J. Biochem. **144,** 477–486 (2008)
doi:10.1093/jb/mvn091

Human HRD1 Promoter Carries a Functional Unfolded Protein Response Element to Which XBP1 but not ATF6 Directly Binds

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Received January 12, 2008; accepted July 8, 2008; published online July 29, 2008

Quality control of proteins in the endoplasmic reticulum (ER) is achieved by two mechanisms, the productive folding mechanism, which is assisted by a number of ER-localized molecular chaperones and folding enzymes (collectively termed ER chaperones), and the ER-associated degradation (ERAD) mechanism, by which misfolded proteins are degraded by the ubiquitin-dependent proteasome system in the cytosol. Accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR), resulting in transcriptional induction of ER chaperones and ERAD components. In mammals, three signalling pathways operate for the UPR, namely the IRE1-XBP1, PERK-ATF4 and ATF6 pathways. Analysis of mouse embryonic fibroblasts deficient in UPR signalling molecule indicates that transcriptional induction of ERAD components depends on the IRE1-XBP1 pathway. However, the molecular basis of this finding remains unclear. Here, we analysed the promoter of human HRD1, which encodes an E3 ubiquitin ligase, an important component of ERAD. We found that induction of HRD1 is mediated by two *cis*-acting elements, a canonical ER stress response element and a novel element we designate as UPR element II. The presence of UPR element II to which XBP1 but not ATF6 directly binds explains at least in part the dependency of HRD1 induction on the IRE1-XBP1 pathway.

Key words: *cis*-element, degradation, ER, protein folding, transcription factor.

Abbreviations: DIG, digoxigenin; EMSA, electrophoretic mobility shift assay; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSE, ER stress response element; MEFs, mouse embryonic fibroblasts; UPR, unfolded protein response; UPRE, UPR element.

Newly synthesized secretory and transmembrane proteins are folded and assembled in the endoplasmic reticulum (ER), which contains a number of molecular chaperones and folding enzymes (collectively termed ER chaperones) to ensure the efficiency and productivity of folding. Proteins which fail to attain native conformation despite assistance from ER chaperones are subjected to ER-associated degradation (ERAD), by which misfolded proteins are delivered to the cytosol and degraded by the ubiquitin-dependent proteasome system (*1*). Protein unfolding or misfolding constitutes a fundamental threat to all living cells and must be counteracted immediately and appropriately. Accumulation of unfolded proteins in the ER under so-called ER stress conditions activates the unfolded protein response (UPR), which in metazoan cells consists of translational and transcriptional control. Upon ER stress, translation is generally attenuated to decrease the burden on the ER. Transcriptional induction of ER chaperones increases the capacity of the productive folding mechanism, whereas transcriptional induction of ERAD components enhances cellular capacity to degrade misfolded proteins. The coordinated activity of these programmes leads to maintenance of the homeostasis of the ER (*2–4*).

Yeast UPR consists of a single signal transduction pathway, the Ire1p-Hac1p pathway, by which transcriptional induction of both ER chaperones and ERAD components is achieved (*5*). In contrast, mammalian cells express three UPR signal transducers as transmembrane proteins in the ER, namely IRE1 (mammalian homologue of yeast Ire1p), PERK and ATF6. Further, IRE1 and ATF6 are duplicated to express ubiquitous IRE1α and gut-specific IRE1β, and both ubiquitous ATF6α and ATF6β, respectively (*6*). Transcriptional induction of mammalian ER chaperones is mediated by *cis*-acting ER stress response element (ERSE), the consensus of which is CCAAT-N9-CCACG (*7*). The two transcription factors ATF6 and XBP1 were isolated as binding proteins to ERSE. Subsequent analyses revealed that ATF6 is activated by ER stress-induced proteolysis (*8*, *9*), while XBP1 functions as a transcription factor downstream of IRE1 and is activated by ER stress-induced mRNA splicing, similarly to yeast Hac1p (*10*, *11*). Active forms of ATF6 and XBP1 bind to the CCACG part of ERSE only when the general transcription factor NF-Y binds to the CCAAT part (*10*, *12*, *13*).

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Ectopic expression of the active form of ATF6 at a physiological level is sufficient to induce transcription of mammalian ER chaperones (*14*). Interestingly, however, transcriptional induction of the major ER chaperones BiP/GRP78 and GRP94 occurs normally in the absence of IRE1α or XBP1 (*15*, *16*), whereas transcriptional induction of ERAD components, such as EDEM and derlins, is lost in cells deficient in IRE1α or XBP1 (*16–18*). These results indicate that the IRE1-XBP1 pathway is specifically involved in induction of ERAD components in mammals. However, the molecular basis of this notion remains unclear, because the *cis*-acting elements responsible for transcriptional induction of mammalian ERAD components have not been identified to date.

HRD1 is an E3 ubiquitin ligase involved in ERAD (*19–21*). It was previously shown that human HRD1 mRNA is induced in response to ER stress and that its induction is blocked by the expression of a dominant negative form of IRE1α (*22*), indicating that human HRD1 is also an ERAD component, the transcription of which is regulated by the IRE1-XBP1 pathway. In this report, we analysed human HRD1 promoter and found that it carries a novel *cis*-acting element we designate as UPR element (UPRE) II. The presence of UPRE II, to which XBP1 but not ATF6 directly binds, explains at least in part the dependency of HRD1 induction on the IRE1-XBP1 pathway.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Recombinant DNA techniques were performed according to standard procedures (*23*). Human HRD1 promoter (−3324 to –1, translational start site set as +1) was cloned by PCR from HeLa cell genomic DNA and then subcloned into pGL3-Basic vector (Promega, Madison, WI, USA) to create pGL3-HRD1P(−3324)-luc. Mutant HRD1 promoters were generated using a QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). A subregion of HRD1 promoter (−1850 to −1285) was also cloned into pGL3-Basic vector to create pGL3-HRD1P (−1850 to −1285)-luc. pcDNA-XBP1(spliced) was described previously (*10*).

