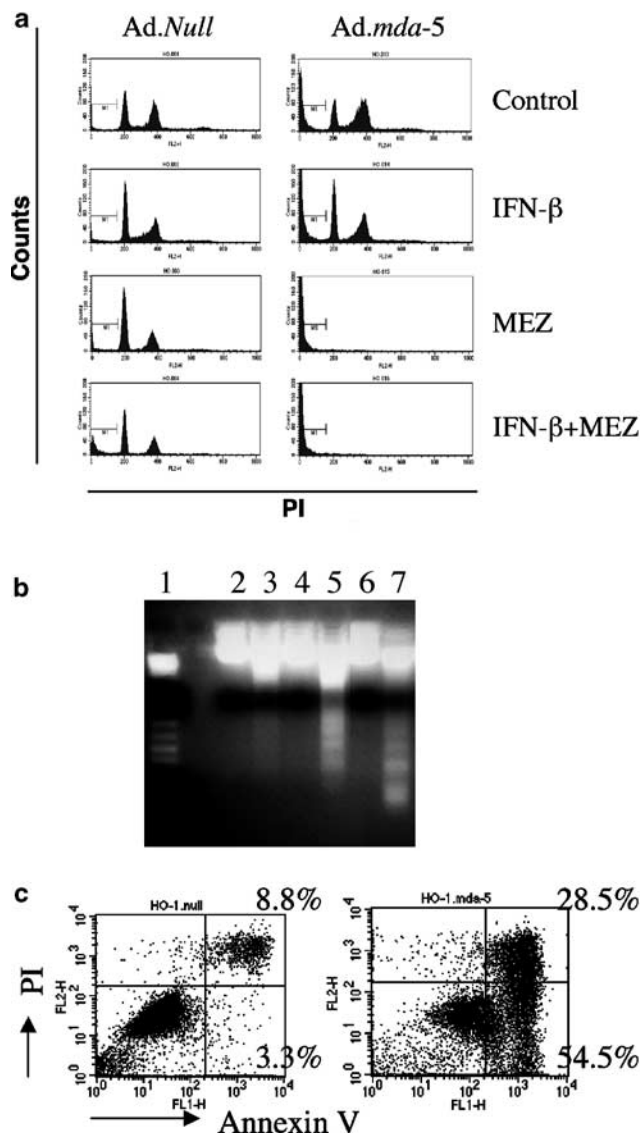
An apoptotic role of *mda-5* was anticipated based on the presence of a CARD-containing motif in the predicted protein (Kang *et al.*, 2002). To confirm this possibility, nucleosomal DNA fragmentation and Annexin V-binding assays were performed (Raffray and Cohen, 1997). Treatment of HO-1 cells with IFN- $\beta$  + MEZ resulted in the formation of a DNA ladder documenting nucleosomal DNA fragmentation and confirming apoptotic cell death. Although less pronounced, minor DNA degradation was also observed in HO-1 cells treated only with IFN- $\beta$  (Figure 5b). In contrast, genomic DNA was not degraded following treatment with MEZ. While infection of Ad.*null* did not induce nucleosomal DNA fragmentation, infection of Ad.*mda-5* induced a DNA ladder indicative of apoptosis (Figure 5b). Additionally, Annexin V binding, which results from phosphatidylserine exposure during the apoptotic process and precedes apoptotic cell death, was analysed by FACS of double stained adenovirus-infected cells with Annexin V and propidium iodide (Figure 5c). Annexin V binding was significantly higher in HO-1 cells infected with Ad.*mda-5* than in Ad.*null*-infected cells (combination of upper and lower right quadrants, 83 and 12.1%, respectively). Furthermore, the Annexin V-positive and propidium iodide-negative cell population (lower-right quadrant), representing apoptotic cell death but not necrotic cell death, also dramatically increased in Ad.*mda-5*-infected cells when compared with Ad.*null*-infected cells. In summary, both DNA ladder formation and Annexin V-binding results provide definitive evidence that Ad.*mda-5* infection can induce apoptotic cell death in HO-1 cells.

#### Effects of specific *mda-5* domains on cellular growth and survival

Adenoviruses expressing deletions of the putative functional domains of *mda-5* were constructed and

