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Human Biliverdin Reductase Is a Leucine Zipper-like DNA-binding Protein and Functions in Transcriptional Activation of Heme Oxygenase-1 by Oxidative Stress*

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Human biliverdin reductase (hBVR) is a serine/threonine kinase that catalyzes reduction of the heme oxygenase (HO) activity product, biliverdin, to bilirubin. A domain of biliverdin reductase (BVR) has primary structural features that resemble leucine zipper proteins. A heptad repeat of five leucines (L_1–L_5), a basic domain, and a conserved alanine characterize the domain. In hBVR, a lysine replaces L_3. The secondary structure model of hBVR predicts an α-helix-turn-β-sheet for this domain. hBVR translated by the rabbit reticulocyte lysate system appears on a nondenaturing gel as a single band with molecular mass of ~69 kDa. The protein on a denaturing gel separates into two anti-hBVR immunoreactive proteins of ~39.9 + 34.6 kDa. The dimeric form, but not purified hBVR, binds to a 100-mer DNA fragment corresponding to the mouse HO-1 (hsp32) promoter region encompassing two activator protein (AP-1) sites. The specificity of DNA binding is suggested by the following: (*a*) hBVR does not bind to the same DNA fragment with one or zero AP-1 sites; (*b*) a 56-bp random DNA with one AP-1 site does not form a complex with hBVR; (*c*) *in vitro* translated HO-1 does not interact with the 100-mer DNA fragment with two AP-1 sites; (*d*) mutation of Lys^{143}, Leu^{150}, or Leu^{157} blocks both the formation of the ~69-kDa specimens and hBVR DNA complex formation; and (*e*) purified preparations of hBVR or hHO-1 do not bind to DNA with two AP-1 sites. The potential significance of the AP-1 binding is suggested by the finding that the response of HO-1, in COS cells stably transfected with antisense hBVR, with 66% reduced BVR activity, to superoxide anion ($O_2^{\cdot-}$) formed by menadione is attenuated, whereas induction by heme is not affected. We propose a role for BVR in the signaling cascade for AP-1 complex activation necessary for HO-1 oxidative stress response.

Biliverdin reductase (BVR)[1] is a recently described serine/threonine kinase (1) that catalyzes reduction of biliverdin IXα at the γ meso bridge to produce bilirubin. Biliverdin is the product of heme (Fe-protoporphyrin IX) oxidation by the heme oxygenase (HO) system. The reductase in response to extracellular stimuli (*e.g.* cGMP, lipopolysaccharides, and free radicals) translocates into the nucleus (2) and is activated by oxygen radicals (1). The mammalian enzyme is highly conserved; the rat and human reductases share 84% amino acid identity (3, 4). Certain features of the reductase are conserved phylogenetically from cyanobacteria to humans including its unique property among all enzymes characterized to date of having dual pH/cofactor requirement (5, 6). Human BVR (hBVR) is a 296-residue-long polypeptide that, based on its predicted amino acid sequence, has a region with certain key residues that are conserved in proteins that have a leucine zipper dimerization domain, such as human Shaker, human c-Myc, *Saccharomyces* GCN4, human c-Jun, human CREB, human c-Fos, and *Saccharomyces* YAP-1 (Fig. 1). This motif is also found in the rat enzyme (Fig. 1). As a rule, the leucine zipper motif consists of repeat of five leucines (L_1–L_5) separated by six amino acids (Fig. 1) (7, 8). Exceptions to this, however, are found, for instance in *Saccharomyces* YAP-1: L_3 is substituted with asparagine; in *Saccharomyces* GCN4 and human CREB, L_5 is substituted by araginine and lysine, respectively; and, in human c-Myc, valine replaces L_1. They all form functional homodimers or heterodimers. In hBVR and rat BVR, L_3 is substituted with lysine at positions 143 and 142, respectively (Fig. 1). Other structural features of the dimerization domain include a secondary structure that in most cases fits the helix-turn-helix model (8–10) and an invariant basic region that starts exactly seven residues N-terminal to L_1 and is flanked by alanine residues (Fig. 1). The basic region is the DNA binding domain (7, 8, 11). An α/β secondary structure with leucine-rich repeats also forms a high affinity protein-protein interaction domain (12, 13). Although the leucine zipper dimerization motif has been identified in several nonnuclear proteins (Fig. 1), the greater numbers of proteins that have these conserved features are transacting factors and play a role in regulation of gene expression.

The AP-1 site is one of the DNA recognition sequences for leucine zipper proteins. The heme oxygenase cognate, HO-1 or hsp32 (14) is activated by increased AP-1 DNA binding in response to certain oxidative stress stimuli (15, 16). Transcriptional activation involves binding of c-Jun and c-Fos homodimers or heterodimers to the AP-1 site (17, 18). Increased AP-1 complex formation is not restricted to HO-1 or oxidative stress; rather, it is identified for activation of several oncogenes and kinases in response to cytokines, growth factors, transformation factors, UV radiation, and other assorted stimuli (19).

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[1] The abbreviations used are: BVR, biliverdin reductase; HO, heme oxygenase; hBVR, human BVR; AP-1, activator protein; MD, menadione.

Using the x-ray diffraction analysis of rat BVR (20)[2] and alignment of the predicted amino acid sequence of hBVR (4), we have identified conserved features of leucine zipper DNA-binding proteins in the reductase. We have questioned whether hBVR recognizes specific sequences of DNA and, if so, whether this binding is of biological significance. We present data that show specific binding of native hBVR to DNA and suggest a role for BVR in regulation of HO-1 oxidative stress response.

