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Production of Three Distinct mRNAs of 150 kDa Oxygen-Regulated Protein (ORP150) by Alternative Promoters: Preferential Induction of One Species under Stress Conditions[1]

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150 kDa oxygen-regulated protein (ORP150) is one of the endoplasmic reticulum (ER)-resident stress proteins. We have cloned and sequenced the entire human ORP150 gene covering over 15-kb. Analyses of transcription initiation sites and transcriptional regulatory sequences revealed that at least three distinct mRNA species were produced by alternative promoters: two of them starting from alternative exon 1 (1A or 1B), and the third one starting from exon 2, six nucleotides upstream of the first AUG initiation codon. Among them, the transcript that begins with exon 1B was preferentially induced by hypoxia or tunicamycin treatment. A *cis*-acting segment involved in the stress-dependent induction was found at the 5′-end of exon 1A, which could account for the selective induction of the transcription from exon 1B. Furthermore, *in vitro* analyses of translation of the third mRNA suggested the constitutive expression of the cytosolic ORP150 due to the lack of the signal peptide resulting from differential translation initiation.

Key words: ER stress, ER stress response element, hypoxia, multiple transcription start sites, ORP150.

Expression of 150 kDa oxygen-regulated protein (ORP150) in cultured rat astrocytes is induced by exposure to hypoxia or hypoxia followed by reoxygenation (*1*). ORP150 has also been reported to be strongly enhanced in mouse brain tissue subjected to ischemia, in macrophages of human atherosclerotic plaques, and in infiltrating human breast cancer cells (*1–3*). We previously cloned the human and rat ORP150 cDNAs (*4*), and nucleotide sequence comparison revealed that they represent orthologs of Chinese hamster GRP170 (*5*, *6*) and mouse CBP140 (*7*). Alignment of the deduced amino acid sequences of human ORP150, rat ORP150, and hamster GRP170 indicated over 90% identity, suggesting that they are functionally homologous proteins. Although the physiological function of these proteins is little understood, their involvement in protein folding, assembly, and insertion into microsomal membranes in conjunction with GRP78 and GRP94 has been suggested (*6*, *8*, *9*). Mammalian ORP150 or GRP170, along with yeast Lhs1p, have been proposed to belong to the same HSP70 subfamily (*10*). Lhs1p is known to be involved in protein folding and protein translocation into the endoplasmic reticulum (ER) (*11*, *12*). Although Lhs1p is not essential for growth, it may interact directly with Kar2p, the yeast counterpart of GRP78, and modulate its activity (*11*).

In mammalian cells, both ORP150 and GRP78 are known to be ER-resident proteins, and are induced by treatment with tunicamycin or 2-deoxyglucose, which causes ER stress, as well as by hypoxia or amino acid analogs (azetidine or canavanine), but not by heat shock, hydrogen peroxide, or cobalt chloride (*4*). The molecular mechanism of Kar2p/GRP78 induction has been well investigated, and revealed to be controlled through the unfolded protein response (UPR) or ER stress response pathway conserved from yeast to man (*13–19*). In the mammalian UPR system, Yoshida *et al.* recently identified a novel *cis*-element, endoplasmic reticulum stress response element (ERSE), necessary and sufficient for induction of GRPs, and isolated a basic-leucine zipper transcription factor, ATF6, as an ERSE-binding protein (*19*). On the other hand, the mechanism of ORP150 induction remains largely unknown. We thus isolated the entire human ORP150 gene and studied the mechanism of its induction by hypoxic stress. Concerning the transcriptional responses to hypoxia, hypoxia-inducible factor 1 (HIF-1) has been reported to be generally involved in the induction of a number of genes (*20*, *21*) by binding to the hypoxia-inducible enhancer element (*22–24*). Because the human OPR150 gene does not contain sequences similar to this enhancer element, another pathway must be involved in the transcriptional activation of ORP150.

In this study, we determined the transcriptional regulatory sequences of the ORP150 gene and the *cis*-acting segment involved in the induction by hypoxia using a transient reporter assay. Our results also show the presence of multiple species of ORP150 mRNA and stress-specific induction of a particular transcript among them.

[1] The nucleotide sequence reported in this paper has been submitted to the DDBJ under accession number AB009979.

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Abbreviations: ER, endoplasmic reticulum; ERSE, endoplasmic reticulum stress response element; HIF-1, hypoxia-inducible factor 1; nt, nucleotide; ORP150, 150 kDa oxygen-regulated protein; SRF, serum response factor; UPR, unfolded-protein response; UTR, untranslated region.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of the Human ORP150 Gene—About 10^6 plaques of a human genomic library (Clontech HL1067j) were screened with human ORP150 cDNA as a probe. The isolated clones were digested with appropriate restriction enzymes, subcloned into pBluescript II SK–, and then subjected to nucleotide sequencing.

Cell Culture and Exposure to Stress—Human astrocytoma U373 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum at 37°C. For hypoxia treatment, cells were cultured in an incubator attached to a hypoxia chamber (85% N_2–5% CO_2–10% H_2, Coy Laboratory Products) for 64 h. For tunicamycin treatment, cells were cultured in the presence of 2 μg/ml tunicamycin for 24 h. Under these conditions ORP150 mRNA was induced by over 5-fold.

Construction of Plasmids—Appropriate DNA fragments of the human ORP150 gene were cloned into the multicloning sites of pGL3-basic or pGL3-promoter (Promega). The expression plasmid of ATF6 (pCMV-ATF6) was a gift from H. Yoshida (*19*).

The DNA fragment [nucleotide (nt)–224 to 1045] of the gene comprising from the promoter region to the 3′-terminus of exon 2 was cloned into pBluescript II SK–. Starting from this plasmid, the segment from nt –38 to 951 or from nt 97 to 951 was deleted by a PCR-based method (*25*) to yield a plasmid containing the promoter region and exon 1A fused to exon 2 (pB-P1A2) or the promoter region, exon 1A, and exon 1B fused to exon 2 (pB-P1B2). From pB-P1B2, exon 1A and exon 2 were deleted to yield a plasmid containing exon 1B (pB-P1B), and from pB-P1A2, exon 2 was deleted to yield a plasmid containing exon 1A (pB-P1A).

The TGATGT sequence overlapping the second ATG of ORP150 cDNA was mutated to CCATGG (*Nco*I recognition sequence) by PCR, and the coding region of the firefly luciferase gene beginning with ATG was inserted at the *Nco*I site. The resulting chimera DNA fragment of the 5′-region of ORP150 cDNA and the luciferase gene were ligated downstream of the T7 promoter of pBluescript II SK–. The three nucleotides just upstream of the second ATG were mutated (TGAGTGATACAC to TGATTGCTGCAC) (pT7-1B2Luc) to prevent cleavage of the signal peptide (*26*). From this plasmid DNA, a fragment containing exon 1B was deleted (pT7-2Luc) to start transcription at six bases upstream of the first ATG, as shown schematically in Fig. 6A.

