Coordinate Regulation of the Human TAP1 and LMP2 Genes from a Shared Bidirectional Promoter

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Summary

Recently, four genes (TAP1, TAP2, LMP2, LMP7) involved or potentially involved in the processing and transport of major histocompatibility complex class I–associated antigen to the endoplasmic reticulum have been identified. We now report the initial characterization of the bidirectional promoter for the human transporter associated with antigen processing 1 (TAP1) and low molecular mass polypeptide 2 (LMP2) genes. These genes are divergently transcribed from a central promoter region of only 593 bp. Functional analysis using a bidirectional reporter system demonstrates the minimal 593-bp promoter is sufficient for concurrent expression in both directions. There is no TATA box homology at either end but there is a prevalence of GC boxes. Transcription is initiated at multiple sites for each gene without any of the TAP1 transcripts overlapping with the LMP2 transcripts. The region proximal to the TAP1 gene is required for maximal basal level expression of not only TAP1 but also LMP2. Furthermore, this region is necessary for tumor necrosis factor α (TNF-α) induction of both genes. Site-specific mutations of an NF-κB element in the TAP1 proximal region blocked induction by TNF-α in both the TAP1 and LMP2 directions. An adjacent GC box was required for basal expression of both genes as well as augmenting the TNF-α induction of the distal LMP2 gene. In vivo genomic footprinting of this region revealed strong protein/DNA interactions at the NF-κB and GC box consensus sequences. In vitro binding studies confirmed the capacity of the NF-κB site to bind p50/p65 and p52/p65 heterodimers and of the GC box to bind Sp1. Thus, the promoter elements proximal to the TAP1 gene play a significant role in regulating basal and induced expression of both TAP1 and LMP2. The findings presented in this report clearly link LMP2 expression with TAP1 expression and provide additional suggestive evidence linking LMP2 to class I antigen presentation.

Presentation of antigenic peptide in the context of MHC class I molecules is required for the intrathymic selection and development of CD8+ T cells (1, 2) and for cytotoxic T cell–mediated killing of infected or transformed cells (3–5). The regulation of class I heavy chain expression on the cell surface occurs at multiple levels, including the retention of empty class I molecules in the endoplasmic reticulum (ER)[1] until association with both β_2-microglobulin (β_2M) and antigenic peptide (6). This stable trimolecular complex is then competent for transport through the Golgi apparatus and to the cell surface. The class I bound antigenic peptides are derived from degraded endogenous proteins in the cytoplasm (7), a process that has been suggested to involve the 20S proteasome complex (8, 9). The proteasome is a multicatalytic, multisubunit complex previously demonstrated to be part of the ubiquitin-dependent protein degradation pathway and is found in all cells studied (10, 11). Two recently identified genes, low molecular mass polypeptide 2 (LMP2) and LMP7, have high homology to the proteasome and are located in the MHC class II locus (9, 12, 13). This region of the class II locus was initially defined as important for class I cell surface expression (14, 15). While early studies indicated LMP2 and LMP7 are not essential for antigen presentation by class I (16, 17), more recent results suggest that

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The first two authors contributed equally to this work and should both be considered first authors.

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[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; CAT, chloramphenicol acetyltransferase; ER, endoplasmic reticulum; HGH, human growth hormone; ISRE, IFN response element; LMP, low molecular mass polypeptide; TAP, transporter associated with antigen processing.

these proteins quantitatively enhance the specificity of the proteasome complex by increasing cleavage after hydrophobic and basic residues (18–20). This specificity is consistent with the characteristics of peptides that are preferentially bound by class I molecules (21, 22). Therefore, the LMP molecules may increase the availability of specific groups of peptides for class I binding.

Transporter associated with antigen presentation (TAP) 1 and TAP2 are closely linked to the LMP genes in the MHC locus, and share homology with the ATP binding cassette family of transporters (14, 23–26). TAP1 and TAP2 form a heterodimer (27, 28) that resides in the ER and cis-Golgi membranes (29) and transports antigenic peptides into the ER lumen for binding by the class I heavy chain (30, 31). The TAP heterodimer is critical for class I function since TAP1- or TAP2-mutant cell lines lack class I on the cell surface (14, 24, 32–35). Reconstitution of the TAP heterodimer rescues class I surface expression in these cells. Furthermore, transgenic mice deficient in the TAP1 gene also lack class I expression on cells and have a severe deficiency in the development of cytotoxic T cells (36). The TAP1/TAP2 complex functions as an ATP-dependent peptide transporter and displays some selectivity toward peptides characteristically eluted from class I molecules (37). Peptides with a minimum length of nine residues and with a hydrophobic COOH-terminal amino acid are transported most efficiently (38). A recent study has demonstrated that the empty class I/β_2M heterodimers physically associate with TAP in the ER (39) and then disassociate from TAP upon peptide binding to class I. This suggests that TAP may have an additional role in directly loading peptide onto class I and/or in retaining empty class I/β_2M in the ER. Significantly, several studies have shown that some tumors and virally infected cells have downregulated TAP expression, potentially as a mechanism to lower class I expression and escape surveillance by the immune system (40–42). Thus, regulation of the TAP genes is critical to the development and integrity of the immune system, while the importance of the LMP genes is less clearly defined.

Analysis of the genomic sequence encompassing the human LMP2 and TAP1 genes revealed that these genes would be transcribed in a divergent orientation with only 593 bp separating the ATG translation initiation codons of the two genes (43). The close proximity of the two genes in reverse orientation suggested that they might share a common promoter region. In this report, we describe the coordinate regulation of the human TAP1 and LMP2 genes. The common 593-bp intergenic region is sufficient to promote transcription of both TAP1 and LMP2. In vivo genomic footprinting and site-directed mutagenesis define a shared Sp1-GC box proximal to the TAP1 gene, which promotes basal level activity of both genes. In addition, a shared nuclear factor (NF)–κB element coordinately induces both TAP1 and LMP2 upon exposure to TNF-α, a cytokine involved in the upregulation of many immune system–related genes. The tight coordinate regulation through these elements suggests that the functions of TAP1 and LMP2 are linked and strengthens the ar-

gument that the LMP2 gene product also plays an important role in the antigen processing pathway.

Materials and Methods

Cell Lines. HeLa is a human cervical carcinoma cell line (CCL2; American Type Culture Collection, Rockville, MD) grown in Eagle's MEM with BSS (Gibco Laboratories, Grand Island, New York) supplemented with 10% FCS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. The cells were plated at 10^6 cells per 150 × 25-mm dish every other day and maintained at 37°C with 5% CO_2. Raji and Namalwa are human EBV-positive Burkitt's lymphoma cell lines, and H9, CEM, and Jurkat are human T lymphoblastoid cell lines. All lymphoid cell lines were maintained in RPMI 1640 supplemented with 8% FCS and 2 mM L-glutamine. U937 is a promyelomonocytic cell line.

