Regulation of Multidrug Resistance 1 (MDR1)/P-glycoprotein Gene Expression and Activity by Heat-Shock Transcription Factor 1 (HSF1)*

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Received for publication, November 10, 1999, and in revised form, April 18, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M909136199

Infection of HeLa cells with adenovirus-carrying HSF1* cDNA, which encodes a mutated form of HSF1 with constitutive transactivation capacity, increased multidrug resistance 1 (MDR1) mRNA level and P-glycoprotein (P-gp) cell surface content and stimulated rhodamine 123 accumulation and vinblastine efflux activity. On the other hand, infection with adenovirus-carrying HSF70 and HSP27 cDNAs did not increase MDR1/P-gp expression. HSF1 regulates MDR1/P-gp expression at the transcriptional level, since HSF1* bound to heat-shock consensus elements (HSEs) in the MDR1 gene promoter and also activated the expression of an MDR1 promoter-driven reporter plasmid (pMDR1(−1202)). In addition, heat-shock increased pMDR1(−1202) promoter activity but not the activity of a similar reporter plasmid with point mutations at specific HSEs, and the heat-induced increase was totally inhibited by co-transfection with an expression plasmid carrying HSF1*, a dominant negative mutant of HSF1. The stress inducers arsenite, butyrate, and etoposide also increased pMDR1(−1202) promoter activity, but the increase was not inhibited (in the case of butyrate) or was only partially inhibited (in the case of arsenite and etoposide) by HSF1*. These results demonstrate that HSF1 regulates MDR1 expression, and that the HSEs present in the −315 to −285 region mediate the heat-induced activation of the MDR1 promoter. However, other factors may also participate in MDR1 induction by stressing agents.

The acquisition of the multidrug resistance (MDR) phenotype, defined as increased resistance against cytotoxic drugs with unrelated structures, represents one of the major obstacles for chemotherapy of tumors and other malignancies. One of the mechanisms that may account for MDR is the surface accumulation of the P-glycoprotein (P-gp), also called the multidrug transporter, which blocks the influx and/or increases the efflux of many hydrophobic agents, including some of the most commonly used anticancer drugs (for a review, see Ref. 1). In humans, P-gp is encoded by the MDR gene family, composed of two members, only one of which (MDR1) seems to be functionally linked to the development of the MDR phenotype (2). P-gp is expressed in some normal tissues, such as epithelial cells of kidney, liver, pancreas, and intestinal mucosa and capillaries of brain and testis. In these tissues, P-gp probably plays a physiological role, promoting the excretion of xenobiotics or preventing their absorption and also possibly acting as a chloride channel (reviewed in Refs. 1 and 3). As a consequence, cancers derived from these tissues may constitutively express P-gp and be intrinsically drug-resistant. However, in most cases P-gp-derived resistance is acquired during chemotherapy, e.g. in breast, bladder, lung, and ovary tumors, among others (for a review, see Ref. 3). Prolonged exposure to cytotoxic drugs may lead to the selection of cells with MDR1 gene amplification, at least under in vitro conditions (2, 4–6). However, even when amplification occurs, it does not always adequately explain the increased P-gp expression and drug resistance (6, 7). On the other hand, P-gp expression may also be de novo induced by short term exposures to cytotoxic agents such as UV light (8), actinomycin D (9), chemotherapeutic drugs (10), and inducers of the stress response (11, 12).

The heat-shock transcription factors (HSFs) were originally characterized as regulators of the expression of heat-shock protein (HSP) genes, through the binding to specific sequences (“heat-shock elements,” or HSEs) present in the promoter of these genes. The HSF family contains three members in humans, namely HSF1, HSF2, and HSF4 (13–15), among which HSF1 is specifically responsible for the stress-mediated HSP induction (16, 17). In unstressed cells, HSF1 is present in the cytoplasm as a monomer or forming heteromeric complexes. Upon treatment with stress inducers, HSF1 homotrimers, translocate to the nucleus, and bind the HSE (16, 17) to further acquire the transactivation capacity (18). Indirect proofs have suggested that HSFs could also participate in MDR1 gene expression. In fact, (a) HSEs have been identified in the MDR1 gene promoter (11, 12); (b) typical stress inducers such as heat-shock and arsenite, which induce HSP gene expression, also induce MDR1 gene expression in some cell types (11, 12); (c) some multidrug-resistant cell lines exhibit constitutively high HSF-DNA binding activity (19); and (d) quercetin, which inhibits HSF-HSE binding, also inhibits HSF-DNA binding and P-gp expression in multidrug-resistant cells (20). However, the problem is far from being clear, since (a) some work suggests that the activation of MDR1 expression by heat-shock and other stressing agents may be mediated by DNA sequences and transcription factors other than HSE and HSFs (21–23); (b) the possibility cannot be excluded that MDR1 gene induc-

