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Promoter Region Architecture and Transcriptional Regulation of the Genes for the MHC Class I-Related Chain A and B Ligands of NKG2D¹

Gopalakrishnan M. Venkataraman,* Dominic Suciú,* Veronika Groh,* Jeremy M. Boss,† and Thomas Spies^{2*}

Ligands of the NKG2D receptor, which activates NK cells and costimulates effector T cells, are inducibly expressed under harmful conditions, such as malignancies and microbial infections. Moreover, aberrant expression in autoimmune disease lesions may contribute to disease progression. Among these ligands are the closely related human MHC class I-related chains (MIC) A and B, which appear to be regulated by cellular stress. Analyses of *MIC* gene 5'-end flanking regions in epithelial tumor cells defined minimal core promoters that directed near maximum heat shock- or oxidative stress-induced transcriptional activation. Considerably larger fully functional promoters were required for maximum proliferation-associated activation. These activities were dependent on core promoter sequences that included heat shock elements, which inducibly bound heat shock factor 1, TATA-like elements, and constitutively occupied Sp1 and inverted CCAAT box factor sites. By contrast, *MIC* gene activation by CMV infection was largely independent of these and upstream promoter sequences, and expression of viral immediate early gene (*IE1* or *IE2*) products was sufficient for induction of transcription and surface protein expression. Altogether, these results reveal distinct modes of activation of the genes for the MIC ligands of NKG2D and provide a molecular framework for analyses of gene regulation under different cellular insult conditions. *The Journal of Immunology*, 2007, 178: 961–969.

Diverse MHC class I-like molecules that have no role in Ag presentation and limited tissue distributions serve as ligands for the NKG2D-DAP10 receptor complex, which activates NK cells and costimulates effector T cell subsets (1, 2). In humans, these ligands include the closely related MHC class I-related chains (MIC)³ A and B (MICA and MICB) transmembrane glycoproteins, which are encoded near *HLA-B* in the MHC and are represented by orthologous sequences in most mammals, except rodents (3). Expression of MIC is mostly restricted to intestinal mucosa, but can be induced by CMV infection in fibroblasts and endothelial cells, and by mycobacterial infection in dendritic and epithelial cells (4–6). Moreover, MIC are frequently associated with epithelial tumors of diverse tissue origins and are aberrantly expressed in rheumatoid arthritis synoviocytes and celiac disease intestinal epithelial cells (7–9). Thus, NKG2D triggering by MIC delivers immunostimulatory signals that can be

beneficial under adverse conditions, such as infections and malignancies, but may exacerbate autoimmune disease progression.

Despite the immunological significance of MIC, molecular mechanisms controlling gene regulation are poorly defined, and it is unknown whether and how different cellular and environmental stimuli converge to induce gene expression. Recent evidence has indicated that activation of DNA damage control pathways results in induction of NKG2D ligands, including UL16-binding protein family members and possibly MICA (10). Moreover, the 5'-end flanking regions of *MICA* and *MICB* contain putative heat shock elements (HSE), which are prototypic transcription inducer sites in *heat shock protein 70* (*HSP70*) genes that bind activated trimeric heat shock factor 1 (HSF1) (4, 11–13). With cell lines, MIC mRNA and protein expression are mostly limited to proliferating epithelial cells. Quiescent epithelial cells grown for extended time at high confluence display relatively small amounts of MIC mRNA and surface proteins that are sharply increased upon exposure to heat shock (14). Oxidative stress has also been found to induce *MIC* gene expression in colon carcinoma cells, although increased cell surface expression was not observed (15). The present study has used molecular and functional approaches to define the architecture of *MIC* gene promoter regions and the significance of transcriptional control elements for cell stress-induced, proliferation-associated, and CMV-mediated transcriptional activation.

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³ Abbreviations used in this paper: MIC, MHC class I-related chain; CBF, CCAAT box factor; ChIP, chromatin immunoprecipitation; HSE, heat shock element; HSF1, heat shock factor 1; HSP, heat shock protein; ICE, inverted CCAAT box-like element; IE, immediate early; Inr, initiator.

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Materials and Methods

Cell culture, heat shock, and flow cytometry

Cell lines were from the American Type Culture Collection. Primary human fibroblasts (passages 4–6) and HeLa S3 cells were grown in Waymouth's and MEM-Joklik medium (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone), glutamine, and antibiotics. Surface expression of MICA, or of MICA and MICB, was monitored by flow cytometry using mAbs 2C10 and 6G6, respectively (4, 14). For heat shock, culture plates with adherent HCT116 cells grown for 7 days at high confluence were sealed with parafilm and floated for 1 h on a 42.5°C water

