The novel MMS-inducible gene *Mif1/KIAA0025* is a target of the unfolded protein response pathway

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Abstract In a search for genes induced by DNA-damaging agents, we identified two genes that are activated by methyl methanesulfonate (MMS). Expression of both genes is regulated after endoplasmic reticulum (ER) stress via the unfolded protein response (UPR) pathway. The first gene of those identified is the molecular chaperone *BiP/GRP78*. The second gene, *Mif1*, is identical to the anonymous cDNA KIAA0025. Treatment with the glycosylation inhibitor tunicamycin both enhances the synthesis of Mif1 mRNA and protein. The Mif1 5′ flanking region contains a functional ER stress-responsive element which is sufficient for induction by tunicamycin. MMS, on the other hand, activates Mif1 via an UPR-independent pathway. The gene encodes a 52 kDa protein with homology to the human DNA repair protein HHR23A and contains an ubiquitin-like domain. Overexpressed Mif1 protein is localized in the ER.

Key words: Unfolded protein response; Tunicamycin; Methyl methanesulfonate; Osmotic shock; Ubiquitin-like; BiP/GRP78

1. Introduction

Treatment of mammalian cells with genotoxic agents elicits a complex response, involving the induction of a variety of genes. Several signal transduction pathways can be activated, depending on the kind of genotoxic agent used. These include the UV response [1], the ATM/p53-dependent [2] and the unfolded protein response (UPR) pathways [3,4]. Proteins induced by genotoxic agents are involved in various cellular processes, such as inter- and intra-cellular signaling, cell cycle regulation and apoptosis. The spectrum of genes induced by genotoxic agents overlaps partially with those activated by other stimuli, like growth factors, serum starvation or non-genotoxic cellular stress [5]. In the course of a search for genes that are induced by DNA-damaging agents, we have applied the method of differential display to identify genes that are induced by the methylating agent methyl methanesulfonate (MMS), a strong mutagen and carcinogen. MMS reacts with the N7 atom of guanine and the N3 atom of adenine in DNA. In addition, it also reacts with -SH groups in proteins [6]. Here we will describe the identification of a novel endoplasmic reticulum (ER) stress-inducible gene, called *Mif1* by us. Enhanced Mif1 mRNA levels were found after treatment with MMS, but also after osmotic shock and exposure to the ER stress inducer tunicamycin. The *Mif1* gene encodes a 52 kDa protein which contains a ubiquitin-like domain and shows resemblance to the human DNA excision repair protein HHR23A.

Proteins destined for secretion, for trafficking to the lysosomes or for integration into the membranes are translocated across the ER membrane as unfolded polypeptide chains during translation. The molecular chaperones BiP, which binds to misfolded proteins, and GRP94 are involved in the proper folding of these nascent proteins. Exposure of cells to ER stress leads to an enhanced level of unfolded proteins and a reduced level of free BiP. This decrease in free BiP protein has been suggested to be the trigger for the activation of UPR, a pathway that is conserved from yeast to mammalian cells [3].

A novel ER stress response element (ERSE) was recently identified, which is present in multiple copies in the promoters of most known mammalian ER stress-inducible genes [7,8]. These ERSEs are necessary and sufficient for induction of BiP after treatment with tunicamycin [7,8]. NF-Y and YY1, two ubiquitously expressed transcription factors, were shown to bind to the CCAAT and the CCACG parts of the ERSE, respectively [9,10]. Regulated expression of the ERSE-containing genes is probably achieved via a still unknown protein. An ER stress-inducible complex (ERSF) has been found in HeLa cells, which binds to the ERSE. Binding of ERSF to ERSE requires a conserved GGC motif within the 9 bp GC-rich region that separates the NF-Y and YY1 binding sites [8]. The identity for ERSF has not yet been established, but the basic leucine-zipper protein ATF6 has been suggested as a component of the ERSF complex [7].

Activation of the UPR leads to enhanced expression of two classes of gene products. One group consists of ER-resident proteins, involved in protein folding, such as the ER chaperones BiP and GRP94 or protein disulfide isomerase. The other class contains non-ER-resident proteins such as the growth arrest and DNA damage-inducible GADD45 and GADD153 [11,12]. We show that Mif1 most likely belongs to the former group.

2. Materials and methods

2.1. Cell culture and treatments

The following cells are used in this study: normal human diploid skin fibroblast strains VH10 (kindly provided by Dr. J.W.I.M. Simons, Leiden University Medical Center) and 1BR2 [13], SV40-transformed normal human skin fibroblasts MRC-5 [13] and VH10SV [14], MMS-sensitive AT4BI skin fibroblasts derived from an ataxia telangiectasia (AT) patient [13], SV40-transformed AT fibroblast strain

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AT5BIVASV [13] and the cervix carcinoma cell line HeLa. Cells were cultured on Dulbecco's MEM (DMEM) (Gibco-BRL, Life Technologies, Paisley, UK), supplemented with 8% fetal calf serum (FCS, Gibco-BRL). Before treatment, all cells (except HeLa) were grown to confluence and serum-starved for 3 days in DMEM supplemented with 0.5% FCS. Prior to UV-A, B or C irradiation, the medium was removed and stored, and the cells were washed twice with phosphate-buffered saline (PBS). Irradiation with UV-A occurred in PBS at a dose rate of 9.8 kJ/m^2/min. The dose rate for UV-B and UV-C irradiation was 10 and 0.5 J/m^2/s, respectively. After UV irradiation, the stored culture medium was added back to the dishes. X-radiation was performed with an Andrex Smart 225 source at a dose rate of 1 Gy/min. Heat shock was performed by incubating the cells in a 42°C water bath for 5 min followed by further incubation in a 37°C incubator. Osmotic stress was applied by adding sodium chloride to the cells up to an added concentration of 0.3 M for 5 min. Then the cells were washed with PBS and further incubated with conditioned medium. All other treatments were performed by incubating the cells as indicated. Tunicamycin was purchased from Sigma Chemical Company. (St. Louis, MO, USA) and MMS from Merck (Darmstadt, Germany).