EXPERIMENTAL PROCEDURES

Materials

All of the chemicals and biochemicals used in this study were of ultrapure quality purchased from Sigma, Aldrich, or Invitrogen. Enzymes used in this study (*Bam*HI, *Blp*I, *Hin*dIII, *Sal*I, *Sma*I, *Xho*I, T4 DNA ligase, DNA polymerase, and polynucleotide kinase) were purchased from New England Biolabs, Invitrogen, or Amersham Biosciences, Inc. [^{35}S]methionine and [^{32}P]ATP Redivue™ radioisotopes were purchased from Amersham Biosciences. We used Redivue™ L-[^{35}S]methionine (catalog no. AG 1094), because this grade of [^{35}S]methionine does not cause the background labeling of the rabbit reticulocyte lysate 42-kDa protein that can occur using other grades of labels (21).

Methods

In Vitro Synthesis of Capped RNA Transcript—The full-length BVR fragment was amplified from the plasmid 494 Gex3 (4) using oligonucleotides OL.507 and OL.508, while HO-1 (22) was amplified using oligonucleotides OL.547 and OL.548 (Table I). They were inserted in the multiple cloning site of pCDNA3 (Invitrogen) between *Bam*HI and *Xho*I. The resultant recombinant DNAs were named as p507 and p547. Methods used in the construction of plasmids, including restriction enzyme digestion, separation of plasmid DNA and restriction fragments on agarose gels, ligation of DNA fragments, and the isolation of plasmid DNA are described in Sambrook *et al.* (23). *Escherichia coli* transformations were performed with $CaCl_2$ (24). PCR was carried out as described by Saiki *et al.* (25). Both plasmid p507 and p547 were transformed in INV-competent cells. The plasmid purification was done with Qiagen Mini Prep plasmid purification kit and was linearized by digesting with *Sma*I. Linearized plasmid was then treated with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol-precipitated. Plasmids were dissolved and stored in RNase-free water. RNA was transcribed by using the RiboProbe *in vitro* Transcription System from Promega. 5 μg of linearized template DNA was used in a 50-μl reaction volume using T7 RNA polymerase in the presence of the m7G cap analog so as to generate the capped transcript. 50 units of ribonuclease inhibitor were also added to the reaction along with required amounts of dithiothreitol and nucleotides. After a 1-h incubation at 37 °C, the reaction mixture was treated with RNase-free DNase (1 μl/μg of template DNA) and was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and ammonium acetate, and resuspended in 20 μl of RNase-free water and kept at −70 °C.

In Vitro Translation—A 5.4-kb pcDNA 3 with 1 kb coding hBVR was used as vector to generate *in vitro* transcribed mRNA with T7 RNA polymerase. The transcribed mRNA was translated in the presence of [^{35}S]methionine using rabbit reticulocyte lysate. *In vitro* translation was performed using micrococcal nuclease-treated rabbit reticulocyte lysate (Promega). A 50-μl reaction mixture was prepared by using 35 μl of lysate, 1 μl of 0.1 M dithiothreitol, 2 μl of 1 mM amino acid mixture minus methionine, 1 μl of RNase inhibitor and 5 μl of translation grade [^{35}S]methionine. 5 μl of transcribed mRNA was added to the above reaction mixture and immediately incubated at 30 °C for 90 min. The *in vitro* translated proteins were resolved on 12% SDS or native polyacrylamide gel along with rainbow or native high molecular weight markers, respectively (Amersham Pharmacia Biotech). The gels were fixed in 10% acetic acid and 30% methanol and then treated with autoradiography enhancer (Amplify; Amersham Biosciences) for 30 min and dried under vacuum at 80 °C for 2 h and autoradiographed at −70 °C.

Preparation of ^{32}P-labeled DNA Fragments—A 56- or 100-bp DNA fragment with and without AP-1 sites was used for the DNA binding assay; their sequences are shown in Table I (OL.619, OL.620; OL.623–OL.630). Complementary oligonucleotides were used to generate double-stranded DNA fragments. 150-ng aliquots of annealed oligonucleotides were radioactively labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The DNA probes were purified with the Qiagen Nucleic Acid Purification Kit.

PCR-generated Site-directed Mutagenesis—A 1-kb hBVR fragment was cut out from plasmid p507 by *Sal*I. This 1-kb fragment was used as the template DNA for site-directed mutagenesis. Oligonucleotides (OL.582–OL.587) used for mutagenesis of hBVR leucine zipper motif at positions Lys^{143}, Leu^{150}, and Leu^{157} are shown in Table I. PCR was carried out in two steps. In the first step, the substitutions were introduced by using OL.621 or OL.622 in combination with oligonucleotides OL.582 and OL.583, OL.584 and OL.585, or OL.586 and OL.587 to generate K143A, L150A, and L157A, respectively. In the second stage of the reaction, the PCR products from the first stage were used as template DNA and were joined together by using oligonucleotides OL.621 and OL.622 (Table I). Another difference in the two-step 30-cycle PCRs was the T_m, which was 48 °C in the first reaction and 43 °C in the second. The PCR products, thus formed, were purified with PCR purification kit (Concert) and digested with *Blp*I and *Hin*dIII. The resultant fragments were inserted in p507, which was used as a vector. Ligation was done within the gel by using 1% low melt agarose. The plasmids were amplified in XL-1 Blue cells and isolated by the Qiagen Mini Prep kit. The DNA sequencing of the mutated hBVR segment was carried out with the oligonucleotides OL.582–OL.587 (Table I) using the ABI PRISM dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Big Dye).

