THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 17, Issue of April 25, pp. 15291–15296, 2003
Printed in U.S.A.

A Single-nucleotide Deletion Leads to Rapid Degradation of *TAP-1* mRNA in a Melanoma Cell Line*

Received for publication, January 29, 2003
Published, JBC Papers in Press, February 11, 2003, DOI 10.1074/jbc.M300954200

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Both viruses and tumors evade cytotoxic T lymphocyte-mediated host immunity by down-regulation of antigen presentation machineries. This can be achieved by either down-regulation of transcription of antigen presentation genes or posttranslational inactivation of proteins involved in antigen presentation. In this study, a major histocompatibility complex (MHC) class I-deficient melanoma cell line, SK-MEL-19, was found deficient in the expression of the transporter associated with antigen processing (*TAP*)-*1* mRNA even after IFN-γ stimulation, despite its active transcription of the *TAP-1* gene. This abnormality was caused by a single-nucleotide deletion at position +1489 of the *TAP-1* gene and was corrected by cycloheximide, which inhibits RNA degradation. Using an inducible Tet-Off system, we demonstrated that deletion of the nucleotide resulted in a >2-fold decrease in the half-life of *TAP-1* mRNA. However, the decrease of the half-life of *TAP-1* mRNA is not mediated by nonsense-mediated mRNA decay because deletions of two additional nucleotides in the region, which corrected the nonsense mutation, did not restore *TAP-1* mRNA stability. To our knowledge, this is the first evidence that the degradation of mRNA of an antigen presentation gene is involved in HLA class I down-regulation in malignant cells.

Recent studies demonstrate that patients with malignant melanoma often have a high number of cytotoxic T lymphocytes specific for melanoma-associated antigens (1, 2). The co-existence of T cells and tumor cells even in the draining lymph nodes suggests that the tumors were able to evade destruction by host cytolytic T lymphocytes. Accumulating evidence supports the notion that both malfunction of T cells and down-regulation of antigen presentation machinery in tumors can be responsible for tumor evasion of host immunity (1–6). In fact, a high proportion of malignant tumors, including melanoma, have severely depressed cell surface expression of class I HLA antigens (7), the target molecules that present tumor antigenic peptide to cytolytic T lymphocytes. Understanding the mechanisms underlying the T-cell malfunction or antigen presentation defects may thus provide insight for immunotherapy of melanoma and other cancers.

Optimal cell surface expression of HLA molecules requires the coordinated expression of several genes, such as transporters associated with antigen processing (*TAP*)[1]-*1*/*2*, low molecular weight peptide (*LMP*)-*2*/*7*, and tapasin, as well as HLA class I heavy chain and β_2-microglobulin (β_2M). In cases of both tumorigenesis and viral infection, expression of these genes and the function of the encoded proteins are often impaired (8–10). The mechanisms for such down-regulation have been studied extensively. Theoretically, gene expression can be modulated by transcriptional, posttranscriptional, translational, and posttranslational mechanisms. The mechanisms that have been shown to underlie the antigen presentation abnormalities are transcriptional suppression of antigen presentation genes and/or functional inactivation of their gene products, either by missense mutation or by protein-protein interactions (11–14). Here we show that actively transcribed *TAP-1* mRNA in the melanoma cell line SK-MEL-19 is rapidly degraded even after stimulation with IFN-γ. Cloning and sequencing analysis have revealed a single-nucleotide deletion at position +1489. This mutation results in substantial reduction of the stability of *TAP-1* mRNA by mechanisms unrelated to nonsense-mediated mRNA decay (NMD). These results reveal a new potential mechanism for tumor evasion of host T-cell recognition.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Human melanoma cell lines 1195, 1102, and SK-MEL-19 were cultured as described previously (15). The breast cancer cell line SK-BR-3 was obtained from ATCC (HTB-30; ATCC, Manassas, VA). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For induction of HLA class I expression, cells were cultured in medium supplemented with recombinant human IFN-γ (1000 units/ml; R&D Systems, Minneapolis, MN). PE-conjugated anti-HLA-A, -B, and -C antibody (clone G46-2.6) and isotype control PE-conjugated mouse IgG1 were purchased from BD PharMingen (San Diego, CA).

Flow Cytometry—Cell surface HLA class I expression was examined by flow cytometry as described previously (4). Briefly, viable cells were incubated with PE-conjugated mouse IgG1 and PE-conjugated anti-HLA-A, -B, and -C antibody at 4 °C for 2 h. After three washes with phosphate-buffered saline containing 1% fetal calf serum, cells were fixed with 1% paraformaldehyde and examined by flow cytometry.

Northern Blot—Cells were either treated with IFN-γ (R&D Systems) at 1000 units/ml for 48 h or left untreated. For cycloheximide (CHX; Sigma) treatment, SK-MEL-19 cells were cultured with IFN-γ at 1000 units/ml for 48 h, and then CHX was added to the cells for a final concentration of either 5 or 10 μg/ml, respectively, for up to 16 h. Total RNA was isolated using TRIzol reagent (Invitrogen). Hybridization conditions followed the instructions of the Northern hybridization kit

* This work was supported by NIH Grants CA82355, CA69091, and CA58033; Department of Defense Grant DAMD17-00-1-0041; and The Ohio State University Comprehensive Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TAP, transporter associated with antigen processing; LMP, low molecular weight peptide; NMD, nonsense-mediated mRNA decay; CHX, cycloheximide; Tet, tetracycline; β_2M, β_2-microglobulin; PE, phycoerythrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green fluorescent protein; WT, wild type.

