

# Identification of Mechanisms Underlying Transporter Associated with Antigen Processing Deficiency in Metastatic Murine Carcinomas

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## Abstract

**Expression of transporter associated with antigen processing (TAP) is often lost in metastatic carcinomas, resulting in defective antigen processing and presentation and escape of the cancer cells from immune surveillance. In this study, the nature of TAP deficiencies in tumors was investigated. By chromatin immunoprecipitation assay, we showed that the recruitment of RNA polymerase II to the *TAP-1* gene was impaired in TAP-deficient cells derived from murine melanoma, prostate, and lung carcinomas, compared with TAP-expressing fibroblasts and lymphoma cells. This suggested that the deficiency in TAP-1 expression resulted, at least partially, from a relatively low level of transcription of the *TAP-1* gene. Furthermore, levels of TAP-1 promoter activity, as assessed by stable transfections with a reporter construct containing the TAP-1 promoter, were relatively low in TAP-deficient cells. To examine genetic heritability of regulators of TAP-1 promoter activity, TAP- and MHC class I-deficient cells of H-2<sup>b</sup> origin were fused with wild-type fibroblasts of H-2<sup>k</sup> origin. Fusion with TAP-expressing cells complemented the low levels of TAP-1 promoter activity in TAP-deficient cells. However, these fused cells exhibited lower levels of TAP-1 mRNA and H-2<sup>k</sup> than unfused fibroblasts. Further analysis showed that TAP-1 mRNA stability was lower in fused carcinoma fibroblasts than in unfused fibroblasts. Based on these results, we propose that TAP deficiency in many carcinomas is caused by a decrease in activity/expression of *trans*-acting factors regulating TAP-1 promoter activity, as well as a decrease in TAP-1 mRNA stability. These results have significant implications for understanding immune evasion mechanisms in tumors.** (Cancer Res 2005; 65(16): 7485-92)

## Introduction

Antigen processing and presentation play a crucial role in immune surveillance. Peptides derived from self, viral, or tumor-related proteins are generated in the cytoplasm by the action of proteasome. Transporter associated with antigen processing (TAP), a member of the ATP-binding cassette transporter family, functions

by transporting these peptides from the cytoplasm to the lumen of the endoplasmic reticulum, where each peptide forms a ternary complex with  $\beta$ -2-microglobulin and MHC class I heavy chain, a process promoted by chaperone proteins (BiP, calnexin, calreticulin, ERp57, and tapasin; refs. 1, 2). These complexes are then transported to the cell surface and recognized by CTLs, which eventually kill cells that present non-self-antigens.

Studies have shown that components of the antigen presentation pathway are impaired in the majority of human tumor cells (3, 4), allowing them to evade immune surveillance. In particular, low expression or absence of TAP (TAP-1 and TAP-2) molecules, a feature common to many tumors (5–7), impairs the formation of the ternary complex in the lumen of endoplasmic reticulum. This results in a lack of MHC class I expression on the cell surface. As a consequence, specific CTLs are unable to recognize and destroy many malignant cells (6).

The importance of TAP-1 function in immunosurveillance has been highlighted in studies using a mouse lung carcinoma cell line, CMT.64 (8). It was shown that the restoration of TAP-1 expression by introducing exogenous TAP-1 or by up-regulating endogenous TAP-1 expression upon IFN- $\gamma$  treatment could correct the MHC class I deficiency, resulting in recognition of these antigen-presenting cells by CTLs *in vitro*, as well as in a decrease of tumor growth and incidence *in vivo* (3, 7, 9). This finding is encouraging for the development of therapeutic approaches that could restore TAP deficiencies in cancer cells, therefore resurrecting immune recognition of neoplastic cells. However, the mechanisms underlying TAP-1 deficiency in tumor cells remain poorly understood. Previous studies reported that down-regulation of TAP-1 expression in many cancer cells likely occurs at the mRNA level (3, 7, 10); however, these studies did not distinguish between defects in transcription or stability of the RNA. Therefore, we investigated the properties and activities of the TAP-1/LMP-2 bidirectional promoter in TAP-expressing and TAP-deficient cells to provide a better understanding of the transcriptional regulation of TAP-1 mRNA in tumor cells.

## Materials and Methods

**Cell lines.** The CMT.64 cell line established from a spontaneous lung carcinoma of a C57BL/6 (H-2<sup>b</sup>) mouse (8) and the Ltk fibroblast cell line derived from a C3H/An (H-2<sup>k</sup>) mouse were grown in DMEM. The LMD cell line derived from a metastatic prostate carcinoma of a 129/Sv mouse (a kind gift of Dr. T.C. Thompson, Scott Department of Urology, Baylor College of Medicine, Houston, TX; ref. 11), as well as the B16F10 (B16) melanoma (12) and RMA lymphoma cell lines, both derived from C57BL/6 mice, were maintained in RPMI 1640. RPMI 1640 and DMEM were supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10 mmol/L HEPES.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-03-3734

**Table 1.** Primers used for RT-PCR analysis

Oligonucleotide	Primer sequence (5' -3')*	T <sub>m</sub> (°C)	bp <sup>†</sup>
Mouse β-actin	F: ATGGATGACGATATCGCTGC R: TTCTCCAGGGAGGAAGAGGAT	54.0	713
TAP-1 5' -end	F: ATGGCTGCGCACGTCTGG R: ACTCAGGCCACCACCCA	63.0	138
TAP-1 3' -end	F: TGGCTCGTTGGCACCCCTCAAA R: TCAGTCTGCAGGAGCCGCAAGA	64.0	775
TAP-1 3' -end (last 155 bp)	F: TTATCACCCAGCAGCTCAGCCT R: TCAGTCTGCAGGAGCCGCAA	61.0	155
TAP-1 promoter <sup>‡</sup>	F: <u>cggaattc</u> GGCTCGGCTTTCCAATCA R: <u>gaggatcc</u> GAGCGTGAGCTGTCCAGAGTCT	60.0	557
pTAP-1-EGFP	F (TAP-1 promoter): TTCTTCCTCTAAACGCCAGCA R (EGFP): CTCGCCCTTGCTACCCAT	61.0	190
CMT.64 TAP-1	F: CTCACTCTGGTCACCTGATCAAC R: TGGTCCAGACTTCAGCCACG	62.0	225
IRF1	F: CAACTTCCAGGTGTCACCCAT R: CACAGGAATGGCCTGGAT	54.9	363
IRF2	F: TGATGAAGAGAACGCAGAGGG R: TTAACAGCTCTTGACACGGGC	54.0	328
S15	F: TTCCGCAAGTTCACCTACC R: CGGGCCGGCCATGCTTTACG	60.0	357
Prion protein	F: ATGGCGAACCTTGGCTACT R: CCAACTACCACCATGAGGTTG	58.0	239

\*F, forward primer; R, reverse primer.