2.2. Reverse transcription, differential display and fragment isolation

Differential display was performed in triplicate essentially as described [15] with a few modifications. In short, cytoplasmic RNA was treated with RNase-free DNase. M-MLV (Gibco-BRL) reverse transcriptase was used for the reverse transcription reaction in the presence of one base-anchored oligo-dT downstream primers extended with 4 nucleotides to create a *Hin*dIII site at the 5′ end of the primers [16]. Amplification reactions were performed in the presence of the same downstream primer, an arbitrary random decamer primer and [^{33}P]α-dATP as the labeling nucleotide under the following PCR protocol: 30 s 94°C, 60 s 40°C and 30 s 72°C for 40 cycles. The following primer combinations were used to identify the MMS-inducible fragments (Mif) described in Section 3: Mif1: downstream primer 5′-AAGCT$_{11}$C and upstream primer 5′-AAGCTTGATTGCC. Mif2: downstream primer 5′-AAGCT$_{11}$A and upstream primer 5′-CTGCTTGATG. PCR products were concentrated in a vacuum centrifuge and separated on 6% denaturing polyacrylamide gels. Gels were dried and, after autoradiography, bands of interest were excised from the gel. DNA was recovered from the gel by overnight elution at 37°C in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH 8.0) and 0.1% SDS) as in [17]. Isolated DNA fragments were re-amplified (30 s 94°C, 60 s 42°C and 30 s 72°C for 30 cycles) with the same upstream and downstream primer combination and then cloned into the pCR2.1 TA-cloning vector (Invitrogen Corporation, Leek, The Netherlands).

2.3. DNA sequencing and database analysis

Double-stranded DNA from the cloned differential display fragments was sequenced with M13 forward and reverse primers and a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden). The isolated differential display fragments were identified with the Wisconsin Package Version 9.1 (Genetics Computer Group (GCG), Madison, WI, USA).

2.4. RNA isolation and Northern blotting

RNA isolation and Northern blotting were performed as described previously [18]. To test for expression of Mifs, the isolated differential display fragments were labeled by random priming with [^{32}P]α-dATP. A 1000 bp KIAA0025 5′ fragment was amplified from a cDNA derived from MMS-treated human skin fibroblasts with the use of the following primer combination: sense 5′-CGTGAACGGTCGTTGCAGA and antisense 5′-GCAGGTACATAACAACGGT. A 667 bp fragment for p21^{WAF-1} was generated from the same cDNA preparation with the following primers: sense 5′-CCCCAGCCTCTGGCATTA and antisense 5′-GTGTCCCTTCCCCTTCCA. A 348 bp fragment of the GRP94 cDNA was amplified by PCR with the primers sense: 5′-TCATCACAGACGACTTCC and antisense: 5′-CCTTCATTCTTTCCACATAC. A 420 bp *Pst*I fragment from pHS208 [19] was used for detection of Hsp27 mRNA expression. cJun expression was tested with a 1 kb *Hin*dIII-*Pst*I fragment from pRSV-cJun [20]. A 600 bp *Pst*I/*Xho*I insert containing the GADD153 cDNA was isolated from pBSIISK (kindly provided by Dr. R. Bernards). A rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe derived from the 1.3 kb *Pst*I fragment from pRGAPDH [21] and a 1500 bp *Pst*I fragment of human elongation factor 1 [22] were used as loading controls.

2.5. Construction of a myc epitope-tagged Mif1 expression vector

The Mif1 cDNA was amplified from pBluescriptKIAA0025 (kindly provided by Dr. T. Nagase) by means of the proofreading *Pfu* DNA polymerase (Stratagene Cloning Systems, La Jolla, CA, USA) and the following primer combination: sense 5′-CGGAATTCCGGCCGCCATGGAGTCCGAGACC and antisense 5′-CGCGGATCCGCGGTTTGCGATGGCTGGGGGGCC. The PCR fragment was cloned into the *Eco*RI/*Xho*I sites of pCDNA3.1A (Invitrogen Corporation, Leek, The Netherlands). The DNA sequence for the resulting myc epitope-tagged Mif1 cDNA was confirmed by sequencing.

2.6. Antibodies and immunoprecipitation

Polyclonal antibodies against Mif1 were raised by immunizing rabbits with the Mif1 peptide H_2N-EPAGSNRGQYPEDSS-$CONH_2$ (Eurogentec Bel. S.A., Herstal, Belgium). The specificity of the antibodies was tested by Western blotting on lysates derived from cells transiently transfected with the myc epitope-tagged Mif1 expression vector. Induction of endogenous Mif1 protein was determined by immunoprecipitation as described before [18].

2.7. Immunofluorescence

Cells transfected with a myc epitope-tagged Mif1 expression vector were fixed with acetone 48 h post-transfection. Immunofluorescence was performed as described before [23]. Mif1 protein was detected by double labeling, with mouse monoclonal anti-myc antibody 9E10 [24] and rabbit polyclonal anti-Mif1 peptide antibody 421, followed by incubation with a rhodamine-conjugated anti-mouse IgG and a FITC-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Nuclear DNA was stained with 2,4-diamidino-2-phenylindole (DAPI).

2.8. Cloning of Mif1 promoter sequences

Screening a flow-sorted chromosome 16 cosmid library from the Los Alamos National Laboratory [25] with a full-length 1860 bp cDNA probe for Mif1 resulted in 13 positive clones. In a re-screen performed by Southern blotting, two of these clones hybridized to both a 5′ and a 3′ probe derived from the Mif1 cDNA and contained a fragment of approximately 10 kb of the *Mif1* gene. FISH analysis assigned both clones to chromosome 16q21. The DNA for the two clones was digested with *Eco*RI, ligated into pIC20H [26] and subclones containing a 4.2 kb insert hybridizing to a 5′ 250 bp probe for Mif1 were isolated. Sequence analysis was performed for two subclones.

2.9. Construction of luciferase reporter plasmids

To obtain a luciferase reporter construct that is regulated by Mif1 promoter sequences, a *Mlu*I/*Nco*I fragment from the pIC20H-Mig1 plasmid, corresponding to nucleotides −765 up to +96 (relative to the KIAA0025 cDNA sequence), was cloned into pGL3basic (Promega Corporation, Madison, WI, USA). The nucleotide sequence of this promoter fragment can be retrieved from the EBI database (accession number AJ250249).

Deletion mutants were generated by digesting the Mif1$^{(-765\rightarrow+96)}$-pGL3basic construct with *Kpn*I in combination with either *Bam*HI, *Pst*I or *Pvu*II, followed by re-ligation according to standard cloning protocols. The control vectors pGL3TATA and 5xJun2TRE were kindly provided by Dr. H. van Dam, Leiden University Medical Center.