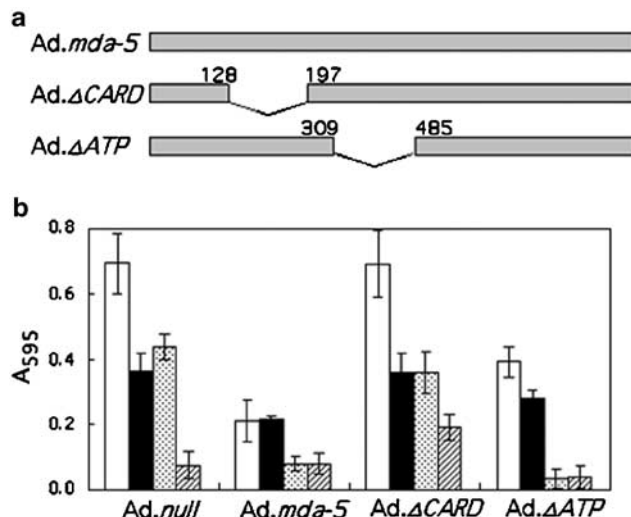


**Figure 4** Apoptotic cell death by overexpression of *mda-5*. (a) Western blot analysis of *MDA-5* expression. HO-1 and FO-1 melanoma cells were infected with Ad.*null* or Ad.*mda-5* at an m.o.i. of 25 PFU/cell and harvested 3 days later. Protein samples and Western blots were prepared as in Materials and methods and were successively probed with mouse anti-HA monoclonal antibody (Covance) and with HRP-conjugated anti-mouse IgG and detected by ECL (Amersham Pharmacia, Piscataway, NJ, USA). (b) MTT cell viability assay. HO-1 cells plated at 2500 per well in a 96-well plate on the previous day were infected with the indicated virus at an m.o.i. of 25 PFU/cell as described in Materials and methods. Infected cells were treated with 2000 U/ml IFN- $\beta$ , 10 ng/ml MEZ or 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ on the following day and MTT assays were performed 3 days after treatment as described in Materials and methods. Filled boxes are untreated; hatched, IFN- $\beta$ ; empty, MEZ; reversely hatched, IFN- $\beta$  + MEZ; y-axis, A<sub>595</sub>. (c) Morphology of Ad.*null*- and Ad.*mda-5*-infected HO-1 cells. HO-1 cells were infected with 25 PFU/cell of Ad.*null* or Ad.*mda-5* and photographed using phase contrast microscopy 3 days later



**Figure 5** Confirmation of apoptosis induction by *mda-5* (a) FACS analysis of sub-G<sub>0</sub> cell populations. HO-1 cells plated on six-well plate (20 000/well) the previous day were infected and treated as in (Figure 4b). After 3 days, sub-G<sub>0</sub> cells were quantitated by FACS as described in Materials and methods. (b) DNA ladder formation by nucleosomal genomic DNA fragmentation. HO-1 cells plated on 10-cm dishes the previous day were treated with 2000 U/ml IFN- $\beta$ , 10 ng/ml MEZ or 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ for 3 days. HO-1 cells were plated on 10-cm plates and infected as described in (Figure 4b) with 25 PFU/cell of *Ad.null* or *Ad.mda-5*. At 3 days after infection or treatment, cells were harvested for genomic DNA preparation and analysed as described in Materials and methods. Lane 1, 1 kDa ladder; lane 2, untreated; lane 3, 2000 U/ml IFN- $\beta$ ; lane 4, 10 ng/ml MEZ; lane 5, 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ; lane 6, 25 PFU/cell of *Ad.null*; lane 7, 25 PFU/cell of *Ad.mda-5*. (c) Annexin V binding to *mda-5* overexpressing cells. HO-1 cells plated on six-well plate (20 000/well) the previous day were infected as in Figure 4b. After 2 days, infected cells were harvested, incubated with FITC-conjugated Annexin V (BD Bioscience, Palo Alto, CA, USA) and analysed as described in Materials and methods

analysed for their ability to suppress growth compared to the wild-type molecule. Since two major functional domains have been identified mutants were constructed

