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NOVEL REGULATORY SITES OF THE HUMAN HEME OXYGENASE-1 PROMOTER REGION

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SUMMARY: Heme oxygenase is the rate-limiting enzyme in heme catabolism, the activity of which is induced by several agents including its substrate heme, u.v. radiation and other oxidant injurious conditions. Its biological function is to provide a specific regulatory mechanism for control of the level of many heme proteins. Using a computer-assisted approach, we examined the presence of different regulatory elements on the human heme oxygenase-1 promoter region which could possibly be involved in its regulation. We identified several NFkB and AP-2-like binding sites in the 5' untranslated region of the human heme oxygenase gene. DNase I footprint analysis confirmed the presence of these two important transcriptional sites. This study is the first to reveal the presence of NFkB and AP-2 binding sites in the heme oxygenase-1 promoter region and thus suggests a role for these transcriptional factors in the regulation of heme oxygenase-1 expression, a stress and heat shock protein, under injury conditions.

Heme oxygenase (HO, EC 1.14.99/3) is the initial and rate-limiting enzyme in heme catabolism. It cleaves heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. The heme molecule has a central role in biological processes since it is the prosthetic moiety of hemoproteins involved in cell respiration, energy generation, oxidative biotransformation and growth differentiation processes. The regulation of HO-1 is thus fundamental for the homeostasis of the cell. In experimental animals, HO-1 increased in conditions such as spontaneous or chemically induced liver tumors and gram positive bacterial infections (1-3). HO-1 activity is also increased in whole animal tissues following treatment with its natural substrate heme, heavy metals, xenobiotics, endocrine factors and synthetic metalloporphyrins (1). Many cells in culture including hemopoietic, hepatic, epithelial and endothelial cells, respond to these agents in a similar way, i.e., a marked increase in HO-1 activity (1-6). Furthermore, HO-1 is

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Abbreviations: Heme oxygenase (HO-1); base pair (bp); polymerase chain reaction (PCR); footprint unit (FPU).

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a heat shock protein, being induced by heat shock (7,8) and also a stress protein induced upon oxidative damage with free radical and UV radiation (9,10). It is therefore possible that the induction of HO-1 may be an essential event for some types of acute reactions and cellular protection.

Two heme oxygenase isozymes, the products of two distinct genes, have been described. HO-1 is the inducible form which is ubiquitously distributed in mammalian tissues, whereas heme oxygenase-2 is believed to be constitutively expressed, not inducible by HO-1 inducers and present in tissues such as the brain and testis (11).

One of the mechanisms by which hormones, growth factors and other stimuli induce the expression of genes is by activating various transcriptional factors. These processes may be part of the mechanism by which various agents, including heme, increase HO-1 expression and activity. Recent studies demonstrated the presence of AP-1 binding sequences, IL-6, metal and heat responsive elements on the HO-1 promoter region (6-8, 11-15).

To address the question of additional regulatory transcriptional sites in the HO-1 promoter which could influence HO-1 activity in oxidative stress, we devised a specific database search program for known transcriptional factors which influences transcriptional regulation of several genes during cell injury and inflammation. We demonstrated that the human HO-1 promoter contains two novel transcriptional sites, NFkB and AP-2, and discussed the potential implication of these findings for the regulation of HO-1 during oxidative stress and inflammation.

MATERIALS AND METHODS

Computer search. The sequence of the human HO-1 promoter region was retrieved from the National Center of Biotechnology Information, NIH, Bethesda, MD. The sequence was analyzed by the DNASIS program using known sequences corresponding to different regulatory sites (16).

DNase I footprint analysis. A 590 bp probe was prepared by PCR using forward primer A (5'-TGACATTTTAGGGAGCTGGA) and reverse primer D (5'-TTGCCTGTCGGGTTGC), corresponding to -480 and +110 positions of the human HO-1 gene, respectively (13, Figure 3). Primers were chosen using the oligo-primers program. PCR was performed by using 100 ng of each primer and 10 ng of plasmid containing the human HO-1 gene (13) as follows: 5 min at 94°C; 30 sec at 94°C; 30 sec at 50°C; 1 min at 72°C for 20 cycles; and 10 min at 72°C. The PCR product was purified on Magic PCR Preps DNA purification System (Promega), end-labelled using γ-[^{32}P]-ATP and polynucleotide kinase. The labelled PCR product was digested with Pst I. Alternatively, the PCR product was first digested with PstI and XhoI and end-labelled using α-[^{32}P]-CTP, dTTP and DNA pol I (Klenow fragment). 10^4 cpm of end-labelled, polyacrylamide gel purified probe was incubated with 1 FPU of AP-1, AP-2 or NFkB (p49 and p50) proteins (Promega) in 20 mM Hepes, pH 8.0, containing 5 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 50 mM KCl, 5 mM $MgCl_2$ and 5% (v/v) glycerol for 15 min on ice and then for 2 min at 25°C. One U/ml of DNase I was added for 1 min at 25°C, and the reactions were terminated by the addition of an equal volume of stop solution (0.5 M