(Eppendorf Scientific, Westbury, NY). The cDNA probes for *TAP-1*, *TAP-2*, *LMP-2*, *LMP-7*, HLA class I heavy chain, and β_2M were made from PCR products using primers listed previously (11). A human splenocyte cDNA library from Invitrogen was used as the template for PCR reactions. All PCR products were subcloned into pBluescript vector (Stratagene, La Jolla, CA) and sequenced and confirmed to be identical to published sequences. The probes were labeled with [α-^{32}P]dCTP (PerkinElmer Life Sciences) using the DECAprime™ II kit (Ambion, Austin, TX).

Generation of TAP-1 cDNA Constructs and Stable Transfection—The human small cell carcinoma H146 cell line (provided by Dr. N. P. Restifo; National Cancer Institute, Bethesda, MD) was incubated with IFN-γ at 1000 units/ml for 48 h. Total RNA was isolated as described above. Reverse transcription was done using the SUPERSCRIPT First-Strand cDNA Synthesis System (Invitrogen). *TAP-1* cDNA was amplified by PCR in three fragments. Primers were as follows: hTAP.f1, 5′-GCGGCCGCTTTCGATTTCGCTTTC-3′; hTAP.r1, 5′-TGCAGTAGCCTGGTGCTATCCG-3′; hTAP.f2, 5′-CTTGCCTTGTTCCGAGAGCTGA-3′; hTAP.r2, 5′-CTCGTTGGCAAAGCTTCGAAC-3′; hTAP.f3, 5′-CGGCCATGCCTACAGTTCGAAG-3′; and hTAP.r3, 5′-ATAAATATCAAGAACCTACAGGG-3′. The three fragments were cloned into pBS-KS vector (Stratagene) at *Not*I/*Sma*I, *Sma*I/*Hin*dIII, and *Hin*dIII/*Xho*I sites, respectively, and sequenced to confirm that the cDNA has a wild type sequence. When SK-MEL-19 cells grew to 70% confluence, 0.2 μg of pcDNA3.1/Hyg(+) vector (Invitrogen) and pcDNA3.1/Hyg(+) vector with wild type *TAP-1* cDNA insert were respectively transfected into each well of a 24-well plate, using 6 μl of FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manual. 48 h later, the transfected cells were replated into 96-well plates and cultured in Dulbecco's modified Eagle's medium and in the presence of 0.5 mg/ml hygromycin B (Invitrogen). Single cell clones were selected for further culture and analyzed for HLA class I antigen expression.

Southern Blot—Genomic DNA was isolated from SK-MEL-19 cells, SK-BR-3 cells, and HeLa cells. Genomic DNA (20 μg) was digested with *Afl*III (Invitrogen) and separated in 0.8% agarose gel. The *TAP-1* promoter probe was made by PCR from normal human lymphocyte genomic DNA with sense primer 5′-TCCCGCCTCGAGCATCCCTGCAAGGCA-3′ and antisense primer 5′-TGCAGTAGCCTGGTGCTATCCG-3′. Probes were labeled as described above.

Generation of Luciferase Reporter Constructs and Assay for Promoter Activity—The *TAP-1* promoter was amplified from SK-MEL-19 cell genomic DNA by PCR using the following primers: hTAP1.Pr1, 5′-GCTCTAGATGGCACTCGGACGCCGTC-3′; and hLMP2.Pf1, 5′-GCTCTAGACCCTGCAAGGCACCGCTC-3′. The PCR products were subcloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and then cloned into pGL2-basic vector (Promega, Madison, WI) at *Xho*I and *Hin*dIII sites. All constructs were confirmed by DNA sequencing. Expression level of the firefly luciferase from the pGL2 constructs (basic, SV40, pTAP1/T, and pTAP1/G) was normalized to the internal control pRL-SV40 *Renilla* luciferase level. Results were shown as the fold increase compared with the pGL2-basic. The dual luciferase assay was carried out according to the manufacturer's instructions (Promega).

Nuclear Run-on Assay—The assay was performed as described elsewhere (16). Briefly, nuclei were extracted from 10^7 to 10^8 SK-MEL-19 cells treated with or without 1000 units/ml IFN-γ. The transcripts were labeled *in vitro* with 40 nM biotin-16-UTP (Roche Molecular Biochemicals) in the presence of 3.75 mM ATP, GTP, and CTP; 25 mM Tris-HCl; 12.5 mM $MgCl_2$; and 750 mM KCl. cDNA fragments of *LMP-2* and *GAPDH* were amplified by PCR from cloned cDNA constructs (11). *TAP-1* cDNA fragment was amplified by PCR from cloned cDNA constructs using primers hTAP1.f1 and hTAP1.r1 described above. The pcDNA3.1/Hyg(+) vector was linearized with *Hin*dIII. All the DNA was immobilized on nitrocellulose membrane using S&S Minifold II slot blot apparatus according to the manual (Schleicher & Schüll). Hybridization conditions were as described previously (16), and the biotin-labeled transcripts were detected using streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals) and CDP-star Ready-To-Use with Nitro-Block-II reagent (Tropix, Bedford, MA).