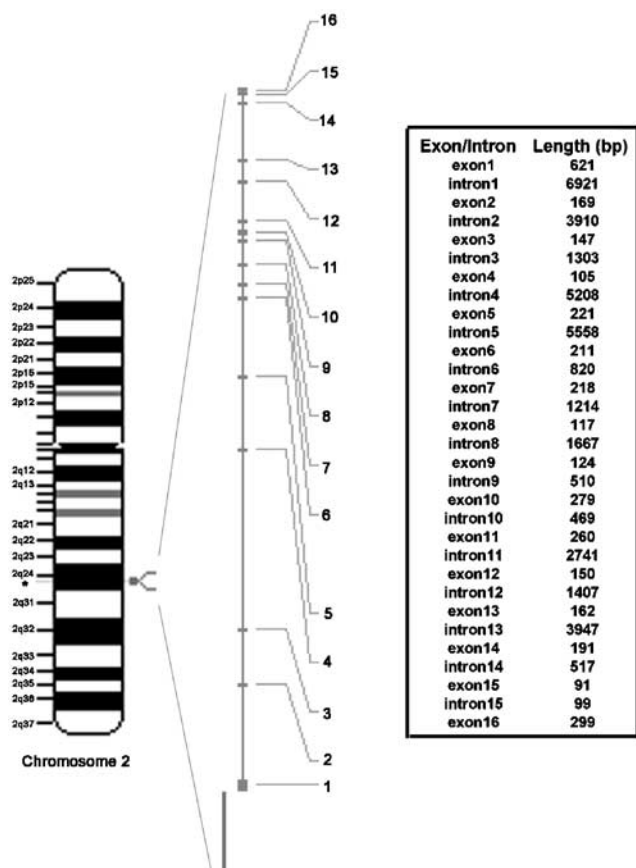


**Figure 6** (a) Schematic diagram of *mda-5* deletion mutants. The residues between indicated regions were deleted in the respective mutants. (b) MTT cell viability assay with *mda-5* deletion mutants. HO-1 cells were plated at 2500 cells per well in 96-well plates, 24 h later cells were infected with 20 PFU/cell, the following day they were untreated or treated with 2000 U/ml IFN- $\beta$ , 10 ng/ml MEZ or 2000 U/ml IFN- $\beta$  + 10 ng/ml MEZ and viable cell numbers were determined by MTT assay on the fourth day after infection as described in Materials and methods. Empty boxes are untreated; filled, IFN- $\beta$ ; dotted, MEZ; hatched, IFN- $\beta$  + MEZ

to delete each of these as shown in Figure 6a. These mutants *Ad.dCARD* deleted for the CARD domain and *Ad.dATP* deleted for the ATPase function of the RNA-helicase were utilized to infect HO-1 melanoma cells and their effect on growth kinetics was monitored utilizing the MTT assay with full-length wild type expressing *Ad.mda-5* and *Ad.null* as controls. When growth was monitored over 7 days either for direct inhibition or in conjunction with IFN- $\beta$ , MEZ or combined treatment, it was apparent that deletion of either domain impacted on the ability of the molecule to cause inhibition compared to *Ad.mda-5*-infected cell populations (data not shown) indicating that they possess functionally relevant activity. Further, it was apparent that both deleted molecules retained some capacity of growth inhibition indicating that they individually possess functionally active domains that possibly combine to contribute to the overall activity of the molecule. Interestingly, deletion of the CARD domain had a greater impact on loss of growth inhibition than deletion of the ATPase domain (Figure 6b). More defined mutational analyses including finer deletion as well as point mutations will be required to address the role and functionality of each domain in better detail.

#### Chromosomal localization and genomic structure of *mda-5*

A GeneBridge4 Radiation Hybrid panel (RH02.05, Research Genetics, Huntsville, AL, USA) was PCR amplified with specific cDNA primers 219 bp apart in the *mda-5* cDNA (TGTATTCATTATGCTACA



**Figure 7** Structure of the *mda-5* genomic locus: a schematic representation of the *mda-5* genomic locus (center) aligned against an ideogram of human chromosome 2 is shown. The specific localization of *mda-5* on 2q24 to 2q31 is shown by a parenthetical symbol. The table on right depicts the exon-intron designations and corresponding lengths in bp. Exons are indicated as rectangles aligned with arrows indicating respective exon number. The introns correspond to thin lines between exons. The lengths of exons and introns are shown to scale. The thicker bar extending downward from exon 1 corresponds to the upstream promoter region

GAACTG/ACTGAGACTGGTACTTTGGATTCT) and the resulting vectors were submitted to <http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper> for genomic mapping. Analysis of the radiation hybrid panel showed that the *mda-5* gene localized to chromosome 2q24, between STS markers WI-6270 and D2S354 (5.01 cR and 1.82 cR apart, respectively). This localization was independently confirmed utilizing a BLAST search against the full human genome sequence. The alignment against human chromosome 2 ideogram shown in Figure 7 corresponds to data generated via the BLAST sequence alignment.

Screening of a bacterial artificial chromosome (BAC) human genomic library (Research Genetics Inc., Huntsville, AL, USA) with a human cDNA probe (corresponding to sequence AF095844) yielded a clone that spanned the entire *mda-5* genomic locus and possibly additional unrelated 5'- and 3'-flanking sequences. This BAC clone was directly sequenced to determine the exon/intron junctions utilizing appropriate cDNA-

derived primers followed by alignment of each sequence to the cDNA as described previously (Gopalkrishnan *et al.*, 1998). Additional subclones were constructed from the BAC clone to facilitate sequencing of difficult regions from BAC DNA and also for cloning specific regions of interest. The results of this analysis are summarized in Figure 7 (middle panel). The locus is comprised of 16 exons and 15 introns (Figure 7, right panel). A variant cDNA 202 bp longer in the 3'-UTR has been identified that likely utilizes an alternative polyadenylation signal and therefore is an alternative polyadenylation product. It was not possible to identify this variant in multiple tissue Northern blots due to lack of resolution and this variant might correspond to a minor tissue specific isoform. The extension in the 3'-UTR occurs in exon 16. An approximately 6 kb upstream, putative transcription regulatory promoter sequence was also present in the BAC clone.