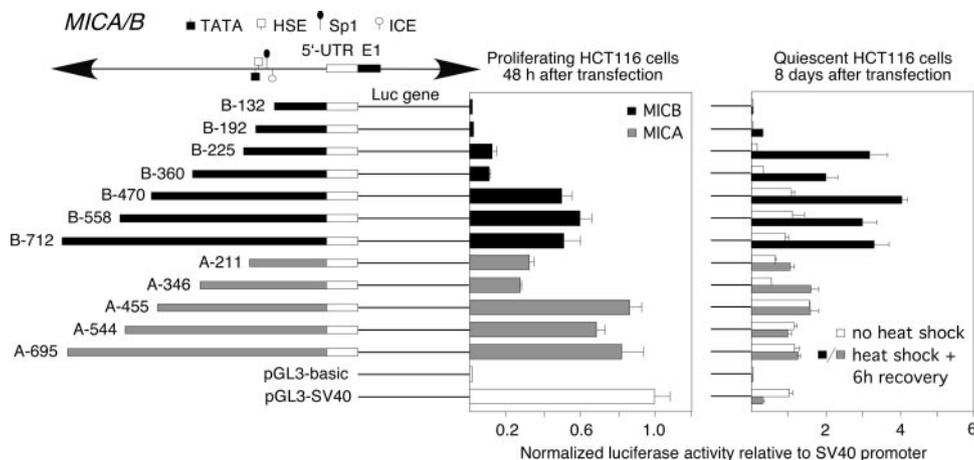


FIGURE 2. Normalized luciferase reporter gene activities driven by *MICB* and *MICA* promoter region-pGL3-basic constructs in transiently transfected proliferating or quiescent and heat shock-treated HCT116 cells. Negative and positive controls were pGL3-basic and pGL3-SV40, which produced absolute luciferase activity units in repeat experiments of $\sim 2,000$ (± 200) and 100,000 ($\pm 20,000$), respectively. Background activities with cell extracts from untransfected cells were ~ 200 . Assays were done in triplicate. Data are representative of at least three independent experiments demonstrating similar activities with each promoter construct relative to those of the SV40 promoter. Bars corresponding to the different *MICB* and *MICA* promoter region construct activities in proliferating or heat shock-treated cells are filled and shaded, respectively. Approximate locations of core promoter region TATA-like, HSE, Sp1, and ICE elements are indicated on the *top line* at left, above the representations of the promoter region fragments inserted in pGL3-basic. 5'-UTR, 5'-untranslated region; E1, exon 1; Luc gene, luciferase gene.

MIC surface expression, human primary dermal fibroblasts were also transfected with pEQ274 and pEQ326 encoding *IE1* and *IE2*, respectively, and with pEQ876 encoding the irrelevant CMV tegument protein (21) (plasmids were a gift from A. Geballe, Fred Hutchinson Cancer Research Center, Seattle, WA). Cells were examined by flow cytometry 72 h after transfection. For the CMV activation experiments, fibroblasts were infected with viral strain AD169 (American Type Culture Collection; multiplicity of infection of 5) 24 h after transfection with *MIC* gene promoter region reporter constructs. Cells were harvested and assayed 40 h after infection. For all reporter enzyme assays, washed cells from individual wells were lysed in 100 μ l of passive lysis buffer (Promega), and firefly and Renilla luciferase activities were measured in 10 μ l of cell extracts in 96-well white luminometer plates (Fisher Scientific) using an EG&G Berthold luminometer with automated dual injectors and time delay. Promoter construct activities were determined by normalizing firefly luciferase activity for transfection efficiency, as measured by Renilla luciferase activity.

Chromatin immunoprecipitation assay (ChIP)

Heat shock-treated or untreated HCT116 cells were processed for ChIP assays, following the kit protocol from Upstate Biotechnology. Immunoprecipitations used mixed rabbit and mouse IgG, anti-HSF1 and anti-CCAAT box factor (CBF)-B mAbs, and a polyclonal rabbit anti-Sp1 antiserum (1 μ g/ml; Santa Cruz Biotechnology). Immunocomplexes were collected with protein A/G agarose beads and sequentially washed, as detailed in the Upstate Biotechnology protocol. Chromatin was eluted with 1% SDS/0.1 M NaHCO₃ and digested with proteinase K, and cross-linking was reversed for 5 h in the presence of 200 mM NaCl at 65°C. DNA was purified using Qiaquick Gel Extraction kit (Qiagen) and dissolved in 100 μ l of Tris-EDTA. Fractions of precleared chromatin were processed similar to immunoprecipitated chromatin for input DNA controls. The forward and reverse primers for PCR amplification (all 5'-3') were GGGCCCTGGC CGTGCTTATGAAGTTGG (-317/-291) and CGCCACCTCTCAGCG GCTCAAGC (-12/-35) for *MICA*; GcGA CAGGTCCAGTCCGT GCTC (-337/-315) and CCCTACGTCGCCACCTTCTCAG CT (-4/-27) for *MICB*; and GTCCCTGTCCCTCCAGTGAAT (-439/-418) and GAACACTGGATCCGCGAGAAGA (-53/-74) for HSP70.

