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# Original Research Communication

# Opposite Expression of the Antioxidant Heme Oxygenase-1 in Primary Cells and Tumor Cells: Regulation by Interaction of USF-2 and Fra-1

ANATOLY SAMOYLENKO,[1] ELITSA Y. DIMOVA,[1] TINA HORBACH,[1] NADIYA TEPLYUK,[2] STEPHAN IMMENSCHUH,[3] and THOMAS KIETZMANN[1]

## ABSTRACT

**Heme oxygenase-1 is the rate-limiting enzyme for the degradation of the prooxidant heme. Previously, we showed that an E-box within the HO-1 promoter is crucial for the regulation of HO-1 expression in primary hepatocytes. Further to investigate the importance of this E-box, we determined the regulatory capacity of the E-box–binding factor USF-2 in primary cells in comparison with transformed cell lines. We found that HO-1 expression was inhibited by USF-2 in primary cells, whereas it was induced in tumor cell lines. Mutation of either the E-box or the AP-1 site within the HO-1 promoter only partially affected the USF-dependent regulation. However, this regulation was dramatically reduced in tumor cells and completely abolished in primary cells transfected with an HO-1 promoter construct containing mutations in both the E-box and the AP-1 site, suggesting that AP-1 factors and USF-2 may act in a cooperative manner. Indeed, protein–protein interaction studies revealed that USF proteins interacted with Fra-1. Further, the USF-dependent HO-1 promoter activity was not detectable with an USF-2 mutant lacking residues of the USF-specific region (USR) or the transactivation domain encoded by exon 4. Together, these data suggest that USF-2 has opposite regulatory roles for HO-1 gene expression in primary cells and tumor cell lines.** *Antioxid. Redox Signal*. 10, 1163–1174.

## INTRODUCTION

Microsomal heme oxygenase (HO; EC 1.14.99.3) catalyzes the oxidative cleavage of heme, which is the initial step of heme degradation. During this reaction, carbon monoxide (CO), $Fe^{2+}$, and biliverdin are produced (61). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase (62). Several HO isoforms have been identified from which HO-1 is the inducible isoform (41). With its substrate heme, HO-1 expression is induced in response to various stimuli (1, 3, 22, 25, 33, 34, 42, 49, 57 68). The enhancement of HO-1 caused by these stimuli appeared to serve as an adaptive and defensive response to cellular stress (12), which was confirmed in experiments (41) with HO-1–deficient mice (41, 46).

The principal mechanism by which most, if not all, agents regulate HO-1 is the modulation of gene transcription (12). Transcriptional regulation of HO-1 is mediated by a network of signaling pathways, which modulate activities of various transcription factors such as AP-1 and Nrf2. We previously found that an E-box in the rat HO-1 promoter played a crucial role for HO-1 expression in primary hepatocytes (29); thereby, the transcription factor Max was identified as an E-box–binding target. However, it remained open whether only the E-box served as a binding site for Max or whether other basic–helix-loop-helix (bHLH) transcription factors such as Myc or USF

[1]Department of Biochemistry, University of Kaiserslautern, Kaiserslautern, Germany.
[2]Institute of Molecular Biology and Genetics, Kiev, Ukraine.
[3]Institute of Clinical Immunology and Transfusion Medicine, Justus-Liebig-Universität Giessen, Giessen, Germany.

can bind. In addition, regulation of HO-1 expression involved an AP-1 site, which suggests a putative crosstalk between E-box–binding bHLH proteins and AP-1 family members. Among the bHLH proteins, USF appears to be important for HO-1 expression, because it was considered to be involved in the induction of HO-1 expression by ultraviolet A light (44, 52) and cadmium (19, 40), as well as to exert cell type–specific effects. Of the two principal USFs known, USF-2 appears to be more important, because its absence in mice causes growth retardation and early death, whereas deficiency of USF-1 was almost without effect (63). Thus, it was the aim of the present study to investigate whether USF-2 may also have a crucial role for rat HO-1 expression in either an inducible or a repressive manner. Because interspecies differences in the regulation of HO-1 gene expression in human and rat cells (30), as well as cell type–specific differences in the function of USF (11, 15, 47) were reported, we used rat primary hepatocytes (PHCs), rat hepatoma H4IIE cells, human primary pulmonary artery smooth muscle cells (PASMCs), human hepatoma HepG2 cells, and human HeLa cells in the present study.

## MATERIALS AND METHODS

All biochemicals and enzymes were of analytic grade and were purchased from commercial suppliers.

### *Animals*

Male Wistar rats (200–260 g) were kept on a 12-h day/night rhythm with free access to water and food. Rats were anesthetized with pentobarbital (60 mg/kg body weight) before preparation of hepatocytes.

### *Cell culture*

Primary rat hepatocytes (PHCs) were isolated by collagenase perfusion as described (18). The cells were cultured at 37°C on 6-cm Falcon culture dishes under air/$CO_2$ (19:5) in medium 199 with Earle's salts containing bovine serum albumin (2 g/L), $NaHCO_3$ (20 m*M*), Hepes (10 m*M*), streptomycin sulfate (117 mg/L), penicillin (60 mg/L), insulin (1 n*M*), and dexamethasone (10 n*M*). Fetal calf serum (FCS) (5%) was present in the initial 5 h, after which cultures were incubated in serum-free medium for another 18 h. Then the medium was changed again, and the cells were further cultured in serum-free medium for 24 h. HepG2, HeLa, and H4IIE cells were cultured at 37°C on 6-cm Falcon culture dishes in MEM supplemented with 10% FCS, 1% nonessential amino acids solution (Gibco), streptomycin sulfate (117 mg/L), and penicillin (60 mg/L). Three hours before transfection and 5 h after transfection of HepG2 and H4IIE cells, as well as 24 h after transfection of HeLa cells the medium was changed. PASMCs were purchased from Cambrex (Verviers, Belgium) and cultured as recommended in Quantum 212 medium (PAA Laboratories, Linz, Austria). Medium was changed 3 h before transfection. HEK293 cells were maintained in MEM supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotics.

### *Plasmid constructs*

The luciferase reporter gene constructs pHO-754, pHO-754ΔA (21), and pHO-754Em (29) were previously described. The luciferase reporter gene construct pHO-754ΔA-Em was generated from pHO-754ΔA with the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) by using the oligonucleotide 5′-GGCTCAGCTGGGCGGCCACctctagACTCGAGTAC-3′. Expression vectors for wild-type, (64) USF2, USF-2-ΔTDU2 (35), U2ΔE4 (36), U2ΔE5 (47), U2ΔUSR (39), and ΔHU2a (64), as well as p-HA-Fra-1 (20), were already described.

