The estrogen receptor (ER) is a ligand-activated transcription factor whose DNA-binding domain (ERDBD) has eight cysteines, which coordinate two zinc atoms, forming two zinc finger-like structures. We demonstrate the capability of iron to replace zinc in iron finger (hereby referred to as iron finger) both in vivo (using Escherichia coli BL21 (DE3)) and in vitro. Iron has the ability to substitute for zinc in the ERDBD as demonstrated by mobility shift and methylation interference assays of iron finger, which show specific recognition of the estrogen response element. The DNA binding constants for both in vivo and in vitro iron-replaced zinc fingers were similar to that of the native finger. Atomic absorption analysis revealed a ratio of 2:1 iron atoms/mol of ERDBD protein, as found for zinc in the crystal structure of native ERDBD. More importantly, we demonstrate that iron finger in the presence of H2O2 and ascorbate generates highly reactive free radicals, causing a reproducible cleavage pattern to the proximate DNA, the estrogen response element. The deoxyribose method, used to detect free radical species generated, and the resultant cleaved DNA ends, caused by iron finger, suggest that the free radicals generated are hydroxyl radicals. Due to the close proximity of the zinc finger to DNA, we postulate that iron-substituted zinc finger may generate free radicals while bound to genetic regulatory response elements, leading to adverse consequences such as iron-induced toxicity and/or carcinogenesis.

The estrogen receptor (ER) is a nuclear hormone receptor belonging to a superfamily of ligand-activated DNA-binding transcription factors. Once ligand-activated, the ER regulates transcription by binding to specific DNA response elements located upstream from its target genes (1, 2). Receptors of the superfamily are divided into comparable, discrete domains known as the ligand-binding, transcriptional activation, and DNA-binding domains (3, 4). The DNA-binding domain is the most highly conserved region and is centrally located in the receptor. Within the DNA-binding domain, eight highly conserved cysteine tetrahedrally coordinate two zinc atoms, forming two nonequivalent zinc finger-like motifs (5). These two nonequivalent zinc fingers fold to form a single structural module, enabling the receptor to bind a specific hormone response element as a monomer or a dimer (6). In the case of the ER the estrogen response element (ERE) is a 15-base pair DNA sequence consisting of two hexameric palindromic sites separated by three base pairs, AGGTCA	TTGACCT (7), facilitating a “head-to-head” homodimerization of two ERDBDs. As well as binding the ERE, the ERDBD includes a nuclear localization signal (8) and a region mediating weak dimerization (9). Earlier in vitro studies have demonstrated the ability of cobalt and cadmium to structurally reconstitute the zinc finger motif of the ERDBD (10). Nickel and copper on the other hand were shown to bind the ERDBD, yet neither restored the DNA binding property. In either case, the biological consequence of heavy metal incorporation into zinc finger may have relevance in the manifestation of metal-induced toxicity (10, 11). Although metals such as iron, copper, or cobalt are essential elements, they are also toxic. Evidence suggests that elevated levels of iron contribute to an increased risk of cancer (12). Primarily, this rise in the risk of malignancy is thought to be due to two reasons: the ability of iron to generate highly reactive free radicals and the increased demand for iron by the rapidly multiplying malignant cells needed for energy production (within the mitochondria) and DNA replication (ribonucleotide reductase) (13). However, at this point, extensive studies investigating the mechanism of iron-induced toxicity have yet to define the true nature of this phenomenon.

This study establishes the ability of iron to replace zinc in the ERDBD both in vivo (using Escherichia coli BL21 (DE3)) and in vitro. We report similar Kd values for DNA binding and metal:protein ratios for native zinc finger and iron-replaced fingers. We also demonstrate the ability of iron finger to generate free radicals that cause cleavage of the proximate DNA, the ERE.