Regional Assignment of the ORP150 Gene—FISH mapping was performed according to Heng *et al.* (*27, 28*) using a biotinylated cDNA probe with the FISH mapping service (SeeDNA Biotech).

Luciferase Reporter Assay—U373 cells (1×10^5) were transfected with 0.3 μg of reporter plasmid and 20 ng of reference plasmid pRL-SV40 carrying the *Renilla* luciferase gene, using Lipofectin (GibcoBRL) according to the manufacturer's instructions. After incubation for 66 h, the cells were washed with PBS and then luciferase activity was measured with a Dual Luciferase Reporter Assay System (Promega). The firefly luciferase activities of reporter plasmids were normalized as to *Renilla* luciferase activities. To examine the effect of expression of ATF6, 60 ng of pCMV-ATF6 was cotransfected with 0.3 μg of reporter plasmid and 20 ng of pRL-SV40, and then luciferase activitiy was determined as above.

Primer Extension Analysis—Twenty micrograms of poly(A)$^+$ RNA was primed with a FITC-labeled oligomer, Rex2-3′ (5′-CACTCAGTGCCAACAGGTCTGCCAGAGCA-3′), which can hybridize to the 3′-end of the second exon in 0.15 M KCl–10 mM Tris-HCl (pH 8.3)–1 mM EDTA. The primer was then extended with Rav2 reverse transcriptase (Takara) at 42°C. After treatment with RNase A and T, the products were precipitated with ethanol and separated on a 10% denatured polyacrylamide gel. For a sample of control cells, a 5-fold excess of the products was loaded on the gel.

S1 Mapping Analysis—Using pB-P1A2 or pB-P1A1B2 as a template and FITC-labeled Rex2-3′ as a primer, the probes were synthesized with Klenow enzyme. The products were digested with *Nhe*I at the 5′-end and purified by alkaline agarose gel electrophoresis. Fifty micrograms of poly(A)$^+$ RNA and about 3 ng of probe were hybridized at 42°C for 16 h, and then digested with S1 nuclease using an S1-assay kit (Ambion). The products were precipitated with ethanol and then separated on the same gel together with the primer extension products. For a sample of control cells, a 5-fold excess of products was loaded.

Northern Blot Analysis—Using pB-P1A or pB-P1B as a template, ^{32}P-labeled RNA probes were synthesized with T3 RNA polymerase and ^{32}P-CTP. Probes for the ORP150 coding region and glyceraldehyde 3-phosphate dehydrogenase gene were labeled using a DNA labeling kit (Pharmacia Biotech). One microgram of poly(A)$^+$ RNA was separated on a 1% agarose gel, transferred to Hybond-N$^+$ (Amersham), and then hybridized with each probe.

Gel Mobility Shift Assay—Nuclear cell extracts were prepared according to the method described by Dignam *et al.* (*29*). DNA probes HX21 and HX30 were labeled with ^{32}P-ATP using T4 polynucleotide kinase (Takara). Nuclear extracts (2 μg of protein) were mixed with ^{32}P-labeled DNA probes in 20 μl binding buffer [10% glycerol–10 mM Hepes (pH 7.9)–50 mM KCl–1 mM EDTA–5 mM $MgCl_2$–0.1 mM phenylmethylsulfonyl fluoride] in the presence or absence of poly(dI-dC) (Boehringer) and competitor DNA, and then incubated for 15 min at room temperature. Antibodies were added if necessary, and further incubation was carried out for 15 min. Then the mixtures were loaded onto a 4.5 or 5% polyacrylamide gel in electrophoresis buffer [45 mM Tris-borate (pH8.3)–1.3 mM EDTA].

In vitro Transcription-Translation—*In vitro* transcription was performed with T7 RNA polymerase using a MEGAscript *in vitro* transcription kit (Ambion). To synthesize capped RNA transcripts, the level of GTP was reduced to 1.5 mM, *i.e.* one-fifth of the normal concentration, and 6 mM cap analog, m^{7}GpppG, was added instead. After incubation for 6 h at 37°C, the template DNAs were removed by the addition of DNaseI, and RNA transcripts were recovered. *In vitro* translation was performed using a Retic Lysate IVT *in vitro* translation kit (Ambion), and the products were analyzed by immunoblot analysis using an antibody against firefly luciferase.

Immunoblot Analysis—*In vitro* translated products were resolved by SDS-PAGE (10% acrylamide) and then transferred to Hybond-ECL nitrocellulose membranes (Amersham). After blocking with nonfat dry milk, the membranes were incubated with firefly luciferase (Biogenesis), followed

by incubation with horseradish anti-rabbit immunoglobulin conjugate (GibcoBRL). An ECL-Plus Western blotting detection system (Amersham) was used to detect the antigen.

RESULTS

Structural Analyses of the Human ORP150 Gene—We isolated, from a human genomic library, three partially overlapping clones covering the entire ORP150 gene and determined the nucleotide sequence of a 16.5-kb segment spanning the gene with extended flanking regions on both sides (Fig. 1A). Comparison with the known sequence of ORP150 cDNA (*4*) revealed that the gene consists of 26 exons. All the exons except for the last one (1,462 nucle-