Cell Culture, Transfection and Luciferase Assay—IRE1α+/+ and IRE1α−/− mouse embryonic fibroblasts (MEFs) (*15*), XBP1+/+ and XBP1−/− MEFs (*16*), and HeLa cells were grown at 37°C in a humidified 5% CO2/95% air atmosphere in Dulbecco's modified Eagle's medium (glucose at 4.5 g/l) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (penicillin at 100 U/ml and streptomycin at 100 μg/ml). Transfection was carried out by the standard calcium phosphate method (*23*) essentially as described (*7*). The luciferase reporter assay was performed according to our published procedures (*12*). To correct transfection efficiency, the reference plasmid pRL-SV40 carrying SV40 enhancer and promoter immediately upstream of the Renilla luciferase gene was cotransfected with reporter plasmid carrying firefly luciferase gene. Relative activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity. pGL3-GRP78P(−132)-luc (*7*) is called the ERSE reporter. p5xUPRE-GL3 is identical to p5xATF6GL3 (*24*) and is called the UPRE reporter.

Northern Blot Hybridization—Total RNA was extracted from MEFs using ISOGEN (Nippon Gene, Tokyo, Japan). Northern blot hybridization was performed according to standard procedures (*23*). Digoxigenin (DIG)-labelled cDNA probes were prepared using PCR according to the manufacturer's instructions (Roche, Basel, Switzerland) and hybridized with RNA electrophoresed and blotted on a membrane. Subsequent reaction with anti-digoxigenin antibody (Roche) and treatment with the chemiluminescent detection reagent CDP-star (GE Healthcare Biosciences, Stockholm, Sweden) were performed according to the manufacturer's specifications. Chemiluminescence was visualized using an LAS-3000mini LuminoImage analyser (Fuji Film, Tokyo, Japan).

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay (EMSA) was performed as described previously (*12*). ATF6α(1-373), corresponding to the active form of ATF6α as well as pXBP1(S), the active form of XBP1, was translated *in vitro* using the TNT T7 quick-coupled transcription/translation system (Promega). NF-Y trimer (NF-YA, NF-YB and NF-YC) was reconstituted from recombinant subunit proteins as described previously (*12*). The sequences of the synthetic double-stranded oligonucleotide probes ERSE, UPRE and Herp ERSE II are 5′-GGAGGGCCTTCACCAATCGG CGGCCTCCACGACGGGGCTGG-3′, 5′-GGTCGAGACAG GTGCTGACGTGGCGATTCCCC-3′, and 5′-GGGGATC CGGACGCCGATTGGGCCACGTTGGGAGAGTGCCT-3′, respectively (underlined sequences match the consensus sequence of ERSE, UPRE or ERSE II). The sequences of the synthetic double-stranded oligonucleotide probes HRD1 ERSE, HRD1 ERSE mut, HRD1 UPRE II and HRD1 UPRE II mut are 5′-GGCTTATCGCAACC AATCAGTGGCAGCCACGGGACCCAACT-3′, 5′-GGCTT ATCGCAACCAATCAGTGGCAGaacatGGACCCAACT-3′, 5′-GGACATTCTTTTTCTTATTGGGCCGCGTAACTTATC GCAAC-3′, and 5′-GGACATTCTTTTTCTTATTGGGaa tatTAACTTATCGCAAC-3′, respectively (underlined sequences match the consensus sequence of ERSE or ERSE II). Radioactive bands were visualized using a FLA-3000G FluoroImage analyser (Fuji Film).

RESULTS

Dependence of HRD1 Induction on IRE1α and XBP1—It was previously shown by reverse transcription-coupled PCR analysis (*22*) that HRD1 mRNA was induced in response to treatment of human embryonic kidney 293 cells with tunicamycin, an inhibitor of protein *N*-glycosylation, or thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase, both known to evoke ER stress (*25*), and that such induction was lost when human embryonic kidney 293 cells stably expressing a dominant negative form of IRE1α, K599A mutant, were treated with tunicamycin, indicating that induction of HRD1 is mediated by the IRE1-XBP1 pathway. To unambiguously show that this is indeed the case, we performed northern blot hybridization analysis of MEFs deficient in IRE1α (*15*) or XBP1 (*16*). As shown in Fig. 1, HRD1 mRNA was