**MATERIALS AND METHODS**

Expression and Purification of ERDBD—E. coli strain BL21 (DE3) was transformed with the plasmid p2R, which encodes for an 84-amino acid polypeptide containing the human ERDBD. The ERDBD expression vector was a gift from P. Chambon (Institut de Chimie Biologique, Strasbourg, France). Expression from this plasmid is driven by T7 RNA polymerase (14). The cells were grown to logarithmic phase in SOC media (A600 = 0.7–0.9), and expression of the polypeptide was induced for 2 h with 1 mM isopropyl-1-thio-β-β-D-galactopyranoside. Cells were collected and resuspended in 10 volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DT, 0.5 mM NaCl and lysed by a freezing and thawing process. Sodium deoxycholate was added to a concentration of 0.05%, and the mixture was centrifuged at 60,000 × g for 45 min at 4 °C. The supernatant containing the ERDBD was brought to 0.2% polyethyleneimine and centrifuged at 35,000 × g for 30 min at 4 °C to remove additional impurities. The extract was dialyzed against loading buffer (100 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 10% (v/v) glycerol, 2 mM DT, 5 mg/liter phenylmethylsulfonfyl fluoride, pH 7.2) and partially purified on a 1-mL Bio-Rex 70 resin (Bio-Rad) column (15). Final separation was achieved on a fast protein liquid chromatography Superdex 75 column (Pharmacia Biotech Inc.) using a 50 mM Tris-HCl, 100 mM NaCl, pH 7.2, buffer.
Metal-free Glassware, Buffers, and Media—All subsequent experiments were performed with acid-washed glassware, and all buffers and media were prepared using double-distilled, deionized, sterile, metal-free water. Any remaining contaminating metal was eliminated with the chelating resin Chelex 100 (Bio-Rad). For the deoxyribonuclease I (ERDBD) with end-labeled ERE (0.01 mg/ml), 250 ng of either metal-replaced HEPES buffer (12 mM HEPES-KOH, 4 mM Tris-HCl, 60 mM KCl, 12% (v/v) glycerol, 1 mM DTT, pH 7.4) for 4 h at 4°C.

In Vivo Apolipoprotein Preparation and Metal Replacement—Apolipoprotein was prepared by dialysis of the native ERDBD against buffer (6x urea, 10 mM 1,10-phenanthroline, 1 mM acetic acid, 5 mM DTT, pH 7.4) for 4 h at room temperature. For metal replacement, the apo-ERDBD was dialyzed against HEPES buffer (12 mM HEPES-KOH, 4 mM Tris-HCl, 60 mM KCl, 12% (v/v) glycerol, 1 mM DTT, pH 7.4) containing 50 μM ferrous ammonium sulfate (Aldrich) after argon gas at 4°C for 3 h.

In Vivo Metal Replacement—The procedure was as described under "Expression and Purification of ERDBD," except metal-free minimal media 9 (8 mM NaCl, 8 mM (NH₄)₂SO₄, Na₂HPO₄, KH₂PO₄, pH 7.4) supplemented with 55 mM glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 20 μM FeCl₂, 100 μg/ml biotin, 100 μg/ml thiamin was used instead of SOC media, with the addition of the appropriate metal to a final concentration of 50 μM.

Gel Mobility Shift Assay—Specific binding of the polypeptide to an ERE was measured by a mobility shift assay with a 5.4% native polyacrylamide gel containing the ERE palindromic double hexamer (5'-AGTACGCGTTAGCTAGCCTGAGCTGTCGAG-3'). Various amounts of ERDBD (250 ng of native ERDBD and 150 ng for each metal-replaced ERDBD) with end-labeled ERE (0.01 μg/ml DNA with a total count of 100,000 cpm) were preincubated for 15 min at room temperature in 30 μl of sample running buffer (20 mM HEPES-KOH, 100 mM NaCl, 20% (v/v) glycerol, 1 mM DTT, pH 7.4) containing 1 μg/ml of nonspecific DNA (poly[dI-dC]). 15 μl of this mixture was then loaded on a low ionic strength 5.4% polyacrylamide gel and electrophoresed for 2 h at 150 V at room temperature. The running buffer (70 mM Tris-HCl, 30 mM sodium acetate, and 10 mM EDTA, pH 7.9) was circulated between chambers, and the gel was prerun for 1.5 h at 100 V at room temperature and was subjected to sample application. The gel was dried and then exposed to x-ray film (Eastman Kodak Co.) overnight and then a Porath gel imager plate.

