

2003

Role of Activator Protein-1 in the Down-Regulation of the Human *CYP2J2* Gene in Hypoxia

Nicole Y. Marden

University of New South Wales, Sydney

Eva Fiala-Beer

University of New South Wales, Sydney

Shi-Hua Xiang

University of New South Wales, Sydney, sxiang2@unl.edu

Michael Murray

University of New South Wales, Sydney, m.murray@unsw.edu.au

Follow this and additional works at: <https://digitalcommons.unl.edu/vetscipapers>



Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#), [Cell and Developmental Biology Commons](#), [Immunology and Infectious Disease Commons](#), [Medical Sciences Commons](#), [Veterinary Microbiology and Immunobiology Commons](#), and the [Veterinary Pathology and Pathobiology Commons](#)

Marden, Nicole Y.; Fiala-Beer, Eva; Xiang, Shi-Hua; and Murray, Michael, "Role of Activator Protein-1 in the Down-Regulation of the Human *CYP2J2* Gene in Hypoxia" (2003). *Papers in Veterinary and Biomedical Science*. 186.

<https://digitalcommons.unl.edu/vetscipapers/186>

This Article is brought to you for free and open access by the Veterinary and Biomedical Sciences, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Veterinary and Biomedical Science by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Published in *Biochemical Journal* 373:3 (2003), pp. 669–680; doi: 10.1042/BJ20021903.

Copyright © 2003 Biochemical Society. Used by permission.

Submitted December 9, 2002; revised April 24, 2003; accepted May 9, 2003; published online May 9, 2003.

Role of Activator Protein-1 in the Down-Regulation of the Human *CYP2J2* Gene in Hypoxia

Nicole Y. Marden, Eva Fiala-Beer, Shi-Hua Xiang, and Michael Murray

Department of Physiology and Pharmacology, School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Corresponding author – Michael Murray, Faculty of Pharmacy, University of Sydney, NSW 2006, Australia, email m.murray@unsw.edu.au

Abstract

The cytochrome P450 (CYP) 2J2 arachidonic acid epoxxygenase gene was down-regulated at a pre-translational level in human hepatoma-derived HepG2 cells incubated in a hypoxic environment; under these conditions, the expression of c-Jun and c-Fos mRNA and protein was increased. The 5'-upstream region of the *CYP2J2* gene was isolated by amplification of a 2341 bp fragment and putative regulatory elements that resembled activator protein-1 (AP-1)-like sequences were identified. From transient transfection analysis, c-Jun was found to strongly activate a *CYP2J2*-luciferase reporter construct, but co-transfection with plasmids encoding c-Fos or c-Fos-related antigens, Fra-1 and -2, abrogated reporter activity. Using a series of deletion-reporter constructs, a c-Jun-responsive module was identified between bp –152 and –50 in *CYP2J2*: this region contained an AP-1-like element between bp –56 and –63. The capacity of this element to interact directly with c-Jun, but not c-Fos, was confirmed by electromobility-shift assay analysis. Mutagenesis of the –56/–63 element abolished most, but not all, of the activation of *CYP2J2* by c-Jun, thus implicating an additional site within the c-Jun-responsive region. The present results establish an important role for c-Jun in the control of *CYP2J2* expression in liver cells. Activation of c-Fos expression by hypoxia promotes the formation of c-Jun/c-Fos heterodimers, which decrease the binding of c-Jun to the *CYP2J2* upstream region, leading to gene down-regulation.