### *Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)*

Nuclear extracts were prepared essentially as described (28). The sequences of the HO-1 oligonucleotides used for the EMSA are 5′-GGCTCAGCTGGGCGGCCACcacgtgACTCGAGTAC-3′ (spanning the wild-type E-box (−47/−42) and 5′-GGCTCAGCTGGGCGGCCACctctagACTCGAGTAC-3′ (containing the mutated E-box) (Fig. 1). Equal amounts of complementary oligonucleotides were annealed and labeled by 5′-end labeling with ($\gamma$-$^{32}$P)ATP (Amersham) and T4 polynucleotide kinase (MBI). They were purified with the Nucleotide Removal Kit (Qiagen). Binding reactions were carried out in a total volume of 20 $\mu$l containing 50 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* EDTA, 5% glycerol, 10 $\mu$g of nuclear extract, 250 ng poly d(I-C), and 5 m*M* DTE. After preincubation for 5 min at room temperature, 1 $\mu$l of the labeled probe ($10^4$ cpm) was added, and the incubation was continued for an additional 10 min. For supershift analysis, 1 $\mu$l of the USF1 (C20), USF2 (N18), Myc (C33), Max (C17), or Sp1 (PEP2-G) antibody (all obtained from Santa Cruz Biotechnology, Heidelberg, Germany) was added to the EMSA reaction, which was then incubated at 4°C for 2 h. The electrophoresis was then performed with a 5% non-denaturing polyacrylamide gel in TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 5 m*M* EDTA) at 200 V. After electrophoresis, the gels were dried and exposed to a phosphoimager screen.

### *RNA isolation, Northern blot analysis, and hybridization*

Isolation of total RNA and Northern blot analysis was performed as described (27). Digoxigenin (DIG)-labelled antisense RNAs served as hybridization probes; they were generated as described (26) by *in vitro* transcription from pBS-HO-1 (800-bp cDNA fragment) by using T3 RNA polymerase or from pBS-$\beta$-actin (550 bp cDNA fragment) by using T7 RNA polymerase and NA labeling mixture containing 3.5 m*M* 11-DIG-UTP, 6.5 m*M* UTP, 10 m*M* GTP, 10 m*M* CTP, 10 m*M* ATP. Hybridizations and detections were carried out essentially as described (27). Blots were quantified as outlined in statistical analysis.

### *Western blotting and immunoprecipitations*

Western blot analyses were performed by using the following antibodies: anti-HO1 (Stressgene), anti-USF-1, anti-USF-2, and anti HA-tag (Santa Cruz Biotechnology). The secondary

antibody was either an anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase (Santa Cruz Biotechnology). The enhanced chemiluminescence (ECL) system (Amersham, Freiburg, Germany) was used for detection. For co-immunoprecipitation experiments, 150-$\mu$g protein samples were cleared with 2 $\mu$g of antibody precoupled to protein-G-Sepharose (Amersham, Freiburg, Germany). The samples were then subjected to Western blot analyses and quantified as outlined in statistical analysis.

### *Cell transfection and luciferase assay*

Freshly isolated rat hepatocytes ($\sim 1 \times 10^6$ cells per dish), HepG2, HeLa, H4IIE cells ($\sim 4 \times 10^5$ cells per dish) were transfected as described (21), thereby controlling transfection efficiency by cotransfection with 0.25 $\mu$g *Renilla* luciferase expression vector (pRLSV40) (Promega). In brief, cells were transiently transfected with 2.5 $\mu$g plasmid DNA containing 500 ng of pRL-SV40 (Promega) to control transfection efficiency and 2 $\mu$g of the appropriate HO-1 promoter *Firefly* luciferase (FL) construct. Every culture experiment was done in duplicate. PASMCs were transfected by using Metafectene (Biontex) according to instructions provided by the supplier. PHC, PASMC, HepG2, H4IIE, and HeLa cells were harvested 48 h after transfection, and Luc activity was recorded in a luminometer by using the dual luciferase assay kit (Promega). From the two dishes in each single experiment, the respective mean of Luc activity was calculated, and according to that, the percentage value of each dish was determined. After three experiments, the respective standard deviation was calculated according to the six percentage values from each dish.

### *Statistical analysis*

Statistical comparisons of absorbance differences were performed by using the Mann–Whitney test (Statview 4.5; Abacus Concepts, Berkeley, CA), and $p$ values $\leq 0.05$ were considered significant. Luc values presented are expressed as mean $\pm$ SEM. Results were compared by ANOVA for repeated Luc measurements followed by the Newman–Keuls test. A probability level $p \leq 0.05$ was accepted as significant.

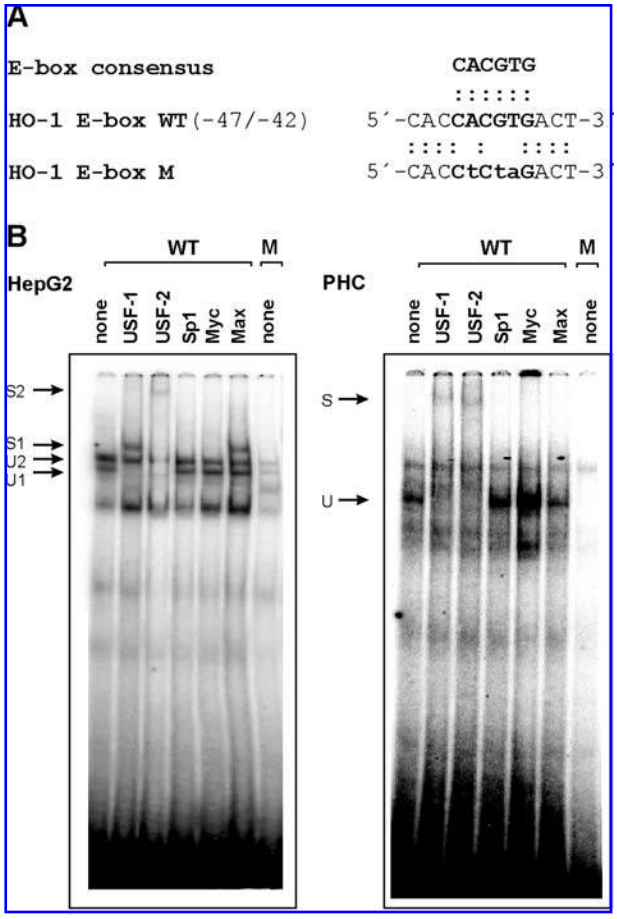


**FIG. 1. Binding of USF to the E-box sequence of the rat HO-1 promoter.** (**A**) The E-box consensus sequence and the oligonucleotides-spanning E-box sequence of the rat HO-1 promoter. Mutations in the oligonucleotide are indicated by lowercase letters. (**B**) The respective $^{32}$P-labeled oligonucleotide was incubated with 7 $\mu$g nuclear extracts. In supershift experiments, the nuclear extracts from HepG2 or PHCs were preincubated on ice for 45 min with 0.5 $\mu$g USF-1, USF-2, Sp1, Myc, or Max antibody before adding the labeled probe. The DNA–protein complexes were separated by electrophoresis on 5% native polyacrylamide gels and visualized with phosphoimaging. U, USF protein complexes, S, supershifted complexes.