Native and Denaturing Gel Analyses—In vitro translated protein was assayed on native gel immediately after synthesis. One μl of *in vitro* translated material was added to 2 μl (25 ng) of annealed, unlabeled control DNA fragment. To this, 0.4 μg of poly(dI·dC) (Amersham Biosciences) in 14 μl of DNA binding buffer (10 mM Tris-chloride (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) was added. It was incubated for 5 min at room temperature, and after adding 5 μl of loading buffer (1.5× DNA binding buffer with bromphenol blue dye), samples were resolved on 12% native polyacrylamide gel in Tris-acetate/EDTA buffer at 35 milliamps. The control DNA helps to prevent the formation of nonspecific protein aggregates, thereby increasing the resolution of protein bands (26). A portion of the translated protein was treated with SDS and analyzed on a denaturing 12% polyacrylamide gel.

DNA Binding Assay—As with native gel analysis, *in vitro* translated proteins were assayed for DNA binding immediately after synthesis. 1 μl of translated material was added to 5000–500,000 cpm of ^{32}P-labeled DNA fragment representing ~2–3 ng of DNA. 0.1 μg of poly(dI·dC) in 10 μl of DNA binding buffer was added to the labeled DNA. After incubating samples for 20 min at room temperature, 5 μl of loading buffer was added. The samples were resolved on 12% native polyacrylamide gel with 35 milliamps at 4 °C. The gels were processed as described above. Dried gels were put on two pieces of film separated by a piece of paper. Autoradiography was done at −70 °C for different time periods.

Western Blot Analysis—For Western blot analysis, the primary antibody was rabbit anti-human kidney BVR (27) with ECL detection system RPN 2106 (Amersham Biosciences). Briefly, *in vitro* translated hBVR was subjected to 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride transfer membrane (Pall Corp.), and subjected to Western blot analysis as described earlier (1).

COS Cell Transfection and BVR Measurement—A cytotoxicity curve for the drug G418 sulfate (Geneticin), used as a marker for the selection of clonal cell lines, was established for exponentially grown COS cells in Dulbecco's modified Eagle's medium (37 °C, 5% CO_2). At a concentration of 440 mg/ml and beyond, the drug was found toxic to the parental cell line. Therefore, the selection medium contained G418 at a concentration of 450 mg/ml. pcDNA3 plasmid containing the antisense sequence was isolated from *E. coli* cultures using Qiagen Midi Prep kit. Transfection was carried out by electroporation. The following day, transfected cells were split 1:2 and seeded on a 100-mm culture dish in the selection medium. The selection process was continued for 8–10 days with a change of selection medium every 2 days. Cells grown in culture flasks to 75% confluence were pooled from three flasks and were used for BVR enzyme activity measurement and mRNA analysis. BVR activity was measured from an increase in absorbance at 450 nm as described before (5) using bilirubin as the substrate and NADH as the cofactor. The activity is expressed as units, a unit representing 1 nmol of bilirubin formed/min/mg of protein.

Northern Blotting—The HO-1 hybridization probe was a 569-base pair HO-1 fragment corresponding to nucleotides 86–654 of rat HO-1 cDNA (28). Cells from a minimum of three culture flasks were pooled and used for each analysis. Total RNA was extracted from COS cells for preparation of poly(A)$^+$ RNA that was separated by electrophoresis on

[2] F. Whitby, J. Phillips, W. K. McCoubrey, C. Hills, and M. D. Maines, unpublished results.

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                    BASIC DOMAIN              LEUCINE ZIPPER
                                                1       2       3       4       5
hBVR        100 MTLSLAAAQELWELAFQKGKVLHEEHVELLMEEFAFLKKEVVGKDLLKGSLLFTSDPL 157
rBVR         99 MTLSFAAAQELWELAAQKGRVLHEEHVELLMEEFEFLRREVLGKELLKGSLRFTASPL 156
hSHAKER     326 GGGGQNGQQAMSLAILRVIRLVRVFRIFKLSRHSKGLQILGKTLQASMRELGLLIFFL 383
hC-MYC      377 LRDQIPELENNEKAPKVVILKKATAYILSVQAEEQKLISEEDLLRKRREQLKHKLEQL 434
sGCN4       224 SSDPAALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER 281
hC-JUN      251 ERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQL 308
hCREB       282 AARKREVRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHK 339
hC-FOS      136 EEPKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKL 193
sYAP-1       63 DPETKQKRTAQNRAAQRAFRERKERKMKELEKKVQSLESIQQQNEVEATFLRDQLITL 120
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FIG. 1. **Amino acid alignment of leucine zipper protein domains.** Key leucine zipper domain molecules (L_1–L_5) and their respective replacements are shown in *boldface type*. Human Shaker, c-Jun, and c-Fos have all five (L_1–L_5) leucine molecules, whereas in the case of hBVR, rat BVR, human c-Myc, *Saccharomyces* GCN4, human CREB, and *Saccharomyces* YAP-1 leucine molecules at positions L_3, L_3, L_1, L_5, L_5, and L_3 are substituted with lysine, lysine, valine, arginine, lysine, and asparagine, respectively. The basic domain is shown as cluster-spacer-cluster structure, and the basic residues are *underlined*. Sequences are derived from *Homo sapiens* (*h*), *Rattus norvegicus* (*r*), and *Saccharomyces cervisiae* (*s*) (14, 47–53).