Keywords: activator protein-1, cytochrome P450, hypoxia, human gene regulation, transcription

Abbreviations used: ANP, atrial natriuretic peptide; AP-1, activator protein-1; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EET, epoxyeicosatrienoic acid; EMSA, electrophoretic mobility-shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; RT-PCR, reverse transcription-PCR

Introduction

The cytochrome P450 (CYP) superfamily of enzymes catalyzes the oxidative metabolism of lipophilic xenobiotics and endogenous compounds, such as fatty acids, vitamins, and steroids [1]. The recently described CYP2J2 enzyme is highly expressed in the human heart and vasculature [2,3], and is also expressed in the lung and other organs [2,4–6]. CYP2J2 catalyzes the oxidative conversion of arachidonic acid into epoxyeicosatrienoic acids (EETs) [2], which have a range of physiological actions, including the relaxation of vascular smooth muscle [7] and anti-inflammatory effects [3]. The cytoprotective role of CYP2J2-derived EETs was established in recent studies that demonstrated that CYP2J2 protein levels are decreased in vascular endothelial cells subjected to hypoxia [8]. Restitution of CYP2J2 function by transfection of the cDNA into cells before exposure to an oxygen-deficient environment enhanced cell survival [8]. The anti-apoptotic actions of EETs have been linked to their capacity to activate a phosphoinositide 3-kinase/Akt signaling pathway [9].

Activator protein-1 (AP-1) is a multiprotein transcription factor complex consisting of leucine-zipper proteins from the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families [10,11]. The AP-1 complex binds to the consensus sequence TGA(G/C)TCA, also termed the PMA response element, to activate target gene transcription [12]. Apart from phorbol esters, AP-1 activity is also induced by stimuli such as hypoxia [13–15], cytokines, growth factors, carcinogens, and UV irradiation [10,16,17]. Thus AP-1 transduces signals that modulate cell survival and apoptosis in the acute-phase response to a range of stress stimuli.

The present study tested the hypothesis that the redox-responsive AP-1 participates in the regulation of *CYP2J2* gene expression in HepG2 human hepatoma-derived cells. Consistent with results in hypoxic endothelial cells, *CYP2J2* was down-regulated in HepG2 cells during culture at 1% O₂. Increased expression of the major AP-1 proteins c-Fos and c-Jun occurred in HepG2 cells cultured under these conditions.

To understand the molecular regulation of the *CYP2J2* gene, the 5' flanking region was cloned and its transactivation by AP-1 gene products was studied by transient transfection analysis. *CYP2J2* reporter constructs were strongly activated by c-Jun, but not by c-Fos or the combination of c-Fos and c-Jun. A c-Jun-responsive module was identified between –152 and –50 bp upstream from the translation start site, and an AP-1-like element (nt –56/–63) within this module was important in c-Jun binding and transactivation of *CYP2J2*. Up-regulation of c-Fos in hypoxic HepG2 cells decreased c-Jun binding to the element. These results implicate c-Jun in the maintenance of *CYP2J2* expression in cells and suggest that the appearance of c-Fos/c-Jun heterodimers during hypoxia mediates the decrease in *CYP2J2* expression.

Experimental

Plasmids and reagents for molecular biology

The pGL3 reporter, pCMV- β galactosidase and pGEM-T easy vectors, recombinant human c-Jun protein, RiboMAX RNA production kit, Steady-Glo Luciferase kit, and β -galactosidase Reporter Lysis assay system were purchased from Promega Corp. (Annandale, NSW, Australia). The Epicentre MasterPure Complete DNA and RNA Purification Kit was obtained from Astral Scientific (Gymea, NSW, Australia). The Clontech Human GenomeWalker Kit was purchased from Progen Industries (Darra, Qld., Australia). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Castle Hill, NSW, Australia). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and [32 P]dCTP were obtained from PerkinElmer (Rowville, Vic., Australia). Plasmid Midi kit, QIAquick Gel Extraction kit, and the OneStep RT-PCR (reverse transcription-PCR) kit were obtained from Qiagen (Clifton Hill, Vic., Australia). The Stratagene QuikChange Site-Directed Mutagenesis Kit was purchased from Integrated Sciences (Willoughby, NSW, Australia). Expression plasmids for murine c-Fos and c-Jun were kindly provided by Dr. K. Imakawa (University of Tokyo, Japan). Expression plasmids for human JunB, JunD, Fra-1, and Fra-2 were provided by Dr. M. Karin (University of California, San Diego, CA, USA). All oligonucleotides were from Geneworks (Adelaide, SA, Australia). The Megaprime DNA Labelling System and ProbeQuant G-50 Micro Columns were from Amersham Biosciences (Castle Hill, NSW, Australia). Enzymes were purchased from Roche Diagnostics unless otherwise specified.