Restriction Fragment Length Polymorphism—Primers hTAP1CE7.f (5′-GCACCCCTCGCTGCCTACCCAGTGGTCT-3′) and hTAP1CE7.r (5′-TACAGGGAGTGGTAGGTTGTACCTG-3′) were used to amplify from the genomic DNA the region of *TAP-1* exon 7 where the single-nucleotide deletion resides. The region was also amplified from cDNA using primers hTAP1CE7.f and hTAP1CE7.r PCR products were separated by gel electrophoresis and purified using Qiagen gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were incubated with *Bsl*I (New England Biolabs, Beverly, MA) at 55 °C overnight and then separated in 5% agarose gel.

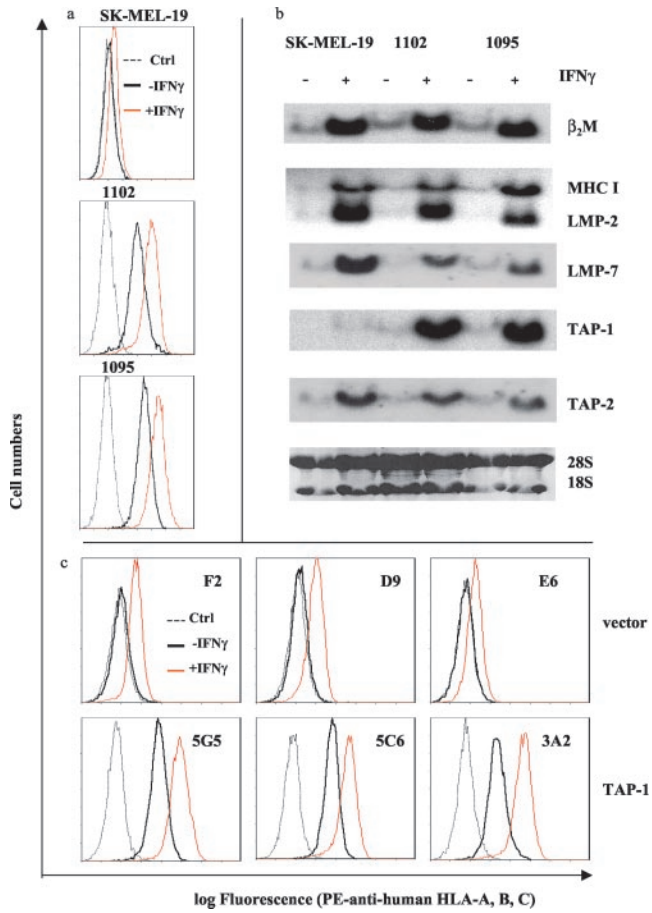

FIG. 1. **Deficiency of surface HLA class I expression in melanoma cell line SK-MEL-19 was due to the *TAP-1* down-regulation.** *a*, HLA class I expression in three melanoma cell lines, SK-MEL-19, 1102, and 1195. *Bold black lines* depict the staining by PE-conjugated anti-human HLA-A, -B, and -C antibody in untreated cells; *dotted lines* represent the staining by PE-conjugated mouse IgG1 as isotype control; and *red lines* represent anti-HLA-A, -B, and -C antibody staining after stimulation with 1000 units/ml IFN-γ for 72 h. *b*, expression of HLA class I heavy chain (*MHC I*), β_2M, *TAP-1*, *TAP-2*, *LMP-2*, and *LMP-7* in each cell line with or without IFN-γ induction (1000 units/ml for 72 h). Total RNA loading to each well was shown as 28 S rRNA (*28S*) and 18 S rRNA (*18S*). *c*, transfection with wild type *TAP-1*, but not vector alone, restored HLA class I expression in the SK-MEL-19 cells. SK-MEL-19 cells were transfected with either vector alone (*top panels*) or vector with *TAP-1* cDNA insert (*bottom panels*). These stable clones from each group were stimulated with or without IFN-γ and analyzed for cell surface HLA-A, -B, and -C, as detailed in *a*.

Generation of TAP-1 cDNA and Tet-Off SK-MEL-19 Cell Lines—Wild type *TAP-1* cDNA was cloned from the human small cell carcinoma H146 cell line as described above. TAP-1 D1489 was generated by PCR using the total cDNA from the SK-MEL-19 cells as the template. Site-directed mutagenesis by overlapping PCR was performed to make TAP-1 Del3 cDNA. After confirming their sequences, all three *TAP-1* cDNAs were inserted into the multiple cloning site of the pBI-EGFP vector (Clontech) by blunt-end ligation. The three constructs, pBI-EGFP-TAP1, pTet-Off (Clontech), and pcDNA3.1/Hyg(+) (Invitrogen), were co-transfected into SK-MEL-19 cells using FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Stably transfected cell clones were selected in 96-well plates in Dulbecco's modified Eagle's medium supplemented with 0.5 mg/ml hygromycin (Invitrogen). To confirm the efficiency of the Tet-Off construct in the tumor cell line, the green fluorescence protein-positive cell clones were treated with 1 μg/ml tetracycline (Roche Molecular Biochemicals) for 24 h. The total RNA was extracted from the cell clones with or without tetracycline treatment. The cell clones in which *TAP-1* mRNA expression was inhibited by at least 95% by tetracycline were used for the study of mRNA stability.

