

The MHC-encoded TAP1/LMP2 bidirectional promoter is down-regulated in highly oncogenic adenovirus type 12 transformed cells

James A. Proffitt, G. Eric Blair*

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

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Abstract Cells transformed by human adenovirus 12 (Ad12) exhibit extremely low surface levels of MHC class I molecules and contain reduced levels of class I heavy chain mRNAs. We report that levels of MHC-encoded TAP1 and LMP2 mRNAs are also down-regulated in Ad12-transformed rat cells, and that transcription of rat TAP1 and LMP2 transcripts is directed from a 564 bp intergenic region which is significantly less active in Ad12-transformed cells compared to those transformed with Ad5. Our results suggest that, in common with MHC class I gene expression, TAP1 and LMP2 gene expression is reduced mainly at the level of transcription in Ad12-transformed cells.

Key words: Gene expression; Nucleotide sequence; Viral suppression of the MHC

1. Introduction

Major histocompatibility complex (MHC) class I molecules are cell surface glycoproteins which play a key role in immune recognition of virally infected and transformed cells [1]. Class I molecules are heterodimers consisting of a polymorphic heavy chain non-covalently linked to an invariant light chain, β_2 -microglobulin. They function by presenting intracellularly processed peptides on the cell surface. Class I molecules loaded with foreign peptide are recognised by cytotoxic T cells which can then lyse the presenting cell [2]. A number of human cancers have been identified with reduced levels of surface class I molecules and it has been postulated that this may represent a mechanism whereby tumour cells can escape immune surveillance [3].

Transport of class I molecules to the cell surface is dependent on their binding to peptide in the endoplasmic reticulum [4,5]. It has been shown that these peptides, which arise from cleavage of proteins in the cytosol, are actively carried into the endoplasmic reticulum by a specific protein complex termed the transporter associated with antigen processing or TAP [6]. TAP consists of two subunits, TAP1 and TAP2, which are encoded by separate genes. Cells deleted for TAP1 and TAP2 have very low levels of surface class I molecules demonstrating that TAP expression is essential for antigen presentation [7,8]. The genes encoding TAP1 and TAP2 are located within the class II region of the MHC and are very closely linked to two other genes called LMP2 and LMP7 [9]. These genes encode γ -interferon inducible components of the proteasome,

a large complex with protease activity thought to be involved in the production of antigenic peptides [10]. Cells deleted for LMP2/7 are still capable of antigen presentation, however they appear to process certain antigens inefficiently [11,12].

Cells transformed by human adenoviruses of different serotype have proven useful in the study of down-regulation of class I antigen expression. Adenovirus 12 (Ad12) transformed cells exhibit reduced levels of MHC class I mRNA and surface class I molecules and are oncogenic when injected into syngeneic animals, whereas cells transformed by Ad5 have unaltered or elevated levels of surface class I molecules and their mRNAs and are non-oncogenic [13–15]. Recently it has been shown that TAP1/2 and LMP2/7 mRNA and protein levels are also reduced in Ad12-transformed mouse cells [16,17], and in a number of human tumours [18,19]. However, the molecular mechanism underlying this down-regulation is unknown. Here we show that TAP1 and LMP2 mRNA levels are coordinately repressed in Ad12-transformed rat cells. Furthermore, we demonstrate that the rat TAP1/LMP2 bidirectional promoter is significantly less active in Ad12-transformed cells compared to Ad5-transformed cells, indicating that the reduction in TAP1/LMP2 mRNA levels occurs at least in part at the level of transcription. Through comparison with previous work on the regulation of MHC class I gene expression, we identify a possible common mechanism for the down-regulation of MHC-encoded genes involved in antigen presentation in Ad12-transformed cells.

2. Materials and methods

2.1. Cell lines

RFC1 is an Ad12-transformed Hooded Lister rat kidney cell line and A12.1 is an Ad12-transformed primary baby rat kidney (BRK) cell line (provided by K. Raska). 5A12 is a BRK cell line transformed with a recombinant Ad5 virus containing the Ad12 E1A gene [20]. A5.2 and Ad5Xho are Ad5-transformed BRK cell lines (provided by K. Raska). 3Y1 cells are established Fischer rat fibroblasts. All cells were cultured in DMEM supplemented with 10% fetal calf serum, in a humidified atmosphere containing 5% CO₂ at 37°C.