## RESULTS

### *Binding of USF to the E-box of the rat HO-1 promoter*

Because the rat HO-1 promoter contains an E-box (5′-CACGTG-3′, −47/−42) matching the canonic USF-binding sequence (see Fig. 1A) (53), the possible binding of USF to this site was investigated with EMSA. When the oligonucleotide was incubated with the nuclear extract from HepG2 cells, two major protein complexes (U1 and U2) were formed; these were no longer detectable when a mutated oligonucleotide was used (Fig. 1B, left). Addition of the USF-1 antibody to the EMSA reaction inhibited formation of complex U1, whereas the USF-2 antibody inhibited the formation of both complexes. Both USF-1 and USF-2 antibodies led to formation of supershifted complexes. To ensure the specificity of the supershift mediated by the USF-1 and USF-2 antibodies, the EMSA was also performed in the presence of an antibody against the GC-box–binding factor SP-1. The SP-1 antibody did not affect complex formation with the E-box oligonucleotide. Further, EMSA was performed with Myc and Max antibodies, from which the Max antibody was previously shown to produce a supershift (29). Again, addition of the Max antibody led to formation of a supershifted complex, whereas incubation of the E-box oligonucleotide with an antibody against Myc did not result in a supershift or inhibition of complex formation.

The binding of nuclear proteins from primary rat hepatocytes (PHCs) to the E-box–containing oligonucleotide from the rat HO-1 promoter also was examined (Fig. 1B, right). Incubation of nuclear extracts from PHC with this oligonucleotide led to the formation of only one strong complex (U). The formation

of the U complex was no longer detectable with the PHC nuclear extracts and hardly detectable when oligonucleotides containing mutations in the HO-1 E-box were used. Again, addition of either USF-1 or USF-2 antibodies to the binding reaction with the PHC nuclear extracts led to disruption of the U complex and to formation of a supershifted complex. The SP-1 and Max antibodies did not affect the U complex, whereas the Myc antibody appeared to form a stronger U complex. Thus, in both HepG2 hepatoma cells and PHCs, USF-1 and USF-2 could bind to the HO-1 E-box.

### *Regulation of HO-1 mRNA and protein expression by USF in different cell types*

Because USF-2 was shown to have a major role *in vivo* (63), we used it predominantly for further experiments. USF-mediated regulation of HO-1 mRNA expression was investigated with Northern blot analysis in human HepG2 hepatoma cells, rat H4IIE hepatoma cells, and rat PHCs. The various cells exhibited different expression levels of HO-1 mRNA when normalized to $\beta$-actin mRNA levels. Whereas human HepG2 and rat H4IIE cells showed low levels of HO-1 mRNA expression, primary rat hepatocytes (PHCs) had higher levels of endogenous HO-1 mRNA expression. When HepG2 and H4IIE cells were transfected with the vector encoding wild-type USF-2, HO-1 mRNA expression was induced to ~350% and 600%, respectively, when compared with the respective control (Fig. 2A). By contrast, in rat PHCs, USF-2 overexpression inhibited HO-1 mRNA expression by ~60% (Fig. 2A). The observed effects of USF-2 appeared to be specific for HO-1, because USF-2 overexpression did not change expression of $\beta$-actin in control experiments (Fig. 2B). These results demonstrated that USF-2 overexpression upregulated HO-1 mRNA expression in both the human and rat hepatoma cell lines but inhibited it in rat PHCs.

Because the cell types under investigation expressed different HO-1 mRNA levels, we also expected a similar type of regulation for HO-1 protein expression. HepG2 cells contained the lowest, and PHCs, the highest amount of HO-1 protein when normalized to $\beta$-actin. However, after transfection of HepG2 and H4IIE cells with the USF-2 vector, HO-1 protein expression was induced by ~400% and 500%, respectively, when compared with the controls (Fig. 2A). By contrast and in line with the mRNA data, overexpression of USF-2 inhibited HO-1 protein expression by ~60% in rat PHCs (Fig. 2A). In addition, USF-1 overexpression had similar effects on HO-1 expression in these cells (data not shown).

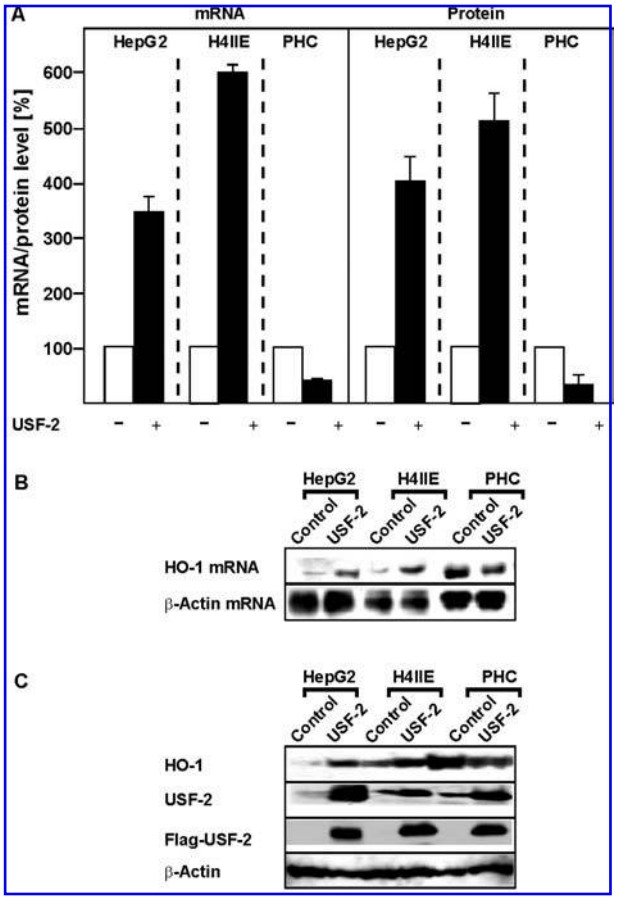


**FIG. 2. USF-dependent regulation of rat HO-1 mRNA levels in different cell types.** HepG2, H4IIE cells, and PHCs were transfected with either an empty vector or a USF-2 expression vector (see Materials and Methods). (**A**) The HO-1 mRNA levels were measured with Northern blot, and HO-1 protein levels, with Western blot. HO-1 mRNA and protein levels in the controls were set to 100%. Values were presented as mean ± SEM of at least three independent experiments. (**B**) Representative Northern blot: 20 $\mu$g of total RNA isolated from cultured HepG2, H4IIE cells, and PHCs were subjected to Northern blot analyses and hybridized with DIG-labeled HO-1 and $\beta$-actin antisense RNA probes. (**C**) Representative Western blot: 100 $\mu$g total protein from HepG2, H4IIE cells and PHCs were subjected to Western blot analyses with HO-1 and $\beta$-actin antibodies, respectively.