Reagents for electrophoresis and immunoblotting

Rabbit anti-(rat CYP2J4) IgG, which is cross-reactive with human CYP2J2, was generously provided by Dr. Qing-Yu Zhang (Wadsworth Centre, New York State Department of Health, New York, NY, USA). Primary antibodies directed against c-Fos and c-Jun were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and secondary antibodies were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Tween 20, reagents for detection of proteins on immunoblots by enhanced chemiluminescence detection and Hyperfilm-MP for autoradiography were from Amersham Biosciences. Schleicher and Schuell Protran nitrocellulose transfer membrane was purchased from Medos Co. (Waverly, Vic., Australia).

Reagents for cell culture

HepG2 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cell-culture media, antibiotics, and fetal calf serum were obtained from Thermotrace (Noble Park, Vic., Australia). Protease inhibitors were from Sigma-Aldrich. Thiazolyl Blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; MTT] and DMSO were also from Sigma-Aldrich.

Cloning of the human CYP2J2 promoter and construction of reporter plasmids

A 2.4 kb fragment of the 5'-flanking region of the CYP2J2 gene was isolated from a human genomic library using the Human GenomeWalker kit and was subcloned into the pGL3

basic luciferase reporter vector (p2J2A; bp -2341/+98). The 5'-truncated constructs p2J2B (-1894/+98), p2J2C (-1228/+98), p2J2D (-574/+98), p2J2E (-152/+98) and p2J2G (-49/+98) were generated from p2J2A by restriction-enzyme digestion and religation using *EcoRV* (to obtain p2J2B), *PvuII* (p2J2C), *NheI* (p2J2D), and *SmaI* (p2J2E and p2J2G) (see fig. 1). The constructs p2J2F and p2J2H, which contain internal mutations of the putative AP-1-like elements spanning -7 to +1 bp (CTGAGCCA) and -56 to -63 bp (CGACGGTC) respectively, were generated from p2J2E (-152/+98) using the QuikChange Site-Directed Mutagenesis Kit and appropriate oligonucleotides (table 1). All constructs were sequenced (ABI Prism Big Dye), and plasmid DNAs were prepared for transfection using a Qiagen Miniprep kit.

```

-2341 AAACTGTCTGCCCTGTTGGTATTACACACAGTCTCTGCTGACTTCCAGCTGATTGCTCTACTGTTTCAACAATGCCTTGGGGCT
-2254 TACATTACTTGAAAATTGATGCAATTAATTTGGGCTTCTCTCAGGAATAGTCTTTGAGGCCAGACTTTGAGATTGTCTGGTC
-2167 CCAAGAGGGTCTTCTAGCTAACTCTTGTGCTGACCGACTTTGGTAAACTTCTAGTTAGCCTGTGGTTATCCTCTGTGTTCTATA
-2080 GAATTACACGCTCTCTATTAAATGCTTACCACCAAAATCTCTATTGTTTACGAGCATTCATCTCTGGTGTGTTGGCTAACCTCTCC
-1993 AGTGTGGAACTCTATCTCATGAGAAAGCTGGGATGAGGGCAATTATGGCCCGGGTAGGTATTATTCTGCCTTA CAAGTCAGG