Primer extension analysis utilizing a 5' <sup>32</sup>P-labeled reverse oriented primer starting at position 228 of AF095844 was performed with conditions described previously (Gopalkrishnan *et al.*, 2000). A sequence reaction utilizing the same primer enabled precise identification of the transcriptional start site as an 'A' residue, 26 bp upstream of the 5'-end of AF095844 (data not shown).

## Discussion

IFNs comprise a family of cytokines initially recognized due to their robust induction upon viral infection and their ability to protect treated cells against viral cytopathic effects via modulation of innate and acquired immune responses (Pestka *et al.*, 1987; Stark *et al.*, 1998). IFNs elicit diverse cell-type-specific responses, including growth inhibition, differentiation and apoptosis, which are frequently exploited in cancer treatment (Fisher and Grant, 1985; Stark *et al.*, 1998; Barber, 2001). Various IFNs have been used as cancer therapeutics alone and in combination with other chemotherapeutics, including etoposide and tamoxifen, for diverse cancers, including melanoma, breast cancer, osteosarcoma and glioblastoma (Packer *et al.*, 1991; Jia *et al.*, 1999; Lindner *et al.*, 2000; Leszczyniecka *et al.*, 2001). However, the efficacy of IFNs as cancer therapeutics has been shown to vary widely depending on cancer type and genetic background (Sangfelt and Strander, 2001).

Two types of IFNs, type I and II, are defined by their interaction with cognate receptors, type I or II receptors, respectively (Pestka, 1997; Stark *et al.*, 1998). Five classes of IFNs, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IFN- $\tau$  (bovine) and IFN- $\omega$ , have been identified based on biological activity, mode of action and primary structure of the protein. IFN- $\alpha$ , IFN- $\beta$ , IFN- $\tau$  and IFN- $\omega$  are type I IFNs interacting with type I receptors and only IFN- $\gamma$  is a type II IFN that binds to type II receptors (Pestka, 2000). Although each receptor requires specific signal transduction components, both receptors interact with JAK family protein tyrosine kinases that phosphorylate

the Signal transducers and activators of transcription (STAT) family of transcription factors (Stark *et al.*, 1998; Samuel, 2001). Phosphorylated STAT proteins translocate into the nucleus and activate transcription of early IFN-responsive genes, which in turn, can modulate the expression of additional genes (Der *et al.*, 1998; Stark *et al.*, 1998). ISGs regulate diverse biological activities, including apoptosis, immune modulation, protein degradation, transcription, translation, etc. (de Veer *et al.*, 2001).

Initial characterization of *mda-5* demonstrated that it was a transcriptionally regulated ISG whose expression was augmented by MEZ-mediated protein kinase C activation (Jiang and Fisher, 1993; Kang *et al.*, 2002). Expression of *mda-5* was also increased by treatment with TNF- $\alpha$  and double stranded RNA (dsRNA) poly[I]:poly[C], although IFN- $\beta$  was the most potent inducer (Kang *et al.*, 2002). The induction of *mda-5* in terminally differentiating HO-1 cells and the signaling pathway of *mda-5* induction by IFN is further detailed in this report by Northern blot hybridization. Induction of *mda-5* by IFN was found to be an early response not requiring prior protein synthesis and to occur in a spectrum of normal and tumor cells. The induction of *mda-5* by IFN requires classical IFN-signal transduction, including the JAK family tyrosine kinases and STAT activation, since mutant cell lines affected for various components in this signaling pathway do not support *mda-5* gene expression (Pellegrini *et al.*, 1989; Darnell *et al.*, 1994; Lutfalla *et al.*, 1995). The primary cellular inducer of *mda-5* gene expression is likely IFN- $\beta$  based on induction kinetics, sensitivity, dependence on IFN-receptor signaling and initial induction by IFN- $\beta$ .