†Length of the PCR amplification product.

‡Restriction enzyme sites are underlined.

**Reverse transcription-PCR analysis.** All primers used for PCR amplifications were purchased from Sigma-Genosys (Oakville, ON) and are listed in Table 1. Total cellular RNA were extracted using RNeasy Mini kit (Qiagen, Mississauga, ON), and contaminating DNA was removed by treating the RNA samples with DNase I (Ambion, Inc., Austin, TX). Reverse transcription of 1 μg of total cellular RNA was done using the reverse transcription kit from Invitrogen (Carlsbad, CA) in a total volume of 20 μL. Two-microliter aliquots of cDNA were used as a template for PCR in a total of 50 μL reaction mixture containing 1× PCR buffer, 250 μmol/L deoxynucleotide triphosphate, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 μmol/L of each primer, and 2.5 units Platinum Taq DNA Polymerase. All PCR reagents were obtained from Invitrogen. cDNA amplifications were carried out with specific primer sets in a T-gradient thermocycler (Biometa, Goettingen, Germany) with 25 to 35 cycles of denaturation (1 minute, 95°C), annealing (1 minute, 54-64°C), and elongation (2 minutes, 72°C). The cycling was concluded with a final extension at 72°C for 10 minutes. Twenty microliters of amplified products were analyzed on agarose gels, stained with ethidium bromide, and photographed under UV light.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation experiments using 2 × 10<sup>7</sup> of CMT.64, LMD, B16, Ltk, or RMA cells were done as previously described (13). Five micrograms of anti-RNA polymerase II antibody (N-20, sc-899, Santa Cruz Biotechnologies, Santa Cruz, CA) were used for the immunoprecipitation. Levels of murine TAP-1 promoter or TAP-1 coding region coimmunoprecipitating with RNA polymerase II from each sample were quantified by real-time PCR using primers specific for the TAP-1 promoter or the last 155 bp of TAP-1 coding region (3' end) as listed in Table 1. Serial dilutions of genomic DNA or plasmid containing the murine TAP-1 promoter were used to generate standard curves for real-time PCR using the corresponding primer sets.

**Cloning of the TAP-1 promoter.** Sequence of the murine *TAP-1* gene region was obtained from the National Center for Biotechnology Information (NCBI) database (Genbank accession no. AF027865). To predict putative transcription factor binding sites, the region between *LMP-2* and

*TAP-1* genes was analyzed using the MatInspector software from Genomatix website. The predicted murine TAP-1 promoter region was then amplified by PCR using genomic DNA from CMT.64 cells as a template and the following primers (Sigma): 5' cggaattcGGCTCGGCTTTCCAATCA3' (forward), 5' gaggatccGAGCGTGAGCTGTCCAGAGTCT3' (reverse). A TAP-1 promoter construct (pTAP-1-EGFP) was then created by inserting the PCR product between the *EcoRI* and *BamHI* sites of pEGFP-1 vector (Clontech, Palo Alto, CA).

**Transfection and selection.** The CMT.64, B16, LMD, RMA, and Ltk cells were transfected with the pTAP-1-EGFP construct or the promoterless pEGFP-1 vector using LipofectAMINE Plus Reagent (Invitrogen). Transfected cells were then selected in the presence of 0.8 to 1 mg/mL G418 (Sigma) for 1 month. Levels of EGFP expression in transfectants, treated or untreated with 50 ng/mL IFN-γ for 48 hours, were assessed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

**Generation of pTAP-1-EGFP-transfected clones by fluorescence-activated cell sorting.** The pTAP-1-EGFP-transfected CMT.64, LMD, and B16 cells that displayed a small level of EGFP were selected by flow cytometry using a FACSVantage DiVa cytometer (Becton Dickinson), grown in bulk culture, and treated with 50 ng/mL recombinant murine IFN-γ (R&D Systems, Minneapolis, MN) for 2 days. Cells that express high fluorescence in response to IFN-γ were then sorted twice and cloned.

**Cell fusion and fluorescence-activated cell sorting analysis.** Twenty million cells from clones of the TAP- and MHC class I-deficient CMT.64, LMD, and B16 cells stably transfected with pTAP-1-EGFP were fused with TAP- and MHC class I-expressing Ltk fibroblasts in a 1:1 ratio, following a polyethylene glycol cell fusion protocol (14). Cells were then incubated with phycoerythrin (PE)-conjugated anti-K<sup>k</sup> mouse monoclonal antibody (mAb) at 4°C for 30 minutes. The fused cells, which displayed both red (PE-anti-K<sup>k</sup>) and green (EGFP) fluorescence, were selected by fluorescence-activated cell sorting analysis (FACS). Flow cytometry analyses of EGFP, K<sup>b</sup>, and K<sup>k</sup> expression were done 1 week after the fusions. PE-conjugated anti-K<sup>b</sup> and anti-K<sup>k</sup> mouse mAbs were purchased from BD Pharmingen (San Diego, CA).

Fusion experiments between a clone of pTAP-1-EGFP-transfected CMT.64 cells and a clone of pEYFP-N1-transfected CMT.64 cells were also done. The fused cells were then selected based on both yellow (EYFP) and green (EGFP) fluorescence. One week after the fusion, levels of EGFP and K<sup>b</sup> in the fused cells were analyzed by FACS.

**Endogenous levels and overexpression of IRF-1 and IRF-2 in cell lines.** Levels of endogenous IRF-1 and IRF-2 in CMT.64, LMD, B16, Ltk, and RMA cells were assessed by RT-PCR using primers specific for IRF-1 and IRF-2, following the conditions described above. To investigate the effects of IRF-1 and IRF-2 overexpression on TAP-1 promoter activity in TAP-deficient carcinomas, a pTAP-1-EGFP-transfected CMT.64 clone was cotransfected with 0.1  $\mu$ g of pEYFP-N1 (enhanced yellow fluorescent protein) vector (Clontech) and 1  $\mu$ g of pCMV/IRF-1 or pCMV/IRF-2 (15) expression vector. Because the pCMV/IRF vectors contained no selection gene, the EYFP served as a marker to select for successfully transfected cells by FACS. Forty-eight hours after transfection, levels of EGFP in the CMT.64 transfectants were analyzed by flow cytometry.