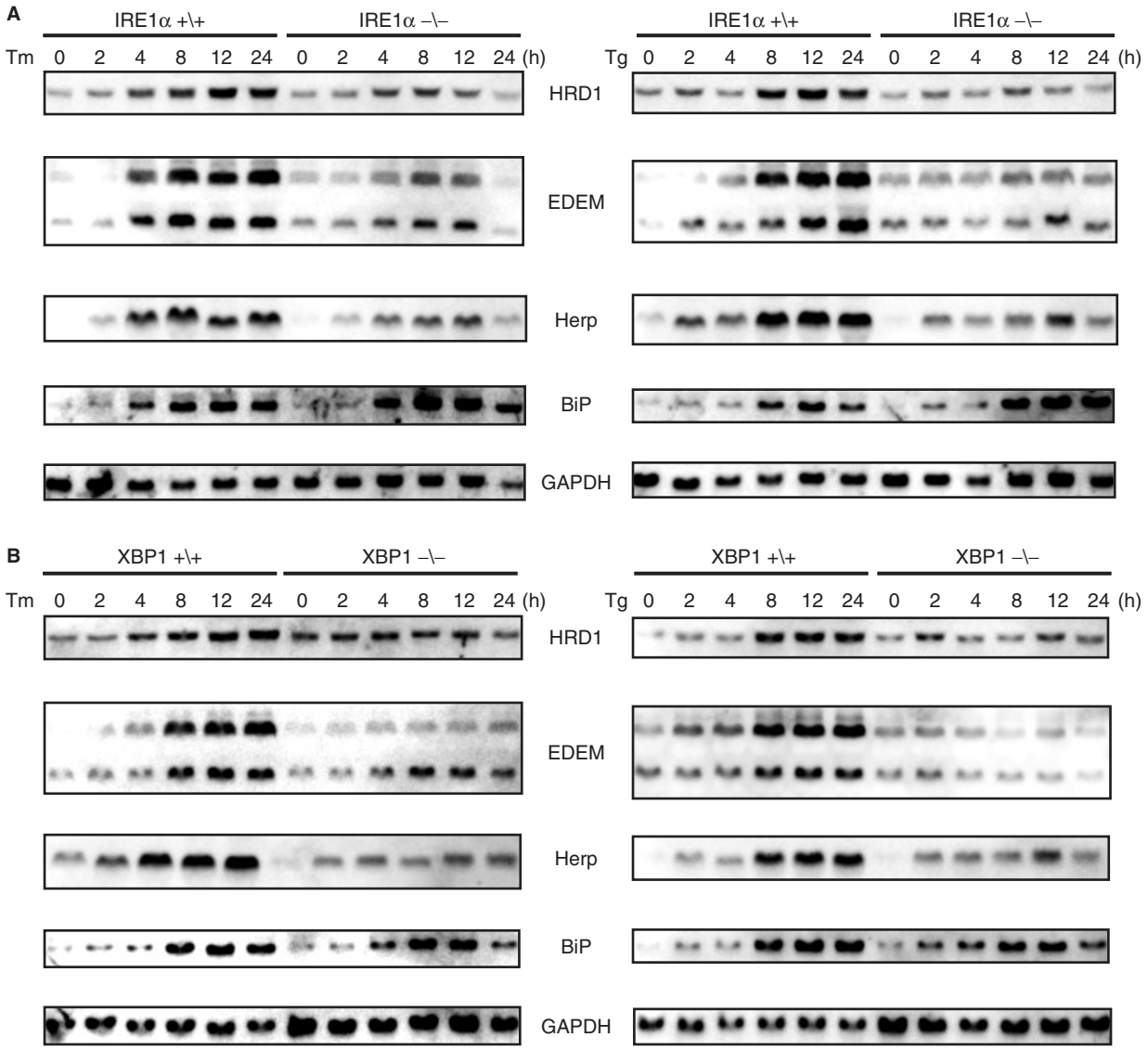

Fig. 1. **Effects of the absence of IRE1α or XBP1 on induction of HRD1.** (A) IRE1α+/+ and IRE1α–/– MEFs were treated with 2 μg/ml tunicamycin (Tm, left panel) or 300 nM thapsigargin (Tg, right panel) for the indicated periods. Total RNA was isolated and analysed by northern blot hybridization using a DIG-labelled cDNA probe specific to mouse HRD1, EDEM, Herp, BiP or GAPDH. (B) XBP1+/+ and XBP1–/– MEFs were treated and analysed as in (A).

induced in IRE1α+/+ or XBP1+/+ MEFs treated with tunicamycin or thapsigargin. However, induction was greatly mitigated in IRE1α–/– or XBP1–/– MEFs treated with tunicamycin or thapsigargin, similarly to other ERAD components, such as EDEM and Herp, consistent with our previous analysis of the induction of EDEM and Herp (*17*, *26*). In contrast, BiP mRNA encoding a major ER chaperone was induced in both IRE1α+/+ and IRE1α–/– MEFs as well as in both XBP1+/+ and XBP1–/– MEFs, as reported previously (*15–17*). Thus, induction of HRD1 depends on IRE1α and XBP1.

Involvement of ERSE in Induction of HRD1—Three *cis*-acting elements are known to respond to ER stress in mammals, namely ERSE (consensus sequence CCAAT-N9-CCACG), ERSE II (consensus sequence ATTGG-N1-CCACGT) and UPRE (consensus sequence TGACG TGG/A). ERSE was identified as a *cis*-acting element responsible for the induction of various ER chaperones (*7*). ERSE II was identified in the promoter of Herp (*27*), one of the most highly inducible proteins during the UPR (*28*). UPRE was selected through artificial binding site selection experiments (*24*). We previously showed that active forms of ATF6 and XBP1 bind to these elements differentially (*26*). Both ATF6 and XBP1 bind to the CCACG part of ERSE only when NF-Y binds to the CCAAT part (Fig. 4B, lanes 1 and 2). ATF6 binds to ERSE II in a NF-Y-dependent manner similarly to the case of ERSE (Fig. 5B, lanes 5 and 7), whereas XBP1 binds to the CCACGT part of ERSE II regardless of the presence or absence of NF-Y (Fig. 5B, lanes 6 and 8). XBP1 but not ATF6 is able to bind to UPRE (Fig. 4B, lane 3 and data not shown). We therefore searched the

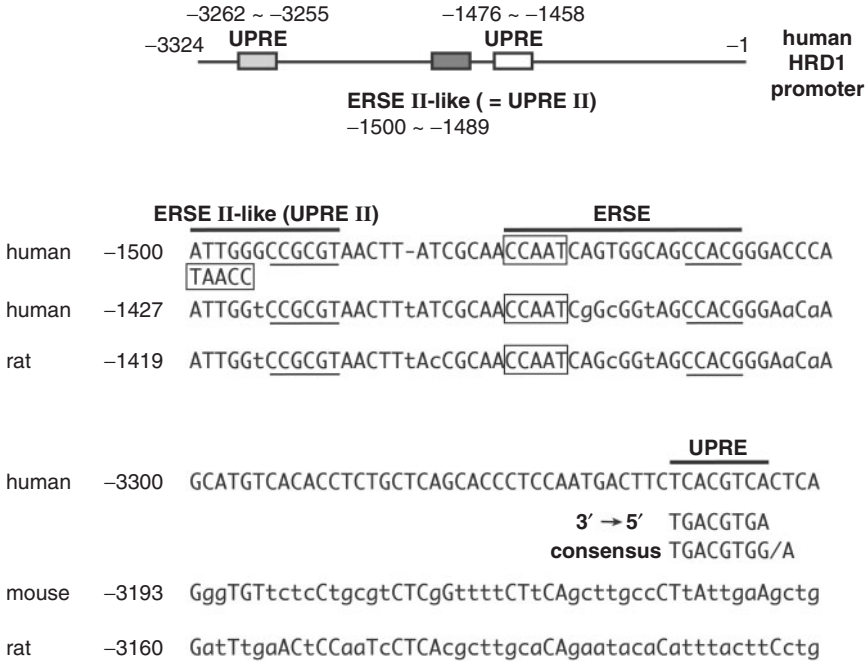

Fig. 2. **Schematic representation and partial sequences of human HRD1 promoter.** Locations of the three potential ER stress-responsive *cis*-acting elements in human HRD1 promoter (−3324 to −1, translational start site set as +1) are shown with boxes at top. Sequences around ERSE (−1476 to −1458), ERSE II-like (−1500 to −1489, renamed later to UPRE II) and UPRE (−3262 to −3255) are shown at bottom together with corresponding sequences of mouse and rat HRD1 promoters, in which nucleotides different from those in human promoter are indicated by small letters. The CCAAT sequences, binding sites of the general transcription factor NF-Y, are boxed. Underlined sequences are identical or similar to the consensus of ERSE or ERSE II. The sequence complementary to that from −3262 to −3255 is identical to the UPRE consensus.

upstream region of the human HRD1 open reading frame and found that HRD1 promoter contains sequences identical or closely similar to the above consensus sequences of the three *cis*-acting element (Fig. 2): a sequence perfectly matching the ERSE consensus is present from −1476 to −1458 (translational start site is set as +1): a second sequence differing from the ERSE II consensus by 1 nt is present from −1500 to −1489: while a third sequence perfectly matching the UPRE consensus is present from −3262 to −3255, although with an opposite orientation. Interestingly, ERSE and ERSE II-like sequences are present in mouse and rat HRD1 promoters at conserved positions with identical orientation, whereas mouse and rat HRD1 promoters do not contain UPRE at similar positions (Fig. 2). No UPRE-like sequence is present in 4 kb upstream regions of mouse and rat HRD1 open reading frames. Importantly, these findings are consistent with the results obtained from functional analysis of these three elements; ERSE and ERSE II-like sequences but not UPRE mediate the induction of HRD1 (see subsequently).