Constructs. The promoterless bidirectional reporter construct, HGHCAT, was constructed by cloning the human growth hormone (HGH) gene, obtained by digestion of pOGH (Nichols Institute, San Juan Capistrano, CA) with BamHI and EcoRI, into the NotI site of pBSCATSK+ (44). Orientation of the insert was confirmed by use of a diagnostic digest. The L-HGH/T-CAT and the reversed T-HGH/L-CAT constructs were generated by cloning the 593-bp LMP2/TAP1 intergenic region into the XbaI site of the HGHCAT vector (see Fig. 2 A). The 593-bp insert was obtained by PCR amplification of a cosmid clone (R4) containing the entire genomic region encompassing LMP2 and TAP1, by use of two flanking 26-bp primers: PCR1 5′GCTCTAGACCCTGCAAGGCACCGCTC3′ and PCR2 5′GCTCTAGATGGCACTCGGACGCCGTC3′. Integrity and orientation of the insert was confirmed by sequencing of both strands. The constructs, T1mtGC and T1mtκB, were constructed by performing site-directed mutagenesis (45) of L-HGH/T-CAT with the following oligonucleotides: T1mtκB, 5′CAGCCTGTTCCTcG tACTagCCGAGAGCCCCGC 3′; T1mtGC, 5′CGAGAGCCCCaaaCTCGTTCCCTCC 3′. Deletion constructs are schematically displayed in Figs. 1 B and 3. Construct names reflect the portion of intergenic promoter region retained in the construct and are numbered relative to the TAP1 ATG codon. The constructs p461-1TAP and p208-1TAP were generated by digestion of L-HGH/T-CAT with unique enzymes that cleave within the promoter region (BssHII or NotI, respectively) and a unique SmaI site within the HGH coding sequence. The resulting recircularized deletion constructs have inactivated HGH reporter genes and thus can only be assayed for TAP-CAT expression. The constructs p593-457LMP and p593-207LMP were generated by digestion of L-HGH/T-CAT with unique enzymes that cleave within the promoter region (BssHII or NotI, respectively) and a unique XhoI site within the CAT coding sequence. The resulting recircularized deletion constructs have inactivated CAT reporter genes and thus can only be assayed for LMP-HGH expression. The junctions for all deletion mutants were confirmed by sequencing.

Transfection. All transfections were performed with 15 μg of total DNA by the standard CaPO$_4$ protocol (46). To minimize sample variability, duplicate DNA precipitates were mixed and then half was added to each plate, one for the uninduced and one for the TNF-α–induced cells. 10 ng/ml of TNF-α (kindly provided by Genentech, South San Francisco, CA) were added to the cells

when changing the media 4 h after the addition of the DNA precipitates. Media aliquots and cells were harvested simultaneously 24 h later. Cell extracts were prepared and protein content determined by the Bradford assay (47). Cell extracts (30 μg) were assayed for chloramphenicol acetyltransferase (CAT) as previously described (48) and quantitated by phospho-imaging (Molecular Dynamics, Sunnyvale, CA). Media aliquots for the HGH assay were analyzed by radioimmunoassay (Nichols Institute). HGH results were normalized for protein concentration to account for differences in cell number.

Primer Extension and RNAse Protection. Poly A⁺ RNA was isolated by use of a modified oligo-dT method (49). HeLa total RNA was isolated by use of RNA STAT-60 (Tel-Test, Friendswood, TX). Primer extension analysis of the TAP1 mRNA was performed essentially as previously described (50) with a distinct 30-nucleotide primer for the endogenous TAP1 mRNA, 5'GCGAGAAGTAGC-AGTACTGTCCCCAGCCAT3', and a 30-nucleotide primer for the transfected TAP1/HGH mRNA, 5'TAGCTAGATGGCACTCGG-ACGCCGTCCCGG3'. Absolute product lengths were obtained by comparison to a sequencing ladder. Position within the promoter was assigned by use of a value obtained by subtracting the size of the coding region 5' to the primer, and the primer size, from the absolute product length. RNase protection was performed essentially as previously described (50). The template for the synthesis of RNase protection probes was constructed by inserting the 593-bp TAP1/LMP2 promoter fragment obtained by PCR amplification into the pCR cloning vector (Invitrogen, San Diego, CA). The orientation was confirmed by sequencing. The TAP1 probe was obtained by in vitro transcription (Promega Corp., Madison, WI) directed by the SP6 promoter from the XhoI linearized template. The LMP2 probe was prepared by in vitro transcription directed by the T7 promoter from the BamHI linearized template.

In vivo Footprinting. In vivo methylation of cells and DNA preparation were performed as described in Pfeifer et al. (51). The ligation-mediated, PCR-amplified in vivo genomic footprinting was performed as originally described by Mueller and Wold (52) and modified by Wright and Ting (53). Three sets of upper strand primers and one set of lower strand primers were used to display the TAP1 proximal promoter. The sequences of the upper strand primers are as follows: Set 1: R412Ap1, 5'AGTCGGCGAGAA-GTAGC3'; R412Ap2, 5'TGTCCCCAGCCATGCGAGAGAAGC3'; R412Ap3, 5'TCCCCAGCCATGCGAGAGAAGCTCCG3'; Set 2: R412Bp1, 5'CATTGGCACTCGGACGC3'; R412Bp2, 5'TGA-AGCTCCGGGTACCGCCGAGTC3'; R412Bp3, 5'TCCGGG-TACCGCCGAGTCCTCCCCTAC3'; Set 3: R412Ep1, 5'CAG-CCATGCGAGAGAAGC3'; R412Ep2, 5'CTAGAGCTAGCCATT-GGCACTCGGAC3'; R412Ep3, 5'AGCTAGCCATTGGCACTC-GGACGCCG3'. The sequences of the lower strand primers are as follows: Set 3: R124Dp1, 5'TGATTTCCACGCTTGCTAC3'; R124Dp2, 5'ATAGTCTGGGCAGGCCACTTTTGGAAG3'; R124Dp3, 5'TCTGGGCAGGCCACTTTTGGAAGTAGGCG3'.