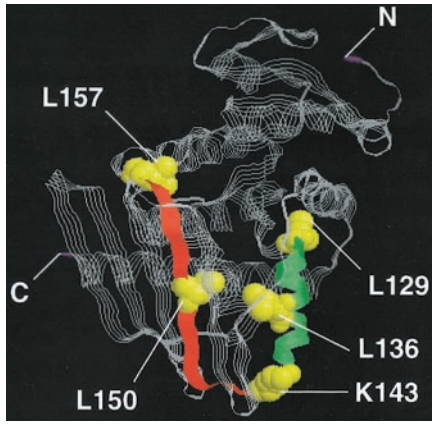

FIG. 2. **The predicted three-dimensional structure of hBVR.** Rat BVR coordinates were used to model the three-dimensional structure of hBVR. The residues of the leucine zipper (*green* and *red*) at key positions Leu^{129}, Leu^{136}, Lys^{143}, Leu^{150}, and Leu^{157} are shown in the space-filling model. Residues between Leu^{129} and Lys^{143} are predicted to form an α-helix; those between Lys^{143} and Leu^{157} form a β-sheet. *N* and *C* denote the N and C terminus, respectively. The *figure* was generated with the molecular graphic program RasMol (36).

denaturing formaldehyde gel, and transferred onto a Nytran membrane. The HO-1 and actin probes were labeled using [α-^{32}P]dCTP with the Random Primers Labeling System (Invitrogen). Prehybridization and hybridization were performed as described previously (29). Blots were probed sequentially with HO-1 and actin. The signals were quantitated using TempDens Platform version 1.0.0 and are expressed relative to that of the control. The control level is arbitrarily given the value of 1.

RESULTS

The comparison of the primary structure of hBVR between amino acids 100 and 157 with known leucine zipper-type DNA binding proteins shows certain common features (Fig. 1). These include the five repeating amino acids L_1, L_2, K_3, L_4, and L_5, spaced every seventh residue, and a basic domain that is flanked by an upstream alanine residue and starts exactly seven residues N-terminal to L_1. There are, however, differences in the primary structure of hBVR and those of most leucine zipper DNA binding proteins; a second basic domain that is present in DNA-binding proteins GCN4, c-Jun, c-Fos, and YAP-1 is not present in BVR. Fig. 2 shows the secondary structure of hBVR, which is modeled after x-ray diffraction analyses of rat BVR crystal structure and shows a U-shaped α-helix-turn-β motif for the leucine zipper motif. Residues that form heptads are identified by a space-filling model. It is noted that a leucine-rich α-helix-turn-β structure is also present in porcine ribonuclease inhibitor and is involved in heterodimer and homodimer formations (12, 13). On the basis of the crystal structure, Kobe and Deisenhofer (12, 13) have shown that the leucine-rich repeat of the ribonuclease inhibitor is also "horseshoe-shaped."

hBVR Forms a Homodimer and Binds DNA—Observations with the primary and secondary features of hBVR were followed by examination of whether hBVR forms a dimer, and if so, whether the dimer interacts with DNA. For DNA interaction analysis, 56-mer and a 100-mer (Table I) DNA fragments encompassing AP-1 sites were used. The 56-mer fragment was a random fragment with one AP-1 site used for investigation of c-Jun and c-Fos DNA binding (26). AP-1 also has been tested for GCN4 binding (30). The 100-mer DNA fragment corresponded to the HO-1 promoter region encompassing two AP-1 sites (31). In order to bind to DNA, leucine zipper type proteins form a dimer, which takes place at the leucine zipper motif (32, 33). Most proteins bearing this structural feature form homodimers, and dimer formation is required for its efficient DNA binding. The only known exception, Fos, forms a stable heterodimer with Jun oncoprotein (17). Therefore, we examined hBVR for homodimer formation immediately after *in vitro* translation of hBVR mRNA, using cold native polyacrylamide gel (4 °C), and employed denaturing/SDS-polyacrylamide gel to dissociate the dimer immediately after *in vitro* translation of hBVR mRNA, should it be formed. On the native gel, the translated protein migrated as an approximately 69-kDa protein (Fig. 3). The protein size was assessed using standard native high molecular weight markers (Amersham Biosciences). Nonspecific protein aggregation was prevented by the addition of control unlabeled DNA (26).

Next, whether the protein synthesized by reticulocyte lysate is in fact hBVR was tested. For this, the *in vitro* translated protein was examined on a 12% SDS-polyacrylamide gel, and the gel was processed either for autoradiography (Fig. 4*A*) or for Western blot analysis (Fig. 4*B*). As shown in the autoradiogram, two prominent bands at ~35 and ~40 kDa were detected. hBVR, based on its predicted amino acid composition, has a molecular mass of ~34 kDa (4). However, because of extensive posttranslational modification, it migrates as a group of size variants with an approximate molecular mass in the range of ~38–42 kDa in SDS gel (4, 34). The Western blot shows, when *in vitro* translated hBVR is probed with antibody to human kidney BVR, two closely migrating bands. The identity of the translated protein was confirmed by comparing its gel migration with wild type hBVR and comparing its immunoreactivity with antibody with that of purified human kidney BVR. As noted in Fig. 4*B*, the pattern of immunostaining of proteins was nearly identical. The control consisted of the rabbit reticulocyte lysate without the addition of transcribed hBVR mRNA. In this lysate, bands near hBVR antibody-immunoreactive bands at the 35–40-kDa region were not detected. Collectively, these findings suggested that hBVR is capable of forming a homodimer.