**Luciferase and  $\beta$ -galactosidase assays.** To show whether the overexpressed IRF-1 and IRF-2 were functional, cells were cotransfected with the IRF-coding constructs, an IFN- $\beta$  promoter-luciferase construct (15), and a pCMV/ $\beta$ -galactosidase vector (Promega, Madison, WI) used to monitor transfection efficiency. A promoterless pGL3-luciferase vector (Promega) was also used as a background control. Forty-eight hours after transfection, the cells were washed twice with PBS and lysed with Reporter Lysis Buffer (Promega). The luciferase and the  $\beta$ -galactosidase activity were measured using the Luciferase Assay System (Promega) and the  $\beta$ -galactosidase Enzyme Assay System (Promega), respectively.

**Western blot.** Fifty micrograms of proteins per sample were separated through 8% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked with 5% skim milk in PBS, probed with anti-mouse TAP-1 rabbit polyclonal antibody (made by Linda Li in Jefferies Lab), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA). For the loading controls, anti- $\beta$ -actin mouse mAb (Sigma) was used, followed by HRP-conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL). Blots were developed using Lumi-light reagents (Pierce).

**Analysis of mRNA stability.** Unfused Ltk fibroblasts and fused CMT.64-Ltk, LMD-Ltk, and Ltk-Ltk cells were treated with 5  $\mu$ g/mL actinomycin D (Sigma) for 2, 4, or 8 hours, or left untreated. Four micrograms of total cellular RNA were used as templates for reverse transcription and amplification by real-time PCR, using TAP-1 (5' end) or S15 or prion specific primer sets listed in Table 1. Serial dilutions of reverse transcription products were used as templates for PCR to generate the corresponding standard curves.

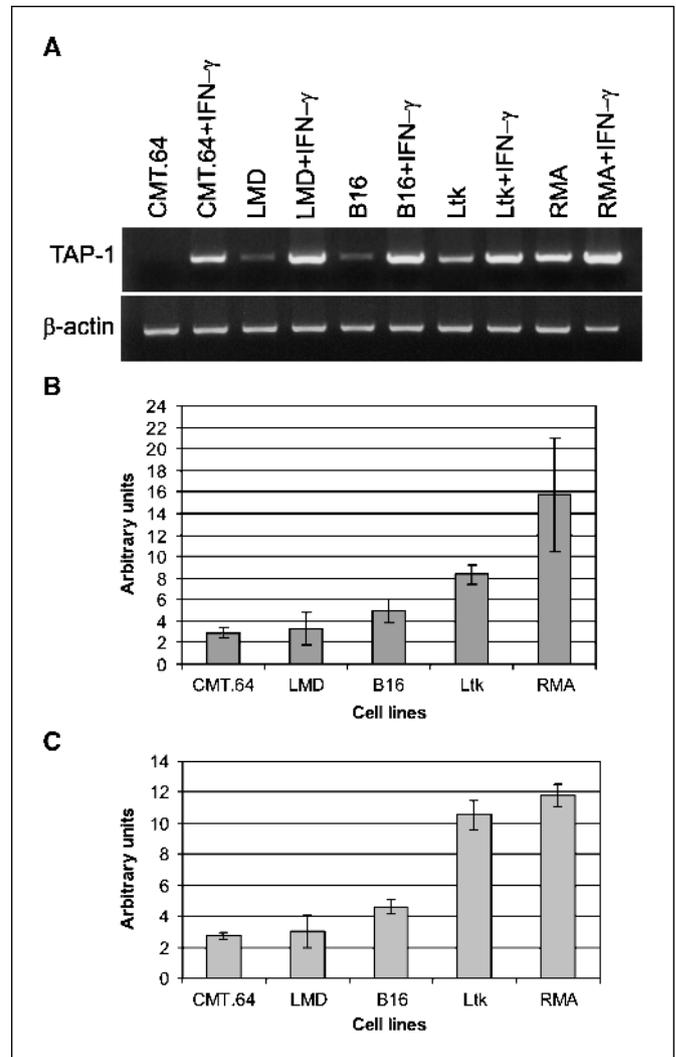
**Real-time quantitative PCR analysis.** In this study, this method was used for quantification of levels of endogenous TAP-1 promoter or TAP-1 coding region coprecipitating with RNA polymerase II in chromatin immunoprecipitation assays, quantification of copy number of the pTAP-1-EGFP construct integrated in stably transfected cells, and measurement of TAP-1 mRNA levels in cells upon actinomycin D treatment. cDNAs reverse-transcribed from 1 to 4  $\mu$ g RNA and genomic DNA were used as templates for amplifications using 200 to 500 nmol/L of each primer and 10  $\mu$ L SYBR Green Taq ReadyMix (Sigma). Thirty-five cycles of denaturation (5 seconds, 95°C), annealing (5 seconds, 61-63°C), and elongation (20 seconds, 72°C) were done using a Roche LightCycler.

## Results

**Levels of TAP-1 messenger RNA in CMT.64, LMD, B16, Ltk, and RMA cells.** To investigate the mechanism underlying the lack of TAP-1 expression in carcinomas, we used CMT.64 (lung), LMD (prostate), and B16 (melanoma) cell lines as models for TAP-deficient cells (3, 7, 11, 16), and Ltk and RMA cell lines (7) as models for TAP-expressing cells. Using reverse transcription-PCR, we confirmed that the levels of TAP-1 mRNA were higher in Ltk and

RMA cells than in CMT.64, LMD, and B16 cells (Fig. 1A). Treatment of the TAP-deficient cells with IFN- $\gamma$  increased the TAP-1 mRNA expression to similar levels as in TAP-expressing cells (Fig. 1A).

**The recruitment of RNA polymerase II to the TAP-1 gene is lower in the CMT.64, LMD, and B16 than in the Ltk and RMA cells.** Two alternative methods that can be used to assess transcription rates are the nuclear run-on assay and the measurement of the recruitment of RNA polymerase II complex to the coding region of genes by chromatin immunoprecipitation assay (17). To investigate whether the lack of TAP-1 mRNA