sodium acetate, 0.1 M EDTA, 1% SDS, 100 μg/ml of yeast tRNA). DNA was extracted with phenol-chloroform, precipitated with ethanol and analyzed by 8 M urea - 6% acrylamide gel electrophoresis in 90 mM Tris/90 mM boric acid/1 mM EDTA, buffer, pH 8.0.

RESULTS AND DISCUSSION

Studies on heme oxygenase transcriptional elements have provided major clues toward understanding of cellular heme homeostasis and significantly contributed to elucidate mechanisms of post-transcriptional regulation in higher eukaryotic cells. HO-1 has been shown to play a role in response to tissue damage and in the acute response to various cytokines. However, the various transcriptional factors involved in the immediate induction of HO-1 following tissue damage are still unknown. To examine the presence of different transcriptional factors whose activation is implicated in response to tissue injury in the HO-1 promoter region, we devised a specific database search program using the National Center of Biotechnology Information and the NIH sequence database. Two NFkB-like regulatory sites were found by computer search using 5' to 3' and 3' to 5' directions for screening (Figure 1). One NFkB transcriptional site (-370 to -379) was found to share the same site with the heat shock responsive element (13). The second one was found more proximately at -156 to -166 (Figure 1). A similar computer analysis indicated the presence of three AP-2-like regulatory sites (Figure 1). The AP-2-like binding sequences were from -116 to -124, from -7 to-15 and from +38 to +52.

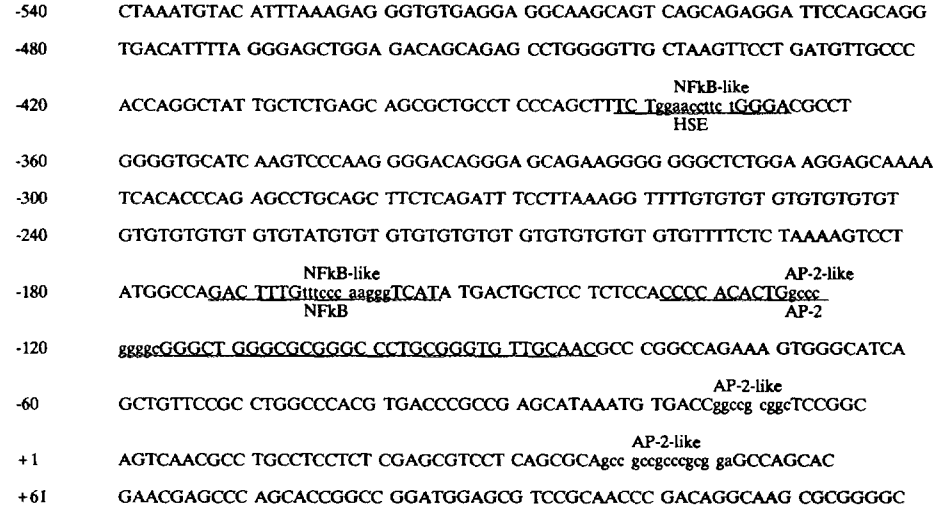

Figure 1. Sequence of the human HO-1 gene upstream region. The sequences marked in lower case letters are found by computer search; the underlined sequences are also confirmed by DNase I footprint assay; HSE, heat shock responsive element.

DNase I footprint assay was used to confirm the presence of NFkB and AP-2 binding sites on the HO-1 promoter region. The end-labelled DNA fragment was incubated with purified transcriptional factors; control experiments include no addition of proteins. As seen in Figure 2, addition of 1 FPU of NFkB (p50 or p49, lane 2 and land 4, respectively) showed the specific protection of the proximal NFkB consensus element (-156 to -166). There was no protection of the distal element by the p50 or p49 proteins suggesting the possibility that this site may be recognized by other rel-like proteins. Addition of double-stranded NFkB oligodeoxynucleotides to the incubation mixture