RNase Protection Assay—The *TAP-1* cDNA construct used to make the *TAP-1* antisense transcript was the same as the one used for the Northern blot. The *GAPDH* cDNA fragment was generated by PCR

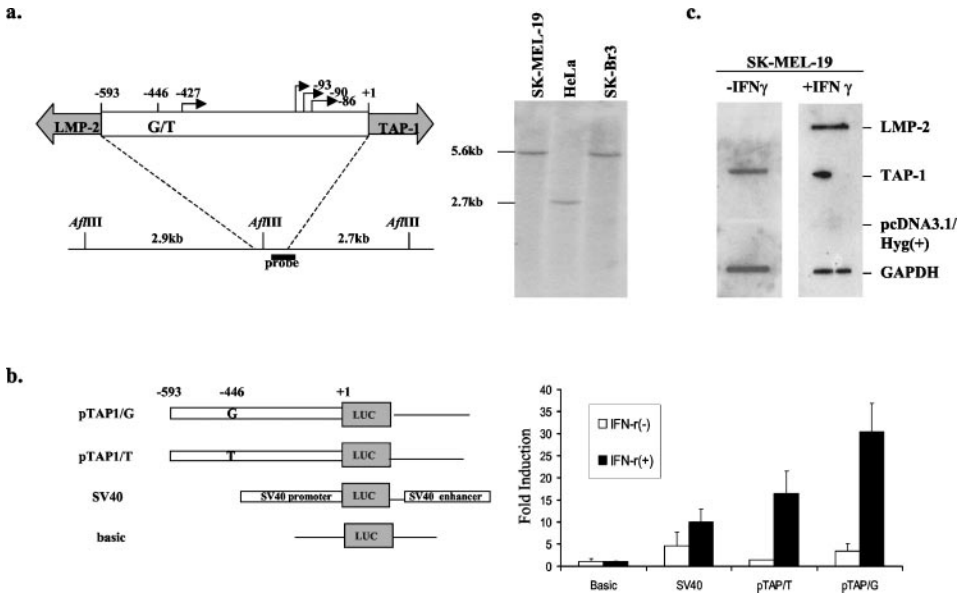

FIG. 2. **Posttranscriptional mechanisms are responsible for poor accumulation of *TAP-1* mRNA.** *a*, a single-nucleotide polymorphism, adjacent to the first transcription start site (−427), was identified at −446 in the bidirectional promoter shared by the *TAP-1* and *LMP-2* genes. The G → T change results in the loss of the *Afl*III restriction site. Southern blot hybridization was performed using *Afl*III and detected by a DNA probe that encompasses the downstream region of the polymorphism site. SK-MEL-19 cells showed one 5.6-kb band that represents homozygous T allele, as did the breast cancer cell line SK-BR-3, which has significant cell surface HLA class I surface expression (data not shown). HeLa cells, in contrast, are homozygous for the G allele. *b*, activities of T and G alleles of *TAP-1* promoter (*pTAP1/T* and *pTAP1/G*, respectively) in SK-MEL-19 cells. The two allelic forms of *TAP-1* promoter were cloned into pGL2-basic vector (*basic*) that did not contain any promoter or enhancer but encoded firefly luciferase. The pGL2-SV40 construct (*SV40*) that had both SV40 promoter and SV40 enhancer as well as the firefly luciferase reporter gene was used as positive control. After transfection, IFN-γ was added to the cell culture at 1000 units/ml. Cells were lysed 48 h after transfection, and luciferase expression was tested using a luminometer. Data shown are representative of at least five independent experiments. *c*, the *TAP-1* gene was actively transcribed in SK-MEL-19 cells in the presence and absence of IFN-γ in nuclear run-on assay. Endogenous *GAPDH* expression was used as a positive control, and the pcDNA3.1/Hyg(+) vector was used as a negative control. The run-on experiments were repeated three times with similar results.

using the primers hGAPDH.f (5′-TGAGAACGGGAAGCTTGTCATCAA-3′) and hGAPDH.r (5′-CAGCCTTCTAGATGGTGGTGAAGA-3′). The EGFP cDNA fragment was also generated by PCR using the primers EGFP.f (5′-TCCAGCAGGATCCTGTGATCGCGCT-3′) and EGFP.r (5′-ACCTACGGCCTCGAGTGCTTCAGCC-3′). The antisense probes were made using the Riboprobe *in vitro* transcription system (Promega). The RNase protection assay was conducted with the RPA III ribonuclease protection assay kit (Ambion) according to the instruction manual. After separation of protected fragments on a 6% sequencing gel, signals were quantified by phosphorimaging (Amersham Biosciences). The *TAP-1* signal intensity was normalized by that of the *GAPDH* signal, which served as a loading control. The percentages of the amount of remaining *TAP-1* mRNA at different time points after tetracycline was added compared with time 0 were calculated.