Analysis of the temporal kinetics of *mda-5* expression indicate potentially novel and interesting features when compared to other IFN-dependent differentiation-induced genes isolated in our subtraction hybridization screens (Lin *et al.*, 1998; Huang *et al.*, 1999b; Kang *et al.*, 2001; Leszczyniecka *et al.*, 2002). Biphasic induction of *mda-5* by IFN- $\beta$  and poly[I]:poly[C] was evident at the mRNA level. In virus-infected and IFN-treated cells a positive feedback loop of IFN regulation occurs via IRF-3 and IRF-7 activity on IFN promoters (Levy *et al.*, 2002). In this context, although the first induction phase might be a direct response to IFN, the second surge could result from additional IFN release through a positive feedback loop. However, we observed that expression of additional IFN- $\beta$ -inducible genes (*hUBP43* and *hPNPase<sup>old-35</sup>*) in the HO-1 system did not exhibit biphasic induction profiles (Kang *et al.*, 2001; Leszczyniecka *et al.*, 2002). Based on these considerations, the possibility of an alternative mechanism for the second surge in gene expression cannot be excluded. Treatment of HO-1 cells with MEZ in the absence of IFN- $\beta$  induces *mda-5* expression with delayed kinetics and the combination of MEZ + IFN- $\beta$  further enhances *mda-5* expression (Figure 1a, b) (Kang *et al.*, 2002). Since MEZ induces TNF- $\alpha$  expression, which itself is a weak inducer of *mda-5*, induction of *mda-5* by MEZ after prolonged treatment may be a consequence of TNF- $\alpha$  production as well as MEZ-mediated protein

kinase C activation (Jiang *et al.*, 2000; Kang *et al.*, 2002). Delayed kinetics of *mda-5* expression by MEZ and serum starvation could also be an indirect effect resulting from inhibition of cell proliferation (Fisher *et al.*, 1985).

Treatment of HO-1 cells with IFN- $\beta$  alone inhibits cells proliferation and increases melanogenesis, while combined treatment with MEZ induces irreversible growth arrest and apoptosis (Fisher *et al.*, 1985; Jiang *et al.*, 1993; Kang *et al.*, 2002). Adenoviral gene transfer has also been used to analyse the effect of *mda-5* on HO-1 melanoma cell growth and survival. Viable cell counts by MTT assay, microscopic observations, DNA nucleosomal ladder formation, FACS analysis for sub-G<sub>0</sub> cell populations and Annexin V binding all confirmed induction of apoptotic cell death by *mda-5* (Figures 4 and 5). MEZ treatment significantly potentiated the induction of apoptosis in Ad.*mda-5* expressing cells in a manner similar to that observed when HO-1 cells were treated with the combination of IFN- $\beta$  + MEZ (Figure 4b). These observations suggest that the apoptotic activity of IFN- $\beta$  in the terminal differentiation process in HO-1 cells might be strongly dependent on *mda-5* expression. Studies are currently in progress to test this possibility.

Based on our previous studies indicating that *mda-5* contains a CARD domain and transfection of an *mda-5* plasmid expression vector into HO-1 cells decreases colony formation (Kang *et al.*, 2002), it was anticipated that *mda-5* would have proapoptotic activity. Further support for this possibility comes from the demonstration that expression of the murine homologue of *mda-5* (*mda-5/helicard*) in 293 T cells accelerated DNA fragmentation stimulated by treatment with the proapoptotic FasL ligand (Kovacsovics *et al.*, 2002). We presently provide definitive evidence that *mda-5* can induce apoptosis in HO-1 human melanoma cells (Figures 4 and 5). In the case of the MDA-5 protein, although both CARD and RNA helicase motifs appear to be genuine functional domains, as initially suggested from bioinformatic analysis, the contribution of each motif to MDA-5 activity remains to be defined though both individually retain some amount of growth suppressive properties displayed by the whole molecule (Figure 6 and data not shown). Studies on murine *mda-5/helicard* demonstrated specific cleavage of this protein upon treatment of cells with apoptotic ligands, including FasL, TRAIL and staurosporine (Kovacsovics *et al.*, 2002). Moreover, a truncated recombinantly expressed mouse *mda-5/helicard* helicase domain, which was shown to localize in the nucleus in transient transfection assays, promoted DNA fragmentation activity in apoptotic cell extracts (Kovacsovics *et al.*, 2002). These findings support the possibility that each domain of MDA-5 may function independently. However, the *in vitro* DNA fragmentation promoting activity of *mda-5/helicard* was unexpected since the corresponding ATPase activity preferred dsRNA to DNA as a cofactor (Kang *et al.*, 2002; Kovacsovics *et al.*, 2002). In addition, nuclear translocation of the helicase domain was observed with the helicase domain alone plus

apoptotic ligand treatment, but did not occur following transfection of a full-length *mda-5*/helicard molecule and similar treatment (Kovacovics *et al.*, 2002). Thus, whether this reflects a difference between the human MDA-5 versus the murine MDA-5/HELICARD molecule or each motif of human MDA-5 functions independently or cooperatively in induction of apoptosis requires further experimentation.