To determine whether the synthesized hBVR binds to DNA, the *in vitro* translated hBVR was incubated with ^{32}P-labeled 56-mer or 100-mer DNA fragments. An identical 56-bp fragment in which the AP-1 site was substituted with an unrelated sequence of equal length was used as control DNA. In addition, two identical 100-bp fragments with one AP-1 or zero AP-1 sites were synthesized and used as controls (OL.619, OL.620; OL.623–OL.630; Table I). After translation, the protein was incubated with DNA fragment, and the protein/DNA mixture was run on a native nondenaturing polyacrylamide gel. To

TABLE I
List of oligonucleotides

The substitutions K143A, L150A, and L157A in oligonucleotides OL.582–OL.587 are shown in boldface type and are underlined. The AP-1 sites are also shown in boldface type and are underlined, while the replacements of AP-1 sites by random sequences are shown in boldface only for the oligonucleotides OL.619, OL.620, and OL.623–OL.630.

Oligonucleotide number	Sequence
OL.507	GGATCCATGAATGCAGAGCCCGAGAG
OL.508	CTCGAGAGCTACATCACCTCCTCCTC
OL.547	GGATCCATGGAGCGCCCACAGCTCG
OL.548	GCTCGAGTGGCGAAGGATCACCATCGCAGGAGCGGTGT
OL.582	GAAAAAAGAAGTGGTGGGG**GCT**GACCTGCTGAAAGGGTCG
OL.583	CGACCCTTTCAGCAGGTC**AGC**CCCCACCACTTCTTTTTTC
OL.584	GACCTGCTGAAAGGGTCG**GCC**CTCTTCACATCTGACCCG
OL.585	CGGGTCAGATGTGAAGAG**GGC**CGACCCTTTCAGCAGGTC
OL.586	CCTCTTCACATCTGACCCG**GCT**GAAGAAGACCGGTTTGGCT
OL.587	AGCCAAACCGGTCTTCTTC**AGC**CGGGTCAGATGTGAAGAGG
OL.619	TCCTCAGCTGCTTTTATGC**TGTGTCA**TGGTTGGGAGGGGTGATTAGCAGACAAAGGGAAGACAGATTTTGC**GATCCTC**CC CTCTGTTCCCTCTGCCTCAG
OL.620	CTGAGGCAGAGGGAACAGAGGG**GAGGATC**GCAAAATCTGTCTTCCCTTTGTCTGCTAATCACCCCTCCCAACCA**TACACA** GCATAAAAGCAGCTGAGGA
OL.621	CAGCCATGAGGACTACATCAG
OL.622	AGCCAGTTCCTTCTCAGAGAA
OL.623	TCCTCAGCTGCTTTTATGC**TGTGTCA**TGGTTGGGAGGGGTGATTAGCAGACAAAGGGAAGACAGATTTTGC**TGAGTCA**CC CTCTGTTCCCTCTGCCTCAG
OL.624	CTGAGGCAGAGGGAACAGAGGG**TGACTCA**GCAAAATCTGTCTTCCCTTTGTCTGCTAATCACCCCTCCCAACCA**TGACACA** GCATAAAAGCAGCTGAGGA
OL.625	TCCTCAGCTGCTTTTATGC**GATCCTC**TGGTTGGGAGGGGTGATTAGCAGACAAAGGGAAGACAGATTTTGC**GATCCTC**CC CTCTGTTCCCTCTGCCTCAG
OL.626	CTGAGGCAGAGGGAACAGAGGG**GAGGATC**GCAAAATCTGTCTTCCCTTTGTCTGCTAATCACCCCTCCCAACCA**GAGGAT C**GCATAAAAGCAGCTGAGGA
OL.627	CACTGAGAGAAACTATTACACAAGCCACATTAGC**ATGACTCAT**TGTTTCTGATCAG
OL.628	CTGATCAGAAACA**ATGAGTCAT**GCTAATGTGGCTTGTGTAATAGTTTCTCTCAGTG
OL.629	CACTGAGAGAAACTATTACACAAGCCACATTAGC**AGATCCTCT**TGTTTCTGATCAG
OL.630	CTGATCAGAAACA**AGAGGATCT**GCTAATGTGGCTTGTGTAATAGTTTCTCTCAGTG

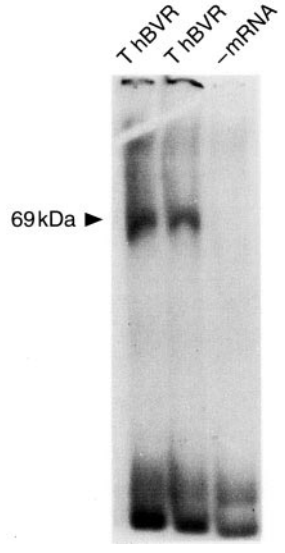

FIG. 3. **Detection of high molecular weight protein synthesized by hBVR mRNA.** *A*, *in vitro* translated hBVR as visualized on a 12% native polyacrylamide gel. From the *left*, the *first two lanes* contain translated hBVR. The molecular mass of the translated protein was approximated to be 69 kDa. This value was obtained using high molecular weight native markers. The *third lane* is that of the control, which consisted of rabbit reticulocyte lysate with all components present in the translation system minus hBVR mRNA.