RESULTS AND DISCUSSION

Down-regulation of TAP-1 mRNA by a Posttranscriptional Mechanism in Melanoma Cell Line SK-MEL-19—Three human melanoma cell lines (1102, 1195, and SK-MEL-19) were examined by flow cytometry for their cell surface HLA class I expression with or without IFN-γ stimulation. A PE-conjugated anti-human HLA-A, -B, and -C antibody was used to detect all HLA class I alleles, and a PE-conjugated mouse IgG1 was used as isotype control. As shown in Fig. 1*a*, 1102 and 1195 cells had significant HLA class I that was further up-regulated by incubation with 1000 units/ml IFN-γ for 3 days. Confirming previous studies (15), we found that SK-MEL-19 cells had no cell surface HLA. Surprisingly, whereas other melanoma cell lines up-regulated their cell surface HLA in response to IFN-γ, very little HLA class I antigen could be found on the SK-MEL-19 cells even after IFN-γ-treatment.

Because optimal cell surface HLA class I expression requires the coordinated expression of multiple genes, including *TAP-1/2*, *LMP-2/7*, and β_2M as well as HLA class I heavy chain, a Northern blot analysis was performed to detect the expression of these genes (Fig. 1*b*). In 1102 and 1195 cells, all six genes were expressed at low but detectable levels. IFN-γ treatment drastically induced expression of all six genes. Interestingly, in the SK-MEL-19 cells, whereas β_2M, HLA heavy chain, *LMP-2*, *LMP-7*, and *TAP-2* were present at low levels without induction, no *TAP-1* mRNA was detected. After IFN-γ treatment, β_2M, HLA heavy chain, *TAP-2*, *LMP-2*, and *LMP-7* were expressed at high levels, yet *TAP-1* was still expressed at low levels.

It has been known that TAP-deficient cells can express HLA class I after transfection with the *TAP-1* or *TAP-2* gene (17–19). To test whether the lack of *TAP-1* expression was responsible for the barely detectable expression of HLA class I antigen on the surface of SK-MEL-19 cells, we transfected the cells with *TAP-1* cDNA. As shown in Fig. 1*c*, the *TAP-1* cDNA-transfected SK-MEL-19 cells expressed significant levels of HLA class I antigen even before IFN-γ-treatment. Moreover, the *TAP-1* transfectants were as responsive to IFN-γ as the other melanoma cell lines. Based on these results, it is likely that the primary defect of antigen presentation in SK-MEL-19 cells is attributable to defects in *TAP-1* expression.

Because the *TAP-1* expression was low at the mRNA level, we hypothesized that the *TAP-1* down-regulation was caused by defective transcription or malfunction in RNA metabolism. The *TAP-1* expression is under the control of a bidirectional promoter, as characterized by Wright *et al.* (20). We cloned and sequenced the 593-bp *TAP-1* promoter from SK-MEL-19 cells. In comparison with the published sequence (20), a single-nucleotide G → T replacement was identified at position −446 (the first ATG of the *TAP-1* gene is designated as +1), which is close to the first transcription start site at −427 (20) (Fig. 2*a*). Because the T allele results in a loss of restriction site *Afl*III, we did a Southern blot hybridization using *Afl*III to confirm the mismatch. As shown in Fig. 2*a*, whereas the HeLa cell line

contained homozygous G alleles as described previously (20), both SK-MEL-19 and the breast cancer cell line SK-BR-3 were homozygous for T alleles that lack the restriction site for *Afl*III. To test whether this single-nucleotide replacement results in reduced promoter activity, both alleles of the *TAP-1* promoter were cloned into the pGL2-basic vector that contains the luciferase reporter gene. As shown in Fig. 2*b*, the T allele *TAP-1* promoter retained 50% of the promoter activity compared with the G allele. However, given the significant variation in transient transfection and luciferase assays, it is unclear whether the G → T change has a significant effect on *TAP-1* transcription. However, both promoters were equally efficiently induced by IFN-γ treatment, whereas the *TAP-1* mRNA in the original SK-MEL-19 cell line was not induced by IFN-γ treatment (Fig. 1*b*). Moreover, our analysis of normal human peripheral blood lymphocyte samples revealed that both alleles were present at a high frequency, and individuals that carry either G or T alleles have equivalent cell surface HLA class I antigen expression (data not shown).

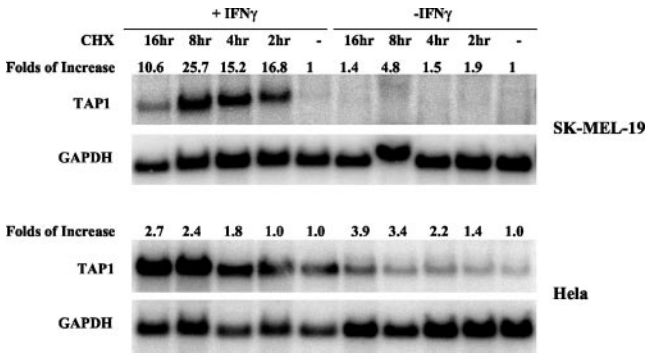

FIG. 3. ***TAP-1* mRNA level in SK-MEL-19 cells was increased by CHX.** The protein synthesis inhibitor CHX was added to the SK-MEL-19 cells and HeLa cells that had normal *TAP-1* and HLA class I expression. Total RNA was isolated from both cells at different time points and subjected to Northern blot hybridization to detect *TAP-1* expression. The blot was exposed to a PhosphorImager, and the signal intensity was quantified using ImageQuant 5.0 software (Amersham Biosciences). After normalization of *TAP-1* signal to endogenous *GAPDH* signal in each sample, the signals in the CHX-treated group were compared with those that received no CHX treatment. These signals were quantitated as fold of those in untreated cells.