### *Regulation of HO-1 promoter–controlled luciferase expression by overexpression of USF-2 in HepG2 hepatoma cells and primary rat hepatocytes*

The role of USF-2 in the regulation of HO-1 expression was investigated in the liver hepatoma cell line, HepG2, and in PHCs. Both cell types were transfected with expression vectors encoding wild-type human USF-2 together with either a wild-type −754-bp HO-1 promoter Luc gene construct (pHO-754) or with –754-bp HO-1 promoter Luc gene constructs in which the E-box (–47/−42) (pHO-754-Em) was mutated. Because previous data indicated that interaction of proteins that bind to the E-box with AP-1 family members is likely (5, 29, 32, 45), we used HO-1 reporter gene constructs, in which the CRE/AP-1 element (–668/−654) (pHO-754ΔA) (21) or both the E-box and the CRE/AP-1 (pHO-754ΔA-Em) binding sites were mutated (Fig. 3A). USF-2 overexpression enhanced Luc activity by about 50-fold when cotransfected with the wild-type pHO-754 Luc construct in HepG2 cells (Fig. 3B). The USF-2–dependent induction was reduced by about 50% when either the construct pHO-754-Em or pHO-754ΔA was used in HepG2 cells. The construct pHO-754ΔA-Em with double mutation additionally reduced USF-2–dependent induction of Luc activity

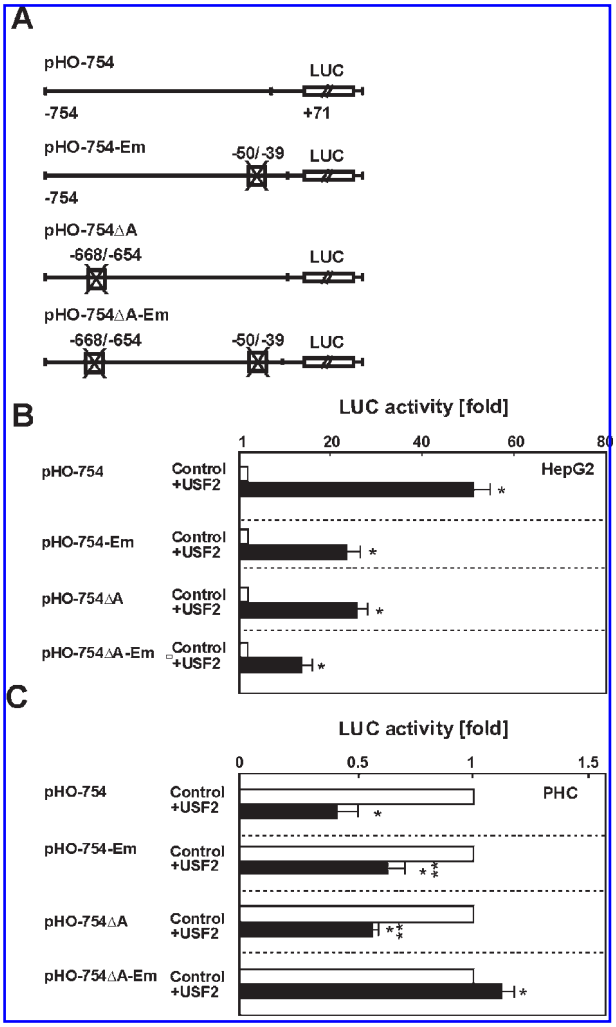


**FIG. 3. Regulation of rat HO-1 promoter constructs by USF-2 in HepG2 and PHCs.** (**A**) The wild-type rat HO-1 promoter (pHO-754), the HO-1 promoter mutated in the classic E-box (pHO-754-Em), the HO-1 promoter mutated in the AP-1 element (pHO-754ΔA), and the HO-1 promoter mutated in both the E-box and AP-1 element (pHO-754ΔA-Em) were used. (**B**) HepG2 and PHC cells were cotransfected with either the USF-2 expression vector or empty control vector and Luc gene constructs given in (**A**), as described in Materials and Methods. The luciferase activity was indicated as fold induction compared with the Luc activity, measured in the respective controls. Values represent means ± SEM of three independent experiments, each performed in duplicate. *Significant difference control *versus* USF-2. **Significant difference pHO-754 + USF2 *versus* pHO-754-Em + USF-2 or pHO-754A + USF-2.

by ~25% (Fig. 3B). By contrast, in PHCs transfected with pHO-754 and USF-2, Luc activity was repressed by ~60% compared with the control (Fig. 3C). Repression by USF-2 was slightly but significantly reduced when PHCs were transfected with the construct pHO-754-Em or pHO-754ΔA (Fig. 3C). However, when PHCs were cotransfected with pHO-754ΔA-Em, USF-2–dependent repression of Luc activity was completely abolished. Thus, these data support that the E-box and the AP-1 site contribute to USF-2–dependent induction of the HO-1 promoter in HepG2 cells and to USF-2–dependent regulation of the HO-1 promoter in PHCs.

### *Regulation of HO-1 promoter–controlled Luc expression by overexpression of USF-2 in primary human pulmonary artery smooth muscle cells, in HeLa, and in H4IIE cell lines*

To investigate whether the observed difference in USF-2–dependent HO-1 gene regulation between primary rat hepatocytes and human HepG2 hepatoma cells exists only in liver-derived cells or whether it is species specific, we further tested primary human pulmonary artery smooth muscle cells (PASMCs), the human cervix carcinoma cell line, HeLa, and the rat hepatoma cell line H4IIE. In line with our findings from PHCs, we found that Luc activity was also repressed by ~50% when PASMCs were transfected with pHO-754 and USF-2 (Fig. 4B). Again, regulation by USF-2 was abolished on the use of the HO-1-Luc construct mutated at both the AP-1 site and E-box. By contrast, in HeLa cells cotransfected with pHO-754 and USF-2, Luc activity was induced by ~45-fold (Fig. 4B). The USF-2–dependent induction of Luc activity was then decreased by ~50% when the construct pHO-754ΔA-Em was cotransfected into HeLa cells. Further, cotransfection of pHO-754 and USF-2 in H4IIE cells resulted in induction of Luc activity, which was also reduced by ~50% when the double-mutant construct pHO-754ΔA-Em was used.

Thus, these data again support the involvement of both the E-box and the AP-1 site in the USF-2–dependent regulation of HO-1 gene expression. In addition, these results demonstrated that USF-2 acted as inhibitor of HO-1 promoter-driven Luc activity in all tested primary cells and as an inducer in all tested tumor cell lines.

### *Regulation of HO-1 promoter activity by overexpression of Fra-1 in HepG2 cells and PHCs*

So far, the only protein from the AP-1 family that may have an effect on E-box–binding proteins is Fra-1 (45). To investigate the effect of Fra-1 and its influence on the HO-1 promoter, HepG2 cells and PHCs were transfected with an Fra-1 expression vector together with pHO-754, pHO-754-Em, pHO-754ΔA, or pHO-754ΔA-Em Luc constructs. Fra-1 overexpression enhanced Luc activity by about twofold when cotransfected with the wild-type pHO-754 Luc construct in HepG2 cells (Fig. 5B). The Fra-1–dependent increase of Luc activity was not changed when the construct pHO-754-Em was used, whereas the mutation of the AP-1 binding site in the construct pHO-754-ΔA completely abolished it. Further, Fra-1 had almost no effect on the double-mutated construct pHO-754ΔA-Em (Fig. 5B). Interestingly, when PHCs were cotransfected with pHO-754 and Fra-1, Luc activity did not change compared with the control (Fig. 5B). Cotransfection of pHO-754-Em and Fra-1 had no effect on Luc activity. However, when the mutant constructs pHO-754ΔA and pHO-754ΔA-Em were used, we observed an Fra-