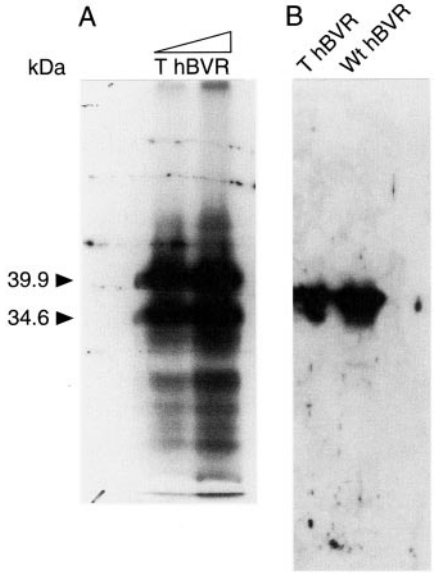

FIG. 4. **Identification of the *in vitro* translated proteins as hBVR by Western blot analysis.** *A*, SDS-polyacrylamide gel electrophoresis of *in vitro* translated BVR with two different amounts of lysate loaded. A 12% SDS gel was used for this experiment. The loading was not intended to be quantitative. Standard molecular mass protein markers indicated the apparent molecular mass of the translated protein bands being 39.9 and 34.6 kDa. *B*, Western blot analysis of *in vitro* translated hBVR. The *first lane* contained the translated hBVR; the *second lane* contained the wild type *E. coli* expressed purified hBVR. The primary antibody was rabbit anti-human kidney BVR. The difference in size of the images shown in *A* and *B* is due to the differential treatment of gels that were required for visualization of translated protein. *T*, *in vitro* translated hBVR; *Wt*, wild type hBVR.

differentiate between ^{35}S-labeled protein and ^{32}P-labeled DNA, the processed gel was exposed to two films separated by an opaque piece of paper, with an enhancing screen against the second film. This was to ensure that the film next to the gel was exposed to both ^{35}S and ^{32}P, while the film next to the screen was exposed only to higher energy ^{32}P radiation. As shown in Fig. 5*A*, the translated hBVR did not bind to a 56-mer DNA fragment having one AP-1 site, while it did bind to the 100-mer DNA fragment having two AP-1 sites. For these experiments, the control contained labeled DNA with rabbit lysate minus hBVR mRNA. As noted in the *figure*, binding complexes were

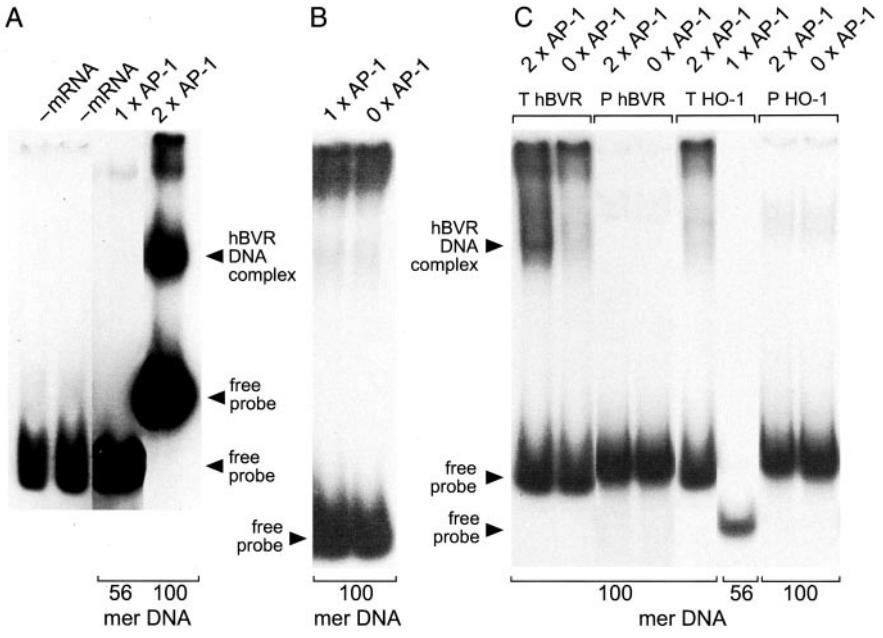

FIG. 5. **hBVR DNA binding assay.** The binding assay was carried out using *in vitro* translated hBVR or HO-1 with modifications denoted for each *lane*. In *A*, the *first two lanes* from the *left* are controls containing the rabbit lysate but without hBVR mRNA. hBVR binding to the 56-mer DNA with one AP-1 site and binding to the 100-mer DNA fragment with two AP-1 sites are shown in the *third* and *fourth lanes*, respectively. The 56-mer DNA used in this experiment has been shown to bind with c-Jun/c-Fos heterodimer (26). The sequence of the 100-mer-long DNA fragment is that of the mouse HO-1 promoter region (39). *B*, analysis of hBVR binding to the 100-mer DNA fragment with one or zero AP-1 sites. *C* shows translated HO-1 binding (THO-1) to the 56- and 100-mer DNA fragments with one or two AP-1 sites, respectively. Also, binding of purified HO-1 to 100-mer DNA with two or zero AP-1 are shown. For comparison, binding of BVR to 100-mer DNA with two or zero AP-1 sites are shown.

not detectable in the control lanes. Also, binding of *E. coli* expressed hBVR protein, which is in monomeric form, to the 100-mer DNA fragment with two AP-1 sites was not detected.

Subsequently, the specificity of DNA binding and the number of AP-1 sites required for binding were examined. For this, hBVR-AP-1 binding was compared between three 100-bp DNA fragments with two, one, or zero AP-1 sites. As shown in Fig. 5*B*, hBVR binding requires two copies of the AP-1 binding sequence, because the interactions of hBVR with 100-bp fragments containing one or zero AP-1 sites were comparable, and the subdued signal appeared to reflect AP-1-unrelated DNA-protein interaction. To further examine the specificity of hBVR DNA binding, binding of *in vitro* translated HO-1 to the same AP-1-containing 56-mer and 100-mer DNA fragments was examined (Fig. 5*C*). The larger DNA had two AP-1 sites. Also, DNA binding was examined using *E. coli* expressed hHO-1 protein. As noted in Fig. 5*C*, neither the *in vitro* translated HO-1 nor the purified protein exhibited binding to the DNA fragments. The specificity of binding was assured by the addition of control unlabeled 100-mer DNA to all DNA binding experiments that used the 100-mer test DNA fragment. The control for the 56-mer test DNA was a 56-mer control unlabeled DNA fragment.