* This work was supported by Dirección General de Enseñanza Superior e Investigación Científica (Spain) Grant PM97–0144 and Comunidad Autónoma de Madrid (Spain) Grant 08.1/0027/1997. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: MDR, multidrug resistance; HSE, heat-shock element; HSF, heat-shock factor; HSP, heat-shock protein; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; PCR, polymerase chain reaction.
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CMV pLpa SR (−) containing, or not containing, the cDNAs for human HSF1d202−316 (Ad−HSF1), inducible rat HSP70 (Ad−HSP70), or human HSP27 (Ad−HSP27), and the infectious plasmid JM17 (25–27). HeLa cells (at about 50% confluence) were infected at a multiplicity of infection of 5–10 PFU/cell.

Flow Cytometry Assays—The cells were detached from culture dishes by a brief incubation with PBS containing 0.04% EDTA at 37 °C, after which they were collected by centrifugation, washed, and resuspended in PBS at the desired concentration.

For determination of P-gp surface expression, indirect immunofluorescence assays were carried out using a mouse anti-human P-gp mAb (clone UIC2, Immunotech, Marseille, France). Cells were incubated for 30 min at 4 °C with the mAb and then washed three times with PBS and incubated again for 15 min at 4 °C with fluorescein isothiocyanate-conjugated anti-mouse IgG (Amersham Pharmacia Biotech). After washing the cells, the fluorescence was estimated by flow cytometry, using an EPICS FL flow cytometer (Coulter, Miami, FL).

The rhodamine 123 accumulation assay was carried out exactly as described by Frommel et al. (28). After 24 h of infection, triplicate cell samples were labeled for 18 h in culture medium containing 0.5 μCi/ml [3H]vinblastine (12.5 Ci/mmol, Amersham Pharmacia Biotech) and then washed three times with PBS and incubated again with fresh, [3H]vinblastine-free culture medium. At 15, 30, and 60 min, the whole medium was removed and replaced, and aliquots of the medium were used to determine the amount of effluxed vinblastine by scintillation counting. To determine the amount of vinblastine remaining in the cells, at the end of the experiment the cells were washed and lysed with PBS containing 0.1% Triton X-100. These data were used to determine the percentage of vinblastine associated with the cells at each time point.

Immunoblot Assays—Cell lysis, electrophoretic separation, blotting onto Immobilon-P membranes (Millipore Corp., Bedford, MA), and immunological detection of proteins were carried out essentially as described previously (29). The antibodies used were mouse anti-human HSP70 monoclonal antibody (clone C292S-5; StressGen Biotechnologies Corp., Victoria, Canada); mouse anti-human HSP27 monoclonal antibody (clone G3.1; StressGen); and mouse anti-chicken β-tubulin monoclonal antibody (Amersham Pharmacia Biotech).

RNA Blot Assays—Total RNA was prepared using an Ultraspec-II RNA isolation kit (Biotech Laboratories, Inc., Houston, TX), following the procedure described by the manufacturer. All other conditions, including the source and preparation of the HSP70-specific cDNA probes, were as described previously (30). Other probes were the 1.4-kilobase MDR1-specific EcoRI fragment of pDR54 plasmid (Ref. 31; American Type Culture Collection, number 61361) and the entire pTRI RNA 28S plasmid, which recognizes 28 S rRNA (Ambion, Inc., Austin, TX).