To obtain luciferase reporter constructs containing only the wild-type or mutated Mif1-C1 (nucleotides −98 to −75) or the first ERSE (nucleotides −65 to −43) of BiP, double-stranded oligonucleotides with the appropriate sequences (Fig. 4B) were ligated into the *Kpn*I and *Bgl*II sites of the pGL3 promoter vector (Promega Corporation, Madison, WI, USA), which contains the SV40 minimal promoter upstream of the luciferase coding sequence. DNA sequences of the inserted region were confirmed by sequencing.

2.10. Luciferase assays

Exponentially growing HeLa cells, cultured on 50 mm Petri dishes, were transiently transfected with the DEAE dextran method [27] with 2 μg plasmid DNA that had been purified by two rounds of CsCl

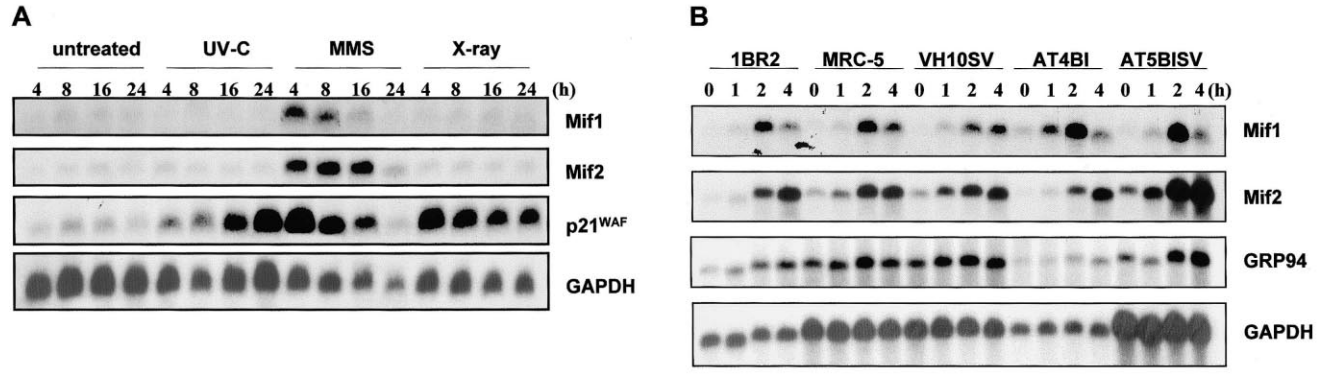

Fig. 1. A: *Mif1* and *Mif2* are MMS-inducible genes. Induction of Mif1 and Mif2 was tested by Northern blotting in VH10 human diploid skin fibroblasts. mRNA was isolated at different time points after treatment with UV-C (15 J/m^2), MMS (1 mM) or X-ray (5 Gy). Induction of p21^{WAF-1} is shown as a positive control for gene induction by the different treatments. Expression of GAPDH is shown as a loading control. B: Mif1, Mif2 and GRP94 are induced by MMS in diploid fibroblasts and SV40-transformed cells. Human diploid (1BR2, AT4BI) and SV40-transformed (MRC-5, VH10SV, AT5BISV) fibroblasts were continuously treated with MMS (1 mM) for 1, 2 or 4 h. Induction of Mif1, Mif2 and GRP94 was tested by Northern blotting. Expression of GAPDH is shown as an internal control. Note that SV40-transformed cells express higher basal levels of the *GAPDH* gene than diploid fibroblasts.

centrifugation. After 24 h, triplicate dishes were either mock-treated or treated as indicated and further cultured for 8 h at 37°C. Then, the cells were lysed in 200 μl lysis buffer (5 mM Tris/phosphate pH 7.8, 400 μM DTT, 400 μM *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (Sigma Chemical Company, St. Louis, MO, USA), 10% glycerol and 1% Triton X-100) at room temperature for 20 min followed by centrifugation. Luciferase activity was measured with Luciferase Assay Substrate (Promega Corporation, Madison, WI, USA) on a Lumat LB9501 luminometer.

3. Results

3.1. Identification of two MMS-inducible cDNA fragments

In order to identify genes that are induced by DNA-damaging agents, human diploid skin fibroblasts were mock-treated or exposed to either UV-C, MMS or ionizing radiation. RNA was isolated 4 h after treatment and differential display was performed as described in Section 2. To confirm that differentially displayed fragments indeed represented differentially expressed genes, they were isolated from the polyacrylamide gel, re-amplified and used as a probe on a Northern blot (Fig. 1A). The nature of the corresponding genes was determined as described in Section 2. Here, we describe the identification of two MMS-inducible fragments (Mifs). Mif1 was 97% identical to an anonymous cDNA, that previously has been described as KIAA0025 (GenBank accession number D14695). Mif2 represents BiP/GRP78 (95% identity, GenBank accession number X87949).

3.2. Induction of Mifs by DNA-damaging agents

Fig. 1A shows the mRNA induction of Mif1 and Mif2 in MMS-treated human diploid skin fibroblasts. Induction occurred with similar kinetics for all non-transformed and transformed cells tested (Fig. 1B and data not shown), indicating that the induction is a general phenomenon. No induction was found for either Mif gene after exposure to UV-C or ionizing radiation, under conditions where the DNA damage-inducible gene *p21^{WAF-1}* was indeed induced. There are several explanations for this observation: e.g. the induction of Mif genes