In Vitro Translation of hBVR Leucine Zipper Mutants and Their Binding to DNA—To establish the role of the leucine zipper motif of hBVR in DNA-protein interaction, site-directed mutagenesis studies were carried out. Mutations were directed to Lys^{143}, Leu^{150}, and Leu^{157} that were changed to alanine, thereby generating K143A, L150A, and L157A, respectively. This was a particularly relevant investigation, because, as noted above, the model of the secondary structure of hBVR (Fig. 2) predicts a β-sheet structure for hBVR between Lys^{143} and Leu^{157}, while the structure common to most leucine zipper DNA-binding proteins is often entirely α-helical. Studies with Jun and Fos oncoproteins suggest that single mutations in the motif are sufficient to abolish specific DNA binding (35). It has also been shown that a single amino acid change in Fos abolishes the DNA binding capabilities of the Fos-Jun dimer complex.

For this set of experiments, the [^{35}S]methionine-labeled mutant BVR proteins were generated by *in vitro* translation and assayed on a 12% native gel for detection of the ~69-kDa protein band and analysis of DNA for complex formation. The 100-mer DNA fragment with two AP-1 sites or without an AP-1 site was used. On the native gel, the high molecular weight band was not detected with the mutated proteins. Also, as shown in Fig. 6, a single mutation in any of the three positions prevented protein-DNA complex formation. As noted, binding of the three mutant proteins with the DNA fragment having two or zero AP-1 sites was essentially comparable and was similar to that of the native hBVR binding to the 100-bp fragment with no AP-1 site. As before, the control, *in vitro* translated hBVR shows clear binding with DNA having two AP-1 sites.

The three-dimensional conformation of hBVR leucine zipper domain, predicted by the RasMol molecular graphic program (36), suggested that substitution of Leu^{143}, Leu^{150}, or Leu^{157} by alanine in the leucine zipper motif apparently does not cause conformational changes in the motif and hence, most likely, does not account for the attenuated DNA binding.

HO-1 Response to Menadione and Heme in COS Cells Transfected with Antisense hBVR—To examine whether DNA binding of hBVR has any bearing on gene expression, induction of HO-1 in COS cells stably transfected with antisense hBVR was examined. HO-1 is transcriptionally regulated by a vast array of stimuli that trigger activation of different regulatory factors. MD and heme are both inducers of HO-1 gene expression but involve distinctly different signaling cascades-activating factors. To determine whether the antisense mRNA affected BVR activity, activity in the transfected cells was measured. As shown in Fig. 7*A*, a 66% decrease in activity was detected. This cell line was then used to examine the response of HO-1 to known inducers, heme and MD, by Northern blot analysis. As noted in Fig. 7*B*, the response of cells carrying antisense hBVR to heme did not differ from that of the control cells, and an increase of ~35-fold in HO-1 mRNA was detected in both sets of cells. In contrast, MD, which is a generator of oxygen radicals, produced a less than remarkable increase in HO-1 mRNA levels in the transfected cells. The control cells, on the other hand, displayed a robust response to MD. The magnitude of increase in HO-1 mRNA in the control and transfected cells was 20-fold *versus* 7-fold, respectively. HO-1 mRNA in COS cells with an absence of inducers was marginally detectable.

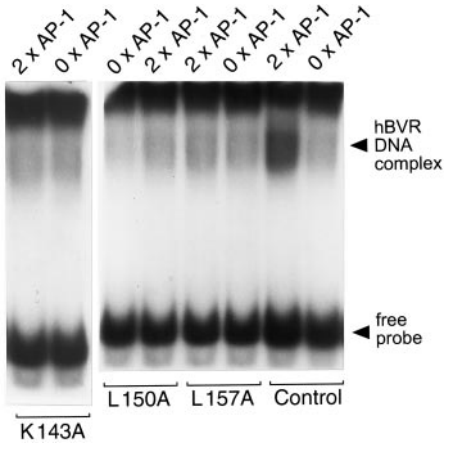

FIG. 6. **Mutant hBVR proteins do not form a DNA complex.** Binding of the three *in vitro* translated hBVR mutants to 100-mer DNA having two AP-1 or zero AP-1 sites is shown. For comparison, binding of native *in vitro* translated hBVR to DNA having two AP-1 sites and with zero AP-1 sites is shown.

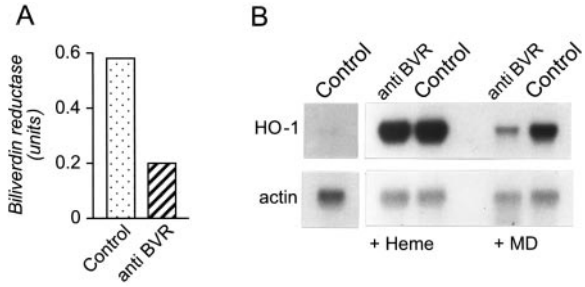

FIG. 7. **Northern blot analysis of HO-1 response to inducers in COS cells transfected with antisense hBVR.** COS cells were stably transfected with hBVR antisense mRNA as described under "Experimental Procedures" and were used for BVR activity analysis and response of HO-1 to inducers. *A*, BVR activity measured in COS cell cytosol fraction prepared from cells pooled from three flasks. Enzyme activity was measured as described under "Methods." *B*, Northern blot analysis was carried out as described under "Experimental Procedures" using three flasks; whole cell preparations were used for isolation of poly(A)$^+$ RNA. The concentration of MD was 100 μM, while the concentration of heme was 10 μM. The duration of treatment for MD was 30 min followed by a 3-h recovery period. The duration of treatment with heme was 3 h (45). The control HO-1 signal intensity is arbitrarily designated as one. Relative intensities, expressed as -fold increase, are as follows: when compared with the control, 1; compared with antisense plus heme, 34.4; compared with control plus heme, 35.4; compared with antisense plus MD, 7.4; and compared with control plus MD, 20.5.