2.2. Ribonuclease protection assay

Total cellular RNA was isolated from Ad-transformed cell lines or 3Y1 cells using the TRIzol reagent (Gibco/BRL) according to the manufacturer's instructions.

To generate a suitably sized riboprobe for the TAP1 mRNA a 222 bp *XhoI*-*PstI* restriction fragment from the rat TAP1 cDNA (provided by G. Butcher) was subcloned into the vector pBluescript SK (Stratagene). For the LMP2 riboprobe a 132 bp PCR product was amplified from the LMP2 gene (described below) and subcloned into the *XbaI* site of pBluescript SK. Uniformly [α -³²P]UTP labeled antisense RNA probes were generated by transcription of linearized plasmid templates with T3 or T7 RNA polymerase.

Purification of full-length RNA probes, hybridisation to cellular RNA, and RNase digestion were performed using the RPAII kit (Ambion Biotechnology) according to the manufacturer's protocols.

*Corresponding author. Fax: (44) (113) 233 3167.

Abbreviations: Ad, adenovirus; CAT, chloramphenicol acetyltransferase; LMP, low molecular mass polypeptide; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing

Briefly, 15 µg of total RNA was coprecipitated with 1×10^5 cpm of antisense probe (TAP1 or LMP2) and resuspended in hybridisation buffer. After incubation at 45°C for 18 h, the solution was digested with RNase A (2.5 U/ml) and RNase T1 (100 U/ml) at 37°C for 30 min. Protected fragments were resolved on 6% polyacrylamide/8 M urea gels and visualised by autoradiography.

Protection assays using a 250 bp mouse β-actin antisense probe (Ambion Biotechnology) were also performed to control for RNA quality and concentration. Size markers were generated by end-labeling pUC118 *Sau*3AI restriction fragments with the Klenow fragment of *E. coli* DNA polymerase and [α -³²P]dGTP.

2.3. PCR amplification of rat TAP1 and LMP2 gene sequences

Genomic DNA was isolated from Wistar rat liver by lysis in 10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS, followed by digestion with RNase A (20 µg/ml) and proteinase K (100 µg/ml), phenol/chloroform extraction and ethanol precipitation. PCR was performed using 250 ng of genomic DNA in a volume of 100 µl containing 20 mM Tris-HCl pH 8.75, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg/ml BSA, 0.2 mM each dNTP, 0.5 µM each primer, and 2.5 U cloned *Pfu* DNA polymerase (Stratagene). All primers used were designed with *Xba*I sites at the 5' end to facilitate subcloning of PCR products (not shown).

Primer pair 5'-GGAACCATGGGAGGGATG and 5'-GTGGT-GGAACGCCGGCA were used to amplify a 132 bp product from exon 5 of the rat LMP2 gene. The PCR cycling conditions were denaturation for 5 min at 95°C followed by 30 cycles of 95°C (1 min), 58°C (30 s), and 72°C (30 s).

Amplification of the rat TAP1/LMP2 intergenic region was performed using primer pairs T1 (5'-GGCCGCAGCAGCAGCCAGT and L1: 5'-AGCCGGCGGTAGGTGCTCC) and T2 (5'-GCTGTC-CAGAGTCCGGTC and L2: 5'-CCTAGCAGTGCGGAGATC). T1 and T2, and L1 and L2 are complementary to sequences within the first exon of the TAP1 and LMP2 genes respectively (see Fig. 2A). PCR cycling conditions were denaturation for 5 min at 95°C followed by 30 cycles of 95°C (1 min), 67°C for T1:L1 or 58°C for T2:L2 (45 s), and 72°C (45 s). The resulting PCR products (681 bp for T1:L1 and 573 bp for T2:L2) were digested with *Xba*I and subcloned into pBluescript. DNA sequence was obtained for the entire intergenic region from a number of independent clones using the Pharmacia T7 polymerase sequencing kit.