Results

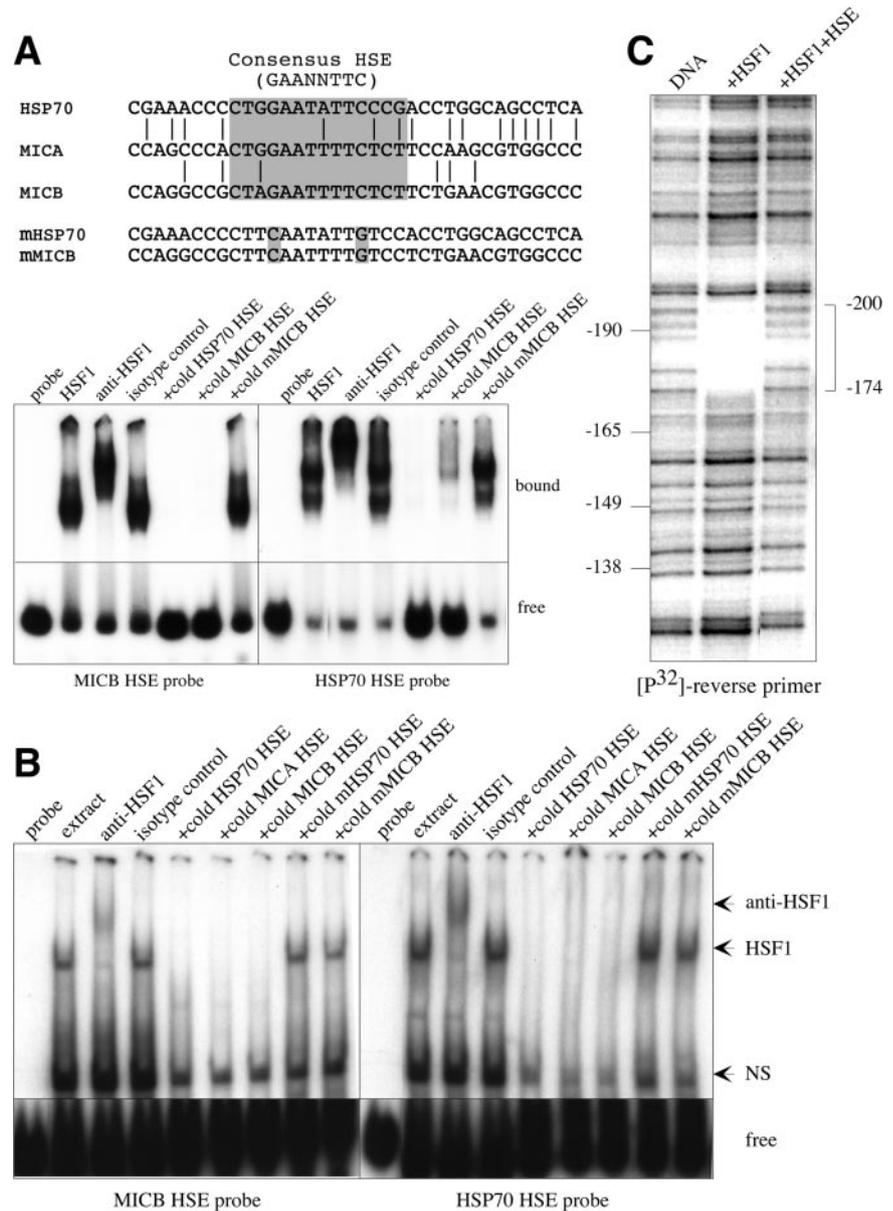
Potential regulatory elements and transcriptional initiation in the 5'-end flanking regions of *MICA* and *MICB*

Aligned cosmid-derived regions 1.5 kb upstream of the translation start codon (ATG) in *MICA* and *MICB* share $\sim 90\%$ sequence identity and include within a 50-bp region proximal sequence motifs for binding of HSF1 (HSE; consensus

GAANNTTC) and Sp1 (consensus GGG(A/C/T)GGG), and an inverted CCAAT box-like element (ICE) (Fig. 1A) (22–24). Additional conserved Sp1 sites are located ~ 160 and 190 bp upstream of the HSE. A potential AP-1 element is located downstream. Although canonical TATA boxes are absent, aligned TATA-like sequences are present in *MICA* (TAAGTTT and TAAATCT, at -67 and -200, respectively) and *MICB* (TA-AATTT at -80 and -215). A sequence of 14 bp in *MICB* (GTTTCATTCAGTTG, from -41 to -54) includes two overlapping motifs matching the consensus for an initiator (Inr) element (YYCANY) (25). This sequence is absent in *MICA*. However, potential Inr sequences occur in *MICA* at -112, -185, and -231; moreover, there is an additional unique Inr in *MICB* at -155 and a conserved Inr in both genes (at -281 and -295, respectively).

Transcriptional initiation was investigated with colon carcinoma HCT116 cells. Similar to other epithelial tumor lines, highly confluent HCT116 cells grown to quiescence contain small amounts of *MICA* and *MICB* mRNAs and display low *MIC* surface protein expression. However, in rapidly proliferating or heat-shocked cells, *MICA* and *MICB* mRNAs and surface proteins are strongly induced (Fig. 1C and see figure 3 in Ref. 14). We failed to obtain informative transcription initiation data using primer extension and RNase protection assays. Therefore, *MICA* and *MICB* 5'-end flanking regions were amplified by RT-PCR using nested sets of primer pairs of which the reverse primers were invariably positioned downstream of the respective ATG. For each gene, the transcript data were similar irrespective of whether proliferating or heat shock-treated HCT116 cells were examined (Fig. 1B). The majority of *MICA* transcripts initiated between about -40 and -100 upstream of the initiation codon, and a minor fraction extended further upstream. These results were corroborated by comparison with 23 *MICA* sequences in GenBank expressed sequence tag database, which localized a major transcription initiation region ~ 30 bp downstream of the proximal TATA-like motif. Among these sequences, 15 initiate between -32 and -42, including 9 sequences that start at -38; 5 sequences initiate between -48

FIGURE 3. Interaction of HSF1 with the HSE of *MICB* shown by EMSA and in vitro genomic footprinting. **A**, Alignment of promoter region sequences containing the HSE in *HSP70*, *MICA*, and *MICB*. Oligonucleotide probes used for EMSA (upper three lines) and nucleotide substitutions in the mutated mHSP70 and mMICB sequences (bottom two lines) are shaded. EMSA experiments (bottom) show binding of rHSF1 to labeled HSE probes, supershifts in the presence of anti-HSF1 Ab, and competition by excess unlabeled HSP70 and *MICB* HSE probes, but not by the mutated oligonucleotides. **B**, Similar EMSA experiment using whole cell extracts from heat shock-treated HeLa S3 cells. NS, nonspecific binding. **C**, DNase I protection assay shows an HSF1 footprint approximately between nucleotide positions -174 and -200 .