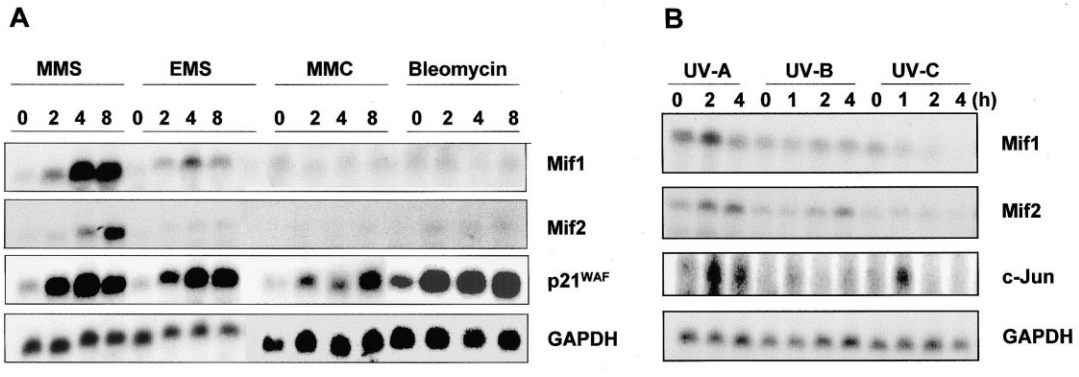

Fig. 2. A: Induction of Mif1 and Mif2 is not a general phenomenon of DNA-damaging agents. Induction of Mif1 and Mif2 by different DNA-damaging agents was assayed by Northern blotting. VH10 cells were treated with the alkylating agents MMS (1 mM) or EMS (20 mM), the crosslinking agent mitomycin C (MMC, 6 μg/ml), or the X-ray-mimicking agent bleomycin (Blm, 40 μg/ml). RNA was isolated after the indicated incubation periods. Lanes marked with time point zero indicate mock-treated cells. A probe for p21^{WAF-1} was used as a positive control for gene induction and a probe for GAPDH was used as a loading control. B: Induction of Mif1 and Mif2 by UV is wavelength-dependent. VH10 diploid fibroblasts were irradiated with either UV-A (150 kJ/m^2), UV-B (200 J/m^2) or UV-C (15 J/m^2). After irradiation, cells were cultured at 37°C as indicated and expression of Mif1 and Mif2 was tested by Northern blotting. Lanes marked with time point zero indicate mock-treated controls. A probe for the early response gene *cJun* was used as positive control.

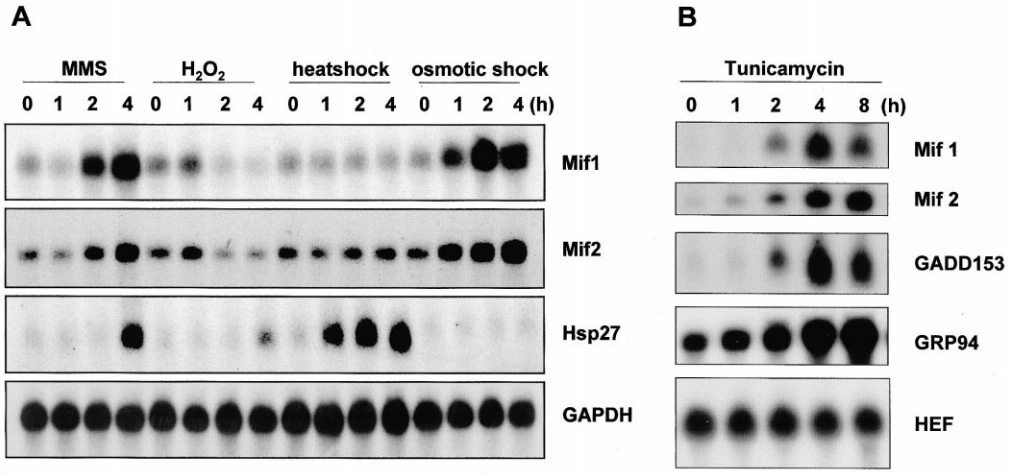

Fig. 3. A: *Mif1* and *Mif2* genes are activated by osmotic stress. Exponentially growing HeLa cells were treated with either MMS (1 mM) or hydrogen peroxide (0.5 mM) for the indicated incubation time. Heat shock was performed by incubating HeLa cells at 42°C for 5 min, followed by a further incubation at 37°C. Osmotic stress was given by adding NaCl to the medium up to an extra concentration of 0.3 M NaCl. After 5 min, the medium was removed and replaced by conditioned medium. The zero time points indicate mock-treated controls. Mif1 and Mif2 expression was tested by Northern blotting using a probe for the first 1000 bp of the KIAA0025 cDNA. Induction of Hsp27 is shown as a control for heat shock treatment. B: Mif1 is induced by tunicamycin. SV40-transformed VH10 cells were incubated with tunicamycin (10 μM) for the indicated period and induction of Mif1, Mif2, GADD153 and GRP94 was tested by Northern blotting. A probe for the human elongation factor 1α is used as a loading control.

after exposure to UV-C and X-ray has different kinetics, or the induction is specific for alkylating agents. To discriminate between these possibilities, human diploid skin fibroblasts were treated with different DNA-damaging agents and Mif expression was tested by Northern blotting. The results shown in Fig. 2A suggest that the induction of the two Mif genes is not a general response to genotoxic agents. Mif1 was induced by MMS and to a lesser extent by EMS. BiP, the gene that corresponds to Mif2, was induced solely after treatment with MMS. Treatment with the crosslinking agent mitomycin C or the X-ray-mimicking agent bleomycin did not result in activation of Mif genes. Induction of the DNA damage-inducible gene $p21^{WAF-1}$ is shown as a control.

To obtain more insight into the spectrum of treatments that induce the Mif genes, human diploid skin fibroblasts were irradiated with UV light of different wavelengths (UV-C: 100–290 nm, UV-B: 290–320 nm, UV-A: 320–400 nm), followed by RNA isolation at early time points (Fig. 2B). As shown, only UV-A irradiation resulted in an enhanced expression of both Mif genes. Furthermore, Mif2 was slightly inducible by UV-B but not by UV-C. Mif1 was not induced by UV-B, whereas UV-C resulted even in a decrease of the basal Mif1 mRNA level. Induction of the early response gene *cJun* is shown as a control.

Irradiation of cells with UV of increasing wavelengths leads to a relative increase in the production of reactive oxygen species, resulting in more damage to protein and membranes than to DNA. Likewise, MMS is supposedly an agent that, in comparison to EMS, produces more damage to proteins, membranes and organelles than to DNA [6]. In addition, apurinic sites, the lesions that remain after repair of methylated bases, are indeed not sufficient for the induction of BiP [12]. Our results suggest that Mif1 activation also requires a DNA damage-independent signal.

3.3. *Mif1 is a stress-inducible gene*

To test the hypothesis that Mif1 is induced by signals independent of DNA damage, HeLa cells were exposed to various kinds of stress. Addition of hydrogen peroxide resulted only in a slight increase in Mif1 mRNA in HeLa cells (Fig. 3A), and this induction was not found in H_2O_2-treated human diploid fibroblasts (data not shown). Heat shock treatment had no effect on Mif1 expression (Fig. 3A), while this treatment clearly induced heat shock protein hsp27 mRNA. In contrast, osmotic shock was found to be a strong inducer of Mif1 (Fig. 3A).