-1908 GCTGGATGGAGGATATCCCTAGACCTCTTAGCCAGTCTTGCCTAGAATAGAGCTTCTGCAACATGACATTCTGGGGCTGGG
-1823 TATAAGAAATACTGGCAGTTTCTCCATCTCAGGGAGATACTGTAGCCTTCATTTGAGAGCTAGGAAAGTGTCTTGATTAACT
-1737 GTCTAGAATGGGTTTCTGTACGTTGATCTGGGGACTAGGAGAAAGA GTGAGTTATGACTCAAAATGCCACAGACTCTTACTGTG
-1652 TTTACTAGCATTTAGTAGATTCTCTTAAATAAAATTTATTTATTTACTGTATGCCTTTAGAACAAATTTTCAGGGACTTAAATTATT
-1565 TTAGTTTATAAATAATTTTAGCAATCACAATTTTACTAGGAAGAGGTTCTGTGGGGTTTCTCATGCTGCCATTATGGAAGTCA
-1478 TTCCCTGCATCAATCTTAATGCAAAAAATAAAAGGAAGGAGAGATAACAAACCTGACGTCCACTGATACGGGAATGAGC
-1394 AAATAAATGTGGCATAGTAATAGAATACTATCCATCTATTAATAAAGTGAATTAGAGCCACATATA TCAGTCAGGATAAACTT
-1309 CAAACACATAACTGAGTTGTAGAATAATCGTGTAAAAACATCATATATAAATATTAGAAATATTGTATGGATGCATAA CAGC
-1224 TGTAGTAAACAGGAAATGTGGACTGGAAGGATGAAAGCTAAGCT CTGACTAGTATTCTCTCTGGACCTTAGAAAGAGAACAA
-1140 AGAGACTTCAACTCTCATTTCTTCTGCTCACACCTGTAATCTAGCCTTTAGGTGGCTGAGGTGGGCAGATCACCT AIGGTC
-1054 AGGAGATTGAGACCGCTGACCAACACGCGCAAAACCCATCTGTACCAAAAAATACGAAATAGCCGGGCATGGTGGCATGTG
-970 CCTGTAGTCCCAGCTACATGGGAGGCTGAAGCAGAAGAATCGCTTGAACCCGGGAGGTGGAAGGTGCACTGAGCTGAATCCAG
-886 CCTGGGCGACAGAGCAAGACTCTGTCTCAAAAATGAAGAAATAAAATATTGTAAGCAGAATAT GTGACTCTATGTTGAGT
-801 ACATGAGTGTGTACTATACTATTCCTGAGCTATGTTTCATGTTTTCAGTCTCACCAATTTGTGAAACCAAGCAGGCACTACCAGAT
-714 GGTATGAAAGGCTGGAACCTGGAGGTCACAGTCTTGGGGCTTTGGAGAGGAGCCTAATAGAGCAAAACGCAAGGATCGGAA
-630 CTAAGACATTCCTTCAACAAATGTTAATTAAGCACTTATTATGGCTGCTGCTCAGT CTAGCAGCTTTGGTGTAGTGCCTTTTAGTC
-543 TTCCAGACATTTCAATACAGGACCTCGTTTAGATGTGTTTTCAGAAGGGTGGATTATTGTTTACCACATACTTAGTACTTAAT
-456 TTTCTGTTTCTCAACAGCAAGATGAGACTACCGAGAGCTTCTCCTTCATAAT TTGTCAAATGAATAATAATCTATGTAAAGAG
-369 GCGGATGTGCTGCCTGGCATATAGTAAGAGCCCAAGAAATGTTAGGTACTGCTGTTGTTATCGCTGTTTACCAGGCACAG TTCCAA
-283 TCATAGGAGAGACGGTGATTGAACCGAACAGAGGCTCAATGTCTGTGGCGCTCTCAGTAAACCCATGAGCTTTCTAAATAGAAA
-208 AGAAACTGCTCTAGCCTGGCCTTTCTGAGACCGGTGCGTGTCTCTCCGGGAATCCAGCGCCTGGCATCTTCGAGGGTGTGCGG
-112 AAGGGGCGGGCTGGGAGGCGGGGACGGCTGGGAGCGAGGCGGGCGGGGACCGTCGCTGCTGGGACCGCGGCTGCTTGGAC
-28 CCGAGAAGAGCAGGAGGACGTCTGAGCCATGCTCGCGCGATGGGCTCTGCGCGCTGCCTCTGGGCAGTGTCCATCTCGG
+58 ACTCTCTACTGGGCACTGTGCGCTTCTGCTGCTGCTGA

```

Figure 1. Nucleotide sequence of the 5' flanking region of the human CYP2J2 gene. Potential transcription factor binding sites, including AP-1, identified by the Genomatix MatInspector software are labeled and underlined. The major transcription start site [24] is marked with an asterisk and the translation start site (+1) is emboldened. The restriction enzyme sites used to create the CYP2J2 5'-flank luciferase deletion constructs are underlined.