and -55 , 1 at -82 , and only 2 sequences initiate far upstream (-2880 and -2932 ; gi:14057251 and gi:82384203, respectively). These sequences were derived from normal human pericardium and an adenocarcinoma cell line. The immediate upstream genomic sequence includes distal TATA-like, Sp1, and CAAT box motifs, but no potential HSE. Their existence tentatively suggests the presence of a second upstream promoter in *MICA*, but supportive functional evidence is lacking, and seeking such evidence was outside the scope of our study. In contrast to *MICA*, transcriptional initiation of *MICB* was localized predominantly between -124 and -156 , upstream of the proximal TATA-like sequence and downstream of the conserved SP1 site (Fig. 1B).

Promoter regions directing proliferation-associated and heat shock-induced transcription

For functional studies of *MICA* and *MICB* gene promoter regions, nested sets of 5'-end flanking sequences immediately upstream of the translation start codons were inserted into pGL3-basic and tested for their ability to drive firefly luciferase reporter gene expression in transiently transfected proliferating HCT116 cells. Transfection efficiencies were normalized by cotransfection of

phRL-TK directing expression of Renilla luciferase. No significant proliferation-associated activities (as little as with the promoterless pGL3-basic) were detected with the shortest *MICB* -132 and -192 fragments, of which the latter includes the conserved HSE, Sp1, and ICE sequence motifs. Activities were increased with fragments -225 and -360 and reached a 5-fold higher near maximum strength with the -470 fragment, which was $\sim 60\%$ of the level recorded with the pGL3-SV40 control (Fig. 2). These results localized *MICB* core and fully functional promoter regions within -225 and -470 bp, which corresponded to *MICA* upstream regions of -211 and -455 bp, respectively. However, *MICA* displayed substantially stronger promoter activities under this study's examined proliferation-induced condition.

To investigate promoter region requirements for heat shock-induced transcription, HCT116 cells cotransfected with reporter constructs and phRL-TK were grown to high confluence past quiescence, heat shock treated or untreated, and tested for luciferase activity after 6 h of recovery. Parallel to functional testing, heat shock-induced MIC surface protein expression was routinely tested by flow cytometry after 12–16 h and was consistently similar to the data shown in Fig. 1C. The recovery time period allowed

for de novo synthesis of the reporter enzymes that were inactivated by heat shock. However, even after recovery, heat shock reduced pGL3-SV40-driven luciferase activity by $\sim 70\%$ (Fig. 2). Therefore, to minimize bias in data representation, MIC promoter region activities in both heat shock-treated and untreated cells were normalized against SV40 promoter activities in untreated cells. This approach enabled accurate relative comparisons of different MIC promoter region activities, but underestimated absolute heat shock-induced promoter region strength. As with proliferating cells, the *MICB* -470 fragment conferred maximum heat shock-induced transcriptional activation, which was ~ 8 -fold stronger. Notably, fragment -225 also showed near maximum activity, which was opposed to its poor activity in proliferating cells (Fig. 2). Thus, sequences required for strong heat shock-induced activation extended ~ 40 bp upstream of the HSE, whereas full proliferation-associated activation was dependent on an additional region up to 240 bp further upstream.

With *MICA*, heat shock-induced promoter region activities were substantially lower than those of equivalent *MICB* constructs, which was consistent with lesser heat shock inducibility of *MICA* mRNA (see figure 3 in Ref. 14). Only fragments -211 and -346 showed significant activation, whereas the -455 promoter region, although comparably active as -346, was of insufficient strength to overcompensate heat shock-induced loss of luciferase activity during the recovery period. Nevertheless, the -455 fragment displayed much stronger induction than the SV40 promoter (Fig. 2). Thus, these results supported heat shock-induced transcriptional activation also of *MICA*, which was similar to *MICB* in its dependence on the core promoter, including a short region upstream of the HSE.

Constitutive and inducible transcription factor binding

As with *HSP70*, the aligned *MICA* and *MICB* core promoter region sequences include inverted repeat 5'-NGAAN-3' pentamers that are characteristic of HSE (Fig. 3A) (22). EMSAs using labeled *MICB* and control *HSP70* oligonucleotide probes and purified recombinant protein confirmed in vitro binding of HSF1. This was indicated by the formation of a predominant single or two separate species of DNA-protein complexes, which were supershifted in the presence of anti-HSF1 Ab. Labeled DNA probes were displaced by excess unlabeled *MICA* or *HSP70* HSE oligonucleotides, but not when these were altered by core consensus mutations (Fig. 3A). Equivalent results were obtained for *MICA* (data not shown) and by using whole cell extract from heat shock-treated HeLa S3 cells (Fig. 3B). A lower m.w. complex presumably corresponds to a constitutive binding factor that is commonly observed with cell extracts (26). Binding of purified HSF1 to an appropriate location within the *MICB* promoter region was visualized by DNase I protection assay, which revealed a footprint located approximately between nt positions -174 and -200 (Fig. 3C).