Nuclear Extract Preparation and Gel Shift Analysis. Nuclear extracts were prepared according to the method of Dignam et al. (54) from HeLa cells grown in suspension to a density of 4 × 10⁵ cells/ml. TNF-α–induced extracts were prepared from HeLa cells treated with 10 ng/ml TNF-α for 15–25 min before harvest. Gel shift analysis was performed as previously described (55). The oligonucleotides used as probes or competitors are as follows: T1wt, 5'GCCTGTTCCTGGGACTTTCCGAGAGCCCCGCCCTCG-TTCCCTCC3'; T1mtκB, 5'GCCTGTTCCTcGtACTagCCGAGA-GCCCCGCCCTCGTTCCCTCC3'; T1mtGC, 5'GCCTGTTCC-TGGGACTTTCCGAGAGCCCCaaaCTCGTTCCCTCC3'; HIV-

κB, 5'CTAGACTACAAGGGACTTTCCGCTGGTCTAG3'; Sp1 con, 5'GTCACCGGGGCGGGGCGGTCAC3'. Antibody supershift experiments were performed by preincubating 1 μg of antibody for 2 h on ice with each binding reaction before the addition of the DNA probe. Antibodies were both a kind gift from Dr. A. Baldwin (University of North Carolina-Chapel Hill, Chapel Hill, NC) and purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

LMP2 and TAP1 Both Initiate Transcription from Multiple Sites within a Common Promoter Region. Primer extension and RNase protection analysis of messenger RNA obtained from several cell lines detected multiple transcription start sites for both genes (Fig. 1 *A* and summarized in Fig. 1 *B*). The TAP1 gene initiated from at least 18 different positions within the first 120 bp upstream of the TAP1 ATG codon. The presence of multiple start sites is consistent with results for other TATA-less, GC-rich promoters (56). The 10 most consistent start sites found by primer extension of Raji cell mRNA are indicated in Fig. 1 and were confirmed in two independent experiments. An additional prominent start site occurs at ∼ −427 bp. Examination of mRNA from CEM cells confirmed the same pattern of proximal initiation start sites in two additional independent primer extension analyses. Only the bands that were consistent in all experiments are marked. These results were also confirmed by RNase protection analysis of mRNA from U937, Jurkat, and HeLa cells (data not shown). The LMP2 gene uses three major start sites clustered at positions −37, −38, and −43 bp relative to the LMP2 ATG codon in U937 cells. At least 10 other weak LMP2 start sites can be detected within the first 100 bp. These results were confirmed in five independent experiments and in two additional cell lines, HeLa and Jurkat. None of the LMP2 transcripts overlap any of the TAP1 transcripts, and all of the initiation sites for both genes lie within the region between the two ATG codons. Therefore, this region could serve as a shared bidirectional promoter.

The Common Promoter Region Is Sufficient to Direct Transcription in Both Directions. The functionality of the region between the two ATG codons was assayed by first cloning this region into a bidirectional reporter gene construct. The 593-bp sequence was amplified by PCR and inserted in either direction between two divergently oriented reporter genes, HGH and CAT (Fig. 2 *A*). The resulting construct when transfected into cells allows the simultaneous measurement of transcriptional activity in both directions. HGH is secreted into the medium and is quantitated by radioimmunoassay, while the CAT enzyme is extracted from the harvested cells and is quantitated by standard thin-layer chromatography CAT enzyme assays. Transfection of HeLa cells with the L-HGH/T-CAT construct results in significant transcription in both the LMP2 direction (HGH) and the TAP1 direction (CAT) compared with the promoterless control (Fig. 2 *B*). Transcription from the reverse construct, T-HGH/L-CAT, also produced significant reporter gene expression in both directions.

A

TAP 1

~ -427 →

-98 →
-93
-90 -86 -83 →
-74 →
-62 -65 →
-59 -53

1 2 G A T C
Raji / control

LMP2

-43 →
-38
-37

3 4 G A T C
U937 / control

B

←
LMP2

CATCCCTGCAAGGCACCGCTCTCCTCGCCGCCTGGGGCACTGGTTTCCAACCTGGGACAG 537
-38 -43
-37

CGCACAACGCGCAGCCGACAGCCCCGCCCCTTCGCGGCGCCGCCAGGAGGCGCCTGGGTG 477

p461-1TAP 427
CTGCGGGGCTGCTTTGCGCGCGGCGCTAACGTGTGTAGGGCAGATCTGCCCCGAGACAAG 417
p593-457LMP

TGACGAGGCAGCCCCGCCCTGAGGCTGGGGTGGGAAAACTGGTGCAAGTGGAAAGGCAGG 357

AGGCAGGGAGAGGCGAGAAGGGTGTGCGTGATGGAGAAAATTGGGCACCAGGGCTGCTCC 297

CGAGATTCTCAGATCTGATTTCCACGCTTGCTACCAAAATAGTCTGGGCAGGCCACTTTT 237

p208-1TAP
GGAAGTAGGCGTTATCTAGTGAGCAGGCGGCCGCTTTCGATTTCGCTTTCCCCTAAATGG 177
p593-207LMP

CTGAGCTTCTCGCCAGCGCAGGATCAGCCTGTTCCTGGGACTTTCCGAGAGCCCCGCCCT 117
NF-κB GC-box

CGTTCCCTCCCCCAGCCGCCAGTAGGGGAGGACTCGGCGGTACCCGGAGCTTCAGGCCCC 57
-93 -90 -86

ACCGGGGCGCGGAGAGTCCCAGGCCCGGCCGGGACCGGGACGGCGTCCGAGTGCCAATG
TAP1 →

Figure 1. (A) TAP1 and LMP2 genes use multiple transcription start sites. (*Left*) TAP1 start sites detected by primer extension analysis. Lane *1*, Raji cell poly A+ mRNA (10 μg) primer extended from a TAP1-specific primer. Lane *2*, control primer extension of the TAP1 primer with 20 μg of tRNA carrier. Prominent bands consistent in all experiments are indicated by arrows, and the position is relative to the TAP1 ATG codon. An irrelevant sequencing ladder (lanes G, A, T, and C) was used to accurately size the protected bands. (*Right*) LMP2 start sites detected by RNase protection. Lane *3*, U937 cell poly A+ mRNA (8 μg) protected by an LMP2-specific probe. Prominent bands are indicated by arrows and position relative to the LMP2 ATG codon. Lane *4*, control RNase digestion of the LMP2 probe with 20 μg of tRNA carrier. A sequence of the LMP2 promoter (lanes G, A, T, and C) was used to size the LMP2-protected bands. The probe extended from the TAP1 ATG to the LMP2 ATG with a short segment of vector sequence flanking both ends. (B) Sequence of the entire 593-bp intergenic region (43). The ATG translation start codons are indicated by a double underline and the gene name. The most prominent transcription start sites determined in A are marked by solid bent arrows for LMP2 and open bent arrows for TAP1. The end points of the deletion constructs used in Figs. 3 and 5 are marked by a solid bracket and the name of the construct. Shaded boxes mark two functional transcription factor binding sites defined in this report. Open box indicates a potential initiator element (69). Dotted underline marks two additional GC boxes, and the solid underline marks the ISRE homology. Numbers along the right side of the figure indicate nucleotide numbering from the TAP1 ATG and are used throughout this report except when referring to the LMP2 start sites, which are relative to the LMP2 ATG.