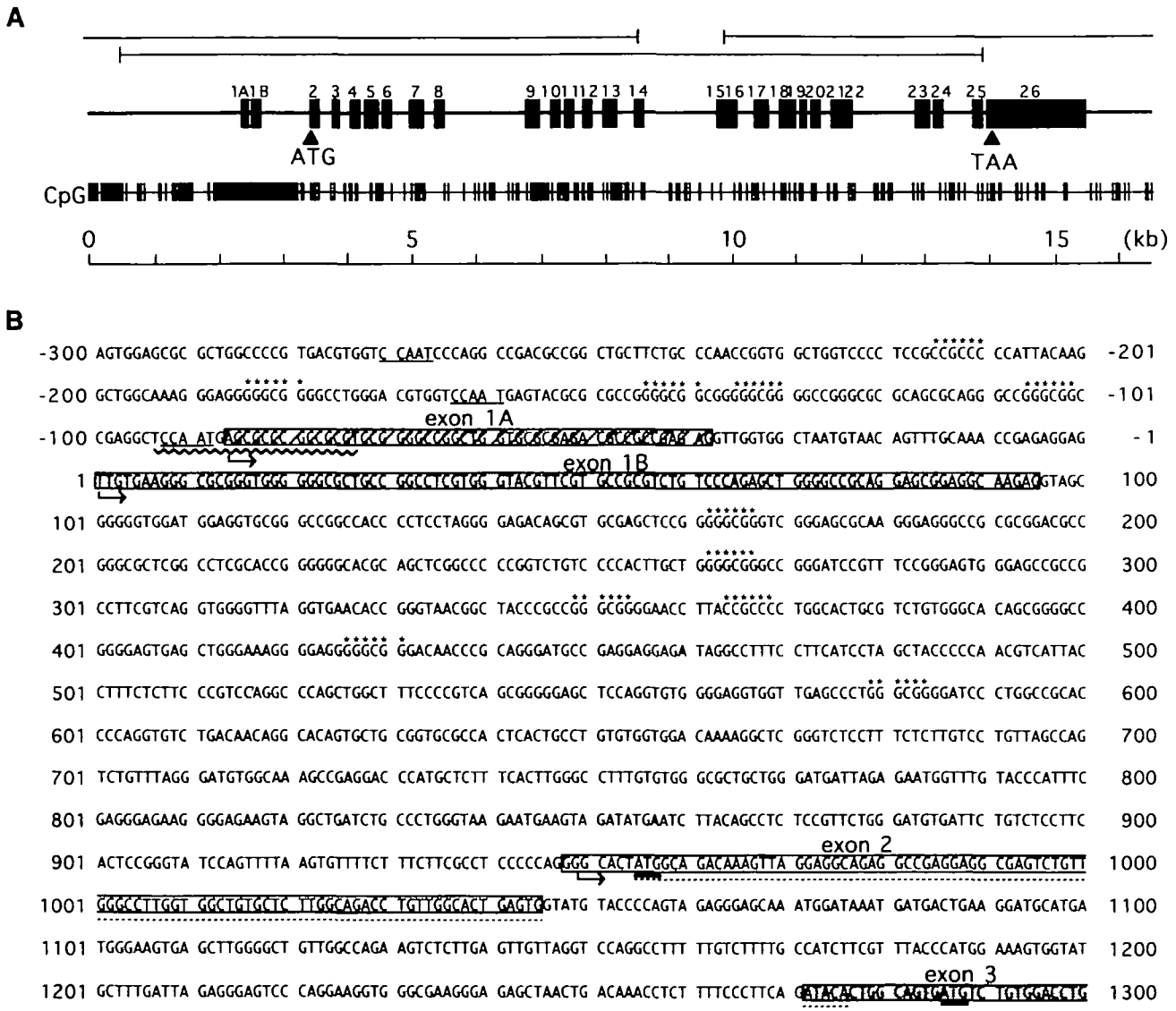

Fig. 1. **Structural features of the human ORP150 gene.** A: Locations of exons and CpG dinucleotides in the entire region of the ORP150 gene. Horizontal lines show the three clones isolated. Exons are represented by boxes. Triangles indicate the translation initiation codon (ATG) and the stop codon (TAA). Short vertical lines denote the positions of CpG dinucleotides. B: Nucleotide sequence of the 5′-portion of the gene, from nt −300 to 1300. Nucleotides are numbered from the 5′-end of cDNA reported previously (*4*). The boxes indicate exons. Asterisks indicate putative Sp1 binding sites. Bold and thin underlines represent the ATG and CCAAT sequences, respectively. The dotted line indicates the sequence coding for the signal peptide. Arrows indicate transcription start sites (see the text). The wavy line represents the hypoxia responsive 21-bp *cis*-acting segment (see the text). C: Alignment of exon 1A of the human ORP150 gene and the 5′-UTR of the hamster GRP170 cDNA. The numbers refer to those in the human ORP150 gene. Identical nucleotides are shaded.

otides) containing a long 3′-untranslated region (UTR) were small (51 to 241 nucleotides long), and the first initiation codon (AUG) was found within the second exon. The sequence of the coding region agreed with that of the cDNA except for one base: T (instead of C) at nt 645, with no change in the predicted amino acid. Some base changes and insertions were observed in the 3′-UTR. All the intron/exon junctions completely matched the known consensus sequences (5′ CT/AG 3′) (*30*).

Whereas the coding region showed high sequence similarity with that of the hamster GRP170 cDNA (88% identity), the 5′-UTR showed much less similarity (44% identity) (*4*). However, a stretch of 48 nucleotides highly similar to the 5′-UTR of hamster GRP170 (85% identity) (*5*) was found upstream of the 5′-untranslated exon 1 (Fig. 1C). In fact, the latter region was shown to be transcribed in human U373 cells (see below). Accordingly, this putative alternative first exon was designated as exon 1A, while the one originally deduced from the cDNA sequence was named exon 1B.

No typical TATA sequence was found, but several CCAAT sequences were found upstream of the transcription start sites (see below) (Fig. 1B). The approximately 1-kb region spanning from this upstream region through the first intron was extremely G+C rich (74%), with a cluster of CpG sequences (Fig. 1A) containing five potential Sp1 binding sites (GGGCGG) upstream of exon 1A and six within the first intron (Fig. 1B). Although the expression of ORP150 is induced by hypoxia (*4*), no sequences similar to that of the hypoxia-inducible enhancer element were found in this region, unlike for some of the other hypoxia-inducible genes (*22–24*). The ORP150 gene was mapped to chromosome 11, region q23.1-23.3, by *in situ* hybridization with the 4.5-kb cDNA as a probe (data not shown).

Analyses of Transcription Start Sites under Normal and Stress Conditions—First, we cloned the 5′-portions of cDNA from cellular poly(A)$^+$ RNA by means of the 5′-RACE technique. Nucleotide sequencing of the resulting cDNA clones revealed that the majority derived from hypoxia- or tunicamycin-treated cells contained sequences corresponding to exon 1B (Table I). In contrast, many of the cDNA clones derived from the control cellular RNA contained exon 2 at their 5′-ends. In addition, the cDNAs containing exon 1A or exon 1B appeared at similar frequencies. These results indicated that exon 1A as well as exon 1B is actually transcribed in U373 cells. Moreover, the induction of ORP150 upon hypoxia or ER stress appeared to depend primarily on transcription initiated from exon 1B.

To determine the transcription initiation sites more precisely, we examined the mRNA cap sites by cloning cDNAs exclusively derived from capped mRNAs (*31*). The cap site for RNA from stressed cells was found to be two nucleotides upstream of the transcription start site (nt +1) deduced on 5′-RACE and cDNA cloning (Table II). In contrast, the cap site for RNA from control cells was mostly found within exon 2 (six nucleotides upstream from the AUG codon), consistent with the results of 5′-RACE; however, some cDNA clones derived from the latter RNA suggested initiation from exon 1A or exon 1B despite slight dislocation. The infrequent occurrence of exon 1-containing clones in this experiment could be due to the differential ligation efficiencies of oligoribonucleotides to the ends of decapped RNA. These results strongly suggested the presence of at least three distinct transcripts with different start sites under normal and stress conditions.