Gel Shift Assays—Whole cell extracts were prepared as described by Zuo et al. (18). The partially complementary oligonucleotides 5′-GGGAGCCACGGATCCGTTCCG-3′ and 5′-GGGAGCCATGTCGACG-3′ (containing AP-1 binding sites) were prepared and, when required, labeled with [32P]dCTP as earlier reported (29). For binding reactions, cell extracts containing 5 μg of proteins were mixed with 10 μl of Kingston buffer (120 mM KCl, 4 mM MgCl2, 0.24 mM EDTA, 0.6 mM phenylmethylsulfonyl fluoride, 10 μg/ml fetal calf serum, and 10% (v/v) glycerol, 24 mM HEPES, pH 7.9), 4 μg of salmon sperm DNA, and water to a final volume of 18 μl. After preincubating for 15 min on ice, 2 μl of labeled probe (approximately 10,000 cpm) was added, and the incubation followed for 15 min at room temperature. To prove the specificity of binding, when required the reactions were carried out in the presence of a 50-fold excess of unlabeled HSP70, MDR1, or AP-1 probes or in the presence of anti-HSF1 antibody (rabbit anti-human HSF1 polyclonal antibody; StressGen) or preimmune serum. The samples were electrophoresed in 4.5% native polyacrylamide gels, and the gels were dried and autoradiographed.

onation by stressing agents, instead of being a direct response, is mediated by increased HSPs expression, and (c) even if HSFs directly regulate MDR1 gene expression, it is not known which member of the family is actually implicated. In the present work, we analyzed the regulation of MDR1 gene expression by HSF1 using direct gene transfer procedures. This was made possible by the use of specific HSF1 mutants, namely a construction encoding an active mutant (HSF1+) (a deleted form of HSF1 with constitutive binding and transcriptional capacities) and a construction encoding a dominant negative mutant (HSF1-) (a deleted form that constitutively binds the HSP gene promoters but is unable to transactivate, even under stressing conditions) (18).

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—All components for cell culture were obtained from Life Technologies, Inc. (Life Technologies). Sodium arsenate and sodium butyrate were obtained from Merck, and etoposide was from Sigma. Human HeLa cells and human 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and antibiotics in a humidified 5% CO2 atmosphere at 37 °C. Sodium arsenate and sodium butyrate were dissolved before use in Dulbecco’s modified Eagle’s medium (DME, 0.1 mM). Etoposide was dissolved in Me2SO at 20 mM and stored at −20 °C. For treatments, HeLa cells were either placed in a bath for 2 h at 43 °C and then allowed to recover for 4 h at 37 °C; treated for 4 h with 100 μM sodium arsenite and then washed twice with Dulbecco’s modified Eagle’s medium and allowed to recover for 4 h in the absence of the drug; or continuously treated for 24 h with 5 mM sodium butyrate or 200 μM etoposide.

Plasmids and Transfection Assays—Expression plasmids were derived from pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) by insertion of cDNA genes for human HSF1 (HSF1wt), HSF1d202−316 (HSF1118), or bacterial β-galactosidase or derived from pcDNA1 (Invitrogen Corp.) by insertion of the cDNA gene for human HSF1d453−523 (HSF1−) (see Ref. 18 and references therein). The reporter plasmid pMDR1 (−1202) was produced by inserting a MDR1 promoter sequence (−1202 to +118) into the luciferase vector pGL2B (Promega, Madison, WI) (24). Mutagenesis of the −315 to −285 sequence of the MDR1 promoter was made by sequential polymerase chain reaction steps from plasmids pMDR1−(1202). The original sequence 5′-GCGGAGACATCTCCTCTGGAAATCCAAACGGT3′ was changed to 5′-GCGGAGACATCTCCTCTGGAATCCAAACGGT-3′, where the mutated bases are underlined. For this purpose complementary oligonucleotides A (5′-GCGGAGACATCTCCTCTGGAAATCCAAACGGTAAGTTC-3′) and B (5′-CACGTTGATTTCGGAGAATGTCGG-3′) were designed. At the same time, oligonucleotides F (5′-GTCATAGGCTAAGCAATGAC-3′) and R (5′-TTTATTAGATCTGGACGCAGTAGC-3′) corresponding to the polylinker flanking regions of plasmid pGL2B, were also synthesized and used as primers. Using the pMDR1−(1202) plasmid as a template, single PCRs with A and R, as well as B and F primers were performed. The amplification products were annealed with each other and extended by mutually primed synthesis. The fragment was then amplified by a second PCR step, in the presence of primers F and R. The product was digested with SnaI and inserted into pGL2B. The resulting reporter plasmid was designated as pMDR1−(1202)/mut. The site-directed mutagenesis was verified by DNA sequencing. pHS70-luc, a plasmid containing the human HSP70 promoter-driven luciferase gene, was kindly supplied by Dr. R. Voellmy (Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL).