The relative levels of TAP1 and LMP2 expression can be determined by comparing CAT expression driven by TAP1 (L-HGH/T-CAT) with that driven by LMP2 (T-HGH/L-CAT) or, analogously, HGH expression driven by either end of the promoter. In both comparisons, TAP1 expression was approximately threefold higher than LMP2 in HeLa cells. Importantly, this analysis also demonstrates that the vector and reporter coding sequences do not influence transcription unequally in one direction. Thus, the TAP1 and LMP2 genes share a common 593-bp promoter region, which is sufficient to promote bidirectional transcription of both genes.

Confirmation that the transfected constructs respond in a similar manner as the endogenous gene was addressed by determining the start sites for the transfected TAP1 gene (Fig. 2 C). Primer extension analysis of RNA isolated from HeLa cells transfected with the T-HGH/L-CAT construct with a primer complementary to the HGH mRNA sequence detected multiple initiation sites. Although the experiment is arduous, especially in TATA-less promoters, the initiation sites correspond well with the endogenous TAP1 initiation sites. The relative intensity of the bands does vary slightly; however, no cryptic start sites are detected. This indicates that

the bidirectional reporter construct is faithfully reproducing TAP1 transcription initiation. Primer extension of the LMP2 transcripts was not successful due to the extremely high G/C content of this region. However, expression of TAP1 and LMP2 was not affected by reversing the promoter (Fig. 2 B). This provides a strong indication that the reporter system is not affecting the results and supports further analysis of the promoter.

Deletions from Either End Diminish Transcription. The initial approach to functionally defining the bidirectional promoter was to construct large deletions from either end and assay expression in HeLa cells (Fig. 3). For ease of discussion, all deletion points are presented relative to the TAP1 ATG translation start codon. Expression of LMP2 from deletion construct p593-207LMP is reduced to only 38% of wild type when ~200 bp are deleted from the TAP1 end. The same 200 bp proximal to the TAP1 gene are also sufficient to promote transcription in the TAP1 direction (p208-1TAP). This indicates that the 207 bp most proximal to the TAP1 gene have a critical role in activating distal LMP2 transcription as well as the proximal TAP1 gene. TAP1 expression is not affected by the inclusion of sequences up to position 461 (p461-

A
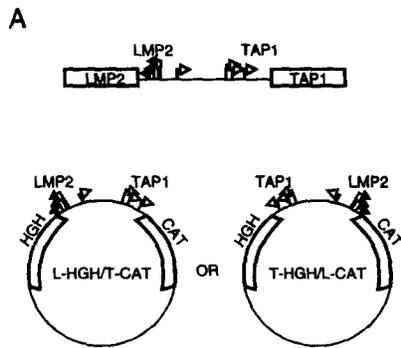
B

Construct	CAT		HGH	
L-HGH/T-CAT	22.3%	(TAP1)	1858	(LMP2)
T-HGH/L-CAT	7.6%	(LMP2)	7096	(TAP1)
HGHCAT	1.2%		0	
TAP/LMP-CAT	2.5 ± .52		—	
TAP/LMP-HGH	—		3.3 ± .66	

Figure 2. The LMP2 and TAP1 intergenic region is sufficient to promote divergent transcription of both genes. (A) Construction of the bidirectional reporter plasmids. The 593-bp intergenic region was amplified by PCR and cloned into the promoterless bidirectional reporter (HGHCAT) in both directions. The prominent transcription start sites as defined in Fig. 1 are indicated by solid bent arrows for LMP2 and open bent arrows for TAP1. (B) Comparison of expression obtained with CAT and HGH as reporters. CAT results are presented as percent acetylation and the HGH results are presented as cpm. Results for L-HGH/T-CAT and T-HGH/L-CAT are of a representative experiment out of a total of three experiments. The ratios of TAP to LMP expression are the averages of two experiments. Regardless of the promoter orientation, TAP1 expression was approximately threefold higher than LMP2. (C) Transcription start sites from the TAP1 promoter of the transfected T-HGH/L-CAT construct closely parallel endogenous start sites. HeLa cells were transfected as described in Materials and Methods, and total RNA was isolated. Start sites were identified by primer extension with an HGH-specific primer. Lane 1, control tRNA (20 μg); lane 2, transfected HeLa cell RNA (10 μg); lanes G, A, T, and C are an irrelevant sequencing ladder for sizing.

C
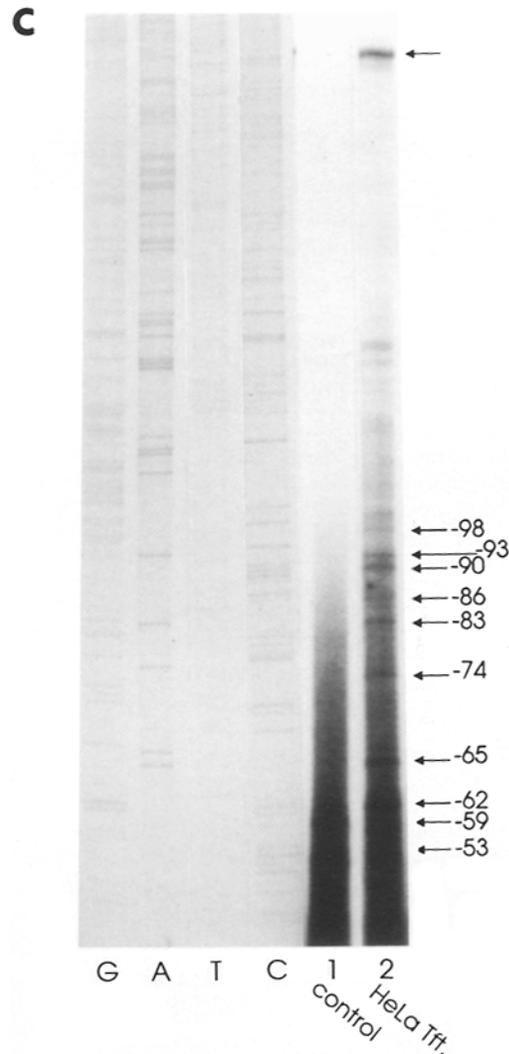
1TAP), indicating that this central region (461–208 bp) is not necessary for basal activity of TAP1 transcription. This region also does not dramatically affect basal LMP2 expression, as demonstrated by the similar levels of activity from constructs p593-207LMP and p593-457LMP. The central region, however, may be important for induction of LMP2 and/or TAP1 transcription, since significant homologies with MHC class I IFN-α/β response elements are located in this region. The smallest LMP2 construct (p593-457LMP) contains all of the LMP2 start sites and is sufficient for a low level of LMP2 transcription. However, the LMP2 proximal region does not have a major affect on distal TAP1 expression (p461-1TAP). These findings reveal that both genes use promoter elements located within the TAP1 proximal region. In addition, the LMP2 proximal region is also essential for LMP2 transcription.