In vivo binding of HSF1, Sp1, and CBF complex to the core promoter regions of *MICA* and *MICB* was investigated by ChIP assay using heat shock-treated or untreated HCT116 cells. Promoter region fragments of ~ 300 bp were amplified from cross-linked and sonicated chromatin preparations after immunoprecipitation with Abs specific for the transcription factors, reverse cross-linking, and DNA purification. As with *HSP70*, heat shock induced rapid HSF1 binding to the core promoter regions of *MICA* and *MICB*. With *MICB*, strong binding persisted during 60 min of heat shock as well as after an additional 90 min of recovery (Fig. 4). With *MICA*, HSF1 binding appeared diminished after 60 min of heat shock, but increased during the recovery period. This discrepancy was most

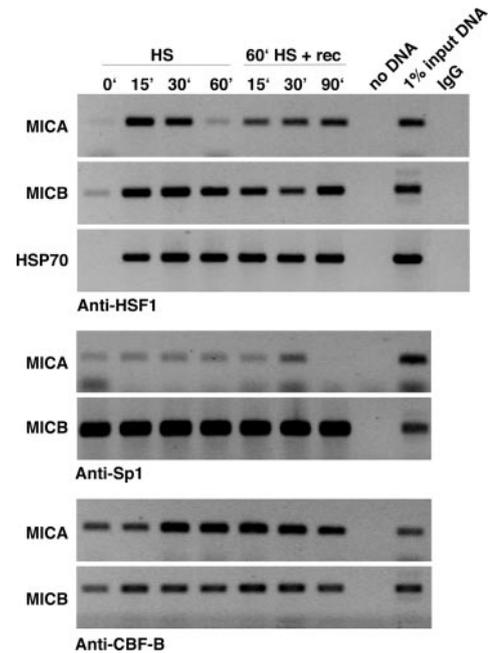


FIGURE 4. ChIP assays using chromatin from heat shock-treated or untreated HCT116 cells demonstrate inducible and constitutive in vivo occupancy of the core promoter regions of *MICA* and *MICB* by HSF1, and Sp1 and CBF, respectively. The inducible binding of HSF1 provides an experimental specificity control, as does the differential binding of Sp1 to the *MICA* and *MICB* promoter regions. Specificity of binding is supported by functional data shown in Fig. 5. All data shown were derived at the same time from the same experiment using the same primers and chromatin preparations. The reduced band intensity suggesting diminished HSF1 binding to *MICA* at the 60-min time point is most likely due to sample loss, as this finding was not reproduced in independent experiments. The immunoprecipitations used anti-HSF1 and anti-CBF-B mAbs, a polyclonal rabbit anti-Sp1 antiserum, and mixed rabbit and mouse IgG control Abs. The IgG control lane in the top panel is thus relevant to all panels. No bands were detected using primer pairs corresponding to irrelevant DNA sequence from the *MICA*-*MICB* intergenic region (data not shown). HS, heat shock; rec, recovery.

likely due to sample loss in this particular experiment, as it was not a reproducible finding. Sp1 and CBF bound constitutively under all conditions, but Sp1 binding appeared much weaker to the *MICA* than to the *MICB* promoter region (Fig. 4). Altogether, these results demonstrated inducible and constitutive transcription factor occupancies of predicted binding elements in the core promoter regions of *MICA* and *MICB*. The apparent weaker binding of HSF1 and Sp1 to the core promoter region of *MICA* might be related to its less inducible expression (see figure 3 in Ref. 14).

Mutational analysis of MIC gene promoter region elements

The functional significance of consensus elements and transcription factor binding was studied by mutational analysis and luciferase reporter gene assays. Constructs with site-directed mutations in the HSE, Sp1, ICE, or TATA-like elements within the *MICA* -455 and *MICB* -470 regions were analyzed for promoter activities in proliferating and quiescent heat shock-treated HCT116 cells. Under these two conditions, mutation of the Sp1 element decreased luciferase activity by 20 and 80% (*MICA*), and by 50 and 85% (*MICB*), respectively. Similar reductions resulted from mutation of the *MICA* and *MICB* HSE (20 and 70%, and 15 and 90% with proliferating and heat-shocked cells, respectively). Thus, these results confirmed the significance of the in vivo transcription