We examined whether these elements contribute to the transcriptional induction of HRD1 by carrying out reporter assays using HRD1 promoter (−3324 to −1) fused to firefly luciferase. Two reporters were employed as controls. The ERSE reporter, in which human BiP promoter containing three functional ERSEs is fused to luciferase, was activated in response to treatment with tunicamycin in both IRE1α+/+ and IRE1α−/− MEFs similarly (Fig. 3B, lanes 7 and 8), because ATF6 is activated similarly in IRE1α+/+ and IRE1α−/− MEFs (*17*). In contrast, the UPRE reporter containing five copies of UPRE upstream of luciferase was well activated in IRE1α+/+ but not at all in IRE1α−/− MEFs (Fig. 3B, lanes 9 and 10), because XBP1 but not ATF6 binds to UPRE (*17*). As shown in Fig. 3B, wild-type HRD1 promoter responded to tunicamycin treatment in IRE1α+/+ MEFs by enhancing luciferase activity, as expected (Fig. 3B, lane 1). Wild-type HRD1 promoter also responded to tunicamycin treatment in IRE1α−/− MEFs (Fig. 3B, lane 2), albeit less extensively than in IRE1α+/+ MEFs (the increase in luciferase activity by tunicamycin treatment in IRE1α−/− MEFs was approximately half that in IRE1α+/+ MEFs). Similar results were obtained with XBP1+/+ and XBP1−/− MEFs (Fig. 3C). These results suggested that HRD1 promoter is regulated by two different *cis*-acting elements.

One element regulating HRD1 induction is certainly the ERSE present from −1476 to −1458. We have shown by EMSA that recombinant NF-Y expressed and purified from *Escherichia coli* binds to the CCAAT part of ^{32}P-labelled BiP ERSE and forms a DNA–protein complex we designate as complex I. This complex is competed out by excess amounts of unlabelled BiP ERSE and supershifted by anti-NF-YA (a subunit of trimeric NF-Y) antibody (*12*). *In vitro*-translated ATF6α(1-373), corresponding to the active form of ATF6α or pXBP1(S), the active form of XBP1, binds to the CCACG part of ^{32}P-labelled BiP ERSE only in the presence of NF-Y and forms a DNA–protein complex we designate as complex II, which

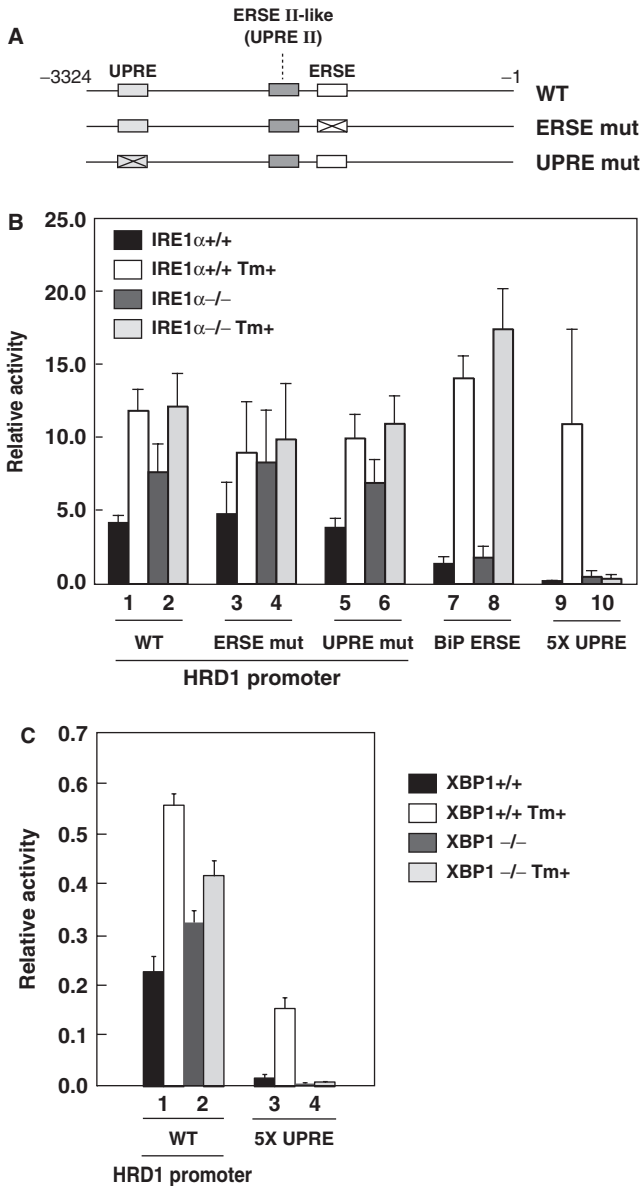

Fig. 3. **Mutational analysis of ERSE and UPRE in HRD1 promoter.** (A) Schematic representation of the wild-type (WT) and mutant HRD1 promoters analysed. Mutant HRD1 promoter in which the CCACG portion of the ERSE (−1476 to −1458) was mutated by transversion is referred to as ERSE mut (Fig. 4A), whereas mutant HRD1 promoter in which TCACGTCA of the UPRE (−3262 to −3255) was mutated by transversion is referred to as UPRE mut. (B) IRE1α+/+ and IRE1α−/− MEFs were transiently transfected with WT or mutant versions of pGL3-HRD1P (−3324)-luc, ERSE reporter containing three functional BiP ERSEs, or UPRE reporter containing five copies of UPRE in tandem, together with the reference plasmid pRL-SV40. Transfected cells were incubated with or without 10 μg/ml tunicamycin (Tm+) for 12 h. Relative luciferase activity was determined as described in EXPERIMENTAL PROCEDURES section and the averages from triplicate determinations of three independent experiments are presented with SDs (error bars). (C) XBP1+/+ and XBP1−/− MEFs were transiently transfected with WT pGL3-HRD1P (−3324)-luc or UPRE reporter together with the reference plasmid pRL-SV40. Reporter assays were carried out and the relative luciferase activity determined is presented as in Fig. 3B.