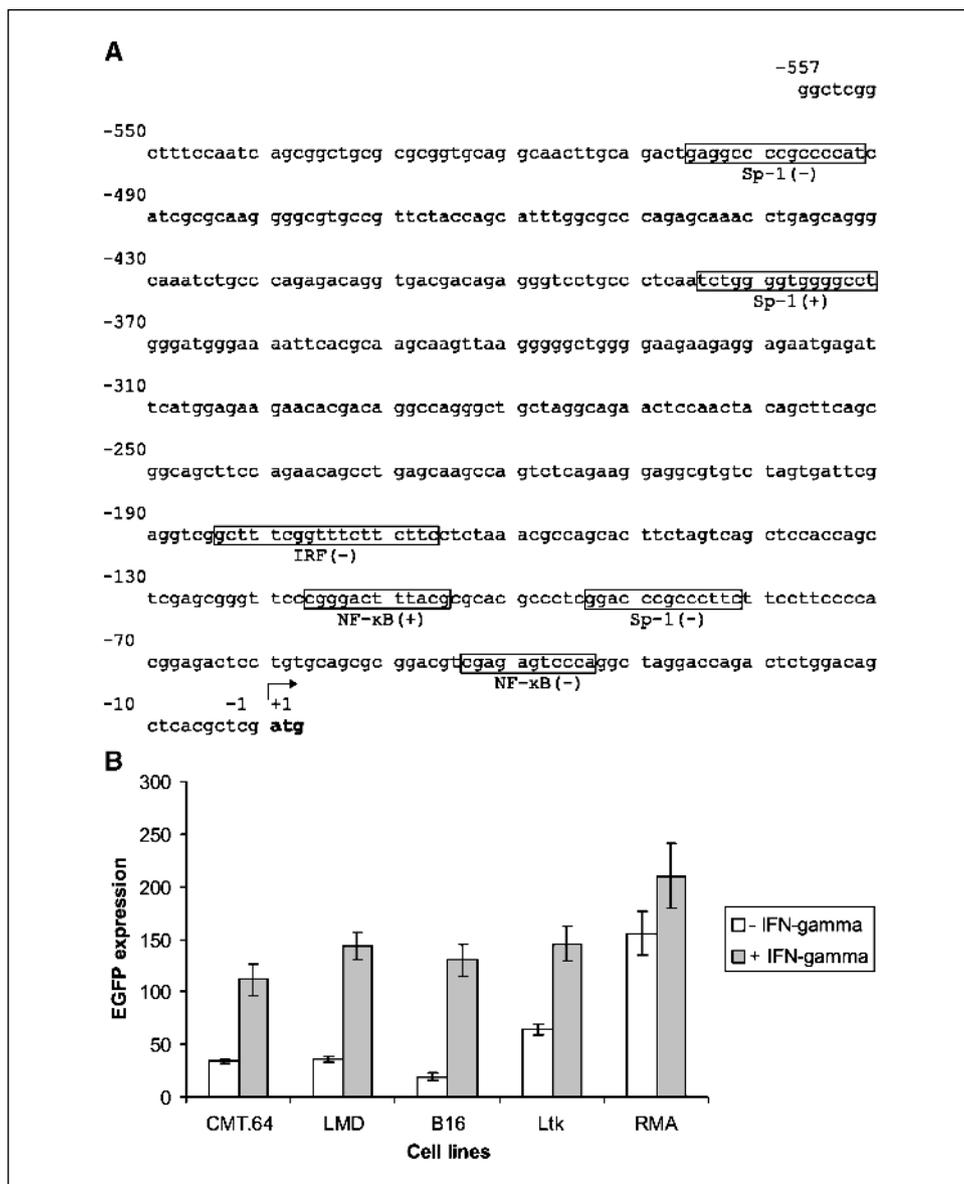
**Figure 1.** Transcription of the TAP-1 gene is low in carcinoma cells. Levels of transcription of the TAP-1 gene were analyzed in CMT.64, LMD, B16, Ltk, and RMA cells. A, RT-PCR analysis of TAP-1 expression in untreated and IFN- $\gamma$ -treated cells (50 ng/mL, 48 hours). Amplification of  $\beta$ -actin cDNA served as an internal control. RT-PCR analyses were done at least thrice and representative data are shown. B, the recruitment of RNA polymerase II to 3' coding region of the TAP-1 gene in CMT.64, LMD, and B16 is impaired compared with that in Ltk and RMA. Chromatin immunoprecipitation using anti-RNA polymerase II antibody was done in each cell line, and the eluted DNA fragments were purified and used as templates for real-time PCR analysis using primers specific for the 3' coding region of the TAP-1 gene. Copy numbers of the eluted 3' coding region of the TAP-1 gene were normalized against the copy numbers in the corresponding inputs and were expressed in arbitrary units. Columns, average of three to six experiments; bars, SEM. C, chromatin immunoprecipitation using anti-RNA polymerase II antibody was done and the relative levels of RNA polymerase II recruitment to the TAP-1 promoter were assessed as described above using primers specific for the promoter region.

expression in CMT.64, LMD, and B16 is due to an impairment of TAP-1 transcription, we compared the levels of RNA polymerase II within the 3' end of the TAP-1 coding region in TAP-deficient and TAP-expressing cells, by means of chromatin immunoprecipitation. We found that the levels of RNA polymerase II within the 3' end of the TAP-1 coding region were lower in CMT.64, LMD and B16 than in Ltk and RMA cells (Fig. 1B), indicating that deficiency in transcription is, at least partially, underlying TAP deficiency in the carcinoma cells. Using the same approach, but with primers specific for the TAP-1 promoter region, we found that the recruitment of RNA polymerase II to the TAP-1 promoter was also relatively lower in TAP-deficient cells (Fig. 1C). Recruitment of RNA polymerase II complex to gene promoters is an important event that supports transcription initiation (18). Therefore, this result directly supported the notion that initiation of TAP-1 transcription was impaired in TAP-deficient cells.

**Absence of mutation in the -557 to +1 region of the CMT.64-derived TAP-1 promoter.** One possible explanation for the

impairment of transcription of the *TAP-1* gene in TAP-deficient cells was the presence of mutation(s) in *cis*-acting elements involved in the regulation of TAP-1 promoter activity. Previous studies have shown that the 593-bp-long region located between *TAP-1* and *LMP-2* genes in humans acts as a bidirectional promoter that drives the transcription of both genes (19). Analysis of the murine TAP-1 promoter had not been reported in prior work; however, we noted that the organization of the murine LMP-2/TAP-1 locus is reminiscent of its human orthologue. Therefore, we used primers flanking the LMP-2/TAP-1 intergenic region to amplify the murine TAP-1 promoter region, using genomic DNA from CMT.64 cells as a template. The resulting PCR product was then cloned and sequenced.