We therefore performed a nuclear run-on assay to directly evaluate the transcription of the *TAP-1* gene. *LMP-2* transcription, which is under the control of the same bidirectional promoter, was also evaluated. As shown in Fig. 2*c*, *TAP-1* was transcribed at high levels in SK-MEL-19 cells under basal conditions, although IFN-γ appeared to up-regulate *TAP-1* transcription somewhat. In contrast, *LMP-2* was transcribed at an undetectable level but was induced to high levels by IFN-γ (Fig. 2*c*). The lack of *LMP-2* transcription at basal condition may reflect the IFN-γ-inducible expression pattern of this gene. These results demonstrate that lack of *TAP-1* mRNA in SK-MEL-19 cells was not due to defective transcription. Taken together, the results demonstrate that a posttranscriptional defect is responsible for poor *TAP-1* expression in SK-MEL-19 cells even after IFN-γ stimulation. Numerous studies have revealed defective *TAP-1* expression among tumor cells (7, 23). To our knowledge, however, this is the first example of a posttranscriptional defect of *TAP-1* expression.

A Single-nucleotide Deletion Leads to Accelerated Decay of TAP-1 mRNA—A major mechanism responsible for posttranscriptional regulation of mRNA is RNA degradation, which can be prevented by CHX, a protein synthesis inhibitor of mammalian cells. It is well established that the turnover of mRNA is closely linked to the translation process and that blocking translation can stabilize mRNA, especially those mRNA with short half-lives (21–23). To test whether accelerated RNA deg-

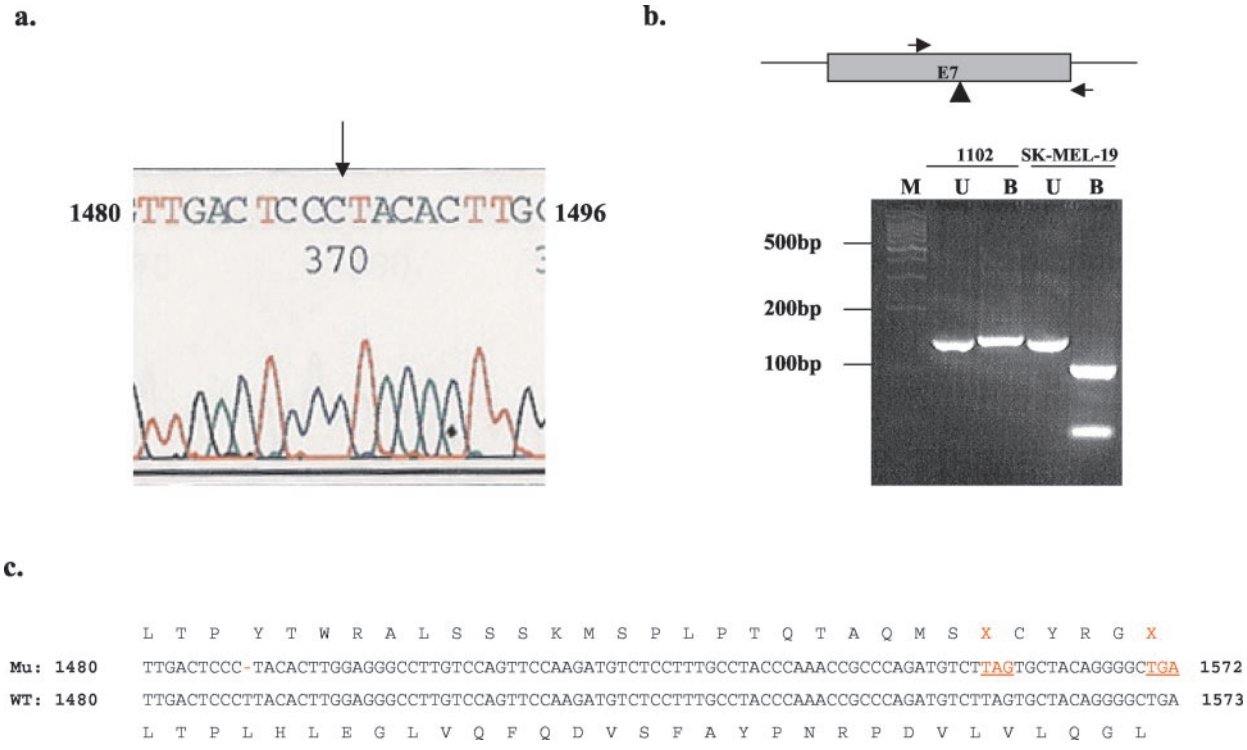

FIG. 4. **A homozygous single-nucleotide deletion was identified in the *TAP-1* gene at position +1489 that resulted in premature termination codons.** *a*, sequencing chromogram. The *arrow* points to the deletion site. *b*, primers hTAP1E7.f and hTAP1E7.r (*arrows*) were used to amplify the deletion region in *TAP-1* exon 7 (*E7*). The *arrowhead* points to the position of the deletion resulting in a new *Bsl*I site. The PCR products of genomic DNA were purified and digested with *Bsl*I. Gel electrophoresis data showed that SK-MEL-19 cells were homozygous for the +1489 deletion. *U*, uncut; *B*, *Bsl*I-digested; *M*, molecular weight. *c*, the sequence of the deletion region is shown, and the downstream premature termination codons (*X*) are *underlined*.