2.4. Transient transfection analysis

The 573 bp PCR product containing the TAP1/LMP2 intergenic region was subcloned in both orientations into the reporter gene vector pBLCAT3. Cell lines were transfected with the resulting constructs using the Lipofectin reagent (Gibco/BRL) and the supplier's recommended conditions. Cells were transfected with a total of 6 µg DNA comprising 4 µg test plasmid along with 2 µg CMVβGal control plasmid. Cells were harvested 40 h post-transfection and β-galactosidase and CAT activities were assayed using standard methods [21]. CAT assays were quantified using a Fuji BAS 1000 phosphorimager. The amount of cell extract used in each CAT assay was standardised by β-galactosidase activity to account for variation in transfection efficiency between cell lines.

3. Results and discussion

3.1. TAP1 and LMP2 mRNA levels are coordinately down-regulated in Ad12-transformed rat cells

Expression of MHC-encoded TAP1 and LMP2 genes was studied in a series of Ad12- and Ad5-transformed rat cell lines by ribonuclease protection assay. Compared to untransformed 3Y1 cells steady-state levels of TAP1 and LMP2 transcripts were undetectable in the Ad12-transformed cell line A12.1 and 10–15-fold lower in the cell line RFC1 (Fig. 1). In contrast, Ad5-transformed cells contained 2–6-fold higher levels of TAP1 and LMP2 mRNAs compared to 3Y1 cells (Fig. 1). No significant differences in the level of β-actin mRNA could be detected between cell lines. Similar levels of TAP1 and LMP2 transcripts were observed in 3Y1 and untransformed BRK cells (results not shown). Rat cells transformed by a

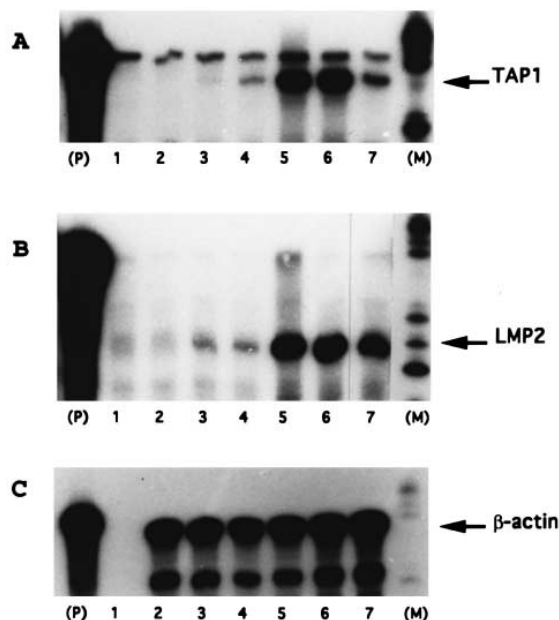


Fig. 1. Analysis of TAP1 and LMP2 mRNA levels in Ad-transformed cells. Steady-state levels of TAP1 (A), LMP2 (B) and β-actin (C) transcripts were determined by ribonuclease protection assay. RNA was analysed from Ad12-transformed cells A12.1, RFC1, and 5A12 (lanes 2–4 respectively), from Ad5-transformed cells A5.2, and Ad5Xho (lanes 5 and 6 respectively), and from untransformed 3Y1 cells (lane 7). RNA from yeast (lane 1) was used as a negative control. Lane P contains undigested probe and lane M DNA size markers.

recombinant adenovirus 5 containing the E1A region from Ad12 also contained reduced levels of TAP1 and LMP2 mRNAs compared to 3Y1- and Ad5-transformed cells, indicating that it is the serotypic origin of the E1A oncogene which determines the level of expression of TAP1 and LMP2 genes. It has previously been shown that levels of MHC class I mRNAs are greatly reduced in RFC1 and 5A12 cells compared to Ad5-transformed cells [22]. Therefore, expression of multiple genes encoding components of the MHC class I antigen presentation pathway is actively repressed in Ad12-transformed cells.