Analysis of the CMT.64-derived TAP-1 promoter region revealed the presence of putative binding sites for various transcription factors, including SP1, NF-κB, and IRF (Fig. 2A). By alignment, we found that the nucleotide sequence of the CMT.64-derived TAP-1 promoter region was identical to the corresponding region in the murine MHC class II locus (NCBI



**Figure 2.** The sequence of the *TAP-1* gene promoter is normal in lung carcinoma cells. However, TAP-deficient cells display relatively low levels of TAP-1 promoter activity. **A**, nucleotide sequence of the CMT.64-derived TAP-1 promoter region is identical to the corresponding region in murine MHC class II locus (NCBI accession no. AF027865). The ATG codon was arbitrarily determined as +1. Motifs located on the sense strand are indicated by a (+) and motifs located on the antisense strand are indicated by a (-). **B**, analysis of TAP-1 promoter-driven EGFP expressions from bulk populations of the pTAP-1-EGFP-transfected CMT.64, LMD, B16, Ltk, and RMA cells after selection in G418 for 1 month. The IFN-γ-treated cells were incubated with 50 ng/mL IFN-γ for 48 hours before FACS analysis. Levels of EGFP in the cells transfected the pTAP-1-EGFP were normalized against corresponding values obtained upon transfection with pEGFP-1 vector alone. *Columns*, average of arbitrary fluorescent units of three independent experiments; *bars*, SEM.

accession no. AF027865). This result showed that there was no mutation present in the -557 to +1 region of the CMT.64-derived TAP-1 promoter.

**Activity of the -557 to +1 region of the TAP-1 promoter is impaired in TAP-deficient cells.** To show that this murine LMP-2/TAP-1 intergenic region indeed displays promoter activity, and to assess whether it contains *cis*-acting elements conferring a differential activity of the TAP-1 promoter between TAP-expressing and TAP-deficient cells, we generated a reporter plasmid containing the -557 to +1 region of the murine TAP-1 promoter upstream of the *EGFP* gene in the pEGFP-1 vector (pTAP-1-EGFP). CMT.64, LMD, B16, Ltk, and RMA cells were then transfected with the pTAP-1-EGFP construct, or the pEGFP-1 vector as a control, and cultured for 1 month under selective pressure. Real-time PCR analysis was done using TAP-1 promoter-specific forward and EGFP-specific reverse primers (Table 1), and 100 ng of genomic DNA from the stable pTAP-1-EGFP transfectants as templates. This showed that, on average, one copy of the pTAP-1-EGFP construct was integrated per cell in all the cell lines used (Supplementary Table 1). EGFP levels in these stable transfectants were then assessed by flow cytometry.

In all the cell lines, the promoterless pEGFP-1 vector transfectants displayed low levels of background EGFP expression; however, the levels of fluorescence were higher in cells transfected with the pTAP-1-EGFP construct than in cells transfected with the vector alone. In addition, the TAP-expressing Ltk and RMA cells expressed higher levels of EGFP than the TAP-deficient CMT.64, LMD, and B16 cells (Fig. 2B). This indicated that the -557 to +1 region indeed displayed promoter activity. Finally, treatment with IFN- $\gamma$  resulted in 3- to 6-fold increases in EGFP expression in CMT.64, B16, and LMD cells (Fig. 2B). This treatment elevated EGFP expression of the TAP-deficient cells to similar or even higher levels than those in untreated TAP-expressing cells, suggesting that treatment with IFN- $\gamma$  was able to overcome the deficiencies responsible for the low activity of the TAP-1 promoter in TAP-deficient cells. Taken together, these results indicated that the cloned TAP-1 promoter region possesses faithful promoter activity and contains *cis*-acting elements conferring the relatively low promoter activity in TAP-deficient cells. Furthermore, based on the observation that the levels of EGFP triggered by the cloned TAP-1 promoter correlated with the levels of recruitment of RNA polymerase II to the endogenous TAP-1 promoter observed by chromatin immunoprecipitation, these transfected cells were proven to be suitable as tools to further investigate the mechanisms underlying the differential activation of TAP-1 promoter in TAP-deficient and TAP-expressing cells.

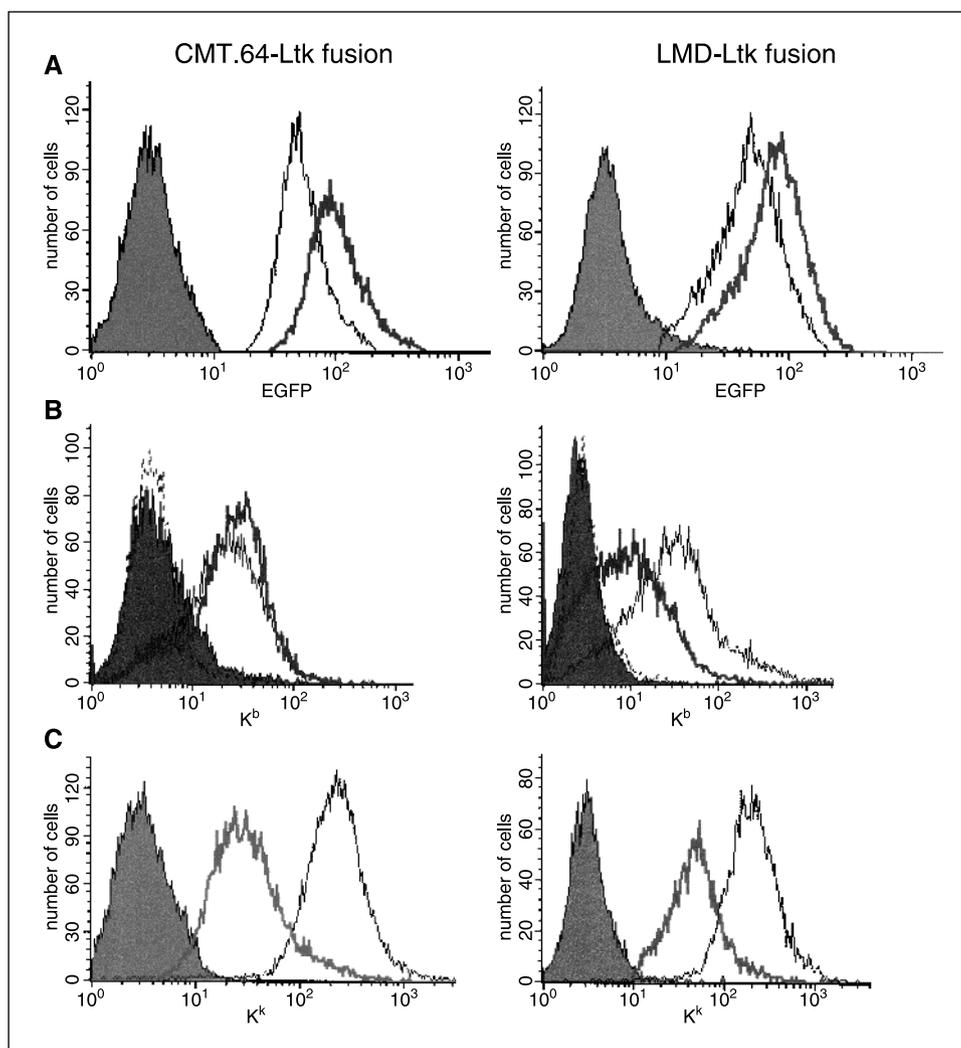
**Effects of fusions between carcinomas and wild-type fibroblasts on TAP-1 promoter activity and MHC class I expression levels.** The relatively low activity of the -557 to +1 region of the TAP-1 promoter in TAP-deficient cells suggested that these cells might be deficient in positive *trans*-acting factors that regulate TAP-1 promoter activity, or that they might display an abnormally high level of activity of *trans*-acting factors that negatively regulate TAP-1 promoter activity. To test these hypotheses, we investigate the effects of fusing the TAP- and MHC class I-expressing Ltk cells with TAP- and MHC class I-deficient carcinomas (CMT.64, LMD, and B16).