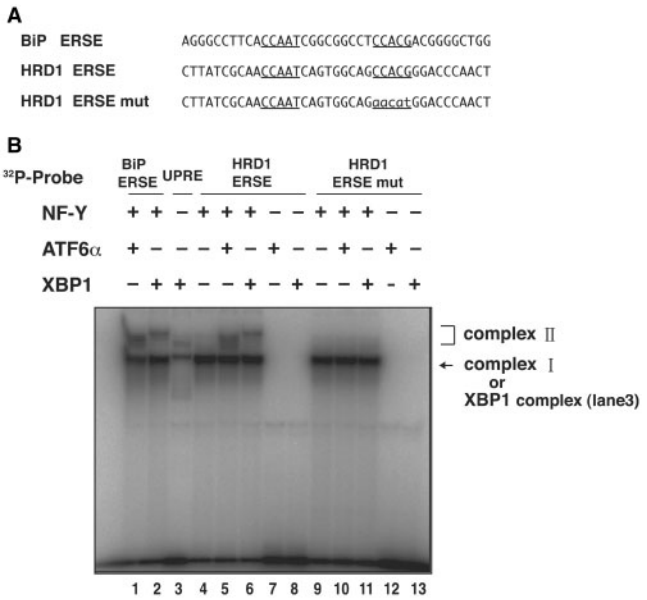

Fig. 4. **Binding of ATF6 and XBP1 to HRD1 ERSE.** (A) Sequences of oligonucleotide probes used for EMSA. (B) 0.1 pmol each of ^{32}P-labelled BiP ERSE, UPRE, HRD1 ERSE and HRD1 ERSE mut was incubated with (+) or without (–) *in vitro*-translated ATF6α(1-373), corresponding to the active form of ATF6α or *in vitro*-translated pXBP1(S), the active form of XBP1, in the presence (+) or absence (–) of recombinant NF-Y as indicated. It should be noted that there is no difference in DNA binding properties between ATF6α and ATF6β. Thus, binding of ATF6α(1-373) represents that of ATF6. Protein–DNA complexes formed were separated from the free DNA probe by electrophoresis on a non-denaturing gel. The migrated positions of complex I, complex II and XBP1 complex are indicated.

migrates more slowly than complex I. Complex II, formed either with NF-Y and ATF6α(1-373) or NF-Y and pXBP1(S), is supershifted by anti-NF-YA antibody, supershifted by anti-ATF6α or anti-XBP1 antibody, respectively, and competed out by excess amounts of unlabelled BiP ERSE (*12*). These results are represented in Fig. 4B (lanes 1 and 2). Similarly, recombinant NF-Y bound to ^{32}P-labelled HRD1 ERSE and formed complex I (lane 4). *In vitro*-translated ATF6α(1-373) or pXBP1(S) bound to ^{32}P-labelled HRD1 ERSE and formed complex II in the presence of NF-Y (lane 5 or 6, respectively) but not in the absence of NF-Y (lane 7 or 8, respectively). Mutation of the CCACG part of ^{32}P-labelled HRD1 ERSE by transversion abolished binding of ATF6α(1-373) or pXBP1(S) without affecting binding of NF-Y (lanes 9–13). Thus, HRD1 ERSE has the same properties as BiP ERSE as far as binding to transcription factors NF-Y, ATF6 and XBP1 is concerned.

Importantly, disruption of HRD1 ERSE by transversion in HRD1 promoter decreased induction to nearly half that of wild-type promoter in IRE1α+/+ MEFs (Fig. 3B, lane 3, compare with lane 1) and abolished it in IRE1α−/− MEFs (Fig. 3B, lane 4) in reporter assays, clearly indicating the involvement of ERSE in HRD1 induction. This also indicates that the other element regulating HRD1 induction is likely to have UPRE-like properties, as HRD1 promoter with ERSE mutated was