Furthermore, we show that not only BiP (Mif2) is induced by MMS ([12] and Fig. 1A,B) but also GRP94, another UPR-responsive ER-resident molecular chaperone (Fig. 1B). Similarly, some of the genes that were originally identified as DNA damage-inducible genes, such as GADD45 and GADD153, are also activated by ER stress ([11,12] and Fig. 3B). We subsequently tested whether Mif1 represents a novel member of the family of ER stress-responsive genes. Indeed we found

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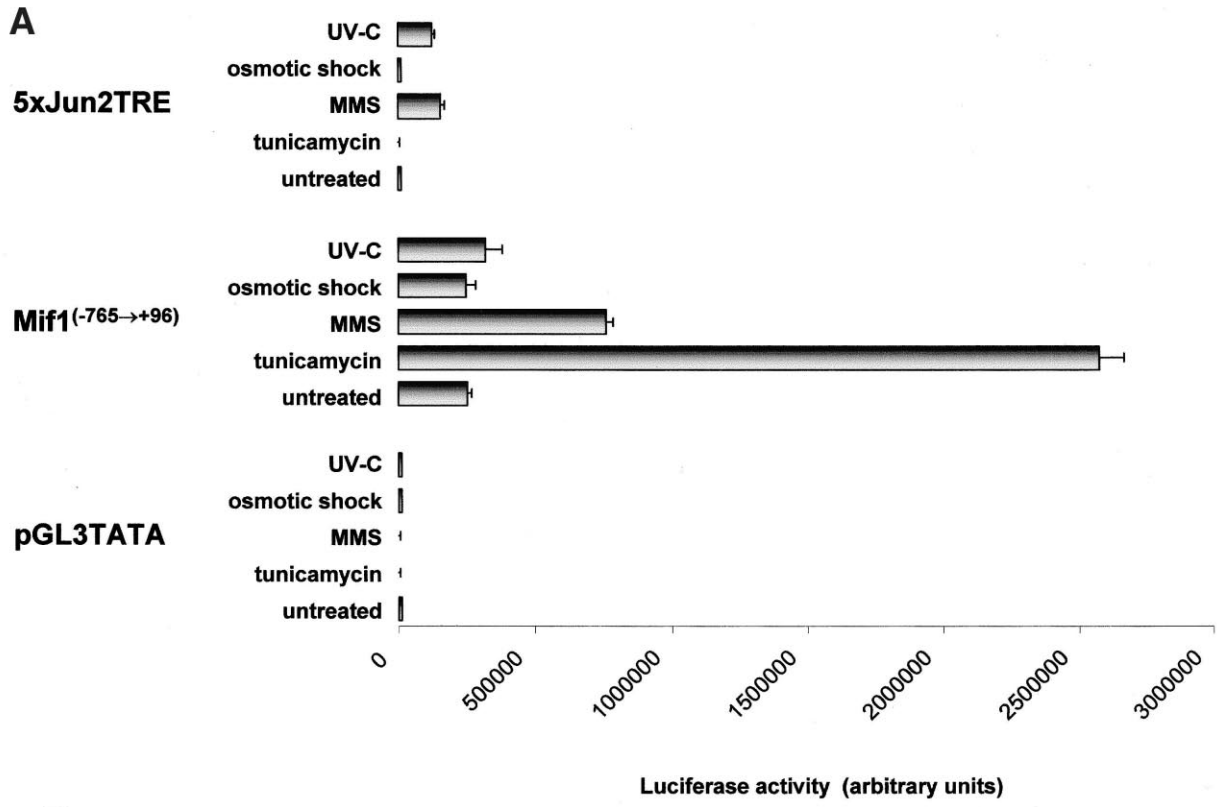
Fig. 4. A: The $Mif1^{(-765\rightarrow+96)}$ promoter fragment is activated by tunicamycin and MMS. HeLa cells were transfected with a luciferase reporter construct under control of the $Mif1^{(-765\rightarrow+96)}$ promoter fragment as described in Section 2. A 5xJun2TRE-luciferase construct was transfected as a positive control for UV irradiation and a pGL3TATA was included as a negative control. After 16 h, cells were either mock-treated or treated in triplicate with tunicamycin (10 μM), MMS (1 mM), osmotic shock (as described in Section 2) or irradiated with UV-C (15 J/m^2) and further incubated at 37°C for 8 h. Finally, the cells were lysed and luciferase activity was measured as indicated in Section 2. B: MMS and tunicamycin differentially regulate the *Mif1* gene. Luciferase reporter constructs under the control of promoter fragments as indicated (see Section 2) were transfected into exponentially growing HeLa cells. Triplicate dishes were mock-treated or exposed to MMS (1 mM) or tunicamycin (10 μM) 16 h after transfection for a period of 8 h. Then the cells were lysed and luciferase activity was measured as described in Section 2.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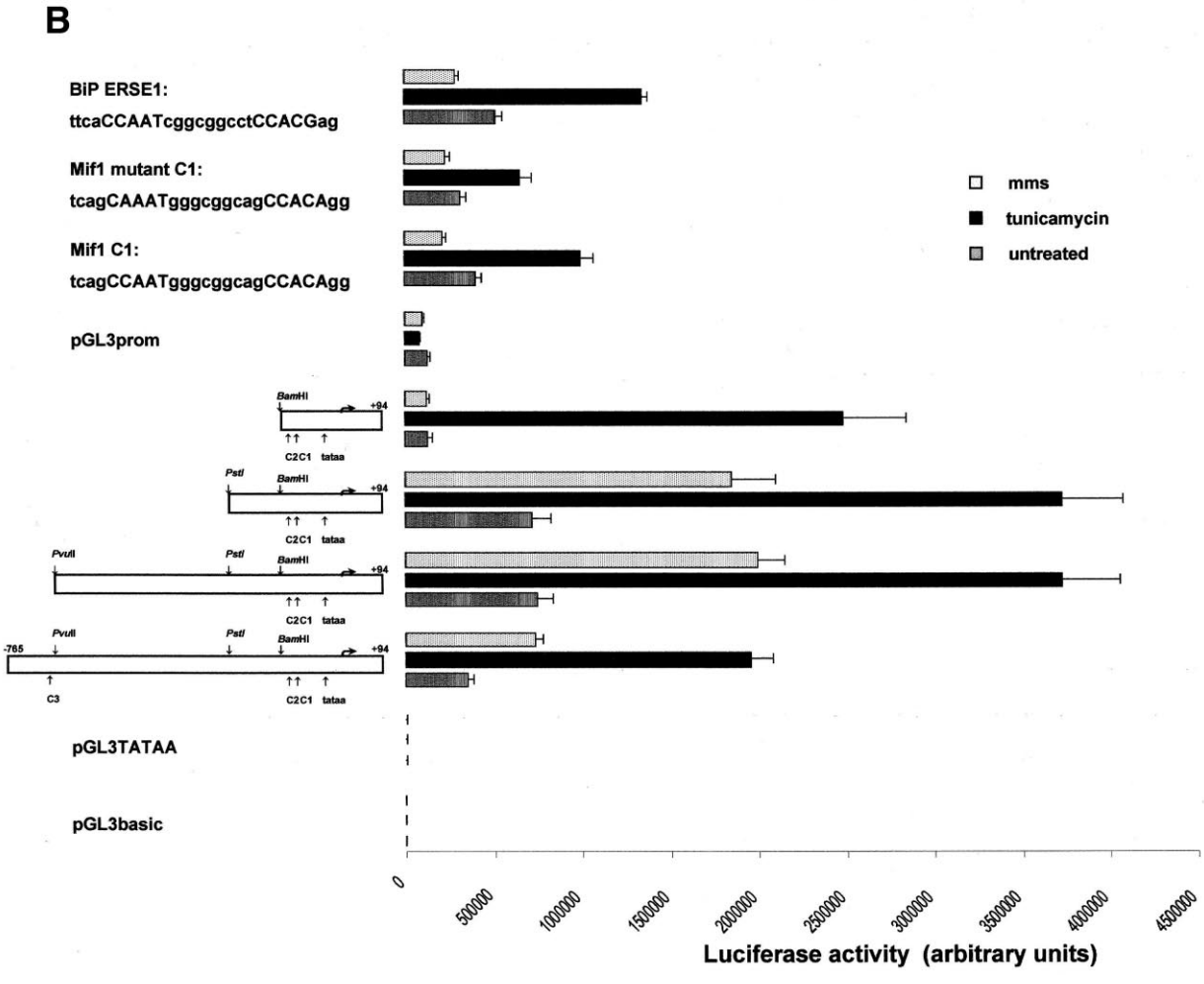
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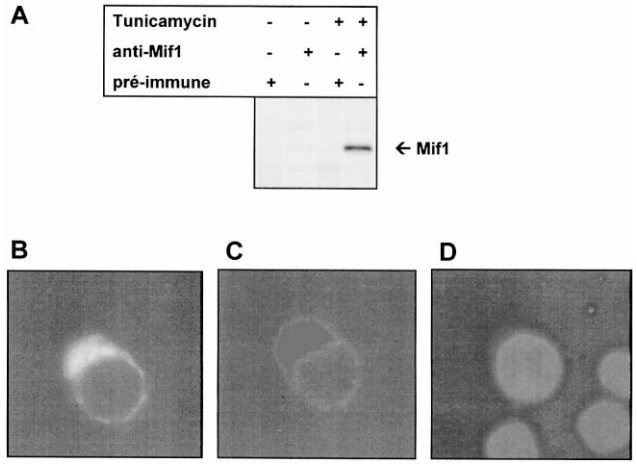