Before fusion, stable pTAP-1-EGFP transfectants of carcinomas were sorted into single-cell clones by FACS, and a clone that displayed high induction of TAP-1 promoter activity and MHC class I expression in response to IFN- $\gamma$  treatment was chosen. By

flow cytometry, we found that levels of EGFP were higher in the fused CMT.64-Ltk and LMD-Ltk cells than in the unfused CMT.64 and LMD cells (Fig. 3A). Further analysis indicated that, whereas the unfused cells did not express K<sup>b</sup>, K<sup>b</sup> was expressed, to some extent, on the surface of the fused CMT.64-Ltk and LMD-Ltk cells (Fig. 3B). Similar results were obtained from a fusion between the B16 and Ltk cells (data not shown). As a control, we fused two groups of TAP-deficient cells, the CMT.64 cells stably transfected with the pTAP-1-EGFP construct with another group of CMT.64 cells expressing EYFP, and showed that there was no induction of EGFP and K<sup>b</sup> surface expression in the fused CMT.64(pTAP-1-EGFP)-CMT.64(pEYFP-N1) cells (data not shown). The increase in EGFP expression of the pTAP-1-EGFP-transfected TAP-deficient carcinomas that were fused with TAP-expressing fibroblasts suggests that the TAP-deficient cell lines studied display a relatively low level of activity of *trans*-acting factor(s) positively regulating the TAP-1 promoter activity. This deficiency could, at least partially, be corrected by a fusion with TAP-expressing cells. We also assessed the expression of MHC class I allotype of the fibroblasts (K<sup>b</sup>) in the fused CMT.64-Ltk, LMD-Ltk and B16-Ltk cells, and found a slight but consistent decrease in the level of K<sup>k</sup> expressed (Fig. 3C).

**Overexpression of IRF-1 and IRF-2 in CMT.64 cells did not result in significant changes in TAP-1 promoter activity.** IFN- $\gamma$  treatment resulted in high induction of TAP-1 promoter activity and could subsequently overcome TAP and MHC class I deficiencies in carcinomas. Therefore, factors that are activated in response to IFN- $\gamma$  are attractive candidates for the future discovery of positive *trans*-acting factors that are either absent or functionally defective in TAP-deficient cells, thus accounting for the impairment of TAP-1 promoter activity in carcinomas. To investigate whether TAP down-regulation might be caused by abnormally low or high levels of IRF-1 and IRF-2, respectively, endogenous levels of IRF-1 and IRF-2 mRNAs in TAP-expressing and TAP-deficient cells were observed by RT-PCR. The results indicated that both IRF-1 and IRF-2 mRNA levels did not correlate with TAP levels in the cell lines (Supplementary Figure 1A). To further investigate the putative role of IRF-1 and IRF-2 in modulating TAP-1 promoter activity in TAP-deficient cells, human IRF-1 and -2 (15) were overexpressed in a clone of CMT.64 cells containing the pTAP-1-EGFP construct. Flow cytometry analysis showed that the overexpression of the IRFs had no significant effect on EGFP levels in the transfectants (Supplementary Figure 1B). To test whether the transfected IRFs were functional, the cells were cotransfected with either an IFN- $\beta$  promoter-luciferase construct or a promoterless pGL3-luciferase vector. As expected (15), we found that the overexpression of exogenous IRF-1 increased the basal level of IFN- $\beta$  promoter activity, whereas the IRF-2 decreased it (Supplementary Figure 1C). This indicated that overexpression of IRF-1 and IRF-2 alone was not sufficient for modulating the TAP-1 promoter activity in CMT.64 cells.

**Analysis of TAP-1 expression in unfused and fused cells.** During the course of these studies, we noted that the TAP-1 promoter displayed a low but detectable activity in TAP-deficient cells, whereas TAP-1 mRNA was barely detectable in these cells. To investigate whether the low levels of TAP-1 mRNA in TAP-deficient cells resulted solely from the deficiency in TAP-1 promoter activity shown above, we compared the levels of TAP-1 mRNA transcribed from the CMT.64 genome and from the Ltk genome in fused CMT.64-Ltk cells. To distinguish between TAP-1 expressed from the



**Figure 3.** The *TAP-1* gene promoter is regulated by positive *trans*-acting factors that are deficient in carcinoma cells. **A**, *TAP-1* promoter-driven EGFP expression increased in the fused CMT.64-Ltk and LMD-Ltk cells (thick lines) compared with that in the unfused pTAP-1-EGFP-transfected CMT.64 and LMD cells (thin lines). Wild-type CMT.64 and LMD were used as negative controls for EGFP expression (shaded areas). **B**, surface expression of K<sup>b</sup> (measured using PE-conjugated anti-K<sup>b</sup> mAb) was increased when CMT.64 or LMD (shaded areas) were fused to Ltk cells (broken lines). Expression of K<sup>b</sup> on fused CMT.64-Ltk and fused LMD-Ltk cells are displayed using thick lines. As a positive control, expression of K<sup>b</sup> was assessed on IFN- $\gamma$ -treated CMT.64 and LMD cells (thin lines). **C**, K<sup>k</sup> surface expression was reduced in the fused CMT.64-Ltk and LMD-Ltk (thick lines) compared with that in the wild-type Ltk cells (thin lines). Wild-type CMT.64 and LMD cells were used as negative controls for K<sup>k</sup> expression (shaded areas). K<sup>k</sup> expressions were assessed using PE-conjugated anti-K<sup>k</sup> mAb. Data are representatives of three fusion experiments.

two genomes, analysis of the *TAP-1* mRNA polymorphism in both cells was carried out. A total of 12 bp differences was found between the nucleotide sequence of CMT.64-derived and Ltk-derived *TAP-1* mRNAs (data not shown). Primers specific to CMT.64-derived *TAP-1* mRNA were then designed to investigate its levels in the fused CMT.64-Ltk cells. Nonpolymorphic primers were used for further analysis of total *TAP-1* expression in the fused cells.