radation is responsible for the lack of *TAP-1* in SK-MEL-19 cells, we treated the SK-MEL-19 and control HeLa cells with CHX after incubation with or without IFN-γ (1000 units/ml) for 48 h at 37 °C. At different time points after the CHX was added to the cell culture, cells were harvested, and the total cellular RNA was analyzed for *TAP-1* mRNA. The intensity of each band was quantified using ImageQuant 5.0 software (Amersham Biosciences) after exposure to a phosphorimaging screen (Fig. 3). For a better comparison, *TAP-1* mRNA levels were normalized to the endogenous housekeeping gene *GAPDH* level, and the fold increase compared with non-CHX-treated cells was calculated. Under basal conditions, the *TAP-1* mRNA was up-regulated 4.8-fold in SK-MEL-19 cells. After IFN-γ induction, the *TAP-1* mRNA was up-regulated 25.7-fold. In comparison, CHX caused a less significant increase of *TAP-1* mRNA in both IFN-γ-treated and untreated HeLa cells. Taken together, the lack of *TAP-1* mRNA, the normal transcription of *TAP-1*, and the rescue of *TAP-1* mRNA by CHX treatment suggest that the *TAP-1* mRNA was rapidly degraded in the SK-MEL-19 cells. It is noteworthy that in SK-MEL-19 cells, the effect of CHX was significantly stronger when used in combination with IFN-γ. This finding cannot be fully explained by the fact the IFN-γ is a transcriptional activator for antigen presentation genes, because its effect on *TAP-1* transcription is not so obvious in SK-MEL-19 cells as shown in Fig. 2*c*. It is likely that IFN-γ stabilized mRNA in SK-MEL-19 cells, although this possibility remains to be tested formally.

The rapid degradation of *TAP-1* mRNA can be due to a genetic lesion in the *TAP-1* gene. Alternatively, it is possible that the tumor cell line expresses factors that can cause *TAP-1* mRNA degradation. The successful rescue of cell surface HLA class I antigen expression by wild type *TAP-1* in SK-MEL-19 cells favors the first hypothesis because a wild type cDNA can be expressed in the tumor cell line. As the first step to test this hypothesis, we cloned *TAP-1* cDNA from SK-MEL-19 cells that were treated with both IFN-γ and CHX as described above. All of the three clones sequenced showed a single-nucleotide deletion at position +1489 (Fig. 4*a*), which resides in exon 7 in the *TAP-1* gene. Further analysis showed that multiple downstream premature termination codons (the closest one is at position +1555) were present due to this nucleotide deletion (two of them are shown in Fig. 4*c*). To confirm that the mutation was in the *TAP-1* gene, we amplified exon 7 of the *TAP-1* gene from the SK-MEL-19 cells by PCR. The PCR products were digested with *Bsl*I because this restriction enzyme recognizes the deletion mutant but does not recognize the wild type exon 7. Because complete digestion was obtained, it appears that the SK-MEL-19 cells are homozygous for the frameshift mutation (Fig. 4*b*), even though the cytogenetic analysis revealed that there are four copies of chromosome 6 present in the SK-MEL-19 cells (data not shown). We subsequently amplified exon 7 of the *TAP-1* gene from 50 normal human peripheral lymphocyte genomic DNA samples by PCR and subjected the PCR products to *Bst*I digestion. Because none of the PCR products from the 50 samples could be digested by *Bsl*I, it is most likely that the single-nucleotide deletion in SK-MEL-19 cells resulted from a somatic mutation (data not shown) and that the apparent homozygosity of the *TAP-1* locus caused aneuploidy.

Premature termination codons resulting from frameshift mutation or nonsense mutation have been shown to interfere with the metabolism of many different mRNAs in mammalian cells, leading to nonsense-mediated altered RNA splicing, such as exon skipping and intron retention and/or NMD (21–23). Alternatively, a mutation may disrupt a cis-element that is necessary for mRNA stability and thereby cause RNA decay. To