Transfection was performed using the LipofectAMINE™ reagent (Life Technologies). Cells were seeded in 12-well plates at a density of 105 cells/well. The next day, the cells were incubated for 5 h with 1 ml of culture medium containing 225 ng of either pGL2B, pMDR1−(1202), pHS70-luc, or pMDR1−(1202)/mut plasmids, 225 ng of expression plasmids (when required), 50 ng of the β-galactosidase plasmid, and 2 μl of LipofectAMINE™ reagent. Following transfection, the mixture was removed, and the cells were incubated under standard conditions for 16 h, after which they were either harvested or subjected to heat shock. Luciferase assays were performed using a Luciferase Assay System (Promega) as a TD-20/20 Luxometer (Turner Designs, Sunnyvale, CA), the values being normalized in relation to protein concentration. Internal normalization of the transfection efficiency was performed using a chemiluminescent reporter gene assay system to detect β-galactosidase (Tropix Inc., Bedford, MA).

Viral Vectors and Infection Assays—Recombinant adenovirus vectors were generated by co-transfection of human 293 cells with plasmid AC-
RESULTS

MDR1/P-gp and HSP Expression—It was earlier reported that the HSF1 mutant was able to constitutively induce the expression of endogenous HSP genes in the absence of stress (27). Hence, this mutant was used to investigate whether HSF1 regulates MDR1/P-gp expression. With this in mind, we measured the MDR1 RNA level at different times after infection with Ade-HSF1+ or the viral vector (Ade), in comparison with untreated cultures (Cont), as determined by indirect immunofluorescence combined with flow cytometry. The profiles corresponding to Ade-infected and control cells totally overlapped. The experiments were repeated twice (A) or three times (B) with similar results.

Since the increase in HSP70 mRNA level apparently preceded the increase in MDR1 RNA level in Ade-HSF1+-infected cells (Fig. 1A), we questioned whether the HSF1-provoked stimulation of MDR1/P-gp expression could be a mere consequence of HSP accumulation. To investigate this possibility, cells were infected with Ade-HSF1+ as well as with viral vectors carrying inducible HSP70 and HSP27 cDNAs (Ade-HSP70 and Ade-HSP27, respectively). Immunoblot assays using anti-HSP70 and anti-HSP27 antibodies proved that, as expected, infection with Ade-HSP70 increased the level of HSP70 protein; infection with Ade-HSP27 increased the level of both HSP70 and HSP27; and Ade-infection was ineffective (Fig. 2A). When P-gp cell surface expression was measured, it was found to be greatly increased by Ade-HSF1+ infection but only slightly augmented by Ade-HSP70 and Ade-HSP27 infection (Fig. 2B). RNA blot assays confirmed the increase in MDR1 mRNA by Ade-HSF1+ but not by Ade-HSP70 or Ade-HSP27 (Fig. 2C). Hence, the increase in MDR1/P-gp expression in HSF1+-infected cells may not be adequately explained as a
consequence of HSPs accumulation.

**Rhodamine Accumulation and Vinblastine Efflux**—Earlier reports indicated that P-gp cell surface expression could be increased without modification in drug accumulation and/or efflux (33, 34). To test the functionality of the HSF1-mediated increase in P-gp, we comparatively measured rhodamine accumulation as well as vinblastine efflux activity in Ade-infected and Ade-HSF1 \(^1\)–infected cells. Some of the obtained results are indicated in Fig. 3. Under the used experimental conditions, a subpopulation of cells with decreased rhodamine 123 accumulation was observed in Ade-HSF1 \(^1\)–infected cultures, in comparison with Ade-infected cultures (Fig. 3A). In a similar manner, Ade-HSF1 \(^1\) infection accelerated the rate of vinblastine efflux, when compared with Ade-infected cultures (Fig. 3B). Hence, HSF1 activity sufficed to stimulate the expression of the multidrug resistance phenotype, as measured by altered P-gp transport activity.