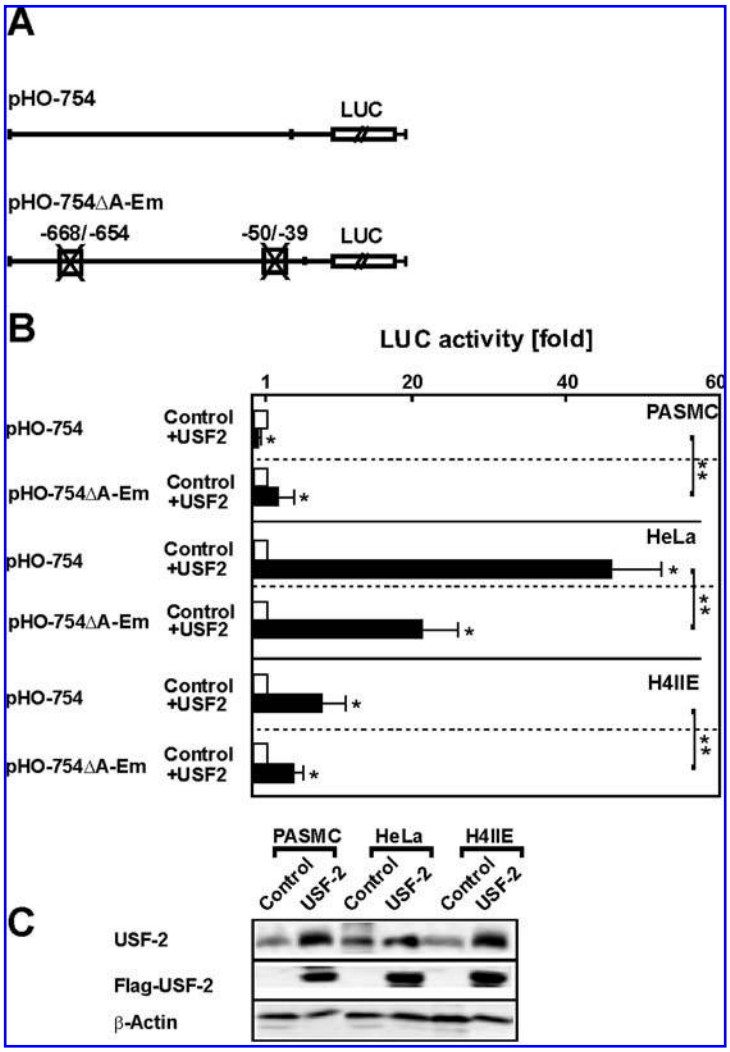


**FIG. 4. Regulation of rat HO-1 promoter constructs by USF-2 in PASMC, HeLa, and H4IIE cells.** (**A**) The wild-type rat HO-1 promoter (pHO-754), the HO-1 promoter mutated in the E-box (pHO-754-Em), and the HO-1 promoter mutated in both the E-box and AP-1 element (pHO-754ΔA-Em) were used. (**B**) PAMSC, HeLa, and H4IIE cells were cotransfected with either the USF-2 expression vector or empty control vector and Luc gene constructs given in (**A**), as described in Materials and Methods. The luciferase activity was indicated as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean ± SEM of three independent experiments, each performed in duplicate. *Significant difference, control *versus* USF-2. **Significant difference, pHO-754+USF2 *versus* pHO-754ΔA-Em+USF-2. (**C**) Representative Western blot: 100 $\mu$g total protein from PASMC, HeLa, and H4IIE cells was subjected to Western blot analyses with USF-2, Flag-tag, and $\beta$-actin antibodies, respectively.

1–dependent induction of Luc activity. Together, these data suggest that the possible interaction between USF-2 and Fra-1 may be responsible for the cell-type–specific effects observed.

## *USF interacts with Fra-1*

Because we detected a complete loss of USF-dependent HO-1 regulation when the mutant pHO-754ÄA-Em lacking both the E-box and the AP-1 binding site was used, we hypothesized that this may be due to an interaction between USF-2 and AP-1. Therefore, we determined whether this may be the case in our system, thus constituting another way for modulating USF-dependent HO-1 regulation. In several co-immunoprecipitation experiments, we tested whether Jun, Fos, and Fra interacted with USF. Whereas Jun and Fos did not display an interaction with USF (data not shown), USF-1 and USF-2 interacted with Fra-1 (Fig. 6). Thus, the interaction of USF proteins with Fra-1 appears to be important for the regulation of HO-1 expression.

## *Identification of the USF-2 functional domain(s) responsible for HO-1 regulation in HepG2 cells and PHCs*

To determine which domains of USF-2 were involved in the regulation of HO-1 gene expression, the wild-type rat HO-1 promoter Luc construct pHO-754 and plasmids expressing wild-type USF-2, as well as various mutants, were cotransfected. These mutants included the protein ΔTDU2 (ΔAA 1-198), which lacks the transactivation domain but contains the USF-specific region (USR); the protein U2ΔE4 (ΔAA 96-158), which does not contain amino acids encoded by exon 4 in the transactivation domain; the protein U2ΔE5 (ΔAA 144-188), which does not contain amino acids encoded by exon 5 in the transactivation domain; the protein U2ΔUSR (ΔAA 208-230) lacking the USR; and the protein ΔHU2a (ΔAA 275-282) lacking the second helix of the HLH domain (Fig. 7A).

In HepG2 cells cotransfected with pHO-754 and USF-2, Luc activity was increased by ~50-fold (Fig. 7B). The Luc activity in pHO-754 and ΔTDU2 cotransfected HepG2 cells was re-