We then carried out S1 mapping and primer extension analyses. When a probe that can hybridize with exon 1A-containing mRNA (P1A2; Fig. 2) was used for S1 mapping (lanes 1, 4, and 7), signals were detected around the 5′-ends

TABLE II. **Determination of mRNA capping sites.** Replacement of the cap structure with a ribo-oligonucleotide was carried out by the method of Maruyama and Sugano (*31*). Poly(A)$^+$ RNA was treated with bacterial alkaline phosphatase (Takara) and the cap structure was removed with tobacco acid pyrophosphatase (Sigma). Then a ribo-oligomer (5′-AGGUACCCGGGAGCUCUAGAUCUCGAGAGG-3′) was ligated to the 5′-end of RNA. Using the oligomer ligated RNA as a template, double strand cDNA was synthesized as in the case of 5′-RACE. The first and nested PCR were performed as described above except for the use of ribo-oligomer specific 5′-primers (first, 5′-AGGTACCCGGGAGCTCTAGGA-3′; nested, 5′-AGGTACCCGGGAGCTCTAGATCTCGAGAGG-3′). The PCR products were digested with *Xho*I and *Sac*I, and then cloned into pBluescript II SK–. The *Xho*I site was in the ribo-oligomers. The nucleotide sequences of independent clones were then analyzed.

RNA source	Number of cDNA clones			
	Sequenced	Cap sites at position (nt)		
		–2 (exon 1B)	949 (exon 2)	others
Control	18	0	14	4[a]
Hypoxia	34	34	0	0
Tunicamycin	19	19	0	0

[a]The cap sites of two of the clones were found at nt –43 in exon 1A, and those of two other clones at nt 3 and 91 in exon 1B.

TABLE I. **Analysis of the 5′-ends of cDNA.** The 5′-ends of cDNA obtained from poly(A)$^+$ RNAs of control, or hypoxia- and tunicamycin-treated cells were cloned according to the protocol for a Marathon cDNA amplification kit (5′-RACE; Clontech) with some modifications. In brief, the first-strand cDNA was synthesized with AMV reverse transcriptase in the presence of 1% DMSO with an ORP150-specific primer, 5′-CCGTTTATTGTGCTTCAGGC-3′. After synthesizing the second-strand cDNA, an adaptor

(5′-GTCGACTAGTAGGTACCCGGGAGCTCTAGATCTCGAGAGGAAGCTTTAAA-OH -3′

3′-OH - AGAGCTCTCCTTCGAAATTT-PO_4-5′)

was ligated to the double stranded cDNA. The first and nested PCR were performed with the adaptor-ligated cDNA as a template using adaptor-specific forward primers (first, 5′-GTCGACTAGTAGGTACCCGGGAGCT-3′; nested, 5′-AGGTACCCGGGAGCTCTAGATCTCGAGAGG-3′) and ORP150-specific reverse primers (first, 5′-ACCAGGATCACCTGCTCAAG-3′; nested, 5′-CTCCTCAAATTCCACACGAG-3′). The PCR products were digested with *Hin*dIII and *Sac*I, and then cloned into pBluescript II SK–. The *Hin*dIII and *Sac*I sites were positioned in the adaptor and cDNA sequences, respectively. Several independent clones were examined by nucleotide sequencing.

RNA source	Number of cDNA clones				
	Sequenced	5′-ends of cDNA			
		exon 1A	exon 1B	exon 2	exon 3
Control	30	5	6	16	3
Hypoxia	21	1	15	3	2
Tunicamycin	24	1	14	3	6

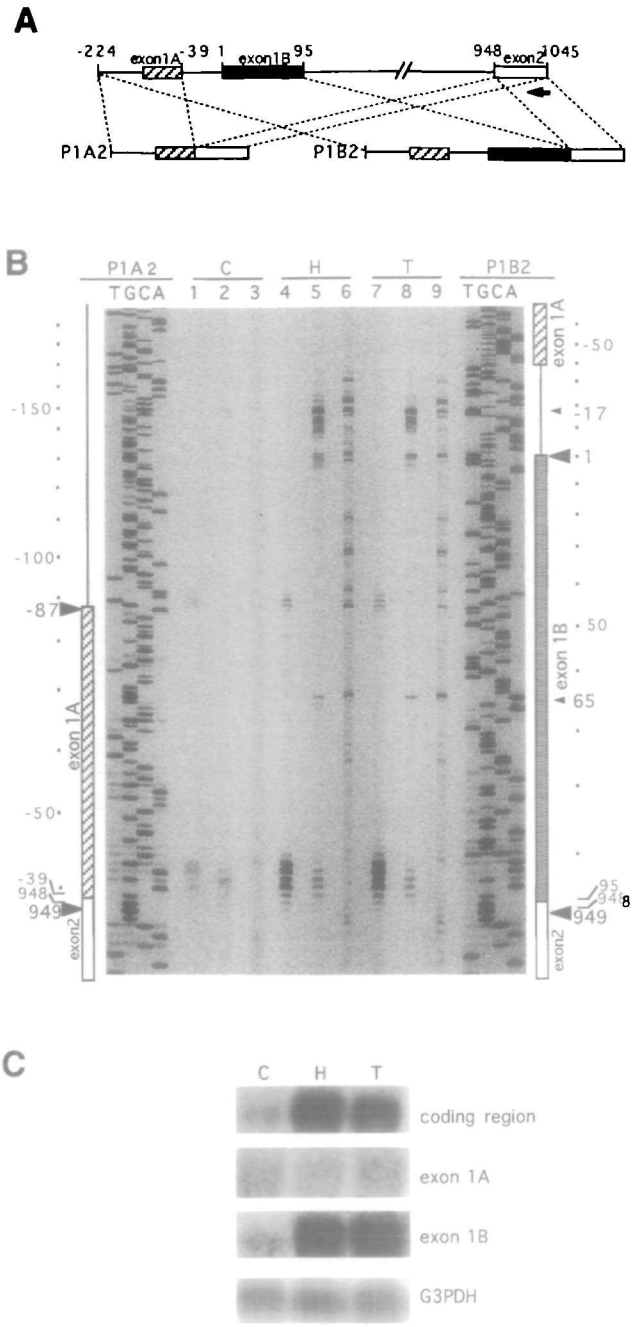

Fig. 2. **Determination of transcription start sites by S1 mapping and primer extension analyses.** A: The upper line indicates the upstream region of the ORP150 gene. The arrow represents the primer, Rex2-3′, used for primer extension analysis. The probes for S1 mapping, P1A2 and P1B2, are shown below. B: Analyses of cDNA products derived from poly(A)$^+$ RNA of nonstressed and stressed cells were carried on the same gel by S1 mapping and primer extension. Lanes 1, 4, and 7, S1 mapping with P1A2 as a probe; lanes 2, 5, and 8, S1 mapping with P1B2 as a probe; lanes 3, 6, and 9, primer extension. The numbers on both sides are the positions in nucleotides. The sequence ladders with each probe were run on each side, as indicated. C, control cells; H, hypoxia-treated cells; T, tunicamycin-treated cells. Large arrowheads indicate sites of transcription initiation, and small arrowheads indicate possible additional sites. C: Northern blot analysis of poly(A)$^+$ RNA from control cells (C), hypoxia-treated cells (H), and tunicamycin-treated cells (T). The probes are shown on the right.

of both exon 1A and exon 2, as expected. The signals around the 5′-end of exon 1A indicated the transcript started from exon 1A, whereas those around the 5′-end of exon 2 indicated either the transcript started from exon 1B or started from near the 5′-end of exon 2. Similarly, when a probe that can hybridize with exon 1B-containing mRNA (P1B2) was used (lanes 2, 5, and 8), signals were detected at the 5′-end of exon 1B, indicating the transcript starting from exon 1B, whereas those around the 5′-end of exon 2 indicated the transcripts from either exon 1A or 2.