Despite the increase in *TAP-1* promoter activity driving the *EGFP* gene in the fused CMT.64-Ltk cells (Fig. 3A), no *TAP-1* mRNA from the CMT.64 genome could be detected in cells that were not treated with IFN- $\gamma$  (Fig. 4A). In fact, analysis of total *TAP-1* expression showed that the fused CMT.64-Ltk and LMD-Ltk cells displayed drastically lower levels of *TAP-1* mRNA and protein than the unfused Ltk fibroblasts did (Fig. 4B). This suggested the existence of posttranscriptional mechanisms that further down-regulate levels of *TAP-1* mRNA in *TAP*-deficient cells. Therefore, we sought to investigate the possibility of a difference in *TAP-1* mRNA stability between *TAP*-expressing and *TAP*-deficient cells.

***TAP-1* messenger RNA stability decreases in carcinoma-fused fibroblasts.** To assess the regulation of *TAP-1* at the level of mRNA stability, unfused Ltk fibroblasts, as well as fused

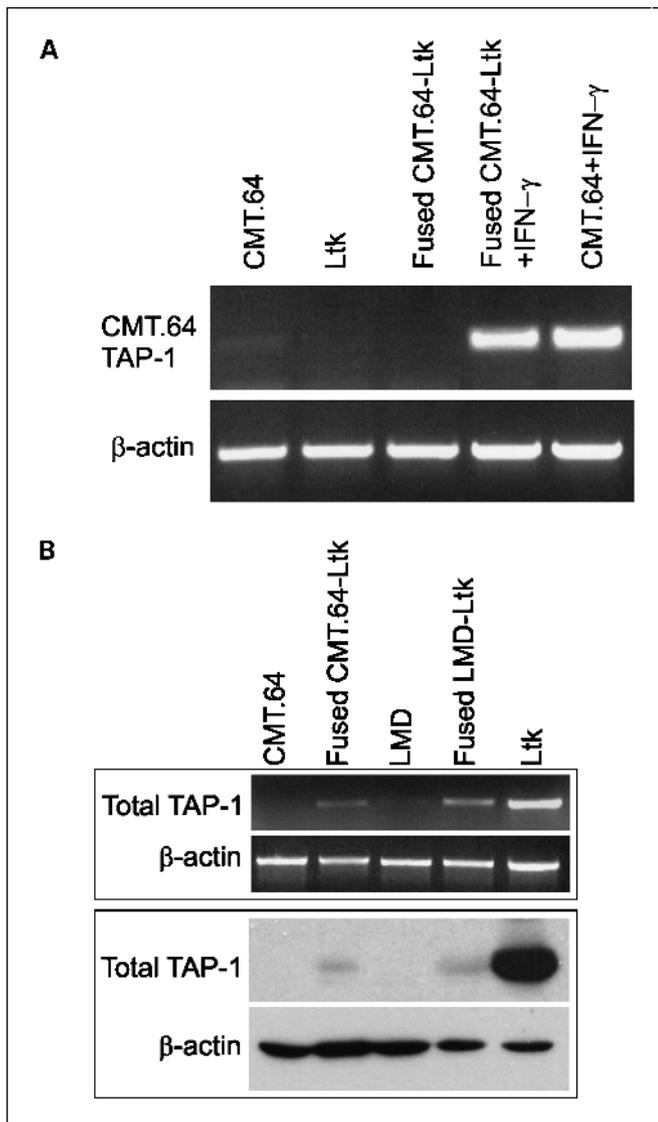
CMT.64-Ltk, LMD-Ltk, and Ltk-Ltk cells were treated with actinomycin D for 2 to 8 hours to block neosynthesis of mRNA. Residual levels of *TAP-1* mRNA after treatment of cells with actinomycin D were then assessed by real-time PCR using constant amounts of total RNA as templates. We found that *TAP-1* mRNA stability decreased when fibroblasts were fused to carcinoma cells (Fig. 5A). As a control, we showed that the stability of unrelated mRNA, such as the mRNA coding for S15 (Fig. 5B) and prion protein (data not shown), was unaffected by the cell fusion events. These results suggest the existence of factors, which remain to be identified, that enhanced *TAP-1* mRNA degradation in carcinoma cells.

## Discussion

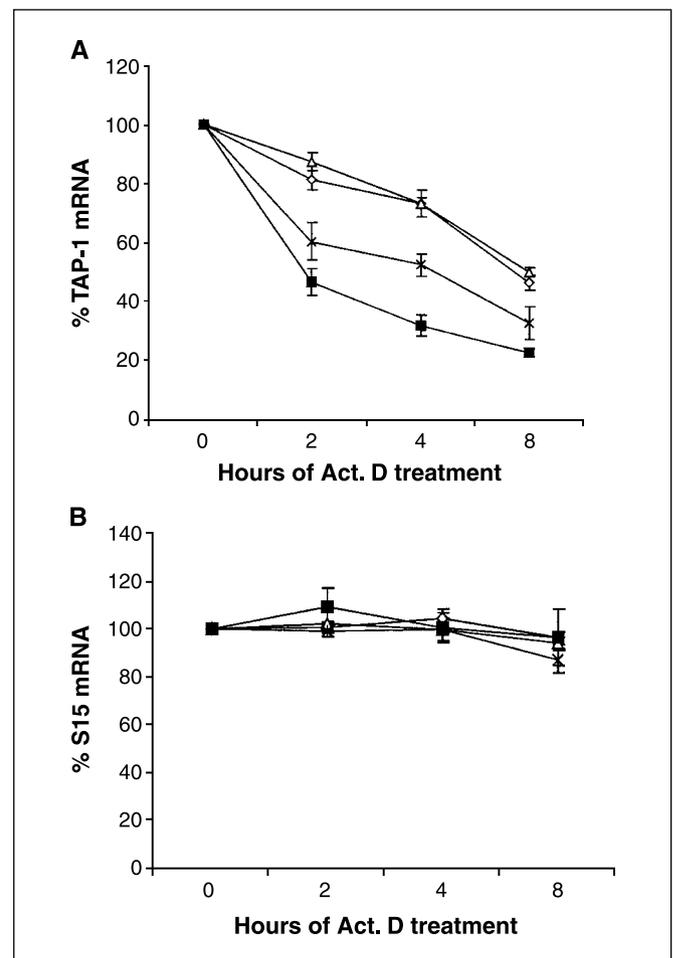
*TAP-1* down-regulation in tumors has been reported in many studies (6, 10, 20–22); however, the molecular mechanisms underlying this defect remain poorly understood. One of the recently proposed mechanisms of antigen processing machinery (APM) down-regulation is the overexpression of oncogenes, e.g., HER-2/neu, that gives rise to the emergence of immune escape phenotype of tumors (23). Related mechanisms of *TAP* down-regulation may include an impairment of *TAP-1* promoter

activity (due to mutation in *cis*-acting elements in the promoter or in distal enhancer/silencer regions, chromatin remodeling at the TAP-1 locus, and/or difference in expression or functionality of *trans*-acting factors), as well as a relatively low stability of TAP-1 mRNA in these cells.