In addition to *mda-5*, IFNs induce expression of several additional proapoptotic molecules, including PKR, 2'-5'OAS, BAK, FAS, TRAIL, TRADD, caspase-4 and -8, thereby activating apoptosis by themselves or in combination with other reagents (de Veer *et al.*, 2001). Activation of PKR and the 2'-5'OAS/RNase L system by dsRNA plays a critical role in IFN-induced apoptosis, although the apoptotic signaling pathway varies with cell type and treatment (Der *et al.*, 1997; Jagus *et al.*, 1999; Saunders and Barber, 2003). The potential for cooperative interactions between *mda-5* and additional IFN-inducible proapoptotic molecules is clearly possible. The requirement for dsRNA to enhance *mda-5* ATPase activity might be a common theme in cell commitment to apoptosis by IFN, as already established in the PKR and the 2'-5'OAS/RNase L systems (Zhou *et al.*, 1997; Saunders and Barber, 2003). The CARD domain in MDA-5 is anticipated to interact with other apoptotic molecules through CARD-mediated protein-protein interactions (Hofmann *et al.*, 1997; Bouchier-Hayes and Martin, 2002). Thus, detailed analysis of the signaling pathways triggered via *mda-5*-induced apoptosis is warranted and will provide significant insights into the mechanism by which IFN mediates apoptosis following viral infection and by direct treatment of sensitive cancer cell types.

Chromosomal localization and genomic structure of human *mda-5* were analysed by experimental and bioinformatic approaches. *mda-5* localized to 2q24 and consists of 16 exons interspersed by 15 introns of variable lengths (Figure 5). The first two exons encode the CARD domain while the RNA helicase motifs are encoded by exons 4-14. The ATPase A motif (TGSGKT) locates to exon 5, ATPase B motif (DECH) and RNA destabilizing motif (GLTAS) to exon 7 and positive charge cluster for potential RNA binding (QARGRARA) to exons 12 and 13, respectively. The homologous position of the site-specific cleavage site observed in mouse *mda-5*/helicard localizes to exon 3. The overall exon distribution of human *mda-5* is also highly conserved in the mouse genome corresponding to the mouse homologue *mda-5*/helicard located at chromosome 2C1.2-3. Both genomes encode a 16-exon and 15-intron gene structure and the exon/intron boundaries are largely conserved. A notable observation made during human genome database searches with the genomic and cDNA sequence was a region on human chromosome 5 that showed >90% homology to the 150 bp exon 12 of *mda-5*. This matches to a part of the coding region corresponding to the positive charge cluster in *mda-5*. The corresponding gene on chromosome 5 is a putative DEAD-box RNA helicase, DDX46 at 5q31. DDX46, also called SF3b, is a stable subunit of

the 17S U2 snRNP, involved in pre-mRNA splicing (Will *et al.*, 2002). Other than the part corresponding to exon 12, DDX46 does not have significant sequence homology with any other region of *mda-5*.

Cytogenetic analysis by comparative genomic hybridization documents alterations in the chromosome 2q24 region in several cancers, although its correlation to cancer etiology is controversial. Frequent gains in chromosome 2q24 were found in hepatoma (Kumon *et al.*, 2001), esophageal carcinoma (Du Plessis *et al.*, 1999), inherited ovarian carcinoma (Tapper *et al.*, 1998) and nasopharyngeal carcinoma (Chien *et al.*, 2001), while losses in 2q24 were reported in basal cell adenocarcinoma of the salivary gland (Toida *et al.*, 2001), colon tumors (Yeatman *et al.*, 1996) and colorectal tumors (Peng *et al.*, 2002). Weak linkage of the 2q24 region in multiple sclerosis of Nordic populations was also observed by microsatellite marker typing (Akesson *et al.*, 2002). Since *mda-5* was induced by treatment with IFN- $\beta$  that has been used for treating patients with multiple sclerosis, *mda-5* might associate with the 2q24 region linkage observed in this disease.

The presence of distinct and unrelated functional domains, CARD and helicase in *mda-5*, a hitherto unique arrangement for any helicase, raises the issue of its evolutionary origin. This is not easily answerable from presently available data including the currently reported information in this manuscript. A possible mechanism is alluded to, by our finding described above that a small region of human *mda-5* (exon 12) has strong homology to a distinct gene locus on human chromosome 5 and therefore might be derived from a common source. It is possible that two distinct genes present in organisms lower down in the evolutionary ladder each containing one of these domains, fused to generate *mda-5*. This possibility is loosely supported by the following observation. Human genome-wide blast searches indicate that *mda-5* does not have any additional region comprising parts of the exonic sequences, such as processed pseudogenes. Genome wide analysis of another major IFN-inducible gene, PKR also indicates a lack of pseudogenes, which might imply, based on one school of thought, a relatively short evolutionary history of such genes. In addition, analyses of currently available genomic and EST databases indicate that *mda-5* homologues might be restricted to vertebrate species or even more restricted to mammalian organisms, but conclusive evidence will only be available when more sequence information accrues in other systems (Wang and Floyd-Smith, 1998; Leszczyniecka *et al.*, 2003).