On primer extension analysis (Fig 2B; lanes 3, 6, and 9), some signals were found at positions identical with those on S1 mapping, probably indicating actual transcription start sites. Taking all the results of 5′-RACE, cap site analysis and cDNA cloning into consideration, the most probable transcription start sites are –87 in exon 1A, +1 in exon 1B, and +949 in exon 2. In addition, transcription might start from nt –17 or +65. Other signals detected only on primer extension are likely to represent products resulting from the interrupted primer extension reaction due to the high G+C contents. The very faint bands for the control transcripts (lanes 1–3) despite higher amounts (5-fold) of samples indicated marked induction of transcription, particularly that initiated from the 5′-end of exon 1B, by hypoxia or tunicamycin treatment. We confirmed this result by Northern blot analysis using a probe specific for exon 1A or exon 1B. Hypoxia or tunicamycin treatment induced the transcript starting from exon 1B by 5-fold, but little affected transcription from exon 1A (Fig. 2C).

Identification of Transcriptional Regulatory Sequences— When the 3.5-kb of DNA from 2.5-kb upstream of exon 1B to exon 2 (nt –2503 to +956) was fused to a firefly luciferase reporter gene, the resulting construct showed about 3-fold higher transcriptional activity than that of the SV40 early promoter (Fig. 3A, lines 1 and 19). Starting with this construct, several deletions lacking 5′-portions of various lengths were examined to localize the transcriptional activities. Deletions up to nt –332 did not affect the activity appreciably (line 2), whereas the extensive 5′-deletion to nt –254 enhanced the activity by 3-fold (line 3), suggesting the negative role played by the region flanked by nt –332 and –255. Further deletion up to –97 (line 4) drastically reduced the activity to a level of less than one-tenth of the maximum activity, suggesting the existence of transcriptional regulatory sequences at just upstream of exon 1A (nt –254 to –98). Additional deletion to +99 further reduced the activity, suggesting that the region from nt –97 to +98 was also involved in the transcription. Significant transcriptional activities were still retained with deletions up to +728 (lines 5–8), indicating that a downstream regulatory sequence for transcription from exon 2 is localized in the segment (nt 728 to 956) just upstream of exon 2. Thus, the segments just upstream of the three transcription start sites were likely essential for each transcription.

To study the upstream regulatory sequence for transcription, the luciferase gene was fused within exon 1A to be transcribed only from exon 1A (lines 9 and 10). The transcriptional activity found in the region from nt –254 to +64 (line 9) was abolished by 5′-deletion to nt –120 (line 10), indicating that the essential sequence for transcription from exon 1A was localized in the just upstream region (nt –254 to –125). Since there were five Sp1 binding sites in this region (from nt –216 to –102), they seemed to be

responsible for the transcription. When the luciferase gene was fused at +32 in exon 1B (lines 11–13), deletion to nt –120 (line 12) also abolished the transcriptional activity despite the presence of the transcription start site from exon 1B. Furthermore, the region from nt –65 to +32 (line 13) did not show any activity. These findings suggested that only the region just upstream of exon 1B was not sufficient for transcription from exon 1B.

To determine the sequence involved in transcription from exon 1B, we examined the luciferase activity upon hypoxia or tunicamycin treatment, because the transcription from exon 1B was most markedly induced under such stress conditions. The luciferase activity of the construct containing the region from nt –254 to +32 was dramatically induced by hypoxia or tunicamycin treatment (Fig. 3B, line 1). On the other hand, deletion of the transcriptional start site of exon 1B reduced the activity to only one-fourth (line 3), which should have represented the transcription from exon 1A. Therefore, most of the activity driven by the region from nt –254 to +32 under stress conditions should have represented the transcription from exon 1B. However, the sequence just upstream region of exon 1B (nt –120 to 32) showed only weak activity even under the stress conditions (line 2), although it contains a *cis*-acting element for the hypoxic response (see below). Therefore, in addition to the just upstream region, a further upstream sequence (nt –254 to –121) seemed necessary for efficient transcription from exon 1B, at least under stress conditions.

It is also worth noting that the activity of the entire region (nt –254 to +956) (Fig. 3A, line 3) is much stronger than the sum of the activities of the upstream and downstream transcriptional regulatory sequences (lines 9 and 11). Although the 5′ region of the first intron did not show detectable transcriptional activity (data not shown), deletion of this sequence from the entire region reduced the activity to only one-tenth, which is just the sum of the upstream and downstream minimal activities (line 14). Six Sp1 sites within the first intron might be involved in the efficient transcription of the entire region.

Cis-Acting Elements for the Hypoxic Response—To identify the region responsible for hypoxic induction, we examined expression of the luciferase reporter gene used above under the stress conditions. Expression of most constructs carrying the region around the 5′-end of exon 1A was enhanced both by hypoxia and tunicamycin treatment (Fig. 4A, lines 1–4, 9, and 10). Despite the lack of the transcription start site for exon 1B, the construct containing the region from nt –254 to –64 showed about 50% enhancement compared with the other active constructs (line 10). On the other hand, the construct containing only the minimal upstream regulatory region (line 11) or first intron (lines 5–8) was virtually inactive. Thus the regulatory element for the hypoxic response was likely localized close to exon 1A (nt –97 to –64).

To identify the sequence responsive to stresses, we then examined various shorter fragments by means of fusion to the SV40 minimal promoter (pGL3-promoter). As shown in Fig. 4B, the segment from nt –133 to –74 specifically conferred the ability to respond to hypoxia or tunicamycin (line 2). Furthermore, the 5′-part of this fragment was completely inactive (line 3), whereas 21-bp fragment HX21 (nt –94 to –74) or 30-bp HX30 (nt –101 to –72) permitted stress