Deoxyribonuclease I Method for Free Radicals—The procedure was performed as described (16). The reaction mixture contained (unless stated otherwise) 20 mM deoxyribose, 1 mM H₂O₂, 20 mM potassium phosphate, pH 7.4, 250 ng of either iron or zinc finger in a total volume of 0.6 ml. After incubation for 1 h at 37°C, 0.5 ml of 1% (w/v) thioribarbituric acid in 50 mM NaOH and 0.5 ml of 2.8% (w/v) trichloroacetic acid were added, and the mixture was heated at 100°C for approximately 20 min. Formation of hydroxyl radicals was measured by an increase in absorbance at 232 nm.

Methylation Interference—The procedure was performed as described (17) using dimethyl sulfate-methylated ERE consensus half-site hexamer (5'-CCGGTACGCTAGCCTGAGCGCC-3'). The DNA (1 × 10⁹ cpm of ERE half-site) was G-methylated by exposing the ERE, which has been 32P-labeled synthetic 37-base pair oligonucleotide containing the ERE palindromic double hexamer (5'-AGTACGCGTTAGCTAGCCTGAGCTGTCGAG-3'). Various amounts of ERE and ERDBD (molar ratio of ERDBD:ERE of 8:1) in 70 mM Tris-HCl, 4 mM MgCl₂, 1 mM NaCl, 10 μM H₂O₂, 200 μM deoxyribose, 1 mM H₂O₂, 200 μM potassium phosphate, pH 7.5) per 10 μl were incubated with 0.6 μl of Cowboys (Johnson et al., 1984) for 15 min at room temperature. The reaction was then stopped with 50 μl of 8 M sodium acetate containing 250 μg/ml carrier RNA and 1 ml of cold ethanol. The sample was then placed in a dry ice bath for 5 min and centrifuged, and the pellet was dried in a Speedvac cassette. Equivalent counts per minute of free and bound ERE were electrophoresed on a 20% urea denaturing gel and electrophoresed at 2000 V for 3 h. The gel was dried and exposed to x-ray film overnight.

DNase Footprint—The procedure was performed as described (18) using a 5'-end 32P-labeled on one strand of a 37-base pair synthetic oligonucleotide containing the ERE (5'-AGTGACGCGTTAGCTAGCCTGAGCTGTCGAG-3'). ERE, at a total concentration of 100,000 cpm, was added to buffer (100 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 1 mM CaCl₂, 20 μM of bovine serum albumin, and 60 mM KCl) with an excess of ERDBD in a molar ratio of 8:1 (ERDBD:ERE) with a final volume of 200 μl. This mixture was preincubated for 20 min at 37°C. One μl of a stock DNase I solution (0.01 mg/ml DNase I, 5 mM sodium acetate, pH 4.5, 1 mM CaCl₂, and 50% glycerol) was added and left for 2 min at 37°C. The reaction was then stopped with 50 μl of 8 M sodium acetate and the DNA was precipitated with ethanol and centrifuged, and the pellet was dried in a Speedvac (Savant SVC100H). Equivalent counts per minute of free and bound ERE were electrophoresed on a 20% urea denaturing gel at 2400 V for 3.5 h. The gel was dried and exposed to x-ray film.