Identification of *mda-5* as an IFN- $\beta$ -mediated differentiation-induced gene in HO-1 cells, having unique attributes, provides a valuable tool in understanding the connections between cellular differentiation, oncogenesis and the dysregulation of normal controls in these processes, particularly in the context of differentiation therapy. Analyzing *mda-5*'s molecular properties offers potential for providing valuable clues relating to gene expression changes and growth control mechanisms induced via the unique combination of helicase-

mediated RNA unwinding activity and CARD-related apoptotic activity. This report addresses many aspects of *mda-5* gene regulation in response to known as well as predicted inducers of expression. In addition, it describes for the first time a detailed genomic characterization and expression profile of a CARD containing helicase molecule. Studies on genomic organization of *mda-5* and particularly its promoter will be useful in improving our understanding of existing or identifying new mechanisms of IFN-mediated gene expression. We also document that this new apoptosis promoting molecule is inducible via IFNs in a variety of cell contexts, both of normal and cancer origins, and is therefore not restricted to specific melanoma systems. This spectrum of induction combined with its apoptosis-inducing capacity suggests that *mda-5* may play a prominent role in IFN-mediated antiviral mechanisms by promoting cell death in the presence of dsRNA. For that reason, *mda-5*'s functional role is likely to be broadly applicable to cellular growth control mechanisms as well as oncogenic and differentiation programs in most cell and tissue contexts.

## Materials and methods

### Reagents

Various IFNs were obtained from PBL Biomedical Laboratories (New Brunswick, NJ, USA): Hu-IFN- $\alpha$ A (Cat. No. 11100-1), Hu-IFN- $\alpha$ B (Cat. No. 11115-1), Hu-IFN- $\alpha$ C (Cat. No. 11120-1), Hu-IFN- $\alpha$ D (Cat. No. 11125-1), Hu-IFN- $\alpha$ F (Cat. No. 11130-1), Hu-IFN- $\alpha$ G (Cat. No. 11135-1), Hu-IFN- $\alpha$ H2 (Cat. No. 11145-1), Hu-IFN- $\alpha$ I (Cat. No. 11150-1), Hu-IFN- $\alpha$ J1 (Cat. No. 11160-1), Hu-IFN- $\alpha$ A/D (*Bgl* II) (Cat. No. 11200-2), Hu-IFN- $\alpha$ 001 (Pestka, US Patent 5789551, 1998; Pestka, US Patent 5869293, 1999), Bovine-IFN- $\tau$  (Cat. No. 19610-1), Hu-IFN- $\omega$  (Cat. No. 11395-1), natural Hu-IFN- $\beta$  (Friesen *et al.*, 1981) and recombinant Hu-IFN- $\beta$  (noted as rIFN- $\beta$  or recombinant IFN- $\beta$  in the text, Cat. No. 11400-2). dsRNA poly[I]:poly[C] was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The reagents whose supplier are not specified were acquired from Sigma Aldrich (St Louis, MO, USA).

### Cell lines and culture conditions

The HO-1 human melanoma cell line was derived from a metastatic inguinal node lesion from a 49-year-old woman and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/100  $\mu$ g/ml) in a 5% CO<sub>2</sub>-95% air-humidified incubator (Fisher *et al.*, 1985). Additional human cell types included, melanoma (MeWo, LO-1, C8161, SK-MEL wt p53, SK-MEL mut p53, WM35, WM278, SH-1, FO-1), SV40-T-antigen immortalized melanocyte (FM516-SV), normal mammary epithelial (HMEC and HBL-100), breast carcinoma (MCF-7, T47D), colorectal carcinoma (SW613), cervical carcinoma (HeLa), glioblastoma multiforme (T98G, G18), prostate carcinoma (DU-145), nasopharyngeal carcinoma (HONE-1), normal cerebellum (NC) and osteosarcoma (Saos-2) (Jiang *et al.*, 1995, 1996; Su *et al.*, 1998). All cancer cells and normal cells, except HMEC, were grown in DMEM-10. HMEC was grown in medium provided by Clonetics (Su *et al.*, 1998). 2fTGH, U1A, U3A, U4A and

USA cells were provided by Dr George Stark (Cleveland Clinic) and grown in DMEM supplemented with 10% FCS and 250  $\mu$ g/ml hygromycin (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991; Lutfalla *et al.*, 1995).

### RNA isolation and Northern blot analysis

Total RNA was isolated by the guanidinium isothiocyanate lysis followed by the acid phenol extraction procedure and analysed by Northern blot hybridization as described previously (Jiang and Fisher, 1993). An *Eco*RI fragment (2.5 kb) of *mda-5* cDNA and 0.7 kb PCR fragment of *gapdh* were used as a probe. Autoradiograms of Northern blots were quantitated by densitometric analysis using a Molecular Dynamics densitometer (Sunnyvale, CA, USA).