Fig. 5. A: Mif1 protein is induced upon treatment with tunicamycin. SV40-transformed VH10 fibroblasts were treated for 3 h with 10 μM tunicamycin followed by pulse labeling with [^{35}S]methionine for 2 h in the presence of tunicamycin. Mif1 protein was immunoprecipitated with rabbit polyclonal αMif1 antibody 421. The pre-immune serum of the corresponding rabbit was used as a negative control. B–D: Overexpressed Mif1 protein is localized in the ER. HeLa cells were transiently transfected with a myc-tagged Mif1 expression vector. 48 h after transfection, Mif1 protein was stained by double labeling with the αMif1 rabbit polyclonal antibody 421 (B) or the αmyc mouse monoclonal antibody 9E10 (C) and visualized with an FITC-conjugated anti-rabbit IgG or a rhodamine-conjugated anti-mouse IgG, respectively. DNA was stained with DAPI (D).

that Mif1 is strongly activated by the glycosylation inhibitor and ER stress inducer tunicamycin, with kinetics comparable to those of BiP, GRP94 and GADD153 (Fig. 3B).

3.4. 5′ Upstream sequence of the Mif1 gene contains an ERSE element that is sufficient for activation by tunicamycin

The *Mif1/KIAA0025* gene has been mapped to chromosome 16 [28]. To isolate a promoter fragment of the gene, we screened a chromosome 16 cosmid library. Two identical 4.2 kb subclones were isolated that contained a 3 kb fragment upstream of the Mif1 coding sequences, exon 1 (248 bp) and part of the first intron. An 861 bp *Mlu*I/*Nco*I fragment, corresponding to nucleotides −765 up to +96 of the *Mif1* gene, was cloned into the pGL3basic luciferase reporter vector. To test the inducibility of this construct, HeLa cells were transfected with this vector or with the control vector pGL3TATA or a 5xJun2TRE-luciferase vector, and were treated with either UV-C, MMS, osmotic stress or tunicamycin. Activation of the transcription activity of the reporter constructs was measured as described in Section 2. In agreement with the results obtained by Northern blotting, we did not observe a significant effect of UV-C on the Mif1 promoter activity, while the 5xJun2TRE, included as a positive control, was clearly induced by UV-C (Fig. 4A). Osmotic shock had no effect on luciferase activity in Mif1$^{(-765\rightarrow+96)}$-pGL3basic-transfected cells (Fig. 4A), suggesting that the element required for induction of Mif1 by osmotic stress is located outside this promoter region. In contrast, MMS or tunicamycin treatment resulted in an enhanced luciferase activity (Fig. 4A), although tunicamycin appears to be a stronger enhancer of the Mif1$^{(-765\rightarrow+96)}$ promoter activity than MMS.

Induction of BiP by tunicamycin requires multiple ERSE sequences in the promoter of the *BiP* gene. The Mif1$^{(-765\rightarrow+96)}$ region contains three ERSE-like sequences (Table 1). We called these ERSE-like sequences C1–C3. Reporter plasmids containing the luciferase gene controlled by the BiP ERSE1, the Mif1-C1 or a mutated mif1-C1 (Fig. 4B) were transfected into HeLa cells, followed by treatment with MMS or tunicamycin. The Mif1-C1 is as active in response to tunicamycin as the BiP ERSE1 (Fig. 4B). In contrast to results described for the BiP ERSE1, mutation of the second cytosine in the CCAAT-box of the Mif1-C1 to adenine had only little effect on induction of luciferase activity by tunicamycin (Fig. 4B). Furthermore, MMS could not activate ERSE1 of BiP or Mif1-C1, whereas the cells transfected with Mif1$^{(-765\rightarrow+96)}$-pGL3basic showed a clear induction after MMS treatment. This shows that the first ERSE of BiP or Mif1 is not sufficient for induction by MMS and suggests that activation of Mif1 by MMS does not involve the UPR.