3.2. Cloning and sequencing of the rat TAP1/LMP2 intergenic region

Sequencing of human and murine genomic DNA has shown that the genes encoding TAP1 and LMP2 are separated by a small intergenic region [9,23], and recently it has been shown that the human sequence can function as a bidirectional promoter directing the divergent transcription of TAP1 and LMP2 transcripts [24]. No genomic sequence data was available for the rat and therefore, to analyse this promoter in Ad-transformed rat cells we isolated the equivalent rat sequence by PCR amplification of genomic DNA using primers complementary to regions within the 5' ends of previously characterised TAP1 and LMP2 rat cDNA sequences (Fig. 2A). A single 681 bp PCR product was obtained and subcloned (results not shown). Sequence analysis identified the rat TAP1/LMP2 intergenic region to be 564 bp in length, 29 bp shorter than the corresponding human sequence (Fig. 2A). The mouse intergenic region was originally predicted to be 1007 bp in size [23]. However, the presence of an in-frame ATG start codon 438 bp upstream from that assigned as the start of the TAP1

A

LMP2

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agccggcggttaggtgtctccggttcagcagccttagcagtcgggagatcTGGTTCCAAT 30
AGCCGGCGGTAGGTGTCTCC          CCTAGCAGTCGGGAGATC (L2)

CAGCAGCGGCGTGCGGCGAAGGCAACTCTCCGCTGCGGCCCGCCCATCGTCGCGCAAG 90

GGGCGTGCCGTTTCTACCGAGCATTTGGCGCCAGAGCTAACTTGAGCAGGGCAGATCTGC 150

CAGAGACAGGTGACGACAGAGGGTCCCGCCCTAAAGCTGGGGTGCGGCTGGGATGGGAA 210

AATTCTCTCAAGCAAGGGGAGAAGACTGGGGAAGGAGAGGAGATGAGAGTCTGGAGAA 270

GAATATGGCATGCCAGGGCTGCTAGGCAGAACAACTCCGACTTCAGCGGCAGCTTCCAGA 330

ATAGCCTGGGCCAGCCAGTCTCAGAAGGGGCGTGTCTAGTGATTGACGGCTGCTTTTCG 390

GTTTCTTCTTCTCTTAAACGCTGGCACTTCTAGTTAGCGACACCAGCTCGACGGGTCTCT 450
CGGGACTTTTCGCGCACGCCCTCGGACCCGCCTTCTTCCCTCCACCGAGACTCCGGTAT 510
NF-κB          GC-box

AGCCCGACgcgaggaggttcaggctgggaccggaactctggacagcgacgctcgatggc 570
          CTGGCTGAGACCTGTGC-5' (T2)  TAP1

tgcgacgcttgccgacggcgcccttgcgtgcgtgcgtggtggactggctgcgtgcgtgcg 630
          TGACCGACGACGACGC

gcc
CGG-5' (T1)

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B

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TCTCGGGACTTTCCGCGCACGCCCTCGGACCCGCCCCCTCT Rat
** ***** * * ***** *
TCTCGGGACTTTCCGAGA-----GCCCGCCCCCTCGT Human

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Fig. 2. Nucleotide sequence of the rat TAP1/LMP2 intergenic region. A: Nucleotide sequence for the entire rat TAP1/LMP2 intergenic region together with partial flanking TAP1 and LMP2 first exon sequences is shown. Predicted TAP1 and LMP2 start codons are underlined and the sequence is numbered from the putative LMP2 ATG. Sequence corresponding to that originally obtained from TAP1 [29] and LMP2 [30] cDNA clones is shown in lower case and the positions of primers used for PCR are marked. Consensus NF-κB and SP1 (GC-box) binding sequences are boxed. This sequence has been deposited in the EMBL data base under the accession number X97611. B: Alignment of rat and human [24] TAP1/LMP2 intergenic sequences containing consensus NF-κB and SP1 binding sequences (underlined).

coding region, suggests that the mouse intergenic region is actually 569 bp in length, just 5 bp longer than the rat sequence. Although there is strong conservation between rat and mouse intergenic regions (approximately 86% identity), overall there is only moderate homology between rodent and human sequences (data not shown). However, functionally important binding sites for the transcription factors NF-κB and SP1 have been identified within the human promoter [24], and these sites are completely conserved in the rat although there is a 9 bp insertion between the two sites in the rat relative to the human (Fig. 2B).