### Western blot analysis

Protein extracts were prepared by lysis in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.5% Na-deoxycholate, 1 mM DTT and protease inhibitors (Roche) and centrifuged at 13 000 r.p.m. for 15 min at 4°C. Protein samples (20  $\mu$ g) were resolved in 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection of the Western blot was performed as described (Lebedeva *et al.*, 2002).

### Adenovirus construction and infection protocol

The replication incompetent adenoviruses with or without *mda-5* (Ad.null, Ad.*mda-5*, Ad. $\Delta$ ATP and Ad. $\Delta$ CARD, respectively) were constructed and amplified as described previously (Holmes *et al.*, 2003). The  $\Delta$ ATP mutant of *mda-5* (aa 310–484 deletion) was constructed by ligating the 5'-*Stu*I fragment to the 3'-*Alw*NI fragment using T4 DNA polymerase. The  $\Delta$ CARD mutant of *mda-5* (aa 129–196 deletion) was produced by ligating the 3'-*Hpa*I fragment to the 5'-*Hind*III fragment and filled using the Klenow reaction. Amplified virus was purified with BD Adeno-X virus purification kit (BD Clontech) and viral titer was determined by plaque assay in HEK 293 cells. Adenovirus was inoculated onto cells plated the previous day at the indicated multiplicity of infection (m.o.i.) with a minimal volume of media providing cell coverage (4 ml for 10-cm dish, 1 ml for 6-cm dish and 50  $\mu$ l for each well of 96-well plate). After 2 h of incubation with rotation, the infected cells were refed with fresh media. Infected cells were treated and analysed as indicated in the figure legends.

### MTT cell growth and viability assay

100  $\mu$ l of complete media containing 0.5 mg/ml MTT were refed to cells plated on 96-well plates and previously untreated or adenovirus-infected as described (Lebedeva *et al.*, 2002). After 3 h, an equal volume of 10% SDS–0.01 N HCl solution was added to the plate and the plate was incubated at 37°C overnight to facilitate lysis of the MTT metabolite. Metabolic conversion of MTT by living cell was measured by A595 with a Model 555 Microplate Reader (BioRad, Hercules, CA, USA).

### Preparation and analysis of apoptotic genomic DNA

Genomic DNA was extracted as described (Lebedeva *et al.*, 2002). Briefly, HO-1 melanoma cells plated on 10-cm dish ( $2 \times 10^6$ /plate) the previous day were treated or infected with virus as indicated in the figure legend. Cells were harvested by collecting media and trypsinizing attached cells, and lysed in 10 mM Tris-HCl (pH 7.9), 5 mM EDTA, 0.1 M NaCl, 0.5%

SDS and 1 mg/ml proteinase K for 2 h in a 37°C water bath. The lysate was incubated at 4°C overnight after salt adjustment to 1 M with 5 M NaCl. The lysate was centrifuged at 18 000 g for 30 min at 4°C and the Hirt supernatant was collected for phenol-chloroform extraction and ethanol precipitation. After treatment with DNA-free RNase A (20 µg/ml) for 10 min at 37°C, 5 µg DNA was resolved in a 2% agarose gel.

#### Fluorescence activated cell-sorting (FACS) analysis

Sub-G<sub>0</sub> cell population and Annexin V binding by apoptotic cells were measured by FACS. For Annexin V binding, cells harvested as in the previous section were washed with PBS once and incubated with FITC-conjugated Annexin V (BD Bioscience, Palo Alto, CA, USA) in 100 µl of 10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl and 5 mM CaCl<sub>2</sub> for 15 min at room temperature. Within 30 min after addition of 400 µl of propidium iodide in the incubation buffer to a final volume of 0.5 µg/ml, the cells were analysed with FACS Calibur and CellQuest software (Becton Dickinson, San Jose, CA, USA). For Sub-G<sub>0</sub> cell population, harvested cells were fixed in 70% ethanol at 4°C for 30 min. After washing in PBS once, the cells were incubated in PBS containing 0.1 mg/ml propidium iodide,

0.6% Nonidet P-40 and 1 mg/ml RNase A for 30 min at room temperature. Stained cells were analysed as above.

#### Determination of genomic structure

Sequence data was generated over exon-intron boundaries using an approximately 200 kb BAC clone (Research Genetics, Huntsville, AL, USA) with primers derived from the cDNA sequence AF095844. The BAC DNA was subcloned and mapped by Southern blot analysis using various regions of the cDNA to facilitate sequencing. All exons and introns were sequenced in their entirety, as were all exon-intron boundaries and this was matched to the published human genome sequence database via BLAST searches (Gopalkrishnan et al., 1998, 2000).

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