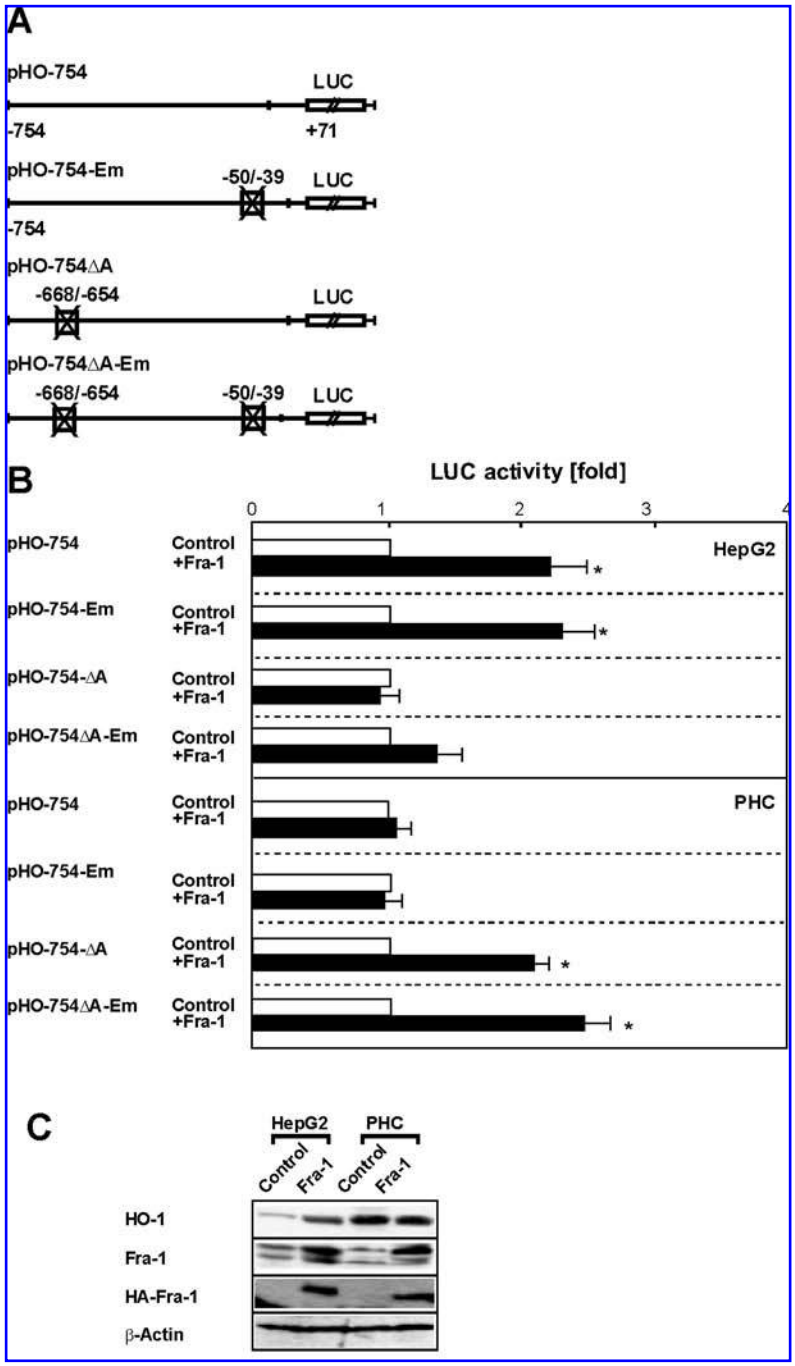


**FIG. 5. Regulation of rat HO-1 promoter constructs by Fra-1 in HepG2 and PHCs.** (**A**) The wild-type rat HO-1 promoter (pHO-754), the HO-1 promoter mutated in the E-box (pHO-754-Em), the HO-1 promoter mutated in the AP-1 element (pHO-754ΔA-Em), and the HO-1 promoter mutated in both the E-box and the AP-1 element (pHO-754ΔA-Em) were used. (**B**) HepG2 cells and PHCs were cotransfected with either the Fra-1 expression vector or empty control vector and Luc gene constructs given in (**A**), as described in Materials and Methods. The luciferase activity was indicated as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean ± SEM of three independent experiments, each performed in duplicate. *Significant difference in control *versus* Fra-1. (**C**) Representative Western blot: 100 $\mu$g total protein from HepG2 cells and PHCs was subjected to Western blot analyses with HO-1, Fra-1, HA-tag, and $\beta$-actin antibodies, respectively.

duced to about sixfold, and in pHO-754 and either U2ΔE4 or U2ΔUSR cotransfected cells, Luc activity was about the same as in the control. In HepG2 cells cotransfected with pHO-754 and the U2ΔE5 vector, Luc activity was still increased by ~3.6-fold. The USF variant ΔHU2a, lacking a part of the HLH domain, was ~20 times less efficient than wild-type USF-2 because of impaired DNA-binding and dimerization activity. However, some transactivation activity remained, because after cotransfection of the ΔHU2a vector and the pHO-754 construct, Luc activity was induced by ~2.3-fold (Fig. 7B). In PHCs cotransfected with pHO-754 and either USF-2 or ΔTDU2, the Luc activity was decreased by ~60%, whereas use of USF2ΔE5 reduced the Luc activity by ~15% (Fig. 7C). By contrast, overexpression of either U2ΔE4, U2ΔUSR, or ΔHU2a mutants did not have inhibitory effects on Luc activity in PHCs.

These data suggest that the USR and the amino acids encoded by exon 4 in the USF-2 transactivation domain are mainly required for the USF-2–dependent regulation of HO-1 expression in both HepG2 hepatoma cells and rat PHCs, whereas the amino acids encoded by exon 5 seemed to play a minor role.

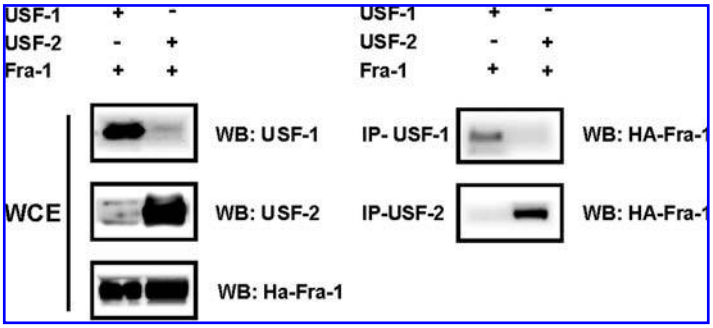


**FIG. 6. Interaction between USF-1 and USF-2 with Fra-1.** Cells were transfected with vectors encoding USF-1, USF-2, and HA-tagged Fra-1. Protein expression levels were checked with Western blot analyses in whole-cell extracts (WCEs). Immunoprecipitation experiments were carried out with USF-1 and USF-2 antibodies, respectively. Fra-1 was detected in the precipitates after Western blot analyses with HA-tag antibody.

## DISCUSSION

In this study, it was demonstrated that the transcription factor USF-2 acts as inhibitor of the rat HO-1 gene expression in rat PHCs and human PASMCs, whereas it acts as inducer in human HepG2, HeLa, and rat H4IIE tumor cell lines. This type of HO-1 regulation is modulated by interaction of USF proteins with the AP-1 family member Fra-1. Further, the effect of USF-2 on HO-1 gene expression appears to be dependent on the presence of the USR and the portion of the transactivation domain encoded by exon 4.