TAP1-proximal NF-κB and GC Sites Coordinately Regulate

Basal and TNF-α-induced Expression of LMP2 and TAP1. The deletion studies suggested that the TAP1-proximal region could contain shared promoter elements involved in coordinate regulation of TAP1 and LMP2 expression. DNA sequence homology searches of this region with a transcription factor data base indicated the presence of a consensus NF-κB site, an adjacent GC box, and an IFN response element (ISRE). These sites were obvious candidates for coordinate basal and/or induced regulation of TAP1 and LMP2. We initially chose to examine basal level regulation by targeting the NF-κB and GC elements by site directed mutagenesis (Fig. 4). Mutation of the GC box (T1mtGC) significantly reduced transcription in both directions. The GC box was necessary for an approximately threefold stimulation of TAP1 expression as well as a greater than twofold stimulation of the distal LMP2 expression. These findings demonstrate an important role for the TAP1-proximal GC box in coordinately activating both
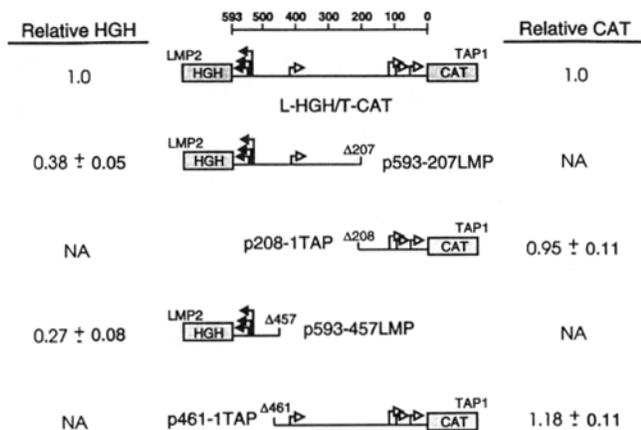
Figure 3. Deletions from either end diminish transcription. Deletion constructs were transfected into adherent HeLa cells as described in Materials and Methods. Results shown for HGH and CAT expression are the average of four and eight independent experiments, respectively, with the SEM, and have been normalized to the wild-type construct, L-HGH/T-CAT. HGH results represent LMP2 transcription and CAT results represent TAP1 transcription. The deletion end points are all presented relative to the TAP1 ATG translation start codon, and a relative scale is shown at the top of the figure. The prominent transcription start sites as defined in Fig. 1 are indicated by solid bent arrows for LMP2 and open bent arrows for TAP1. *NA*, not assayed because that reporter was deleted in the construct.

genes. Mutation of the NF-κB site (T1mtκB) did not alter expression of either gene in the uninduced HeLa cells. This result is consistent with the absence of active NF-κB in the nucleus of uninduced HeLa cells (57).

TNF-α is a potent inducer of TAP1 expression (58) and HLA class I genes (59). However, as yet no published studies of LMP2 regulation by TNF-α are available. One mechanism by which TNF-α exerts its effect on many genes is
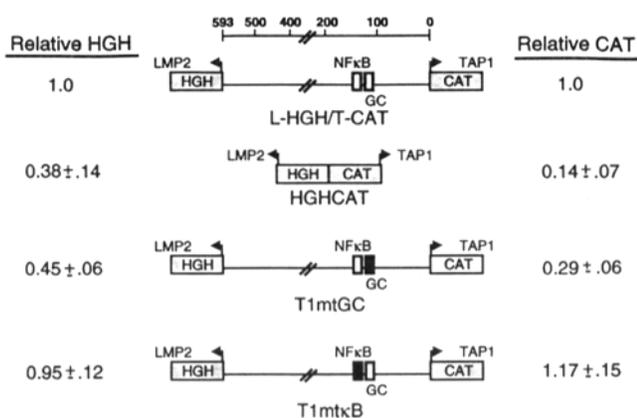
Figure 4. The TAP proximal GC box is required for both TAP1 and LMP2 basal expression. Relative positions of the NF-κB site and the adjacent GC box are indicated by open boxes. Mutated elements are indicated by solid boxes. The constructs were assayed in adherent HeLa cells as described in Materials and Methods. Results shown are the averages of six independent experiments with the SEM and have been normalized to the wild-type construct, L-HGH/T-CAT.

through the activation of the NF-κB transcription factors (60). We examined the TAP1 and LMP2 deletion and point mutation constructs to determine if, upon activation with TNF-α, the putative NF-κB site functions to promote TAP1 and LMP2 transcription. The wild type promoter construct (L-HGH/T-CAT) is inducible ~2.5-fold in both the TAP1 and LMP2 directions upon TNF-α treatment (Fig. 5). All LMP2 inducibility is lost upon deletion of the TAP1-proximal region (p593-207LMP), suggesting that elements proximal to the TAP1 gene are required for LMP2 induction. Concordantly, the same TAP1-proximal region is sufficient to promote TNF-α induction of TAP1 (p208-1TAP). Mutation of the NF-κB site located in this region (T1mtκB) abrogates inducibility of both genes. Interestingly, the adjacent GC box (T1mtGC) is also required for the maximal induction of the LMP2 gene but not the proximal TAP1 gene. The effect only on the distal gene suggests that the GC box may function to amplify the adjacent NF-κB element over larger distances. These findings define a central role of NF-κB in mediating TNF-α induction of TAP1 and demonstrates that LMP2 is also regulated by this cytokine. Most importantly, it describes conditions of coordinate and inducible regulation of TAP1 and LMP2 mediated by identical elements.