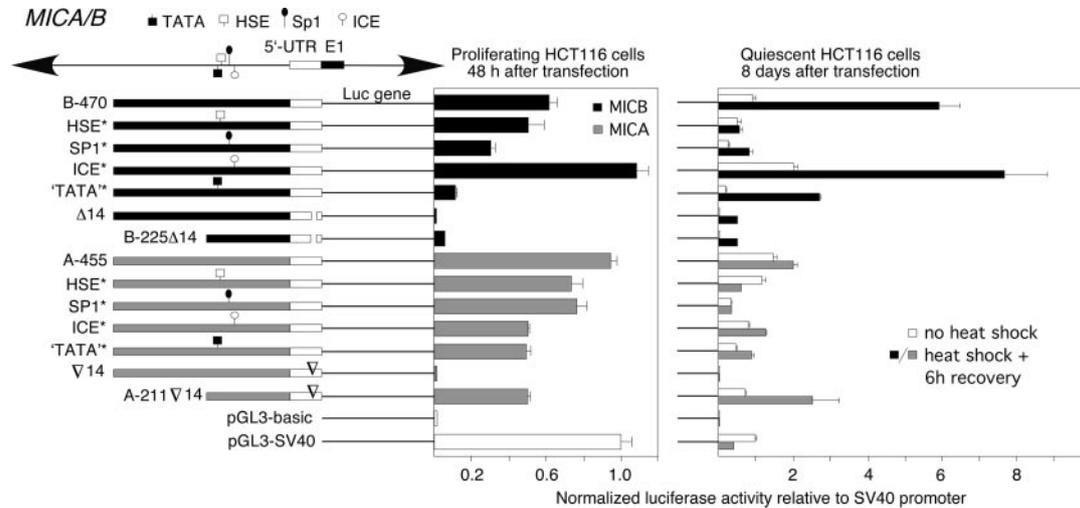


FIGURE 5. Mutational analysis of MIC gene promoter regions. Effects of mutations in transcription factor binding sites and of reciprocal deletions (Δ) and insertions (∇) of the 14-bp *MICB* core promoter region sequence on transcriptional activities of *MICA* and *MICB* functional and core promoter regions represented by fragments A-455 and B-470, and A-211 and B-225, respectively. Compare activity bars corresponding to the A-211 ∇ 14 and B-225 Δ 14 insertion and deletion variants to wild-type data shown in Fig. 2. Mutated transcription factor-binding elements are denoted by asterisks. Reporter constructs, controls, transfections, and heat shock treatment of HCT116 cells, and luciferase assays were as in Fig. 2. Assays were done in triplicate, and data shown are representative of at least three independent experiments demonstrating similar activities of each reporter construct relative to those of the SV40 promoter. Bars corresponding to the different *MICB* and *MICA* promoter region construct activities in proliferating or heat shock-treated cells are filled or shaded, respectively. Approximate locations of core promoter region TATA-like, HSE, Sp1, and ICE elements are indicated on the *top line*, above the representations of the wild-type and mutated promoter region fragments inserted in pGL3-basic. 5'-UTR, 5'-untranslated region; E1, exon 1; Luc gene, luciferase gene.

factor-binding data shown in Fig. 4 and demonstrated critical roles of Sp1 and HSF1 in heat shock-induced and to lesser extents in proliferation-associated transcriptional activation (Fig. 5). By contrast, mutation of the conserved ICE sequence produced opposite effects with *MICA* and *MICB*, significantly reducing and enhancing luciferase activities, respectively. Thus, in the context of the *MICB* promoter, the CBF complex may function as a negative regulator. These discordant effects might be related to the presence of an additional potential ICE in the *MICA* promoter region 26 bp downstream (Fig. 1A). Proximal to the conserved ICE is a TATA-like element (at -67 in *MICA* and -80 in *MICB*) that was unlikely to have functional significance because the short B-132 and B-192 promoter region fragments showed no or little activity in the reporter gene assays (Fig. 2). However, the unusually positioned TATA-like element 28 bp upstream of the HSE was included in the core promoter region constructs from both genes. Mutation of this element substantially lowered luciferase activities, with reductions of $\sim 45\%$ (*MICA*) and 75% (*MICB*) in proliferating cells, and 45% (*MICA* and *MICB*) in heat shock-treated HCT116 cells (Fig. 5). Thus, as with the conserved HSE, Sp1, and ICE elements, this distal TATA-like sequence was essential for optimal *MIC* gene transcription under both experimental conditions.

The most conspicuous difference between the *MIC* gene promoters is the 14-bp sequence in *MICB* (-41 to -54) that includes two overlapping Inr-like elements and is absent in *MICA* (Fig. 1). Reciprocal insertion (∇) and deletion (Δ) of this sequence in the A-455 and B-470 fully functional, and in the B-225 core promoter constructs, abrogated or severely reduced proliferation-associated and heat shock-induced transcriptional activation. By contrast, insertion of this sequence in the A-211 core promoter construct had a moderately enhancing effect in proliferating cells and resulted in substantially increased activity after heat shock induction (Figs. 2 and 5). Thus, these results revealed profound differences in promoter context-dependent regulation, indicating the presence of *MICA* and *MICB* gene-specific sequences that may be associated with negative or positive modulation.

Transcriptional induction by oxidative stress

Exposure of HCT116 cells to hydrogen peroxide after transfection with promoter region reporter constructs revealed activation patterns that were similar to those caused by heat shock treatment, although some variances occurred. Moreover, the dependencies on transcription factor-binding elements were comparable (Fig. 6A). Thus, oxidative stress, which could be associated with *MIC* expression in tumors and the intestinal mucosa, was a condition that strongly activated *MIC* gene transcription. Physiological significance was supported by marked increases of *MIC* surface expression on near confluent HCT116 cells after 72 h of exposure to 0.1 or 1 mM hydrogen peroxide (Fig. 6B).

MIC gene regulation by CMV

CMV infection of human fibroblasts or endothelial cells results in up to 10-fold increases of cell surface *MIC* and is associated with induced HSP70 expression (see figure 1 in Ref. 6) (27). We therefore anticipated an involvement of HSF1 in CMV-mediated *MIC* gene activation. Reporter gene assays were carried out with primary human fibroblasts infected or mock infected with CMV strain AD169. Infection had no noticeable effect on the synthesis of control Renilla luciferase used for normalization. The core (A-211 and B-225) and fully functional (A-455 and B-470) promoter regions of *MICA* and *MICB* displayed potently induced activities, with ~ 20 - and 40 -, and 10 - and 20 -fold increases, respectively, over the mock infection expression levels (Fig. 7A). Strong activation (~ 25 -fold increase) was also conferred by the *MICB* -192 region, which produced no or minimal activity in proliferating or quiescent heat shock-treated HCT116 cells (Fig. 2). Moreover, the mutations of the HSE, Sp1, or ICE elements in the fully functional promoter constructs failed to severely suppress activation. Thus, *MIC* gene induction by CMV was not critically dependent on HSF1 and had no discernible promoter sequence requirements (Fig. 7A). Cotransfection of reporter constructs with pEQ276 di-