DISCUSSION

When a leucine zipper motif in the primary sequence of hBVR was detected, we considered that in BVR the motif could either be involved in dimerization, DNA binding, or some other functions related to its kinase activity. Of course, the possibility that the motif is of no apparent biological significance was not ruled out. A unifying feature of sequence-specific DNA-binding proteins is dimerization. Presently, evidence is provided that indicates formation of a homodimer by hBVR that binds to DNA and involves the leucine repeat region; the DNA binding sites are identified as two AP-1 recognition sequences. The finding that the single form of the nascent protein (Fig. 3) dissociates in two species (Fig. 4) under denaturing conditions and identification of the proteins based on their immunoreactivity as BVR (Fig. 4*B*) are indicative of a BVR homodimer formation. Moreover, the reductase contains the characteristic putative dimerization interface made of L_1, L_2, K_3, L_4, and L_5, which is found in several proteins that bind nucleic acids (Fig. 1). The finding that site-directed mutation of these residues blocks the ability of hBVR to form a complex with 100-mer DNA with two AP-1 sites is indicative of their participation in the formation of the hBVR DNA complex. It is not known whether hBVR also interacts with other proteins to form heterodimers. Previous studies have shown that in many instances the DNA binding property of proteins with the leucine zipper motif is lost with single or double mutations in the motif, which may or may not alter the dimer formation (26, 35, 37). In the case of hBVR, individual mutations at the K_3, L_4, and L_5 prevent dimer formation.

Although hBVR has similarities in structure to a number of DNA-binding proteins with a leucine zipper motif, it also has divergent features. Moreover, based on the predicted secondary structure of hBVR, the sequence of amino acids between Leu^{129} and Lys^{143} forms an α-helical structure, while the sequence between Lys^{143} and Leu^{157} is mainly β-sheet. Notably, the predicted secondary structure for many leucine zipper DNA-binding proteins is two α-helices separated by a β-turn. Also, GCN4, a leucine zipper type DNA-binding protein falls short of such a helix-turn-helix motif (30). The DNA contact region in many of the leucine zipper proteins is the sequence immediately NH_2-terminal to the leucine zipper with a notable degree of basicity that starts seven residues N-terminal to L_1. In BVR, however, the content of basic amino acids in this region is low in comparison with that of other DNA-binding proteins, and unlike those proteins that have two clusters of basic residues linked by a spacer sequence with an invariant alanine spacer, only one basic cluster is present in BVR (Fig. 1). A second N-terminal basic domain is also absent from c-Myc, which is a helix-loop-helix DNA-binding protein. It has, however, a basic domain near the C terminus of the protein. The reductase has a basic domain near the carboxyl terminus of the protein: KKRILH (residues 275–280), which plausibly could also interact with DNA. In addition, the second basic domain is also absent in the leucine zipper protein human Shaker K^+ channel 3 β-subunit (Fig. 1), which interestingly is also an oxidoreductase (38). In Shaker, which is a member of the aldo-ketoreductase superfamily, the leucine zipper motif is involved in interaction of K^+ channel subunits and to our knowledge has not been reported to bind to DNA.

Observations with COS transfected with antisense BVR are supportive of the suggestion that hBVR DNA binding is probably of biological consequence as far as the regulation of HO-1 by free radicals is concerned. An inference as to the possibility of sequence-specific DNA binding involving the AP-1 sites of HO-1 is drawn from two pieces of data: (*a*) BVR-DNA complex formation was observed with a DNA fragment of HO-1 promoter region, and (*b*) cells transfected with antisense BVR displayed an attenuated increase in HO-1 gene expression in response to oxidative stress, whereas their response to heme was similar to that of control. As reported, mutations in AP-1 binding sites block HO-1 gene activation by oxidative stimuli (15, 16, 39). Also, the leucine zipper transcription factors, Jun and Fos, which constitute the AP-1 family, are activated by oxidative events (40, 41). In addition, several other DNA binding sites for transcriptional activation of HO-1, which is responsive to a wide assortment of stimuli (reviewed in Ref. 42), have been identified (15, 16, 28, 43).

On the basis of the denoted observations, it is reasonable to suspect that BVR may have a function of sorts in the AP-1 pathway of cell signaling. MD has long been used as an oxidative stress model. It stimulates the rate of NADPH oxidation, H_2O_2 production, and redox cycling that results in formation of superoxide anions (44). The previous findings, that the reductase is activated by the oxidant, H_2O_2, and is a serine/threonine kinase (1), lend support to this idea. Noteworthy is that H_2O_2 is an activator of HO-1 gene expression (45, 46). The suggestion

that hBVR-DNA binding is linked to the activation of the HO-1 gene is also consistent with previous observations that, in HeLa cells in response to cGMP and in intact rats in response to lipopolysaccharides or to the free radical generating compound, bromobenzene, reductase translocates from the cytosol to the nucleus (2). All mentioned stimuli are inducers of HO-1 gene expression.

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