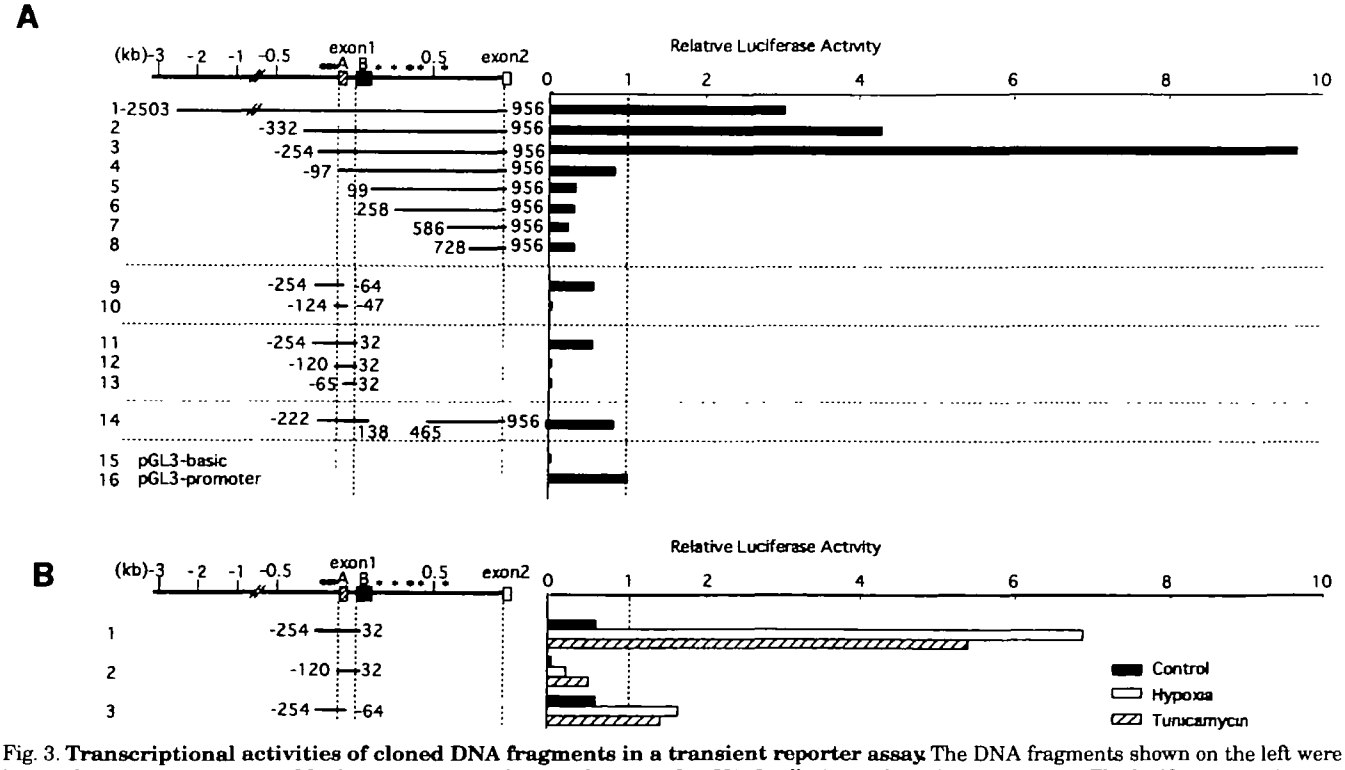

Fig. 3. **Transcriptional activities of cloned DNA fragments in a transient reporter assay.** The DNA fragments shown on the left were inserted into a promoterless pGL3-basic vector and then used to transfect U373 cells. Asterisks indicate Sp1 sites. The luciferase activity was normalized as to that of pGL3-promoter under non-stress conditions. The mean values (SEM <20%) for three independent assays are presented.

induction of about 3-fold (lines 4 and 5). In addition, tandem repeats of the HX30 fragment in both normal and opposite directions markedly enhanced the inducibility (lines 6 and 7) consistent with the known properties of enhancers. Thus the element required for the hypoxic response of the ORP150 gene appeared to be located within the 21-bp sequence (HX21). Interestingly, this sequence contains a stretch of 19-bp (Fig. 4C) almost identical (1 base mismatch) with the recently identified ERSE (CCAATN$_9$CCACG) for the human GRP78 gene (*19*). These results strongly suggested the involvement of the ERSE-like sequence in the hypoxic induction of ORP150.

Possible Trans-Acting Factors for the Hypoxic Response—A transcription factor, ATF6, was recently shown to activate the transcription of mammalian GRP78 and other GRP genes in an ERSE-dependent manner (*19, 32*). As shown in Fig. 4D, cotransfection of an ATF6 expression plasmid with each of the reporter plasmids carrying the 21-bp sequence markedly enhanced the expression, without any external stress, to levels comparable to that obtained upon hypoxic induction (lines 1, 2, and 4–7). These results strongly suggested that the hypoxia-inducible expression of ORP150 is primarily regulated by ERSE and transcription factors including ATF6. Surprisingly, the 21-bp *cis*-acting segment identified above overlaps with the 5′-end of exon 1A (Fig. 1B). This probably explains the above results of selective induction of transcription from exon 1B upon hypoxia or tunicamycin treatment, because when this sequence is occupied by transcription factors under stress conditions, transcription from exon 1A would be difficult to initiate.

We then examined factors that could bind to the above element (HX21 or HX30) by means of gel mobility shift assays. The complex detected as band 1 in Fig. 5A, unaffected by hypoxia or tunicamycin treatment (lanes 1–3), was effectively competed out by unlabeled DNA probes, HX30 (but not HX21) or ERSE of human GRP78 (lanes 4, 5, 8, and 9): this band was specifically supershifted on the addition of antibodies against NF-Y, a transcription factor known to bind to the CCAAT motif within the C1 region of the GRP78 promoter (*33*) that contains ERSE with some flanking sequences (lane 11). The failure of competition by unlabeled HX21 is likely due to a lack of at least several adjacent residues on the 5′-side of CCAAT. On the other hand, three distinct bands (2, 3, and 4) were observed with lower concentrations of poly(dI-dC), which strikingly increased under the stress conditions (Fig. 5, B and C). These bands were effectively competed out by HX21 or HX30 but