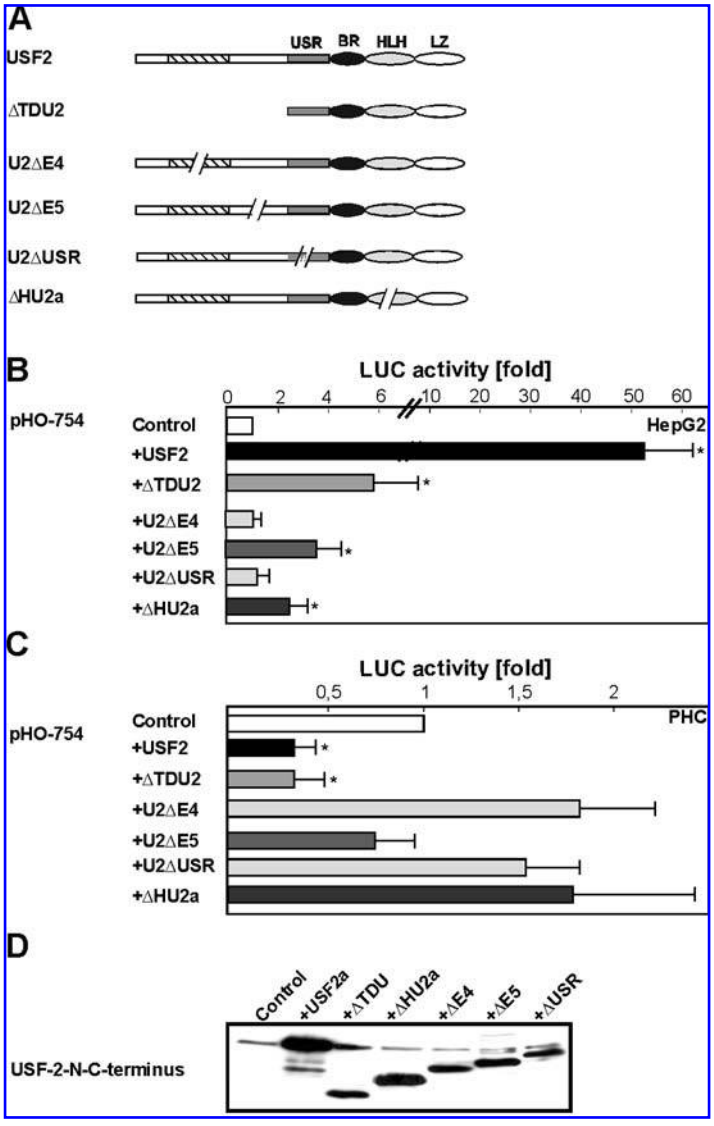


**FIG. 7. Regulation of rat HO-1 promoter constructs by wild-type and mutant USF-2 in human and rat hepatoma cells.** (**A**) Scheme of wild-type and mutant USF-2, ΔTDU2, U2ΔE4, U2ΔE5, U2ΔUSR, and ΔHU2a proteins. (**B, C**) The rat HO-1 promoter construct pHO-754 was cotransfected either with an empty vector or with expression vectors encoding wild-type or different USF-2 mutants displayed in **A** into HepG2 cells and PHCs. The luciferase activity was expressed as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean ± SEM of four independent experiments, each performed in duplicate. Statistics used Student's *t* test for paired values. *Significant difference control *versus* USF-2 variant. (**D**) Expression of USF-2 and its mutant forms. The 100-$\mu$g cell lysates from the luciferase assays (**B**) were analyzed with Western blotting with a mixture of antibodies recognizing the USF-2 N-terminus and USF-2 C-terminus to monitor the expression levels of USF-2 or USF-2 mutants, respectively.

### *Differential regulation of HO-1 gene expression*

Although the capability to induce HO-1 was demonstrated for a wide variety of agents, such as heme (61), UV light (25), heavy metals (42), and inflammatory cytokines (1, 49), much less is known about agents mediating HO-1 repression. The repression of heme- and IL-6–induced HO-1 gene expression by dexamethasone was demonstrated in rabbit coronary endothelial cells (14). Interferon-$\gamma$ also decreased HO-1 mRNA levels in the human glioblastoma cell line T98G (59). Controversial effects have been reported for the regulation of HO-1 by hypoxia. Hypoxia was shown to inhibit HO-1 expression in human umbilical vein endothelial cells (HUVECs), human coronary arterial endothelial cells, human astrocytes (43), the human glioblastoma cell line T98G, and the human lung cancer cell line A549 (31), whereas, conversely, hypoxia induced HO-1 expression in rat aortic vascular smooth muscle cells (VSMCs) (34), rat myocytes (16), rat PHCs (37), and rat C6 glioma cells (31).

Whereas the transcriptional mechanisms responsible for HO-1 induction appear to involve several transcription factors, such as Nrf-2, AP-1, STAT-3, or Max, which can bind to either the proximal promoter region or distal promoter elements (50), so far only one repressor is known. This repressor, known as Bach-1, targets two distal mouse HO-1 enhancer regions. These enhancer regions, which are located at $-4$ and $-12$ kb of the mouse HO-1 gene promoter, have been shown to play a major role for the inducible HO-1 expression, because they contain several copies of the antioxidant response element (ARE), which serves as a nuclear target for the transcription factor Nrf2 (2, 50). The repressor Bach-1 regulates the availability of the ARE for the transcriptional activator Nrf2, as demonstrated in bach1/nrf2-deficient mice (58), and thereby inhibits HO-1 transcription.

We demonstrated that the cell-specific repressive effect of USF-2 on HO-1 expression is mediated *via* the AP-1 site and the E-box within the proximal promoter region of the rat HO-1 gene. This would add USF-2 as an additional repressor of HO-1 gene expression.

Because a number of studies propose an interspecies difference between HO-1 gene regulation in human and rat cells (31), we assumed that this might be relevant also for the effects of USF. Although we found that the cells under study expressed different levels of HO-1, our study did not reveal an interspecies difference in HO-1 regulation by USF-2, because in rat PHCs and primary human PASMCs, HO-1 was repressed, but in rat H4IIE hepatoma cells, in human hepatoma HepG2, and in HeLa cells, HO-1 was induced. Instead, our experimental results imply that a different mode of regulation by USF-2 exists between primary cells and proliferating cell lines. Our findings that the USF-mediated effects on HO-1 gene expression are not species specific are supported by other studies showing that USF proteins are involved in upregulation of HO-1 by ultraviolet A light (44) and cadmium (19, 40).

### *The upstream stimulatory factors as inhibitors and activators*

USF was originally identified from HeLa cell nuclei as an activator of the adenovirus major late promoter (53) and was later shown to activate a number of genes, including regulators involved in cellular proliferation, such as p53 (48), cyclin B1 (13), and transforming growth factor $\beta_2$ (54), as well as glucose-controlled genes such as fatty acid synthetase (65) and L-type pyruvate kinase (35). Although an inhibitory role of USF in the regulation of gene expression is much less investigated, it was also observed in experiments studying the immunoglobulin heavy-chain enhancer gene in NIH 3T3 cells (8), the rabbit CYP1A1 gene in RK13 cells (60), rat plasminogen activator inhibitor-1 (PAI-1) in rat PHC (51), and aortic preferentially expressed gene-1 (APEG-1) in rat aortic smooth muscle cells (10). Thus, the findings of our study with the HO-1 gene in rat PHCs and PASMCs are another example for the role of USF as an inhibitor of gene expression. The findings of our study with the HO-1 gene support the idea that USF transcriptional activity is cell-type dependent. This is supported by findings with the PAI-1 gene, in which USF inhibited PAI-1 expression in rat PHCs (51), whereas USF was shown to induce PAI-1 expression in renal epithelial cells (NRK-52E, clone EC-1) (67) and HepG2 cells (15). Thus, it appears that in primary cells, USF acts as an inhibitor of gene expression, whereas in most cell lines, it acts as an activator or, as in Saos osteosarcoma cells, remains transcriptionally inactive. The results of this study, that the HO-1 promoter regulation by USF-2 required especially two regions of the transactivation domain (the USR and the portion of the transactivation domain encoded by exon 4) support a model in which the activity of USF proteins is controlled by interaction with a coactivator.