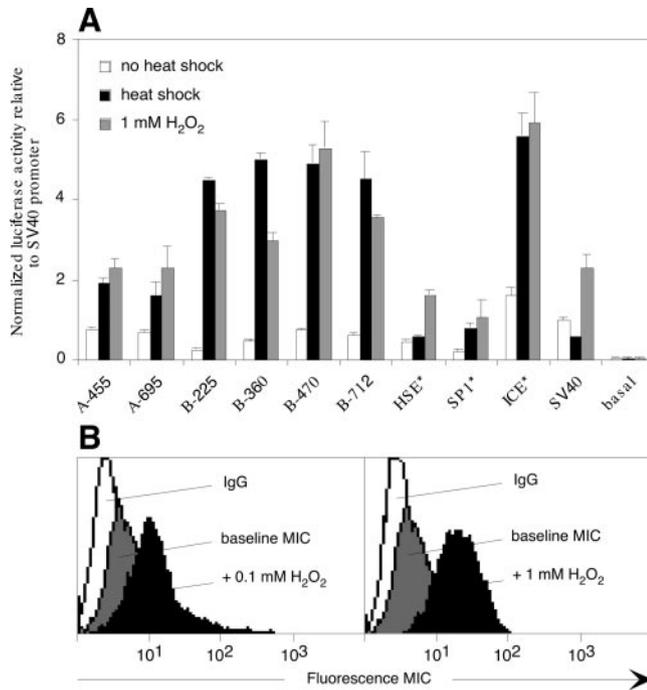


FIGURE 6. Analysis of gene regulation and surface protein induction by oxidative stress. *A*, Oxidative stress-mediated transcriptional activation of *MIC* gene promoter region reporter constructs and effects of mutations in transcription factor-binding elements. HCT116 cells were exposed to hydrogen peroxide (1 mM for 72 h) 24 h after transfections and assayed as in the experiments shown in Figs. 2 and 5. The HSE*, Sp1*, and ICE* mutations were all in the *MICB* B-470 promoter region fragment. Assays were done in triplicate, and data shown are representative of three independent experiments. Luciferase activities of untreated cells (□), and of cells exposed to heat shock (■) or H₂O₂ (▒) are plotted relative to those of the SV40 promoter. Untreated cells were near confluence and proliferating at the beginning of experiments, but reached high confluence and quiescence when assays were done. *B*, Flow cytometry using mAb 6G6 shows increased MIC surface expression after 72-h exposure of HCT116 cells to 0.1 or 1 mM H₂O₂.

recting the expression of the CMV IE1 and IE2 gene products resulted in 20- and 10-, and 10- and 5-fold inductions of the *MICA* and *MICB* core and fully functional promoter regions, respectively, as compared with transfection of negative control pEQ336, which contains the *IE2* promoter/enhancer, but no coding region (Fig. 7*B*). Moreover, transient expression of IE1 and IE2 together or individually induced cell surface MIC expression, thus indicating that viral *trans* activation was largely, if not completely, mediated by these CMV gene products (Fig. 7*C*).

Discussion

The present results provide a basic analysis of the architecture and function of the promoter/regulatory regions of the *MICA* and *MICB* genes, and of the involvement of transcription factor binding sites under various activating conditions. Similar to *HSP70* genes, the conserved *MIC* gene HSE inducibly binds HSF1 and is critical for activation by heat shock and oxidative stress. Constitutively occupied Sp1 sites and TATA-like elements, which are located unusually far upstream, moderately or profoundly affect stress-induced and proliferation-associated induction. Typically, Sp1 recruits basal transcription machinery in TATA-less CpG is-