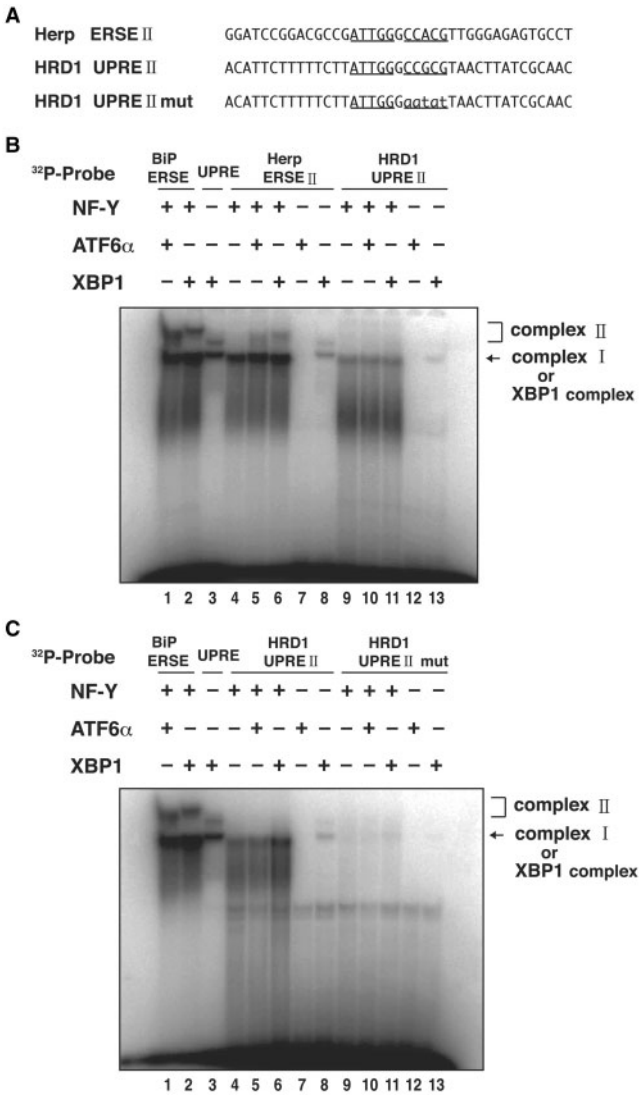

Fig. 5. **Binding of ATF6 and XBP1 to HRD1 UPRE II.** (A) Sequences of oligonucleotide probes used for EMSA. (B) A 0.1 pmol each of ^{32}P-labelled BiP ERSE, UPRE, Herp ERSE II and HRD1 UPRE II was incubated with (+) or without (−) *in vitro*-translated ATF6α(1-373) or pXBP1(S) in the presence (+) or absence (−) of recombinant NF-Y as indicated, and analysed as in Fig. 4B. (C) A 0.1 pmol each of ^{32}P-labelled BiP ERSE, UPRE, HRD1 UPRE II and HRD1 UPRE II mut was incubated with (+) or without (−) *in vitro*-translated ATF6α(1-373) or pXBP1(S) in the presence (+) or absence (−) of recombinant NF-Y as indicated, and analysed as in Fig. 4B.

activated in IRE1α+/+ MEFs but not in IRE1α−/− MEFs (Fig. 3B, lanes 3 and 4). Unexpectedly, however, disruption of the perfect UPRE from −3262 to −3255 by transversion had almost no effect on induction in both IRE1α+/+ and IRE1α−/− MEFs (Fig. 3B, compare lanes 5 and 6 with lanes 1 and 2). This indicates that there must be inhibitory activity or an inhibitory chromatin structure around the UPRE from −3262 to −3255 that blocks UPRE-mediated transactivation, because XBP1 is able to bind to ^{32}P-labelled UPRE and form a DNA–protein complex we designate as XBP1 complex in EMSA (Fig. 4B, lane 3), which is competed out by an excess amount of unlabelled UPRE and supershifted by anti-XBP1 antibody (*10*).

Identification of UPRE II—We therefore examined whether the ERSE II-like sequence from −1500 to −1489 contributes to the induction of HRD1. First, its DNA-binding properties were determined by EMSA in comparison with those of ERSE II present in the Herp promoter. As reported previously (*26*), recombinant NF-Y bound to ^{32}P-labelled Herp ERSE II and formed complex I (Fig. 5B, lane 4). *In vitro*-translated ATF6α(1-373) bound to ^{32}P-labelled Herp ERSE II and formed complex II in the presence of NF-Y (lanes 5) but did not form a DNA–protein complex in the absence of NF-Y (lane 7), whereas *in vitro*-translated pXBP1(S) bound to ^{32}P-labelled Herp ERSE II regardless of the presence or absence of NF-Y: complex II was formed in the presence of NF-Y (lane 6), while XBP1 complex was formed in the absence of NF-Y (lane 8). These results are consistent with our notion that ERSE II is a site of NF-Y-dependent ATF6 binding and of NF-Y-dependent as well as -independent XBP1 binding.

The HRD1 ERSE II-like sequence differed from the ERSE II consensus by 1 nt, and we found that this A to G change at the third position of the CCACGT dramatically affected binding of transcription factors, which led us to rename it HRD1 UPRE II. Although recombinant NF-Y bound to ^{32}P-labelled HRD1 UPRE II and formed complex I (Fig. 5B, lane 9), neither *in vitro*-translated ATF6α(1-373) nor pXBP1(S) bound to ^{32}P-labelled HRD1 UPRE II in the presence of NF-Y to form complex II (Fig. 5B, lanes 10 and 11), in marked contrast to the case of their binding to Herp ERSE II. *In vitro*-translated ATF6α(1-373) also failed to bind to ^{32}P-labelled HRD1 UPRE II in the absence of NF-Y (lane 12). On the other hand, *in vitro*-translated pXBP1(S) was able to bind to ^{32}P-labelled HRD1 UPRE II in the absence of NF-Y and formed XBP1 complex, albeit weakly (lane 13). We speculate that this weak NF-Y-independent binding of pXBP1(S) might have been overwhelmed by stronger binding of NF-Y, explaining why it is not visible in lane 11, as complex I and XBP1 complex migrated to similar positions. Mutation of the CCGCG in HRD1 UPRE II by transversion abolished binding of pXBP1(S) (Fig. 5C, compare lane 13 with lane 8) as expected, although this mutation also abolished binding of NF-Y (lanes 9–11). NF-Y thus appeared to recognize not only the core CCAAT but also flanking sequences of this particular element, which may be the reason why complex II was not formed when pXBP1(S) was incubated with ^{32}P-labelled HRD1 UPRE II in the presence of NF-Y (lane 6); pXBP1(S) associated with NF-Y may be unable to find its binding sequence due to the occupation of NF-Y. These properties of UPRE II are identical to those of artificially selected UPRE, as mentioned above, in the sense that it is not a binding site of ATF6 regardless of the presence or absence of NF-Y but a site of NF-Y-independent XBP1 binding. Nevertheless, as its sequence is different from that of UPRE, we have employed the name UPRE II.

Induction of HRD1 via ERSE and UPRE II—We next assessed the contribution of HRD1 UPRE II to the induction of HRD1. Disruption of UPRE II by