In Vivo Genomic Footprinting Reveals Bound NF-κB and GC Boxes at the TAP1-proximal End. Studies of the bidirectional reporter constructs clearly defined a functional importance for the NF-κB site and GC box. To directly address the im-
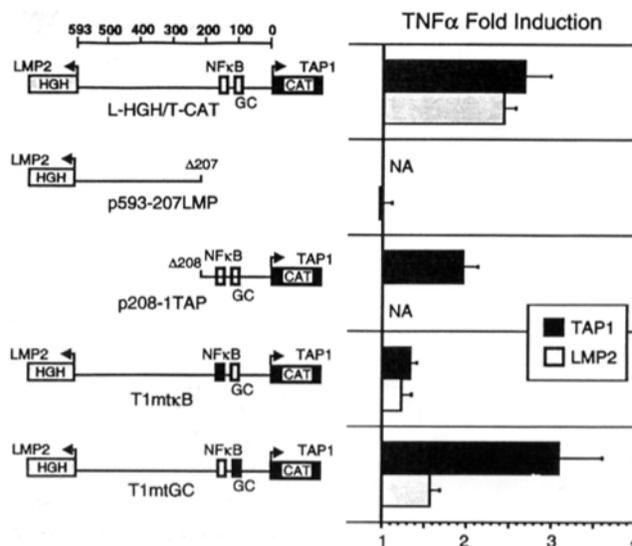
Figure 5. The NF-κB site is required for TNF-α induction of TAP1 and LMP2 expression, and, adjacent to the GC box, is also required for LMP2 induction. Black bars indicate TAP1 induction. Shaded bars indicate LMP2 induction. TNF-α was added for 24 h to 10 ng/ml. Fold induction for each construct was determined by dividing the uninduced CAT or HGH result by the induced CAT or HGH result, respectively. *NA*, not assayed because the reporter gene was deleted in that construct. The result for the deletion mutants, p593-207LMP and p208-1TAP, is the average of four and eight independent experiments, respectively. The result for the wild-type and site-specific mutants is the average of six independent experiments. Error bars represent the SEM.

portance of these sites in transcription of the endogenous genes in an intact cell, we examined the in vivo promoter occupancy by in vivo genomic footprinting. In vivo genomic footprinting reveals the sequences bound by proteins when the genes are in their normal in vivo state. This approach therefore delineates the in vivo utilized elements and provides information on protein/DNA interactions that are physiologically relevant. Examination of the TAP1-proximal 200 bp with four separate primer sets revealed no in vivo contacts within the first 98 bp upstream of the TAP1 initiation codon. However, a large cluster of DNA/protein interactions were revealed between −98 and −146 bp on both the lower strand (Fig. 6) and the upper strand (data not shown, summarized in Fig. 6). The cluster of contact points was found in both B lymphoblastoid cell lines (Raji) and T lymphoblastoid cell lines (H9). However, the contacts differ slightly between cell lines, with the most abundant contacts in the H9 cell line. This region contains the NF-κB site and the adjacent GC box as well as an additional protected GC box (labeled GC₂ box). The most consistently bound site in all cells is the GC box, which was functionally defined above to be important for the expression of both TAP1 and LMP2. Significantly, the putative NF-κB site is strongly protected in the H9 cell line (Fig. 6), but not at all in the Jurkat cell line (data not shown).

This corresponds well to the constitutively high levels of NF-κB–related subunits found in H9 cells and their virtual absence in uninduced Jurkat cells (61). The different pattern of NF-κB contacts seen in the B cell line may be indicative of different NF-κB subunits present in these cells. Occupancy of the GC₂ box varies between cell lines examined. The site is strongly protected in H9 cells, not at all in Raji cells (Fig. 6), and weakly in HeLa cells (Fig. 7). Binding at this site may have importance in modulating the expression levels in different cell types.

Induction by TNF-α Alters In Vivo Binding at the NF-κB Site. NF-κB is regulated by controlling the ability of the active subunits to be transported to the nucleus (62); thus induction by TNF-α would be predicted to alter in vivo binding at the TAP1 proximal NF-κB element. We directly examined in vivo occupancy at this site in HeLa cells during TNF-α induction (Fig. 7). Before the addition of TNF-α, HeLa cells show strong contacts on both strands of the TAP1-proximal GC box and weak contacts at the GC₂ box. In addition, there are two enhancements on the lower strand of the NF-κB element (lane *1* vs. lane *2* and lane *4* vs. lane *5*). Treatment with TNF-α for 15–20 min resulted in a shift of binding at the NF-κB element to two partial protections on the lower strand and two additional partial protections on the upper strand (lane *2* vs. lane *3* and *5* vs. lane *6*). Binding at the GC boxes remained unchanged. The weak NF-κB protection in vivo is similar to that described previously in the

Figure 6. In vivo footprint of the TAP1-proximal promoter reveals protein/DNA contacts at an NF-κB site and two GC boxes. The lower strand in vivo genomic sequence is displayed for the TAP1-proximal region by use of primer Set 3 (see Materials and Methods). The upper strand contacts were revealed with primer Sets 1 and 2 (data not shown). Raji is a B lymphoblastoid cell line. H9 is a T cell line with constitutively high NF-κB. *cont.,* in vitro methylated deproteinized DNA; *DMS,* in vivo methylated DNA. Open arrows indicate protections; solid arrows indicate enhancement. Weak protections or enhancements are indicated by open or closed arrowheads, respectively. Brackets on the left side of each sequence delineate binding site homologies as described in the text and the sequence summary. Sequence summary at bottom is marked with the H9 contact points. Shaded boxes represent transcription factor binding site homologies as indicated in Fig. 1 *B.*

Figure 7. In vivo binding at the NF-κB element is altered upon TNF-α induction of TAP1 and LMP2 transcription. Lower strand (lanes *1–3*) and upper strand (lanes *4–6*) in vivo genomic sequence of the TAP1-proximal promoter from HeLa cells. Lanes *1* and *4,* in vitro methylated deproteinized DNA; lanes *2* and *5,* in vivo methylated DNA from uninduced HeLa cells; lanes *3* and *6,* in vivo methylated DNA after a 20-min TNF-α induction. The arrows with stars indicate the new protections arising after TNF-α induction. All other markings are as described in Fig. 6.