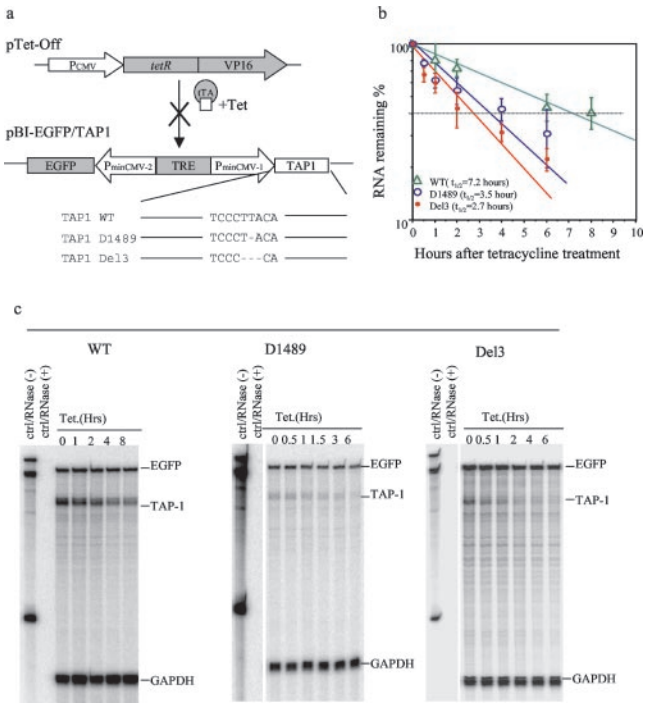

FIG. 5. **The single-nucleotide deletion D1489 in the *TAP-1* gene results in accelerated decay of the TAP-1 mRNA by a non-NMD-related mechanism.** RNase protection assay of three Tet-Off SK-MEL-19 cell clones stably transfected with TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 cDNA constructs was conducted using the antisense transcripts as probe. *a*, depiction of the Tet-Off system and the pBI-EGFP/TAP1 constructs containing TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 used to measure the mRNA half-lives. The nucleotide sequences around the mutation site in the cDNA constructs are denoted. *b*, decay kinetics of the TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 mRNA. Means ± S.D. of four experiments are presented. The half-lives of the mRNA were determined by manual best-fit regression analysis. *c*, RNase protection assay to demonstrate the accelerated decay of TAP-1 D1489 and TAP-1 Del3 message compared with that of the wild type TAP-1. The antisense probe protects a 345-nucleotide *TAP-1* mRNA. One representative assay for each *TAP-1* construct is shown.

differentiate the two possible mechanisms, we designed another *TAP-1* mutant (TAP-1 Del3) that has an in-frame 3-nucleotide deletion at the same position as TAP-1 D1489 (Fig. 5*a*) and compared the mRNA half-lives of TAP-1 WT, TAP-1 D1489, and TAP-1 Del3. If the degradation of TAP-1 D1489 is via the NMD pathway, then the half-life of the TAP-1 Del3 message should be comparable with that of TAP-1 WT. Otherwise, if the message of TAP-1 Del3 is comparable with or even more unstable than that of the TAP-1 D1489, then it is the deletion itself, but not the resulting premature termination codons, that leads to the accelerated decay of *TAP-1* mRNA in SK-MEL-19 cells. The Tet-Off gene expression system was adapted (Fig. 5*a*) to compare the stabilities of various *TAP-1* mRNAs. We first selected the SK-MEL-19 transfectants with an induction ratio (Tet 0/Tet 24 h) of 20 or greater to test mRNA half-lives. This ensured that more than 95% of transcription was blocked by the tetracycline after 24 h. The selected cell clones were then treated with 1 μg/ml tetracycline for different lengths of time before the total RNA was harvested. The amounts of the mRNA were quantified by phosphorimaging. In addition, the intensity of the *TAP-1* signal was normalized to that of *GAPDH* before the percentage of remaining mRNA was calculated. Four experiments were conducted, and an average percentage value was used to derive the mRNA half-lives ($t_{1/2}$) via regression analysis. As shown in Fig. 5, *b* and *c*, with a $t_{1/2}$ of 7.2 h, the TAP-1 WT mRNA is considerably more

stable than that of the TAP-1 D1489 ($t_{1/2}$ = 3.5 h). However, the mRNA derived from TAP-1 Del3 ($t_{1/2}$ = 2.7 h), which has an in-frame 3-nucleotide deletion, was degraded at least as fast as that of TAP-1 D1489. Because the TAP-1 Del3 mRNA has no premature termination codon downstream to the deletion, the accelerated decay in mutant *TAP-1* mRNA is most likely through mechanisms other than NMD. It is more likely that the mutation disrupts a cis-element critical for the stability of *TAP-1* mRNA. Whereas few cis-elements that help to stabilize mRNA have been reported, at least two have been reported by others (24, 25) and our group (26). Preliminary analysis showed no similarity between the region surrounding the mutation and the previously reported cis-element.

Nevertheless, the NMD is a well-conserved cellular surveillance mechanism. Whereas our work revealed a non-NMD mechanism for *TAP-1* mRNA degradation, it is still possible that mRNA derived from the endogenous mutant *TAP-1* gene can also be degraded by NMD. It has been shown recently that several criteria have to be met for the pathway to degrade a premature termination codon-containing message. First, at least one downstream spliceable intron is required for optimal NMD (27–29). The intron is thought to help recruit NMD factors, such as hUpf3, to the mRNA via the spliceosome (30–32). Second, the premature termination codon should be at least 45–55 nucleotides away from the next spliceable intron (27, 28). The lack of introns in our constructs may have prevented us from revealing NMD in *TAP-1* mRNA degradation. However, intronless premature termination codon-containing HEXA mRNA was shown to be subject to NMD, although at a lower efficiency than that seen when multiple downstream introns are present (33). In preliminary studies, when we made pBI-EGFP/TAP1 constructs with intron 7 or 8, the results also failed to support a role for NMD in degradation of the mutated *TAP-1* mRNA (data not shown).

Posttranscriptional regulations of other genes involved in antigen presentation have been reported previously (34). The increased turnover of HLA-C heavy chain mRNA has been suggested to contribute to the low level of HLA-C surface expression (34). Our work shows that mutations in the *TAP-1* gene in a tumor cell line can modulate its mRNA stability. This mechanism may be exploited by tumors to evade host immunity.

Acknowledgments—We thank Dr. Jian-Xin Gao for flow cytometry and Jennifer Kiel and Lynde Shaw for secretarial assistance.

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