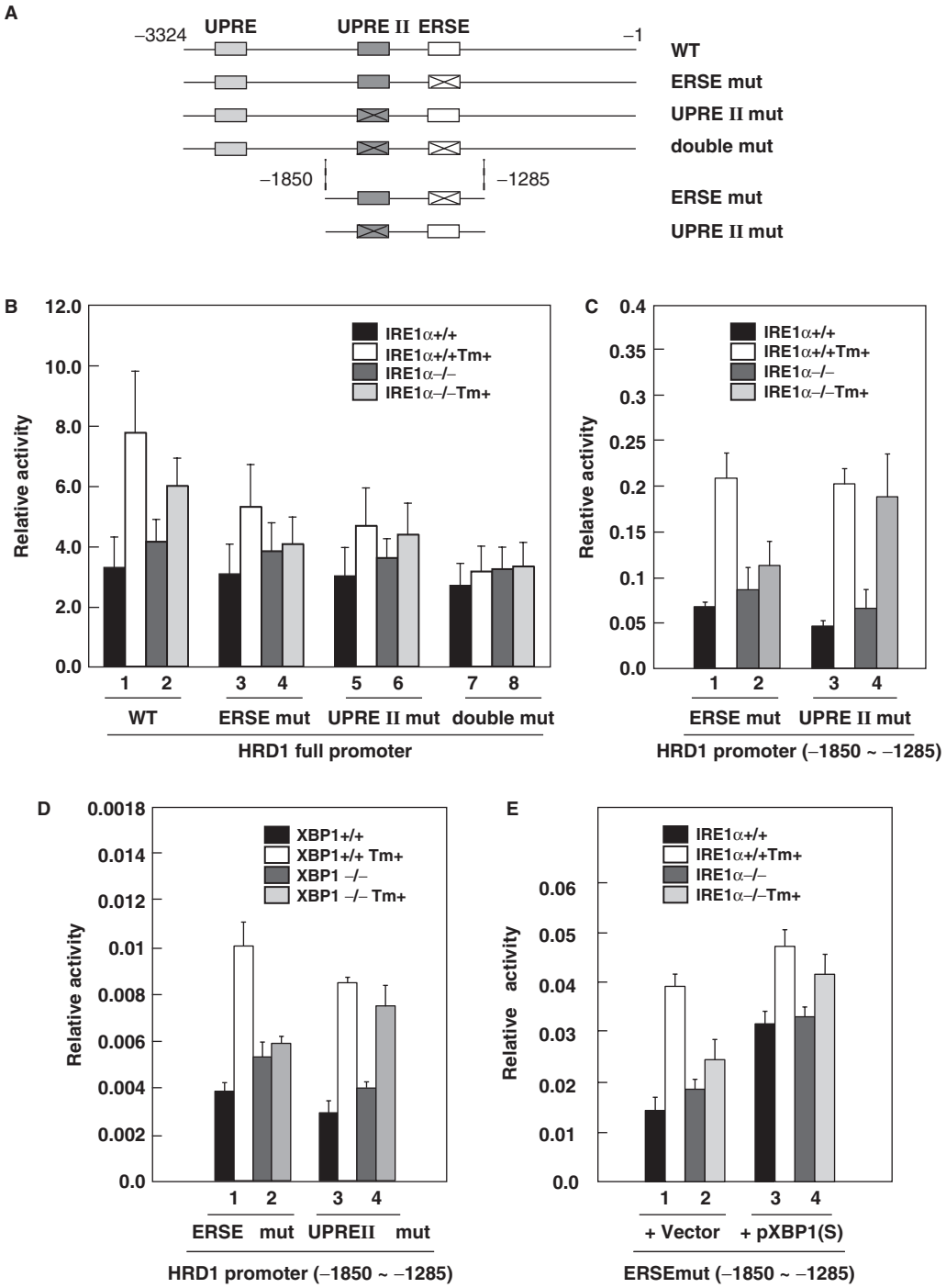

Fig. 6. **Mutational analysis of ERSE and UPRE II in HRD1 promoter.** (A) Schematic representation of the wild-type (WT) and mutant HRD1 promoters analysed. Mutant HRD1 promoter in which CCGCG of the UPRE II (−1500 to −1489) was mutated by transversion is referred to as UPRE II mut (Fig. 5A), whereas mutant HRD1 promoter in which both ERSE and UPRE II were mutated simultaneously by transversion is referred to as double mut. A subregion of HRD1 promoter (−1850 to −1285) with either ERSE or UPRE II mutated was also analysed. (B) IRE1α+/+ and IRE1α−/− MEFs were transiently transfected with WT or mutant versions of pGL3-HRD1P(−3324)-luc together with the reference plasmid pRL-SV40. Reporter assays were carried out and the relative luciferase activity determined is presented as in Fig. 3B. (C) IRE1α+/+ and IRE1 α−/− MEFs were transiently transfected with mutant versions of pGL3-HRD1P(−1850 to −1285)-luc together with the reference plasmid pRL-SV40. Reporter assays were carried out and the relative luciferase activity determined is presented as in Fig. 3B. (D) XBP1+/+ and XBP1−/− MEFs were transiently transfected with mutant versions of pGL3-HRD1P(−1850 to −1285)-luc together with the reference plasmid pRL-SV40. Reporter assays were carried out and the relative luciferase activity determined is presented as in Fig. 3B. (E) IRE1α+/+ and IRE1α−/− MEFs were transiently transfected with vector (pcDNA3.1) or pcDNA-XBP1(spliced) to express pXBP1(S) together with ERSE mutant of pGL3-HRD1P(−1850 to −1285)-luc and the reference plasmid pRL-SV40. Reporter assays were carried out and the relative luciferase activity determined is presented as in Fig. 3B.

transversion in HRD1 promoter decreased the induction in response to tunicamycin treatment to nearly half that of wild-type promoter in IRE1α+/+ MEFs (Fig. 6B, compare lane 5 with lane 1), as in the case of disruption of ERSE (lane 3) in reporter assays. Importantly, mutant HRD1 promoter with the disrupted UPRE II still responded to tunicamycin treatment in IRE1α−/− MEFs (lane 6), in contrast to the case of mutant HRD1 promoter with disrupted ERSE (lane 4). As we were aware that high basal activity of HRD1 promoter obscured the response and made comparison difficult, we analysed a region from −1850 to −1285 that encompassed UPRE II and ERSE with reporter assays. As shown Fig. 6C, the −1850 to −1285 region with disrupted ERSE responded to tunicamycin treatment in IRE1α+/+ but not in IRE1α−/− MEFs (lanes 1 and 2), while the −1850 to −1285 region with UPRE II disrupted responded to tunicamycin treatment in both IRE1α+/+ and IRE1α−/− MEFs similarly (lanes 3 and 4). Essentially identical results were obtained with XBP1+/+ and XBP1−/− MEFs (Fig. 6D). Thus, ERSE mutant promoter (−1850 to −1285) behaved like UPRE, whereas UPRE II mutant promoter (−1850 to −1285) behaved like ERSE. Introduction of the active form of XBP1, pXBP1(S), into MEFs by transfection enhanced transcription from ERSE mutant promoter (−1850 to −1285) containing UPRE II constitutively, regardless of the presence or absence of IRE1α, as expected (Fig. 6E, compare lane 3 with lane 1 and lane 4 with lane 2). Furthermore, simultaneous disruption of ERSE and UPRE II rendered the full HRD1 promoter unresponsive to tunicamycin treatment (Fig. 6B, lanes 7 and 8). We concluded that HRD1 promoter is regulated by ERSE and UPRE II.