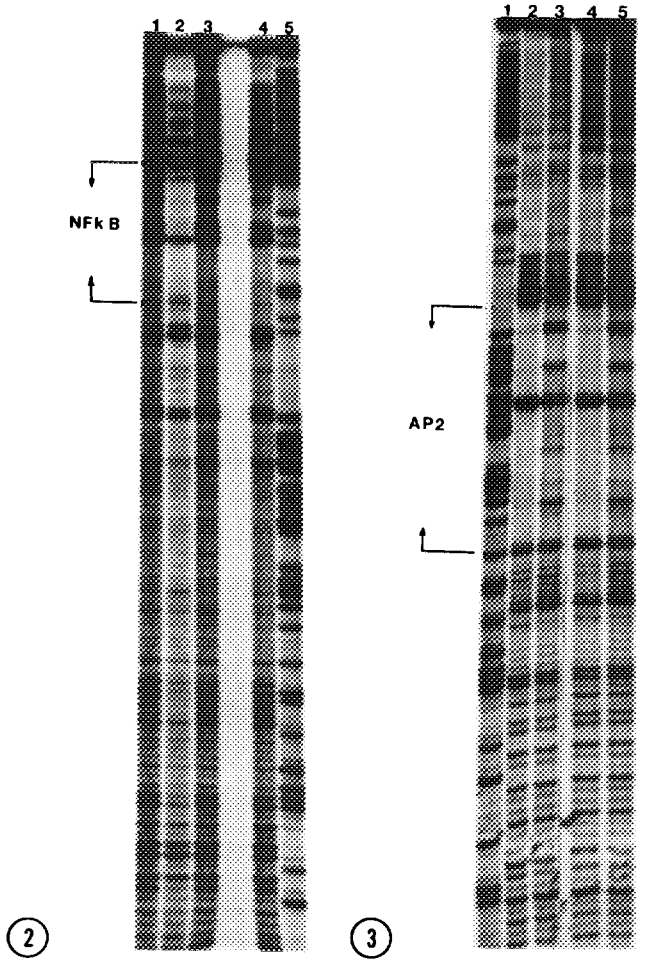

Figure 2. DNase I footprinting analysis of the human HO-1 promoter in the presence of NFkB protein. Lane 1, no protein; lane 2, 1 FPU of purified NFkB transcriptional factor (p50, Promega); lane 3, the same as lane 2 plus specific competitor; lane 4, 1 FPU of NFkB transcriptional factor (p49, Promega); lane 5, G sequencing reaction (19).

Figure 3. DNase I footprinting analysis of the human HO-1 promoter in the presence of AP-2 protein. Lane 1, G sequencing reaction; lane 2, 1 FPU of purified AP-2 transcriptional factor (Promega); lane 3, the same as lane 2 plus specific competitor; lane 4, the same as lane 2 plus nonspecific competitor; lane 5, no protein.

showed the specificity of the binding, i.e., not protection of the proximal sequence by the p50 protein (Figure 2, lane 3).

A similar footprint assay was performed using AP-2 protein. As seen in Figure 3, addition of 1 FPU of AP-2 (lane 2) showed the specific protection of the distal AP-2 consensus element (116 to -124). There was no protection of the other two computer-predicted AP-2 binding sites. Addition of double-stranded AP-2 oligodeoxynucleotides to the incubation mixture showed the specificity of the binding, i.e., no protection of the distal sequence by the AP-2 protein (Figure 3, lane 3). Addition of a nonspecific competitor (NFkB oligodeoxynucleotides) showed no protection of this sequence (Figure 3, lane 4), suggesting binding specificity for AP-2.

In this report, we have demonstrated by computer analysis and DNA footprinting that the HO-1 promoter region contains NFkB and AP-2 transcriptional sites. This demonstration provides a starting point for the exploration of the possible complex biological consequences of this NFkB/AP-2 interaction during the course of inflammation or stress. Taken at face value, it suggests that the expression of HO-1 mRNA levels is transcriptionally controlled by factors which increase or decrease NFkB during environmental stress and injury. It also implies an unexpected twist to our perception of the molecular signals that regulate heme oxidation during inflammation. Heme oxygenase catalyzes the first step in the heme degradation pathway leading to biliverdin formation which is completed by the conversion of biliverdin to bilirubin (17). Many reports during the past few years have suggested that the conversion of heme to bilirubin may be of great benefit to cells to cope with oxidative stress (18). Several lines of evidence have indicated that heme oxygenase is a stress protein and is expressed in the same condition as other heat shock proteins, but the available data did not permit a direct demonstration of the presence of these important factors, NFkB and AP-2, in the heme oxygenase promoter. The demonstration of the presence of these regulatory sites may help to investigate further the importance of expression of this enzyme protein during inflammation and oxidative stress.

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