**Transcriptional Regulation**—We queried whether the HSF1-mediated increase in MDR1/P-gp expression was regulated at the transcriptional level. This was first investigated by means of gel shift assays, using a MDR1 promoter-derived oligonucleotide. Although the MDR1 promoter contains multiple HSEs (11), it was reported that those present in the –315 to –285 region seem to mediate the induction of MDR1 by arsenite, an inducer of the stress response (12), and hence this sequence was selected as the MDR1 probe (MDR1wt probe) (Fig. 4A). In addition, a similar sequence in which point mutations at the HSEs were introduced (MDR1mut probe) and a HSE-containing HSP70 promoter-derived oligonucleotide (HSP70 probe) were used as negative and positive controls, respectively (Fig. 4A). The obtained results are represented in Fig. 4B. Extracts from Ade-HSF1 \(^1\)–infected cells bound the MDR1wt probe with a similar pattern as in the case of the HSP70 probe, while no binding was observed using the the MDR1mut probe. The binding to the MDR1wt and HSP70 probes was specific, since it was not detected when using extracts of Ade-infected cells or when the incubation was carried out in the presence of anti-HSF1 antibody or excess homologous (MDR1wt or HSP70) probes. On the other hand, it was not affected by excess MDR1mut or heterologous (AP-1) probes.

The results obtained by gel shift assays were corroborated and extended by transient transfection assays. For this purpose, we used a MDR1 promoter-driven luciferase gene construction (pMDR1(–1202) plasmid) and a similar construction with the same point mutations in the –315 to –285 region, which disrupted HSF1 binding (pMDR1(–1202mut plasmid). It was found that pMDR1(–1202) activity was greatly increased by co-transfection with HSF1, while the same assay caused a much lower increase of pMDR1(–1202mut activity (Fig. 5). Taken together, these results demonstrate that HSF1 regulates MDR1 expression at the transcriptional level and suggest that the HSE sites located in the –315 to –285 region are critical for such a transcriptional regulation.

**Effect of Stress Inducers**—While the preceding results demonstrated that MDR1/P-gp expression is susceptible to regulation by HSF1, they did not prove that HSF1 is in fact responsible for MDR1 induction under stressing conditions. To investigate this possibility, cells transfected with either pMDR1(–1202) or pMDR1(–1202mut were subjected to heat-shock, which is the most typical stress inducer. It was observed that heat effectively increased pMDR1(–1202) promoter activity but not pMDR1(–1202mut activity (Fig. 6A). This proved that the HSEs present in the –315 to –285 region are specifically required for the heat-provoked induction of MDR1 expression. As a second experimental approach, heat-shock was applied to cells co-transfected with pMDR1(–1202) plus one of
the following expression plasmids: HSF1 wild type (HSF1wt), which binds the HSE but does not transactivate in the absence of stress (18); a dominant negative HSF1 (HSF1−); or the empty vector (pcDNA3.1). The results are represented in Fig. 6B. It was observed that heat-shock activated pMDR1(−1202) promoter activity in both pcDNA3.1- and HSF1wt-transfected cells, while the activation was totally inhibited by HSF1− transfection. In addition, it was observed that the basal pMDR1(−1202) activity reached similar values in nonheated HSF1wt-transfected and pcDNA3.1-transfected cells. Taken together, these results indicate that HSF1 mediates the heat-provoked activation of MDR1 expression and that the mere binding of HSF1 does not suffice to transactivate the MDR1 promoter, as was reported in the case of HSP gene promoters (18).

Finally, we wanted to know whether HSF1 could also mediate the induction of MDR1/P-gp by other agents. With this aim, cells co-transfected with pMDR1(−1202) plus either HSF1− or pcDNA3.1 were treated with the stress inducer sodium arsenite, the differentiation inducer sodium butyrate, and the antitumor drug etoposide, all of which were reported to activate MDR1 expression in different cell types (10–12, 23, 34). Parallel determinations were carried out using a HSP70 promoter-driven reporter plasmid (pHSP70-luc) instead of pMDR1(−1202). The obtained results are indicated in Fig. 7. It was observed that arsenite, butyrate and etoposide increased HSP70 promoter activity, and the increase was totally inhibited by co-transfection with HSF1− (Fig. 7A). In this sense, not only arsenite, but also butyrate and etoposide, may be strictly considered inducers of the stress response. These agents also increased pMDR1(−1202) promoter activity, but in this case the increase was not inhibited (butyrate) or was only partially inhibited (arsenite and etoposide) by HSF1− (Fig. 7B).