### *The involvement of the E-box and the AP-1 site in the USF-dependent regulation of HO-1 expression*

Transcription factors of the bHLH-zip family such as USF, Myc, Max, and TFE3 (4, 8, 24), as well as the bHLH-PAS family, such as HIF-1$\alpha$, ARNT, and AHR (51, 60), have the ability to bind E-boxes in promoter regions of different genes. The HO-1 promoter contains an E-box (5′-CACGTG-3′) at $-47/-42$, and it was previously, as well as in this study, shown that Max could bind to this E-box (29). Whereas in the previous study, Max binding was found to be involved in mediating the p38 MAPK effects, we did not resolve all bound protein complexes. Thus, in the present study, by continuing these investigations, the binding of USF to the E-box of HO-1 promoter in both HepG2 cells and PHCs was demonstrated. Mutation of this E-box abolished binding of both Max and USF.

However, in the functional transfection assays, mutation of the E-box significantly affected but did not completely abolish the USF-dependent HO-1 promoter-driven Luc gene expression in rat PHCs, PASMCs, H4IIE, HepG2, and HeLa (Fig. 3). The USF-dependent induction of Luc activity in the tumor cells was even more dramatically reduced but still not completely abolished with the AP-1/E-box double mutant (pHO-754ΔA-Em) (Fig. 3B). By contrast, the USF-dependent inhibition of HO-1 promoter activity was lost when the double mutant pHO-754ÄA-Em, lacking both E-box and AP-1 binding sites, was used in the primary cells (PHCs and PASMCs) (Fig. 3B).

These phenomena could be explained by a possible interaction between USF and some AP-1 dimers belonging to the basic leucine-zipper domain (bZIP)–containing transcription factors

(55). Although it was already reported that heterodimerization between USF and AP-1 family members such as c-Fos (5), c-Maf (32), and Fra-1 (45) could occur, the role of this interaction remained unknown. Our findings now show that this interaction appears to be important for the modulation of USF-dependent gene regulation. This appears to be of particular importance for a number of certain physiologic stimuli that have been shown to induce USF-2 expression, such as glucose (56) or even harmful agents like cadmium and UVA, from which the latter are powerful inducers of HO-1 expression. These findings about USF-2, along with the results of the present study, underline the notion that the HO-1 gene expression is regulated by a complex network of transcriptional regulators and coactivators. This could allow not only a cell-specific regulation of HO-1 expression but also a fine-tuning in response to certain activators, such as UVA and cadmium.

Interestingly, our data in tumor cells show that USF-2 appears to have some remaining inducible potential even when the E-box and the AP-1 site are mutated. This may imply that another uncharacterized site(s) in the HO-1 promoter could mediate a portion of the USF2-dependent induction. However, our sequence analyses so far revealed that no other E-box sequences are present in the HO-1 promoter, thus making another direct effect of USF-2 unlikely. Thus, it is tempting to speculate that another factor(s) could act in an indirect manner. This factor could then be induced by USF-2 and bind to the HO-1 promoter, or it could be another cofactor capable of interacting with USF-2. The presence or absence of those factors in either tumor cells or primary cells may then contribute to additional effects on HO-1 gene expression in different cells.

This appears to be relevant for primary cells, because the present study shows that Fra-1 has no effect on HO-1 but induces the HO-1 promoter when E-box and AP-1 sites are mutated. Although the reason that Fra-1 induces the HO-1 promoter without E-box and AP-1 sites in primary cells is at present not entirely clear, we hypothesize that the complex consisting of USF proteins and Fra could interact with other transcription factors and would recruit cofactors like p300 (7). The latter is not only interacting with USF and Fra proteins but also with CREB, C/EBP, or FoxO proteins. Thus, we speculate that loss of the Fra/USF binding to the HO-1 promoter will disturb recruitment of their coactivator complex, which may then disable appropriate formation of another complex, which would inhibit HO-1 expression. This will then induce HO-1 expression, even when the E-box and the AP-1 site are mutated. Thus, the results from the present study underline even more the differences between tumor and primary cells.

### *USF and HO-1 as putative targets for antitumor therapies*

The findings of this study may be important for growth and proliferation processes. It was suggested that when cells progress from quiescence into the S phase, the Myc/Max heterodimer competed with USF proteins for binding to the E-box (6). Accordingly, USF-1 and USF-2 mediated antiproliferative properties; when overexpressed in REF cells, they inhibited c-Myc–induced cellular transformation (38). The Myc-dependent cellular transformation was inhibited only when wild-type USF-1 and USF-2 or vectors for USF mutants lacking the N-terminal transactivation domain were transfected. The DNA-binding–deficient forms of USF-1 and USF-2 did not affect the Myc-dependent foci formation in REF cells (38). This is in line with the results of the present study in which the cotransfection experiments with the USF-2a mutants and the HO-1 Luc constructs demonstrated the necessity of the N-terminal part encoded by exon 4, the USR, and the DNA-binding domain for the inhibitory action of USF (Fig. 7).

The antiproliferative activities of USF suggested that inactive USF could promote carcinogenesis. Indeed, inactivation of USF-2 in knockout mice led to the generation of prostate adenomas (9). In addition, USF-2 was found to be completely inactive in several cell lines from breast tumors (23). Thus, the loss of the repression by USF with the HO-1 promoter gene constructs with the mutated USF and AP-1 site, as shown here (Fig. 4), would also coincide with the observation that HO-1 was found to exert antiapoptotic effects, which would promote growth of tumor cells. The antiapoptotic action of HO-1 involves its antioxidative effects and the production of CO production that was shown to inhibit both expression of p53 and release of mitochondrial cytochrome *c* (17). In addition, mice subcutaneously injected with HO-1–overexpressing melanoma cells displayed development of more dense tumors, augmented metastasis, and a significantly shortened lifetime (66). Thus, as shown in this study, it appears that USF controls HO-1 expression, which both may then be considered as new targets in antitumor therapy.

In summary, our study shows that USF-2 acted as inhibitor of HO-1 expression in rat and human primary cells and as inducer in rat and human proliferating cells. The USR and the portion of the transactivation domain encoded by exon 4 were necessary for both the inducible and repressive effects of USF-2 on HO-1 gene expression. Thereby, the interaction between USF-2 and Fra-1, as well as the integrity of the AP-1 site and the E-box within the HO-1 promoter, are critically involved.

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## ABBREVIATIONS

AP-1, activator protein-1; E-box, enhancer box; FCS, fetal calf serum; Fra, fos-related activator; HO-1, heme oxygenase-1; HUVECs, human umbilical vein endothelial cells; PASMCs, pulmonary artery smooth muscle cells; PHCs, primary rat hepatocytes; USF, TDU, transactivation domain of USF; USF, upstream stimulatory factor; USR, USF-specific region; VSMCs, vascular smooth muscle cells.

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Address reprint requests to:
*Dr. Thomas Kietzmann*
*Department of Chemistry/Biochemistry*
*Erwin-Schrödinger Strasse Geb. 54*
*D-67663 Kaiserslautern, Germany*

*E-mail:* tkietzm@gwdg.de

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