A

B
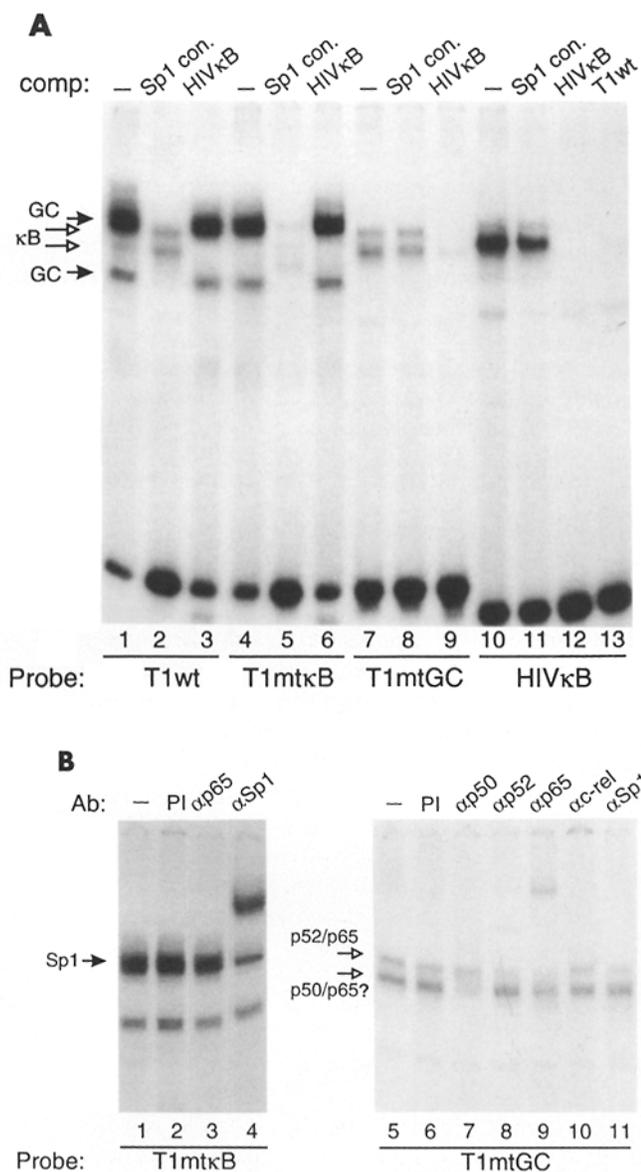
Figure 8. Sp1 binds the TAP1-proximal GC box, while heterodimers of p50/p65 and p52/p65 can bind to the NF-κB element. (A) Gel shift analysis of TNF-α–induced HeLa nuclear extract binding to the TAP1-proximal region. Probes are as indicated at the bottom and span the NF-κB site and the adjacent GC box (see Materials and Methods). Solid arrows mark the bands binding at the GC box; open arrows mark bands binding to the NF-κB site. Oligonucleotide competitors were used at ∼100-fold molar excess and are indicated at the top of the gel. (B) Identity of the gel shift bands were confirmed by antibody super-shift analysis. DNA probe is as indicated at the bottom, and the antiserum used is marked at the top of the gel. Nuclear extract is the same as in A. PI, preimmune sera; αp65, αp50, αp52, αc-rel, antibodies directed to specific subunits of NF-κB; αSp1, antibody against human transcription factor Sp1.

human invariant chain gene promoter (63). The resulting TAP1 in vivo binding pattern closely resembles that shown for the H9 cell line (Fig. 6), known to have high levels of constitutive NF-κB activity. These results suggest that TNF-α treatment of HeLa cells induces a change in NF-κB binding, leading to a more active transcriptional complex.

TAP1-proximal NF-κB Site Preferentially Binds p50/p65 and p52/p65 Heterodimers While the GC Box Binds Sp1. As a complement to the in vivo binding studies, we used in vitro gel shift analysis to define the protein factors binding to the TAP1-proximal promoter elements. A DNA probe spanning the NF-κB site and the adjacent GC box revealed one very prominent band and several weaker bands (Fig. 8 A, lane 1). The TAP1 GC box has strong homology to a high-affinity binding site for the abundant Sp1 transcription factor. Oligonucleotide competition of the wild-type probe with a consensus Sp1 element removes the most prominent band and the fastest migrating band (lane 2). This competition reveals two other binding activities, labeled κB. Likewise, mutation of the GC box allows detection of the same two κB bands (lane 7). These two κB bands are specifically competed by a consensus HIV NF-κB oligonucleotide but not a consensus Sp1 element (lanes 9 and 8, respectively). Thus, two complexes of different mobility interact with the TAP1 NF-κB site. The two bands labeled GC in lane 1 show specific affinity for the TAP1 GC box. The two GC bands bind to the wild-type probe and also to a probe mutated in the NF-κB site (lanes 1 and 4). Competition of either of these probes with a consensus Sp1 element removes the GC bands, while competition with the NF-κB oligonucleotide has no effect (lanes 2 and 5 vs. 3 and 6). Thus, the TAP1 GC box forms two specific complexes, while the NF-κB site forms two additional specific complexes. Although the in vivo studies clearly displayed cooccupancy of the NF-κB and GC boxes, an in vitro complex containing both GC and NF-κB binding activities is not easily discerned in these conditions. The diffuse complex migrating slightly slower than the prominent GC band is visible only with the wild-type probe and is probably the trimolecular complex (lane 1). In addition, other multicomplexes may be masked by the large signal from the upper GC band.

Antibodies specific for the GC box–binding protein Sp1 or several distinct NF-κB subunits were used to directly assess the identity of the proteins bound (Fig. 8 B). The upper prominent GC band is specifically recognized by antibodies directed to Sp1, while neither preimmune nor anti-NF-κB-p65 have any effect (lanes 1–4). A small amount of upper GC band remains after reaction with the anti-Sp1 antibody and may be residual Sp1 or an antigenically distinct GC box–binding protein. The lower GC-specific band did not react with the anti-Sp1 antibody and thus may be a different GC box–binding protein. This observation is consistent with previous findings of antigenically unrelated Sp1 family members binding in gel shift assays (64). The specificity of Sp1 binding to the TAP1-proximal GC boxes was confirmed by DNase I in vitro footprinting. Purified, recombinant Sp1 as well as crude HeLa nuclear extract strongly protect the GC box region in a similar manner (data not shown). Thus, it is likely that Sp1 is the major protein bound at the GC box; however, other GC box–binding proteins can not be excluded at this point.

The panel of NF-κB subunit-specific antibodies shows that the lower κB band is diminished by anti-NF-κB-p50 (lane 7), while the upper band is diminished and partially super-shifted by anti-NF-κB-p52 (lane 8). Both the upper and lower

bands are diminished by anti-NF-κB-p65 (lane 9), and a supershifted complex is generated. While the upper band is completely abolished by the anti-NF-κB-p65, the lower band is only partially diminished. Neither band was recognized by the control preimmune sera nor anti-c-rel or anti-Sp1 (lanes 6, 10 and 11, respectively). These findings indicate that the upper band is composed of p52 and p65 subunits, while the lower band is composed at least in part of p50 and p65. These findings together suggest that Sp1 in conjunction with either NF-κB p50/p65 or p52/p65 bind to the TAP1-proximal promoter and mediate transcriptional activation of both TAP1 and LMP2.