**DISCUSSION**

The role of MDR1/P-gp expression as one of the main determinants of the MDR phenotype is of great importance in understanding the mechanisms of the regulation of this gene and to generate strategies to inhibit its expression and/or function. The human MDR1 promoter contains multiple elements, which point to a complex regulation. Thus, it has been demonstrated...
Although the only goal of the present work was to analyze the MDR1 gene regulation by HSF1, the obtained results may also have some clinical relevance. Among the different strategies conceived to overcome the P-gp-mediated MDR (for a review, see Ref. 44), those commonly used consist in the administration of agents such as verapamil, cyclosporin A, and related compounds, which bind P-gp and block its transport activity. By contrast, the attempts to specifically inhibit MDR1 expression have been of little effect until now. In this regard, our present results indicate that it could be possible to prevent MDR1/P-gp expression by inhibiting HSF1-HSE binding or HSF1-directed transactivation. For instance, antitumor drugs rapidly induce MDR1/P-gp expression in cultured cells (10), and MDR1/P-gp is actually increased after chemotherapy in different cancers (e.g. acute leukemia, breast cancer, neuroblastoma, and pheochromocytoma, among others). If such induction is mediated at least in part by HSF1, as indicated by our experiments using etoposide, then it could be attenuated by gene transfer procedures using the HSF1' mutant. The same procedure might help to prevent MDR1 induction by other stressful albeit common clinical situations (e.g. fever, which might mimic in vivo the effect of heat-shock in vitro). Moreover, HSF1 might help to prevent the undesirable induction of Hsp70 expression. This later aspect is also interesting, since Hsp70 and other Hsps may reduce the lethality of antitumor drugs and other cytotoxic insults by preventing apoptosis (for a review, see Ref. 45) and hence provide an alternative mechanism for drug resistance. A detailed analysis of the involvement of HSF1 in the regulation of MDR1/P-gp expression in different tumor cells, and of the different factors that may contribute to drug resistance is required to define the feasibility of this hypothesis.

Acknowledgments—We are greatly indebted to Dr. R. Voellmy (Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL) and Dr. R. Mestril (The Cardiovascular Institute, Loyola University Medical Center, Maywood, IL) for Hsp70 reporter plasmid, HSF1 expression vectors, and adenoviral vectors, to Dr. K. W. Scotto (Memorial Sloan-Kettering Cancer Center, New York) for the pMDR1(−1202) reporter plasmid, to Dr. C. Cale’s (Dept. of Bioquímica, Universidad Autónoma, e Instituto de Investigaciones Biomédicas, CSIC, Madrid) and Dr. E. Páez (Centro de Investigaciones Biológicas, CSIC, Madrid) for laboratory facilities, and to Drs. R. Voellmy and A. Corbi (Centro de Investigaciones Biológicas, CSIC) for critical reading of the manuscript.

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that MDR1 is susceptible to positive regulation by the Sp1 (35, 36), AP-1 (37), NF-IL6 (38), NF-Y (24), EGR1 (39), YB-1 (23), and MEF-1 (40) transcription factors; to negative regulation by cross-coupling of the NF-xBp65 and c-Fos factors (41); and to either positive or negative regulation by p53 (42, 43). Although earlier reports identified the presence of HSEs in the MDR1 promoter, the present work provides the first direct demonstration that HSF1 regulates MDR1/P-gp expression and drug transport activity. Moreover, the HSF1-mediated regulation of MDR1 exhibits some characteristics earlier reported for HSP genes, such as activation by heat-shock and separate regulation of the HSF1 binding and transactivation capacities (18). This indicates that MDR1 may be strictly considered a stress gene. However, the present results also indicate that the regulation of MDR1 and HSP70 by stress inducers is not identical and suggest that factors other than HSF1 may account, at least in part, for the induction of MDR1 by some stress agents. In fact, while HSF1 sufficed to explain the activation of the HSP70 promoter by butyrate, arsenite, and etoposide, this factor was either irrelevant (in the case of butyrate) or insufficient (in the case or arsenite and etoposide) to fully explain the activation of the MDR1 promoter by these agents. In this regard, a recent report indicated that the activation of the MDR1 promoter by butyrate is mediated by the NF-Y (24), and our preliminary observations indicate that its activation by etoposide is partially regulated by AP-1.2

N. E. Vilabon, A. Galán, A. Troyano, E. de Blas, and P. Aller, unpublished results.
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