3.3. Expression of the rat TAP1/LMP2 intergenic region in Ad-transformed cells

The rat TAP1/LMP2 intergenic region was subcloned in both orientations into pBLCAT3 to generate the reporter constructs pTAP1cat and pLMP2cat (Fig. 3A). Transfection of Ad5Xho and A5.2 cells with each construct generated significant levels of CAT activity compared with the promoterless control indicating that the 564 bp intergenic region functions as a bidirectional promoter and is sufficient to mediate transcription of both TAP1 and LMP2 genes in Ad5-trans-

formed rat cells (Fig. 3B and results not shown). Levels of expression from the TAP1 end of the promoter were higher than those from the LMP2 end in both Ad5Xho and A5.2 cells. Similar results were obtained analysing the activity of the human TAP1/LMP2 intergenic region in HeLa cells [24]. Transfection of pTAP1cat and pLMP2cat into RFC1 and A12.1 cells resulted in 5–8-fold lower levels of CAT activity compared to Ad5Xho cells indicating that activity of the TAP1/LMP2 bidirectional promoter is significantly reduced in Ad12-transformed cells (Fig. 3B). Similar levels of repression have been observed for a MHC class I heavy chain gene promoter (H-2K^b) transfected into Ad12- and Ad5-transformed cells [25–27], and it has been shown that down-regulation of class I mRNA levels in Ad12-transformed cells occurs predominantly at the level of transcriptional initiation [22]. These results imply that reduction in TAP1/LMP2 mRNA levels also occurs primarily at the level of transcription.

3.4. Conclusions

It has been shown that compared to Ad5-transformed cell lines, Ad12-transformed cells contain extremely low levels of NF-κB capable of binding to DNA, possibly due either to

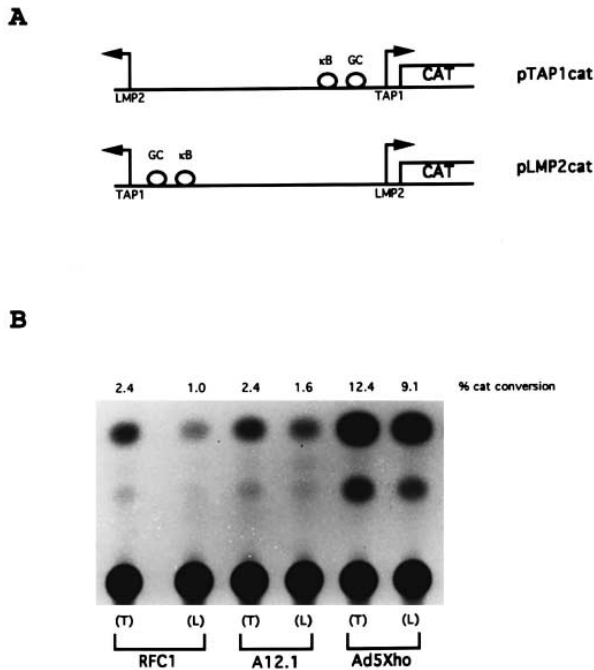


Fig. 3. Activity of the TAP1/LMP2 bidirectional promoter in Ad-transformed cells. A: Schematic diagram of reporter gene constructs obtained by cloning the TAP1/LMP2 intergenic region into pBLCAT3 in both orientations. B: Constructs pTAP1cat (T) and pLMP2cat (L) were each co-transfected with the control plasmid CMVβGal into Ad12- and Ad5-transformed cell lines. Resulting CAT activity (normalised for β-galactosidase activity) is presented as percentage conversion of [¹⁴C]chloramphenicol to its acetylated derivatives. Transfections were performed at least three times with more than one batch of each plasmid and representative results are shown.

induction of a nuclear inhibitor of NF-κB function or inhibition of processing of the p105-NF-κB precursor by the Ad12 E1A protein [25,26,28]. The difference in MHC class I mRNA levels between Ad12- and Ad5-transformed cells has been attributed to the resulting differential binding of NF-κB to an enhancer element within the MHC class I promoter. As described above, a consensus binding site for NF-κB is also present within the rat TAP1/LMP2 promoter, and this sequence is fully conserved between rat and human (see Fig. 2B). Mutational analysis of the human TAP1/LMP2 promoter has demonstrated that this site is important for expression [24], and initial protein binding studies show that the rat sequence is capable of binding NF-κB family members in vitro (results not shown). We suggest that by targeting NF-κB, Ad12 can repress multiple components of the MHC class I peptide presenting pathway at the level of transcription, thus resulting in greatly reduced expression of class I molecules at the cell surface and escape from immune surveillance.

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