We used murine lung, prostate, and skin carcinomas (CMT.64, LMD, and B16) as models for TAP- and MHC class I-deficient cancer cells, and propose that TAP deficiency in these cells is caused by the lack of activation or expression of TAP-1 transcriptional activators as well as a decrease in TAP-1 mRNA stability. Our results showed that (a) treatment of the TAP-deficient cells with IFN- $\gamma$  increased the TAP-1 mRNA expression to similar levels as in TAP-expressing cells; (b) the initiation of TAP-1 transcription was impaired in TAP-



**Figure 4.** Fusion of fibroblasts with carcinoma cells results in a decrease in the expression of TAP-1 mRNA and protein. **A**, no expression of TAP-1 mRNA from the CMT.64 genome was detectable by RT-PCR in the fused CMT.64-Ltk cells. Amplifications of reverse-transcribed CMT.64 TAP-1 mRNA from IFN- $\gamma$ -treated cells and  $\beta$ -actin mRNA were used as positive and loading controls, respectively. Data are representatives of three experiments. **B**, total TAP-1 expression was reduced when CMT.64 and LMD cells were fused with wild-type Ltk cells, as shown by RT-PCR (*top*) and Western blot (*bottom*). Levels of  $\beta$ -actin were used as loading controls. Data are representatives of three experiments.



**Figure 5.** The TAP-1 mRNA is unstable in carcinomas. Cells were treated with actinomycin D for 2 to 8 hours as indicated. Constant amounts of RNA purified from these cells were used as templates for real-time RT-PCR using primers specific for TAP-1 (**A**) and S15 (**B**). Levels of TAP-1 and S15 mRNA in actinomycin D-treated cells are expressed as percentages of the levels of the corresponding mRNA in untreated cells. *Points*, average of three to six experiments; *bars*, SEM.  $\diamond$ , Ltk;  $\triangle$ , Fused Ltk-Ltk;  $\ast$ , Fused LMD-Ltk;  $\blacksquare$ , Fused CMT-Ltk.

deficient cells; (c) the relatively low activity of TAP-1 promoter in the carcinomas is due to regulatory defects rather than mutations in the TAP-1 promoter; (d) low TAP-1 promoter activity and MHC class I deficiency in carcinomas could be corrected, at least partially, by fusions with wild-type fibroblasts; and (e) a decrease in TAP-1 mRNA stability also contributed to TAP-1 deficiency in murine lung carcinomas.

Based on the structural and functional analysis of TAP-1 promoter of TAP-deficient cells, as well as results obtained from chromatin immunoprecipitation assays of endogenous TAP-1 promoter in various TAP-expressing and TAP-deficient cells, we propose that one mechanism underlying TAP deficiency in these cells is the impairment of the ability of TAP-1 promoter to drive transcription. As no mutation was observed in the CMT.64-derived TAP-1 promoter, this deficiency was likely to be caused by the lack of transcriptional activators necessary for optimal binding of the RNA polymerase II complex to the TAP-1 promoter or, conversely, by the presence of transcriptional inhibitors that prevent the binding.

FACS analysis of the fusions between TAP- and MHC class I-deficient carcinomas (CMT.64, LMD, and B16) of H-2<sup>b</sup> origin and

TAP- and MHC class I-expressing fibroblasts (Ltk) of H-2<sup>k</sup> origin showed an increase of TAP-1 promoter activity and some increase in K<sup>b</sup> expression. However, despite the increase of the promoter activity in the fused CMT.64-Ltk, no TAP-1 mRNA from the CMT.64 genome could be detected by RT-PCR (Fig. 4A); instead, total levels of TAP-1 mRNA and protein in the fused CMT.64-Ltk and LMD-Ltk cells were lower than in the unfused Ltk cells (Fig. 4B). This might have accounted for the decrease in surface expression of K<sup>k</sup> that we observed in the fused cells (Fig. 3). These unexpectedly low levels of TAP-1 mRNA and protein in the fused fibroblasts-carcinoma cells prompted us to investigate whether other mechanisms contribute to the TAP-1 deficiency in carcinoma cells, in addition to the impairment of TAP-1 promoter activity, that would also account for the disappearance of TAP-1 in the fused cells. We found that the stability of TAP-1 mRNA was decreased when fibroblasts were fused with carcinoma cells. This result suggests that the stability of TAP-1 mRNA is lower in CMT.64 and LMD cells than in the Ltk cells. Unfortunately, the extremely low levels of TAP-1 mRNA in the carcinoma cells, even in absence of actinomycin D,

preclude direct confirmation of this hypothesis. Further studies will be required to precisely characterize the positive regulatory factors that are lacking or defective in carcinomas, as well as the mechanism that leads to a reduction of TAP-1 mRNA stability in carcinomas.

These studies are of fundamental importance as they will significantly contribute to a better understanding of the underlying cause of antigen processing deficiency in many tumor types. This will, in turn, lead to new approaches to modify the immunogenicity and antigenicity of tumor cells, thereby allowing recognition of tumors by immune surveillance mechanisms.

## Acknowledgments

Received 12/1/2003; revised 4/19/2005; accepted 5/27/2005.

**Grant support:** National Cancer Institute of Canada, Canadian Institutes of Health Research, Canadian Network for Vaccines and Immunotherapeutics, and Genemax, Inc.

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We thank Dr. T.C. Thompson for providing the LMD cell line, Andy Johnson for assistance with the cell sorting, Kyung Bok Choi for helpful discussions, Linda Li for excellent technical assistance, and Cheryl Pfeifer for critical review of the manuscript.

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## Identification of Mechanisms Underlying Transporter Associated with Antigen Processing Deficiency in Metastatic Murine Carcinomas

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*Cancer Res* 2005;65:7485-7492.

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