Hydroyxl Radical Footprint—The procedure was performed as described (19) using a 5'-end 32P-labeled on one strand of a 37-base pair synthetic oligonucleotide containing the ERE (5'-AGTGACGCGTTAGCTAGCCTGAGCTGTCGAG-3'). A mixture of 1 × 10⁹ cpm of ERE and ERDBD (molar ratio of ERDBD:ERE of 8:1) in 70 mM of binding buffer (70 mM Tris-HCl, pH 8, 60 mM NaCl, 20% (v/v) glycerol, pH 7.5) was preincubated for 15 min at room temperature. A solution of 0.1 mM [Fe(EDTA)]²⁻ was prepared by mixing equal volumes of 0.2 mM ferrous ammonium sulfate and 0.4 mM of EDTA. Then 10 μl each of 0.1 mM [Fe(EDTA)]²⁻, 0.01 M sodium ascorbate, and 0.3% (v/v) H₂O₂ were added to the binding buffer mixture. The reaction proceeded for 1 min at room temperature and was quenched with 43 μl of sample running buffer (50 mM NaOH and 0.5 ml of 2.8% trichloroacetic acid in 50 mM NaOH and 0.5 ml of 2.8% trichloroacetic acid were added, and the mixture was heated at 100°C for approximately 20 min. Formation of hydroxyl radicals was measured by an increase in absorbance at 232 nm.

RESULTS AND DISCUSSION

Iron-replaced Zinc Finger

In Vivo and in Vitro Metal Replacement—To obtain structural metal replacement of the ERDBD, the metal atom of the zinc finger must be coordinated tetrahedrally by four cysteines of the individual zinc finger (5). The zinc atom does not directly interact with the ERE but instead acts as a type of scaffolding that confers the required conformation to the overall structure, enabling the ERDBD to bind the ERE. This suggests that any metal with similar coordination properties and ligand affinities to zinc may be able to replace it in the zinc finger. Iron, as Fe(Ii), possesses the appropriate reconstitution criteria. These include tetrahedral geometry, with a coordination number of 4 and an affinity for sulfhydryl groups. A precedent has been set by the protein rubredoxin, found in Clostridium pasteurianum, which tetrahedrally coordinates iron with four cysteines (20). Also, in vitro metal replacement experiments, in which the first zinc finger of the erythroid tran...
scription factor GATA1 was successfully reconstituted with Fe(II) had been reported (21). The GATA1 zinc finger is similar in design to the ER zinc finger in that they both contain zinc tetrahedrally coordinated to four cysteines. Our results demonstrate the ability of iron to replace zinc within the ERDBD. The gel mobility shift assay shown in Fig. 1 demonstrates the ability of iron to replace zinc in the ERDBD in vitro. When apolypeptide of ERDBD was dialyzed against buffer containing iron as Fe(II), the specific DNA binding activity was restored to about the same level of the native, zinc finger (Fig. 1, lanes a and c, respectively), while the control, apolypeptide dialyzed against metal-free buffer, showed no binding activity (Fig. 1, lane d).

The iron finger of ERDBD could be synthesized in vivo when the transformed E. coli were grown in minimal medium supplemented with 50 μM ferrous ammonium sulfate. As seen in Fig. 2, transformed cells displayed similar growth rates when grown in either a metal-depleted or a zinc-supplemented medium, while the cells clearly showed a much faster growth rate in the iron-supplemented medium. This result is in accord with the assumption that a continuous supply of iron is essential for the growth and reproduction of these organisms (13). The ERDBD purified from E. coli culture grown in the iron-supplemented minimal medium had similar DNA binding specificity to ERE as that of native ERDBD, and the iron finger formed in vitro (See Fig. 1, lane e). Metal analysis of purified in vivo iron

**Fig. 1.** Mobility shift experiments with native, apolypeptide, iron in vitro and in vivo replaced ERDBD, and the ERE. Lane a, iron in vitro substituted finger; lane b, apolypeptide; lane c, native ERDBD zinc finger; lane d, apolypeptide control; lane e, in vivo iron finger; lane f, free DNA. Experimental details are described under “Materials and Methods.”

**Fig. 2.** Growth rate curves of transformed E. coli BL21 (DE3). The bacteria were grown in minimal media supplemented with 50 μM iron or zinc or no metal (control). See details under “Materials and Methods.”
finger revealed a 2:1 molar ratio of iron to ERDBD peptide (2.3 ± 0.2 atoms of Fe/mol of peptide), confirming that the peptide was synthesized as iron finger. Zinc content of this peptide was only 0.02 ± 0.01 atoms/mol of ERDBD.