Discussion

This report provides the initial characterization of the transcriptional regulation of the human TAP1 and LMP2 genes. Extensive evidence has demonstrated that the MHC class II-linked TAP1 and TAP2 gene products are peptide transporters required to provide peptides to the ER for class I binding. Furthermore, the TAP messenger RNAs can be induced by cytokines consistent with class I expression (1, 24). However, no studies on the transcriptional regulation of these genes have been published. Interestingly, many tumors and virally infected cells downregulate class I, and this phenomenon is proposed to allow these cells to escape from immune surveillance (65). Some of these class I-deficient, virally infected cells clearly have class I mRNA. In addition, some tumors have a class I deficiency despite the presence of class I mRNA (66). Recently, the TAP1 molecule has been shown to be downregulated in some of these tumors, suggesting a mechanism for the loss of class I surface expression (40, 42). It will be important to determine if this downregulation occurs at the level of transcription or later in synthesis. Although no studies have been published regarding the levels of TAP in virally infected cells, it will not be surprising to find some viruses that also target TAP1 and/or TAP2 to escape immune surveillance. Thus, investigations into the regulation of TAP gene expression will be critical to the understanding of tumor development and, potentially, viral infection.

The genes for the proteasome complex subunits LMP2 and LMP7 are found in the MHC class II locus closely linked to the TAP genes. The significance of LMP2 and LMP7 in antigen presentation is not as well defined as the TAP genes. Cell lines deficient in both LMP genes are still capable of antigen presentation. However, a close examination of peptides produced when LMP2 and LMP7 were present indicated a shift in the endopeptidase specificity of the proteasome. The characteristics of the resulting peptides are structurally more related to those typically bound by class I. The LMP proteins are found in a subset of the total proteasomes present in cells, and their representation can be increased by cytokines also responsible for class I upregulation. Thus, LMP2 and LMP7 may function to direct the ubiquitous proteasome complex to directly serve the immune system. The findings presented in this report clearly link LMP2 transcription with

TAP1 transcription and provide additional suggestive evidence linking LMP2 to class I antigen presentation.

The human TAP1 and LMP2 genes are divergently transcribed with only 593 bp separating the two ATG translation initiation codons. The close proximity of the two genes suggests coordinate regulation, but this cannot be assumed. Several examples of bidirectional discordant regulation have been described. The human genes COL4A1 and COL4A2 are separated by only 127 bp and are divergently transcribed. Mutation of several cis-acting elements within the shared region has differential effects on the transcription of the two genes (67). The divergently arranged H3-II and H3-III histone genes are also differentially regulated (68). Although only 900 bp separate the two genes, each gene has its own set of cis-acting elements, which have no effect on the other gene. Therefore, thorough characterization of the cis-acting sequences within the TAP1/LMP2 intergenic region is necessary to understand the relationship between TAP1 and LMP2 expression.

We have shown in this report that the intergenic region is sufficient to promote transcription in both directions of a bidirectional reporter. The LMP2 gene initiates transcription predominantly from three sites clustered at −40 bp relative to the LMP2 ATG codon. This site is occupied in vivo (Wright, K. L., unpublished observation) and shows partial homology to the initiator element of the erythroid porphobilinogen deaminase gene (69). This is also the predominant initiation site of the mouse LMP2 gene (70). The TAP1 gene uses multiple start sites spread throughout the first 120 bp relative to the TAP1 ATG codon. An additional start site occurs at a far upstream location (∼ −427 bp). The upstream start site aligns with the major mouse TAP1 transcript; however, it has not yet been determined if the mouse TAP1 gene also uses the proximal start sites (70). Significantly, there is strong homology between mice and humans in this TAP1-proximal region.

The human TAP1 and LMP2 genes are coordinately regulated by the common use of at least two TAP1-proximal elements, an Sp1-GC box and NF-κB site. In vivo genomic footprinting revealed that both of these sites are occupied in cells. The Sp1-GC box upregulates basal transcription of both genes two- to threefold. Furthermore, the adjacent NF-κB element mediates induction of both TAP1 and LMP2 in response to TNF-α treatment. The Sp1-GC box is also required for the full induction of the distal LMP2 gene. One possible role for the Sp1-GC box in this induction is to amplify the effects of the adjacent NF-κB site over a greater distance. This would be consistent with the observation that the proximal TAP1 gene requires only the NF-κB site but not the Sp1-GC box for TNF-α induction. In addition, in vivo occupancy of the Sp1-GC box is constitutive, while the NF-κB binding is modulated on TNF-α induction. This region is also highly conserved in the mouse promoter, with a 7-bp insert between the two elements (71). This conservation suggests that the mouse genes may also use these sites for coordinate regulation of TAP1 and LMP2.

TNF-α treatment of HeLa cells induces the binding of at

least two forms of NF-κB to bind the TAP1 proximal site, p50/p65 and p52/p65. Both of these heterodimers have previously been shown to be activators of transcription (72, 73). Uninduced HeLa cells contain nuclear p50/p50 homodimers (57), and this may correlate with the distinct pattern of in vivo NF-κB occupancy observed on the uninduced TAP1 promoter. Interestingly, p50/p50 homodimers have been implicated in repressing transcription (74). However, there may be a complex interplay between the NF-κB element and the SP1-GC box since mutation of the NF-κB site in the context of a wild-type GC box did not significantly increase transcription.

TNF-α is also one of the cytokines that induces class I transcription (75). Fine mapping of the class I response element has previously identified a highly conserved NF-κB site located in the enhancer A domain (57). The common use of NF-κB to induce TAP1 and LMP2 concordantly with class I activation suggests a direct mechanism to link transporter levels with increases in class I production. Importantly, it also provides further credence for a significant role for LMP2 in the immune response. Likewise, class I and TAP1 are both upregulated by IFN-γ (24, 75). The putative ISRE element, located in the intergenic promoter (Fig. 1 B), appears to be important for upregulation of both LMP2 and TAP1 by IFN-γ (White, L. C., manuscript in preparation) and could also serve to increase transporter levels during the activation of an immune response.

These findings now provide a basis to examine the transcriptional control of TAP1 and LMP2 and have demonstrated that these genes are coordinately regulated. Coordinate regulation of LMP2 with TAP1 is an important finding, suggesting a functional linkage between the two gene products and thereby placing LMP2 in the class I antigen presentation pathway. Furthermore, the study of TAP1 and LMP2 regulation may enable the design of new strategies to upregulate class I during cancer and viral infections.

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