DISCUSSION

When unfolded proteins are accumulated in the ER, eukaryotic cells from yeast to humans induce transcription of ER chaperones and ERAD components to maintain the homeostasis of the ER. In yeast, ER chaperones and ERAD components are induced by the same mechanism via the Ire1p-Hac1p pathway. In contrast, mechanisms underlying their induction have diverged in mammals, as transcriptional induction of ERAD components but not ER chaperones depends on the IRE1-XBP1 pathway, the mammalian counterpart of the yeast Ire1p-Hac1p pathway (Fig. 1). However, the molecular basis of this divergence remains unclear, mainly because the *cis*-acting elements responsible for induction of mammalian ERAD components have not been identified to date. This is in marked contrast to the situation of mammalian ER chaperones, whose induction is known to be mediated by *cis*-acting ERSE (*7*). UPRE has the properties of those expected of a *cis*-acting element responsible for the transcriptional induction of mammalian ERAD components, as UPRE-mediated transactivation depends on the IRE1-XBP1 pathway (*17*). Nonetheless, UPRE is an artificially selected sequence and its presence has yet to be demonstrated in any promoters of mammalian ERAD components.

In this report, we analysed the promoter of human HRD1, an important component of mammalian ERAD, and found that induction of HRD1 upon ER stress is mediated by two *cis*-acting elements. One is a canonical ERSE and the other is a novel element we designate as UPRE II, on the basis that it has properties closely similar to those of UPRE but differs from UPRE in sequence. The presence of UPRE II, to which XBP1 but not ATF6 binds directly (Fig. 5), explains at least in part the dependency of HRD1 induction on the IRE1-XBP1 pathway. We emphasize that the identification of XBP1 binding sites is not straightforward. A recent report (*29*) identified several XBP1 binding sites using chromatin immunoprecipitation on chip analysis, namely GCCACG (underlined sequence is identical to CCACG of ERSE), GACGTG (part of UPRE consensus), ACGT core and CGGAAG. However, the UPRE II we identified in HRD1 promoter (CCGCGT) differed from all of the above sequences. Careful work is necessary to unravel XBP1 binding sites. Perhaps the most unexpected finding of this study is that a sequence perfectly matching the consensus of UPRE at −3262 to −3255 plays almost no role in the induction of HRD1 in response to ER stress (Fig. 3). There must be inhibitory activity or an inhibitory chromatin structure around the UPRE from −3262 to −3255 that blocks UPRE-mediated transactivation, because XBP1 is able to bind to the UPRE when tested in EMSA (Fig. 4). Solving this discrepancy will require analysis at the chromatin level. Nonetheless, given that it is not present in mouse and rat promoters (Fig. 2), the UPRE sequence may have been created in human HRD1 promoter by chance, without functional implications.

We showed here that the induction of HRD1 is mediated by ERSE and UPRE II (Fig. 6). While this article was under revision, Kaneko *et al*. (*30*) reported the characterization of human HRD1 promoter. They found that human HRD1 promoter carries a complete ERSE (designated ERSE2) and an incomplete ERSE (designated ERSE1) downstream of ERSE2; their ERSE2 is identical to our ERSE at −1476 to −1458 (Fig. 2). Using reporter luciferase assays, they showed that ERSE2 but not ERSE1 is involved in the induction of HRD1 (*30*), consistent with our results (Fig. 3). Nevertheless, notwithstanding their conclusion that ER stress-induced HRD1 expression is mediated by the IRE1-XBP1 pathway, the molecular basis of their conclusion remains obscure, because ATF6 but not XBP1 bound to ERSE2 in EMSA (*30*). Because XBP1 but not ATF6 bound in EMSA to the UPRE II we identified in this report (Fig. 5), our results explain at least in part the dependency of HRD1 induction on the IRE1-XBP1 pathway.

Given that the induction of HRD1 is mediated by ERSE and UPRE II (Fig. 6), induction should be considerable even in the absence of IRE1α (to an extent approximately half that observed in IRE1α+/+ MEFs, based on the results of Fig. 6B, lanes 1 and 2), as ATF6 binds to ERSE and activates transcription. However, induction of HRD1 mRNA on ER stress was markedly reduced in IRE1α−/− or XBP1−/− MEFs (Fig. 1). The promoter of Herp, a component of ERAD (*31*, *32*), is well characterized; induction of Herp on ER stress is

mediated by ERSE and ERSE II (*27*). Given this, induction of Herp should occur normally even in the absence of IRE1, as ATF6 binds to both ERSE and ERSE II and activates transcription. However, induction of Herp is greatly diminished in IRE1α−/− or XBP1−/− MEFs as compared with that in IRE1α+/+ or XBP1+/+ MEFs [Fig. 1 and (*26*)]. It was recently reported that Herp ERSE II-mediated transactivation is not affected by the absence of IRE1α (*33*), contrary to our previous results (*26*). However, we consistently and reproducibly observe diminished transactivation through Herp ERSE II in IRE1α−/− MEFs, and the results of Liang *et al*. (*33*) are not consistent with the greatly mitigated induction of Herp mRNA in IRE1α−/− MEFs as compared with that in IRE1α+/+ MEFs [Fig. 1 and (*26*)]. Further, the analysis of Liang *et al*. (*33*) did not include necessary controls such as measurement of the UPRE reporter, the transcriptional activity of which is absolutely dependent on IRE1α.

These results require consideration of why the induction of Herp or HRD1 is affected by the absence of IRE1 more severely than the extent expected from promoter analysis. One common feature is that the two *cis*-acting elements regulating the induction of Herp or HRD1 are close to each other: ERSE II is only 23 bp upstream of ERSE in the Herp promoter, while UPRE II is only 13 bp upstream of ERSE in the HRD1 promoter. Chromatin structures around closely spaced regions may affect the recruitment of certain transcription factors. Further analysis combined with chromatin-level analysis will improve our understanding of the molecular mechanisms underpinning the specific regulation of promoters of mammalian ERAD components during the UPR.

ACKNOWLEDGEMENTS

We are grateful to Dr L. Glimcher of Harvard Medical School for her provision of XBP1+/+ and XBP1−/− MEFs. We thank Kaoru Miyagawa for her technical and secretarial assistance.

FUNDING

The Ministry of Education, Culture, Sports, Science and Technology of Japan (15GS0310 and 19058009 to K.M.) in part.

CONFLICT OF INTEREST

None declared.

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