The methylation interference assays with the half-site ERE oligonucleotide, performed with zinc- or iron-ERDBD, each displayed specific interactions only with the guanine of the ERE half-site hexamer (TGGACT) (Fig. 3) as previously reported (10). The above results, combined with the comparable ERE-ERDBD Kd values obtained for native ERDBD and in vivo and in vitro iron fingers (Kd values of 10, 29, and 39 nM, respectively) evidently show that iron can substitute for zinc in the ERDBD and maintain ERE-specific binding and affinity. The zinc content of iron finger formed in vivo is too low to account for the above results, and in vitro formed iron finger had no detectable zinc present yet showed identical results to in vivo iron finger.

Iron Finger Hydroxyl Radical Generation—Our subsequent studies show that iron finger in ERDBD in the presence of H2O2 and ascorbate in phosphate buffer at pH 7.4 generates hydroxyl radicals as determined by the deoxyribose method (Table I). Mannitol, thiourea, and formate are well known scavengers of hydroxyl radicals and therefore they all reduce the absorbance at 532 nm as seen in Table I. Catalase converts H2O2 into H2O, preventing hydroxyl radical generation, and hence causes a reduction of absorbance. On the other hand, superoxide dismutase converts superoxide to H2O2 and does not prevent hydroxyl radical generation. Therefore iron does replace zinc finger zinc, and iron finger, thus formed, generates highly reactive hydroxyl radicals.

It is known that iron induces mutagenesis/carcinogenesis (22–24), but the exact mechanism of iron-induced toxicity is unknown. An excess of iron in the circulatory plasma, which exceeds the iron saturation capacity of serum, results in abnormal deposition of iron in body organs, which may lead to deleterious effects. Stevens et al. (12) found that levels of transferrin saturation between 50 and 60% increase the risk of cancer by 37%. The ability of iron to promote malignancy is believed to be a result of at least two possible mechanisms. One is the catalytic interaction of iron and oxygen, generating free radicals and resulting in cellular consequences such as DNA mutations, sister chromatid exchange, and carcinogenesis. This mechanism is supported by the observations that cells are protected against iron-induced toxicity by either sequestering iron with iron chelators or eliminating hydrogen peroxide with catalase and thereby preventing the induction of free radicals (25, 26).

The hydroxyl radical can travel a maximum distance of 60 Å (25). Therefore, the resultant cellular damage occurs proximate to the site of formation. Hydroxyl radicals produced near DNA may cause strand breakage, while damage done to protein that is present in excess may have no biological consequences. Therefore, the biological importance of hydroxyl radical formation is related to the site of action. To investigate the damage done by the iron finger to the ERE, a 32P-3′-labeled 37-base pair oligonucleotide, containing the ERE, was incubated with iron finger in the presence of 0.03% H2O2 and 1.0 mM ascorbate in 10 mM Tris-HCl buffer, pH 7.4, and run on a 20% urea denaturing gel. Free iron and Fe-EDTA cleaves both DNA strands randomly (Fig. 4, lanes c–f), while we find a reproducible scraggly cleavage pattern (Fig. 4, lanes i and j) when iron finger is incubated with the ERE. In Fig. 4, the bands caused by free iron, Fe-EDTA, and iron finger scission are aligned. These results suggest that the mechanism of scission and, therefore, the species responsible are the same in each case, involving specifically the hydroxyl radical (29). This is consistent with the results obtained from the deoxyribose method. This raises the question: are the observed iron finger-ERE scissions caused by iron finger or a result of a footprint, such as one that would be obtained by incubating free iron with a protein-DNA complex? Here again we have a 32P-3′-labeled 37-base pair oligonucleotide containing the ERE, used for a zinc finger DNase footprint or incubated with iron finger or zinc finger in the presence of 0.03% H2O2 and 1.0 mM ascorbate in 20 mM phosphate or 10 mM Tris-HCl, pH 7.4, with or without 10 μM Fe(II), and the cleavage products were analyzed on a 20% urea denaturing gel.
turing gel. As seen in Fig. 5 (lane b), the DNase footprint of native finger has no scissions occurring within the ERE site, in contrast to iron finger cleavage (Fig. 5, lane c). Moreover, zinc finger incubated with free Fe(II) (10 μM) (Fig. 5, lane d) has a cleavage pattern distinct from that of iron finger. DNA labeled at the 5'-end incubated with free Fe(II) and under the appropriate conditions creates DNA fragments that terminate in a 3'-phosphate and 3'-phosphoglycolate groups, and chelated iron, such as Fe-EDTA, leaves fragments ending in only 3'-phosphate groups (29). The same cleavage experiments performed with phosphate buffer instead of Tris-HCl is shown in lanes d–h. Lane d, native finger and free iron; lane e, native alone; lane f, G-methylation Maxam-Gilbert sequencing of ERE(I). See details under “Materials and Methods.”