3.5. Identification of an 122 bp promoter region that facilitates induction of Mif1 by MMS via an UPR-independent mechanism

To dissect the signalling pathways used to activate Mif1 by MMS or tunicamycin, deletion mutants were generated of the Mif1$^{(-765\rightarrow+96)}$ promoter fragment (Fig. 4B). Luciferase activity of lysates derived from HeLa cells transfected with these mutant promoter luciferase plasmids showed that the *Pvu*II$^{-654\rightarrow+96}$ and the *Pst*I$^{-262\rightarrow+96}$ fragments possessed an MMS-inducible promoter activity that is comparable to that of Mif1$^{(-765\rightarrow+96)}$ (Fig. 4B). Deletion up to the *Bam*HI site (*Bam*HI$^{-140\rightarrow+96}$) resulted in a strong decrease of basal activity and loss of MMS inducibility, while the activation of luciferase by tunicamycin was not affected (Fig. 4B). Thus, the fragment of 122 bp (*Pst*I-*Bam*HI) that lacks an ERSE is required for the induction of luciferase activity by MMS.

3.6. Mif1 protein level is enhanced by tunicamycin

To test whether induction of Mif1 mRNA results in an enhanced protein level, Mif1 protein was immunoprecipitated from a [^{35}S]methionine-labeled lysate derived from tunicamycin or mock-treated VH10SV cells. In untreated cells, Mif1 was detectable as a faint band of approximately 52 kDa (Fig. 5). Treatment with tunicamycin resulted in a 5–10-fold rise of the Mif1 protein level.

3.7. Overexpressed myc-tagged Mif1 is localized in the ER

We showed that the *Mif1* gene is activated by tunicamycin, a strong inducer of ER stress. In addition, we showed that the C1 region of the promoter has properties of a functional ERSE, indicating that *Mif1* is a UPR-regulated gene. Two classes of genes are controlled by UPR: those encoding ER-resident proteins or nuclear proteins. Therefore, we wished to determine the cellular localization of Mif1 protein. Since we failed to detect endogenous Mif1 protein by immunofluorescence, we transiently transfected HeLa cells with a myc-tagged Mif1 expression vector. Localization was established by immunofluorescence with both an anti-Mif1 polyclonal antibody

Table 1
ERSE-like sequences in the 5′ flanking region of the *Mif1* gene

BiP ERSE1 [7,8]:	CCAAT cggcggcct CCACG
Mif1-C3: −661:	CCAAT ttccctggg CCTCA strand: (−)
Mif1-C2: −123:	CCAAT cggcgtccg GATCC strand: (−)
Mif1-C1: −93:	CCAAT gggcggcag CCACA

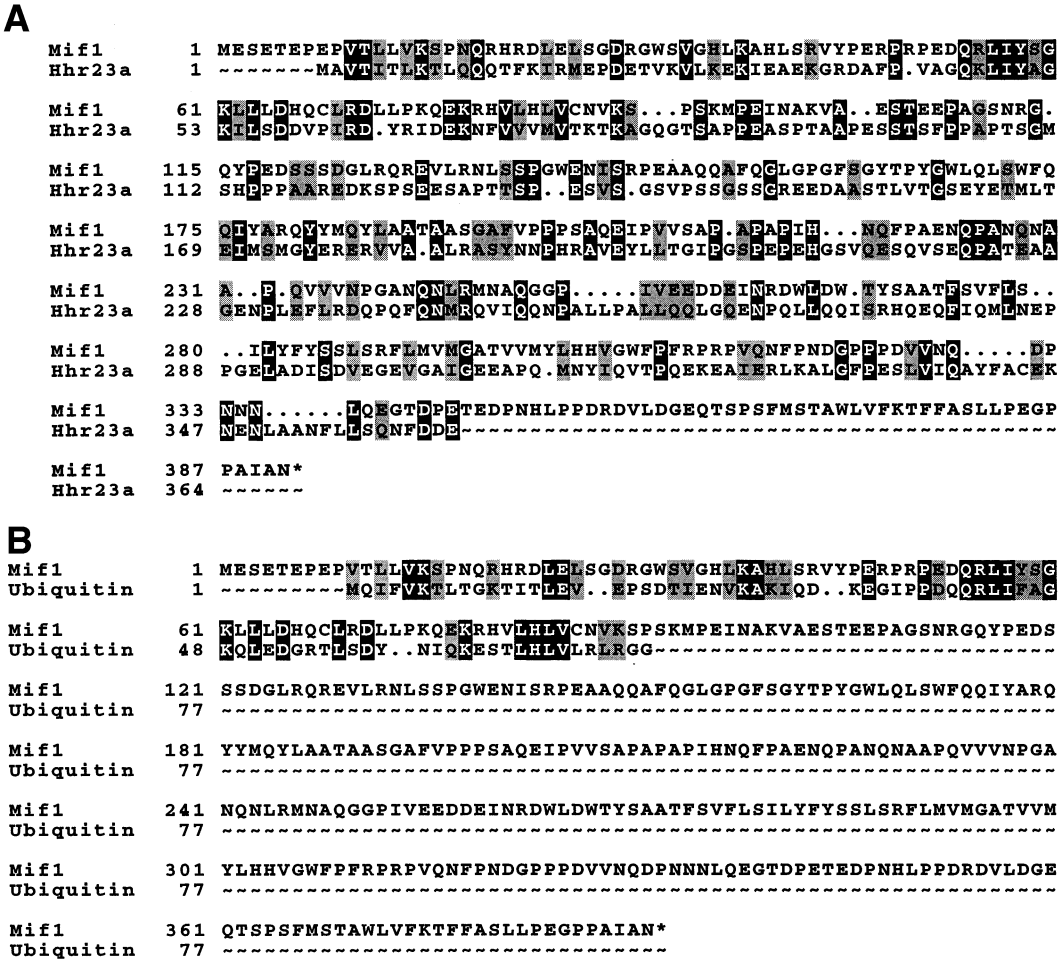

Fig. 6. Alignment of Mif1 and HHR23A protein sequences (A) or Mif1 and ubiquitin (B). Identical amino acids are presented in black boxes, whereas similar residues are show in gray boxes.

and an anti-myc monoclonal antibody. Mif1 was found around the nucleus (Fig. 5B,C), in a structure resembling the ER, just like the ER-resident protein GRP94 [29].