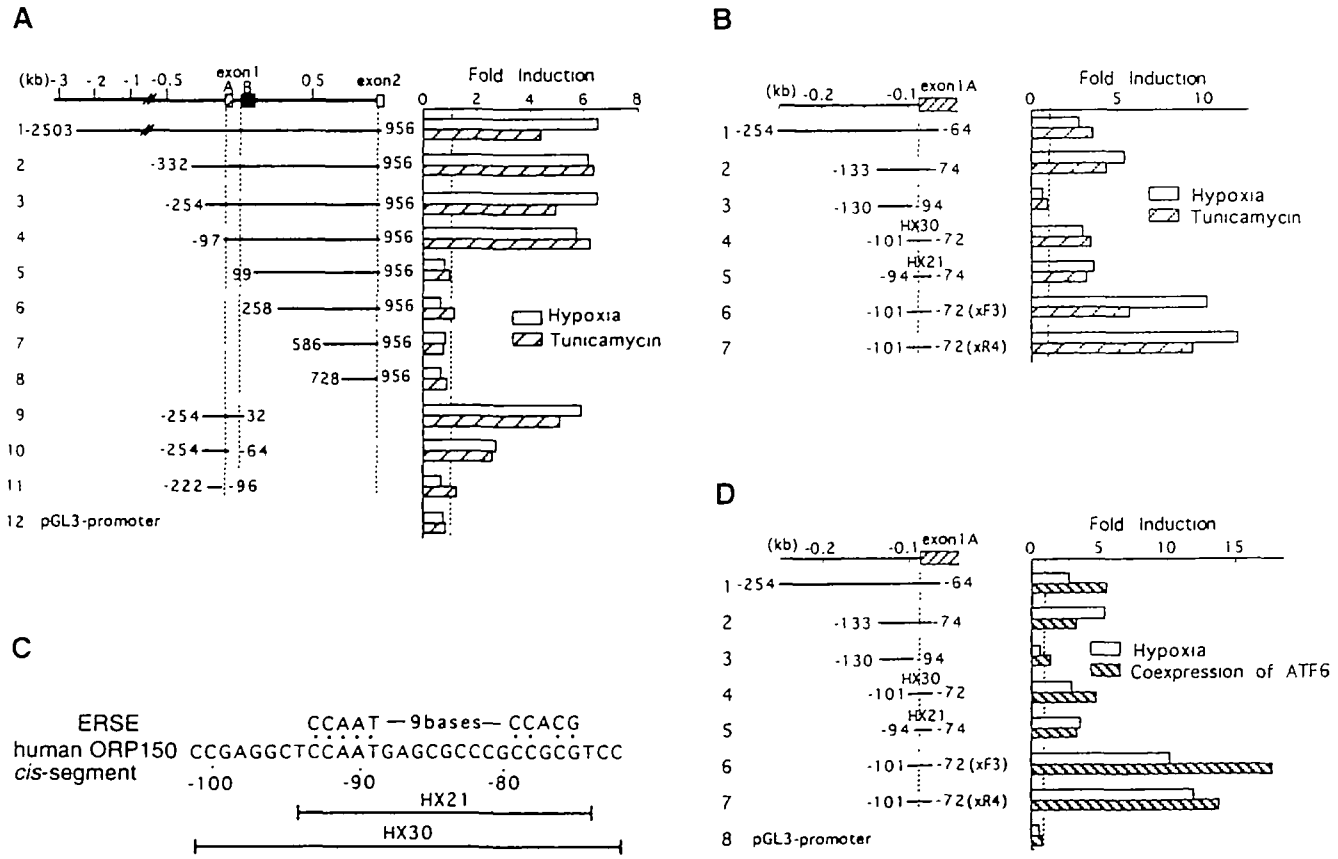

Fig. 4. **Determination of the regulatory *cis*-acting segment for the hypoxic response in the human ORP150 gene.** The DNA fragments inserted into promoterless pGL3-basic (A) or pGL3-promoter (B and D) are shown schematically on the left. xF3 means three tandem repeats of the fragment in the normal direction, whereas xR4 means four tandem repeats in the opposite direction. Fold induction is defined as the ratio of induced to basal expression of the reporter luciferase. The mean values (SEM <30%) for three independent assays are shown. C: Comparison of the *cis*-acting segment for the hypoxic response of ORP150 with the ERSE consensus sequence (*19*). The numbers below indicate positions (nt) in the human ORP150 gene. HX21 and HX30 represent the regions used in the reporter assay (B and D) and a gel mobility shift assay (Fig. 5), respectively.

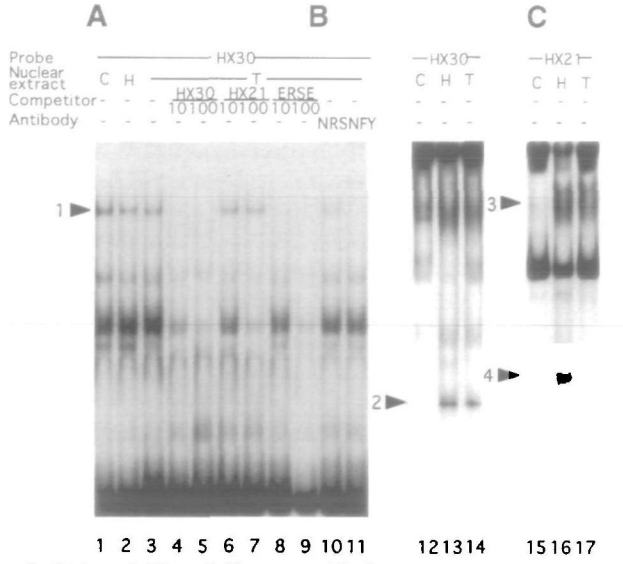

Fig. 5. **Gel mobility shift assay.** Nuclear extracts (2 μg protein) prepared from control cells (C), hypoxia-treated cells (H), and tunicamycin-treated cells (T) were incubated with ^{32}P-labeled DNA probe HX30 or HX21 in the presence of poly(dI-dC) (A, 0.1 μg; B and C, 0.01 μg), and then resolved on native polyacrylamide gels (A, 4.5%; B and C, 5%). The sequence of the competitor DNA fragment, GRP78 ERSE, was CCTTCACCAATCGGCGGCCTCCACGA. The ratios of the competitor to the probe used are indicated. NRS and NFY mean nonimmune rabbit serum (Sigma) and rabbit antibody against NF-Y (Rockland), respectively.

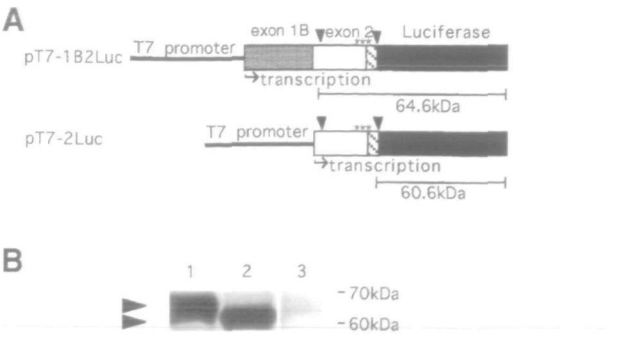

Fig. 6. ***In vitro* transcription-translation of ORP150-luciferase fused genes.** A: The template DNAs used are shown schematically. The coding region of firefly luciferase cDNA (solid box) was ligated immediately downstream to the second AUG codon of ORP150 cDNA present in exon 3. The shaded, open, and hatched boxes represent exon 1B, exon 2, and exon 3, respectively. Triangles indicate the positions of AUG, and asterisks show the mutations (amino acid changes) introduced to prevent cleavage of the signal peptide. The translation products starting from the first and second AUGs are predicted to be 64.6 and 60.6 kDa, respectively. B: Immunoblot analysis of *in vitro* transcription-translation products using antibody against firefly luciferase. Lane 1, pT7-1B2Luc; lane 2, pT7-2Luc; lane 3, no mRNA. The positions of marker proteins are indicated to the right.

not by ERSE, and were not recognized by antibodies against NF-Y or ATF6 (data not shown), suggesting that these stress-specific complexes are composed of other unidentified factor(s).