Therefore, we conclude that the DNA strand breaks are mediated through iron finger generating free radicals while coordinated to the ERDBD and not from the oxidative release of iron finger iron.

The specificity of iron-ERDBD binding and cleavage at the specific DNA binding target versus other DNA sequences were evaluated. Iron-ERDBD protein demonstrates specific binding and cleavage to the target DNA only (data not shown).

The ERDBD binds to the ERE as a “head-to-head” dimer. Therefore, when iron-substituted ERDBD binds the ERE there are four individual iron fingers proximate to the DNA. To investigate in detail the specific damage done by iron finger to the ERE, the resultant cleavage pattern is aligned to a schematic of the dimerized ERDBD bound to the ERE using the crystal structure coordinates (Fig. 6). The SETOR program (30) was used to produce the molecular diagram of the ERDBD bound to DNA. The cleavage pattern was sequenced and aligned using G-methylation cleavage with piperidine and DNA size markers. Below the cleavage pattern a histogram demonstrates the relative damage done to the ERE. The data points were calculated using NIH Image 1.52 computer software. These results demonstrate that possibly all four iron fingers are involved, to some extent, in cleaving the ERE. The ERDBD has two nonequivalent zinc fingers, each with a distinct function. One finger mediates the specific binding of the ER to the ERE by binding within the major groove of the DNA. It is this iron-substituted finger, binding within the major groove of the ERE and therefore proximate to the DNA, that causes the most extensive damage to the DNA. Furthermore, as seen from the histogram of Fig. 6, the damage done by the ironfinger on the DNA is specific to the DNA binding target.
iron finger, within the major groove, has a gaussian distribution, demonstrating that metal-induced DNA cleavage is not site-specific. The second ER finger includes a dimerization face responsible for the ER head-to-head dimer interface. The dimerization finger hovers above the ERE and hence is further away from the DNA, yet it still cleaves the ERE, but to a lesser extent.

In the case of an excess of iron, the metal may be incorporated into the zinc fingers directly during its synthesis and folding or by means of a zinc/iron exchange. Zinc, in zinc finger, appears to be kinetically labile and is exchangeable (31, 32). NMR studies have revealed that a chemical exchange process occurs in the zinc binding site (33). Iron finger, along with hydrogen peroxide, produced from cellular processes, and a reducing agent, such as ascorbate or superoxide, will drive the Fenton/Haber and Weiss reactions forward, augmenting the rate of hydroxyl radical generation. The hydroxyl radicals generated, that escape through the iron finger peptide, may cause extensive damage to the proximate DNA, the ERE. Therefore, we postulate that an iron-substituted zinc finger may generate free radicals while bound to the ERE, leading to adverse consequences such as iron-induced toxicity and/or carcinogenesis.

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In Vivo and in Vitro Iron-replaced Zinc Finger Generates Free Radicals and Causes DNA Damage

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