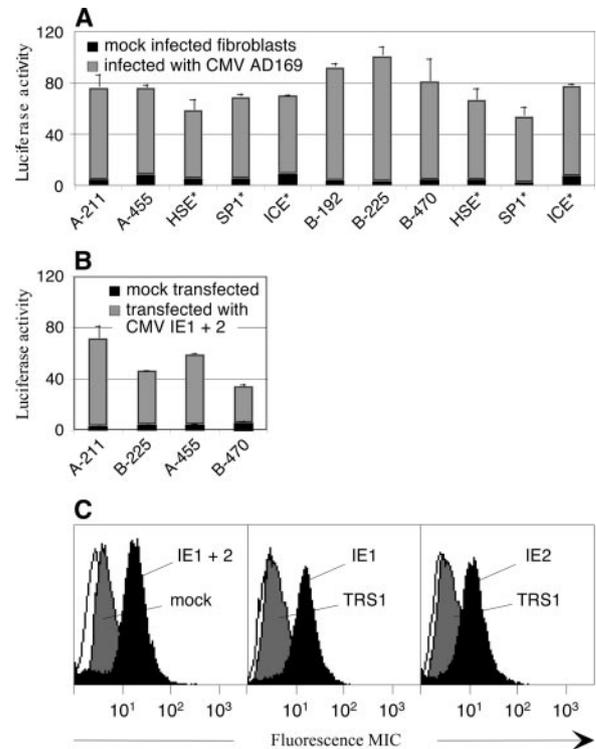


FIGURE 7. Regulation of *MIC* gene induction by CMV. *A*, Relative lack of involvement of specific promoter regions and transcription factor binding sites in CMV-mediated transcriptional activation. Luciferase activities of wild-type and mutated *MIC* promoter region constructs in transfected and CMV AD169-infected primary human fibroblasts are plotted relative to activities of the SV40 promoter in mock-infected cells. Mutated binding elements are denoted by asterisks. The HSE*, Sp1*, and ICE* mutations were all in the *MICA* A-455 and *MICB* B-470 promoter region fragments. *B*, *Trans* activation by the CMV IE1 and IE2 gene products 24 h after cotransfection of reporter constructs and pEQ276. Mock transfections were with plasmid pEQ336, which contains the CMV *IE2* promoter/enhancer, but lacks the coding region (21). *C*, Transfection of primary human fibroblasts with pEQ276, pEQ274, or pEQ326, encoding CMV IE1 and IE2 together and each of these genes individually, respectively, resulted in induced MIC surface expression 72 h after transfection (filled profiles). Negative controls were transfections with pEQ336 (mock) and pEQ876 encoding the irrelevant viral tegument protein (shaded profiles). Open profiles are IgG control stainings. As is commonly observed, the fibroblasts display small amounts of basal surface MIC expression.

land promoters of housekeeping genes (28, 29). As with *MIC*, Sp1 is required for constitutive and inducible expression of human *HSP70* (30). A common ICE appears to be a negative regulator of *MICB*, but not of *MICA*. The interacting CBF complex contributes significantly to basal and stress-induced expression of *HSP70* and activates genes involved in diverse cellular processes (25, 31); however, there is precedence for negative regulation of a number of genes (32). Although the *MICA* and *MICB* promoters are very similar, reflecting the close evolutionary relationship of the two genes, our results provide evidence for differential regulation, which is best exemplified by the context-dependent enhancing function of the 14-bp sequence in *MICB*. Generally, in accord with mRNA data (see figure 3 in Ref. 14), the baseline transcriptional activities of *MICA* were higher than those of *MICB*, which was more stringently regulated and displayed stronger inductions. With regard to transcriptional initiation, our data suggest that the majority of *MICA* transcripts initiate downstream of the conserved

AP-1-TATA-like motifs, a region that lacks a recognizable Inr. By contrast, most MICB transcripts initiate further upstream, proximal of the HSE-Sp1-ICE elements. The Inr at -231 may represent a minor initiation site in *MICA* and could be driven by potential Sp1 binding sites located at -364 upstream. Although these elements are conserved in *MICB*, the corresponding Inr is missing because of a critical cytosine for adenosine substitution (Fig. 1). There was no evidence for an involvement of separate promoters during proliferation or heat shock-associated gene activation.

Not surprisingly, *MIC* lack the prototypic MHC class I gene regulatory elements located between about -95 to -220 upstream of the translation initiation codons. These are constituted by the S-X-Y module, which binds RFX, CREB, and CBF complexes representing the MHC enhanceosome, and by the IFN-stimulated response element and enhancer A, which interact with IFN-regulated factor and NF- κ B, respectively (33). However, MHC class I gene core promoters include TATA- and Inr-like motifs and a CA/GT-rich region that binds Sp1, but these elements are differently organized. By contrast, considerable symmetry is shared between *MIC* and *HSP70* promoters in the arrangement of the AP-1/TATA-like (TATA in *HSP70*), CBE, Sp1, and HSE elements, although intervals vary (11, 12).

In the induction of *MIC* by CMV, there were only modest effects of mutations in the HSF1-, Sp1-, and CBF-binding elements, although combinatorial effects were not assessed, and activation was independent of sequences upstream in the fully functional promoter regions. Expression of CMV IE1 or IE2 alone was sufficient for *trans* activation. These nuclear phosphoproteins are promiscuous activators of many viral and cellular genes, such as of *c-fos*, *c-myc*, and *HSP70*, and interact with various transcription factors within very short basal promoter regions (34). The activation mode by IE1 and presumably IE2 involves chromatin remodeling through displacement of histone deacetylases (35). This mechanism may also operate in the *trans* activation of *MIC* gene expression in CD4 and CD8 T cells by the viral Tax regulator protein in human T cell leukemia virus-1-associated neurologic disease (36). Histone deacetylase inhibitors have been shown to induce *MIC* gene expression (37).

The similarities in regulation between *HSP70* and *MIC* genes suggest that conditions in tumor environments that lead to *HSP70* induction, such as oxidative stress, hypoxia, and hypoglycemia, may also favor *MIC* expression. Moreover, oxidative stress could be a critical condition underlying the characteristic *MIC* expression in normal intestinal epithelium (4). Our results provide no insights into the regulation of *MIC* in autoimmune lesions. However, in rheumatoid arthritis, proliferating, but not quiescent synovocytes express *MIC*, resulting in costimulation of autoreactive CD28⁻NKG2D⁺ CD4 T cells, and thus presumably in exacerbation of disease progression (8). In this regard, the present results demonstrate the requirement of *MIC* fully functional promoter region sequences and of defined core promoter elements for maximum proliferation-associated transcriptional induction. It remains unknown, however, whether chromatin modifications may also be involved. Altogether, our results provide a molecular framework of *MIC* gene regulation that may be applicable to future studies of gene expression in malignancies, infections, and autoimmune disease.

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Disclosures

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