Alternative Translation Initiation among the Three Species of mRNA—The exon 1A- or exon 1B-containing mRNA is probably translated from the first AUG codon located in exon 2 (Fig. 1B). However, the question arose as to whether or not the transcript starting from exon 2 can be translated from the same AUG located so close to its 5′-end (six nucleotides apart). To address this issue, we constructed the pair of ORP150-luciferase chimera genes depicted in Fig. 6A and carried out *in vitro* transcription-translation studies. The mRNA from one construct (pT7-1B2Luc) should be transcribed from the 5′-end of exon 1B, whereas the mRNA from the other (pT7-2Luc) should be transcribed from six nucleotides upstream of the first ATG in exon 2. Immunoblot analysis of the protein products revealed that the first construct produces a protein of about 65 kDa, as expected (Fig. 6B, lane 1), but the second construct produced a protein of about 60 kDa, the size expected for translation starting from the second AUG codon in the same reading frame (lane 2). These results strongly suggested that the ORP150 mRNA transcribed from exon 2 is translated not from the first but the second AUG located in exon 3, to generate a protein lacking the N-terminal signal peptide of 32 amino acids encoded by the sequence flanked by the first and second AUGs (Fig. 1B).

DISCUSSION

In this study, we showed the transcription initiation of the ORP150 gene from multiple sites by alternative promoters, producing at least three species of mRNA; ones starting from exon 1A, exon 1B, and exon 2. Translation of the first two mRNAs starts from the first AUG codon within exon 2, whereas translation of the third mRNA starts most likely from the second AUG, resulting in the synthesis of proteins containing or lacking the signal peptide, respectively. All three transcripts seem to be observed under normal conditions, whereas the transcript starting from exon 1B is preferentially induced through activation of the UPR pathway under stress conditions, resulting in the accumulation of ORP150 in the ER.

Transcription of Three Species of mRNA—Promoters are often located at the upstream edge of the CpG islands generally near the 5′-end of housekeeping genes (*34*). In the ORP150 gene, the region extending over 1-kb from the upstream region of exon 1A to the first intron contains a cluster of CpG sequences (Fig. 1A), and three species of mRNA are synthesized. Distinct basic transcriptional activity seemed to be located in the just upstream region of each transcription start site. For efficient transcription from exon 1B, however, a further upstream region was required, which overlapped the multiple Sp1 site-containing region essential for transcription from exons 1A. The locations of the upstream regulatory regions that direct transcription from exon 1A and 1B are consistent with the above criterion for the promoter. On the other hand, the downstream regulatory sequence for transcription from exon 2 was unusually located near the 3′-end of the G+C rich region. Furthermore, the entire G+C rich region through the 3′-end of the first intron was essential for high-level transcription (Fig. 3A), suggesting the presence of an enhancer-like segment(s) which increases the entire transcription level. Possible candidates are the multiple Sp1 sites located in the first intron. Thus, the Sp1 sites in both the upstream region and the first intron are likely to play an important role in the transcription of ORP150.

The expression of many genes containing multiple promoters is controlled in very diverse and complex ways: the level and/or tissue specificity of transcription initiation as well as the translation efficiency or stability of mRNA isoforms with different leader exons can vary extensively, and protein isoforms with distinct N-termini can be generated (*35*). Furthermore, some genes have downstream CpG islands in addition to upstream ones, leading to alternative transcription initiation and subsequent translation of functionally disordered truncated proteins (*36–38*). In the case of the ORP150 gene, however, transcription from exon 2 results in translation initiation from the second AUG in the same reading frame (Fig. 6). Since the sequence between the first and second AUGs encodes the signal peptide, the translation product from the second AUG will not be translocated into the ER. This signal peptide-lacking isoform, which is only 3 amino acids shorter than mature ORP150 in the ER, could be functional if not extremely unstable in the cytosol. A similar example, in which subcellular localization is regulated by transcription from alternative initiation sites in a single gene, has been reported for serine: pyruvate aminotransferase, indicating that the localization of this enzyme in mitochondria or peroxisomes depends on whether the transcript encodes the N-terminal mitochondrial targeting signal or not (*39*).

Hypoxic Induction of ORP150 Is Regulated by the UPR Pathway—In response to hypoxia or tunicamycin treatment, the mRNA containing exon 1B was strongly induced (Fig. 2). This is in good agreement with the observation that the 21-bp segment, TCCAATGAGCGCCCGCCGCGT, responsible for hypoxia-dependent expression of the reporter gene is located at the 5′-end of exon 1A (Fig. 4). If some transcription factor(s) binds to this *cis*-acting segment under stress conditions, transcription initiation from exon 1A would be difficult to initiate. This segment contains the sequence matching, except for one base, ERSE, which was recently identified as a *cis*-element responsible for ER stress-specific transcriptional induction of mammalian glucose-regulated proteins (*19*). We observed that transcription factor NF-Y, which had been shown to bind to the CCAAT motif present in the GRP78 promoter (*33*), binds to the ERSE-like segment of the ORP150 gene in a gel mobility shift assay (Fig. 5A). NF-Y, however, is a ubiquitous transcription factor (*40*), and its binding to the ORP150 gene was not affected by the stresses (Fig. 5A), suggesting the involvement of other specific factor(s) in stress-dependent transcription activation. As a most likely candidate of such a factor, bZip transcription factor ATF6, which had been reported to interact with serum response factor (SRF) and to activate SRF-mediated transcription (*41*), was recently identified and shown to enhance transcription through ERSE in response to ER stress (*19*, *32*). The results of the ATF6 coexpression experiment (Fig. 4D) indicate that induction of ORP150 can be mediated by ATF6 through the ERSE-like segment in a manner similar to in the cases of other ER chaperone proteins. However, the 3′-portion of the 21-bp segment of the ORP150 promoter is not completely identical to the proposed ATF6-binding site, CCACG (*19*). Thus we cannot exclude the possibility that an unidentified factor(s) is involved in the hypoxia-responsive induction of ORP150. In fact, some factors that bind to the segment only under the stress conditions were detected in a gel mobility shift assay (Fig. 5, B and C).

These observations together with the previous findings that the stress inducibility and tissue specificity of ORP150 expression are quite similar to those of GRP78 (*4*) strongly suggest that hypoxic induction of ORP150 is dependent upon the UPR distinct from the HIF-1-mediated pathway. Consistent with this notion, the ORP150 gene contains no hypoxia-inducible enhancer element such as those found in human erythropoietin (*24*), vascular endothelial growth factor (*23*), and other hypoxia-responsible genes (*22*). As regards the basal expression of ORP150 under non-stress conditions, another pathway distinct from the UPR pathway could be involved in analogy with those of GRP78 and GRP94 regulated through a mitogenic pathway (*42*).

While little is known about the function of ORP150 at present, the selective accumulation in the ER with the stress-dependent increase in transcription from exon 1B is quite consistent with the expected function in the ER to cope with deleterious ER stresses. Our observation that the ORP150 and GRP78 proteins in U373 cells can be coprecipitated with ORP150 antibodies (data not shown), as reported for Chinese hamster ovary cells (*6*), suggests their cooperative functioning as molecular chaperones in the ER. Furthermore, we raised the possibility of the different subcellular localization of ORP150 based on the existence of multiple transcripts. The cytosolic ORP150, whose mRNA was not induced significantly upon stress, might have a housekeeping function in the cytosol.

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