4. Discussion

4.1. Identification of MMS-inducible genes by differential display

MMS and other alkylating agents are well-known carcinogens which induce cellular stress by causing damage to DNA and proteins. When exposed to alkylating agents, cells respond in several ways, e.g. by the activation of transcription of stress-responsive genes. Among such genes are the DNA repair gene O6-methylguanine-DNA methyl-transferase [30], the early response genes c-*fos* and c-*jun* [31], and the late response gene collagenase [5]. These genes have in common that they are also induced by other classes of DNA-damaging treatments, such as ionizing radiation. A separate group of genes activated by alkylating agents also shows specificity towards protein-damaging treatments. In this report, we describe the identification of two MMS-inducible genes that probably belong to the latter group. One of the genes we identified by differential display is *BiP/GRP78*, which we called *Mif2*. This gene is known to be induced by agents that affect the ER, including alkylating agents. In this report, we show that also *GRP94*, another ER stress-inducible gene, is activated by MMS. In addition to *GRP94*, we identified *Mif1* as a novel MMS-inducible gene. Induction of this gene was detected after treatment with MMS, but not after exposure to UV-C or ionizing radiation.

4.2. Mif1 is a stress-inducible gene whose product is similar to the human DNA excision repair protein HHR23A

Database analysis revealed that Mif1 corresponds to KIAA0025, an anonymous ORF that was originally identified in the human immature myeloid cell line KG-1. Expression of this gene was found in all tissues tested [28]. No function or functional domains were reported for the putative protein.

In this paper, we report that activation of the *Mif1* gene may be involved in a stress response pathway. Induction of Mif1 by MMS was found in all cell lines tested. In addition, we showed that induction of Mif1 was not restricted to MMS. Osmotic stress, UV-A irradiation and treatment with the glycosylation inhibitor tunicamycin also led to the activation of the *Mif1* gene. Mif1 was not induced in diploid fibroblasts by either TNFα or H_2O_2, under conditions that we could detect activation of the stress-inducible transcription factor cJun (data not shown). This indicates that a recently identified

UPR-independent signal transduction pathway from the ER to the nucleus mediated by NFκB [32] will not stimulate Mif1 expression.

A Pearson and Lipman search for protein similarity using FastA revealed that the predicted protein, encoded by the *Mif1* gene, contains an N-terminal ubiquitin-like domain (UbL) and is similar (25% identity and 51% similarity over 261 residues) to the human nucleotide excision repair (NER) protein HHR23A (Fig. 6).

The function of the UbL remains unknown, although an increasing number of proteins containing such a sequence have been described. However, it has been shown that the UbL is essential for the function of yeast RAD23. Yeast cells became UV-sensitive upon deletion of the UbL from *RAD23*, while replacement of the UbL with ubiquitin rendered the cells UV-resistant [33]. Recently, it was shown that RAD23 protein interacts with the 26S proteasome via the N-terminal UbL and was rapidly degraded via the proteasome pathway. Consequently, RAD23 was stabilized in yeast strains bearing mutations in the proteasome subunits [34]. However, proteasomal proteolysis of RAD23 protein is not required for efficient NER [35]. Rather, it appears that binding of RAD23 to the 19S regulatory complex of the proteasome increases the rate and/or efficiency of NER. Further studies will have to elucidate whether the UbL of Mif1 associates to similar proteins.

4.3. *Mif1 is activated by UPR-dependent and UPR-independent mechanisms*

We showed that the *Mif1* gene is strongly activated upon treatment with the alkylating agent MMS, the glycosylation inhibitor tunicamycin or by osmotic shock.

Recently, two laboratories independently defined a consensus sequence in the promoter for most mammalian genes that are activated via the UPR upon ER stress. This sequence consists of a CCAAT-box, followed by a 9 bp GC-rich spacer region and a less well conserved CCACG motif. These ERSE sequences are found in multiple copies and in both orientations in the promoter of most mammalian ER stress-responsive genes [7,8]. The Mif1$^{(-765\rightarrow+96)}$ region contains three CCAAT motifs, followed by a 9 bp CG-rich region. The first ERSE sequence, which ends with a CCACA motif, is found close to the TATAA-box in the (+) strand of the *Mif1* gene. We showed that first ERSE indeed functions as a bonafide ERSE. In analogy to other UPR-responsive genes, two other ERSE-like sequences are found in the non-coding strand of the first 765 bp promoter region of Mif1. These results strongly suggest that Mif1 belongs to the group of UPR-responsive genes. This assumption is further underscored by the fact that overexpressed Mif1 was localized to the ER, although we are aware of the fact that an overexpressed protein will not necessarily reside in the same compartment of the cell as its endogenous counterpart, which is expressed at lower levels.

Induction of Mif1 by MMS or tunicamycin reaches similar levels when tested by Northern blotting. However, when tested in a luciferase assay, tunicamycin is a much more potent activator of Mif1$^{(-765\rightarrow+96)}$ than MMS. A simple explanation could be that induction of Mif1 by MMS requires multiple sites, of which at least one is present in the Mif1$^{(-765\rightarrow+96)}$ fragment, while other activating transcription factors bind outside this region. However, a disruption of the secondary structure in the DNA of the Mif1$^{(-765\rightarrow+96)}$ fragment could also be a likely explanation.

Studies with deletion mutants of the Mif1 promoter suggested that ERSE-like sequences may not be involved in the activation of Mif1$^{(-765\rightarrow+96)}$ by MMS. A 122 bp fragment lacking an ERSE was required for the enhanced activity after MMS treatment. Deletion of this fragment resulted in a loss of basal activity and inducibility by MMS. A TFSEARCH [36] using this 122 bp fragment as query revealed the presence of an E-box class B motif. The group of B-class regulatory proteins includes the upstream stimulatory factor (USF). USF is an ubiquitously expressed complex consisting of two polypeptides, USF1 and 2, that can bind to the E-box as either homo- or heterodimers. USF is involved in both basal transcription and induction of transcription upon treatment with insulin. Further studies will have to elucidate whether USF is indeed required for the activation of Mif1 by MMS.

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