Table 1. Sequences of double-stranded oligonucleotides used in EMSA analysis

Oligonucleotide	Sequence (5'→3')
AP-1 consensus (sense)*	CTAGT GATGAGTC AGCCGGATC
AP-1 consensus (antisense)	GATCGATCCGGCTGACTCATCA
2J2-(− 56/−63) (sense)†	CGGGGCGGGGACCGTCGCTGCTGGG
2J2-(− 56/−63) (antisense)	CCCAGCAGG CGACGGT CCCCGCCCG
2J2-mt-(− 56/−63) (sense)‡	CGGGGCGGGG <u>AAAAAAAA</u> ACCTGCTGGG
2J2-mt-(− 56/−63) (antisense)	CCCAGCAGG <u>TTTTTTT</u> CCCCGCCCG
Stat5 β -casein promoter (sense)	GGACTTCTTGGA ATTAAGGGA
Stat5 β -casein promoter (antisense)§	GTCCCTTA AATCCA AGAAGTCC

* The sense probe has a 5'-CTAG overhang and the antisense probe has a 5'-GATC overhang; the AP-1-like binding element is emboldened.

† AP-1-like element on the antisense strand is emboldened.

‡ Mutagenized bases are underlined.

§ STAT5 element emboldened; the antisense probe has a 5'-G overhang for labeling purposes.

Cell culture

The HepG2 human hepatoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin antibiotic mix, 26 mM sodium hydrogen bicarbonate, and 20 mM Hepes. Cells were cultured at 37°C using a mixture of air/CO₂ (19:1) and were passaged twice weekly. Cells at passage 4 were used for all experiments.

Cells were seeded in 75 cm³ flasks at a density of 2×10^5 /ml for 48 h before hypoxic exposure. For hypoxic incubations, flasks were transferred to an oxygen-regulated incubator containing a mixture of N₂/CO₂/O₂ (94:5:1) for 16 h. Control (normoxic) flasks were cultured for the same time period in 21% O₂ (air/CO₂, 19:1). For reoxygenation, flasks were removed from 1% O₂ and returned to 21% O₂ for a further 30 min. After treatments, cells were harvested and RNA, total cell lysates, and nuclear protein fractions were prepared.

MTT assay to assess cell viability

HepG2 cells were seeded (in triplicate) at 2×10^5 /ml in six-well plates and incubated at 21% O₂ for 48 h before exposure to 1% O₂ for 16 h; normoxic controls were incubated at 21% O₂ for 16 h. At the end of this time, MTT (625 μ g/250 μ l) was added, and cells were incubated for 2 h at 37°C. The media was aspirated, DMSO (1 ml) was added to each well, plates were shaken at room temperature for 30 min, and the absorbance at 540 nm was measured. The absorbance of cells cultured in normoxia (21% O₂) was taken to represent 100% viability. Viability was also determined after a longer period of hypoxia (40 h) and at several times after return to normoxic conditions.

Preparation of a recombinant CYP2J2 RNA internal standard

A 254 bp product spanning nt 724–749 and 953–978 was amplified by PCR from CYP2J2 cDNA using the forward and reverse primers shown in table 2, and was then cloned into the pGEM-T Easy vector [(2J2-254)-pGEM]. Two HindIII sites separated by 57 bp were cre-

ated within (2J2-254)-pGEM by site-directed mutagenesis (using the QuikChange kit). After digestion with *HindIII*, the larger CYP2J2-plasmid fragment was purified by gel electrophoresis and religated with T4 DNA ligase to generate (2J2-197)-pGEM. Recombinant (2J2-197)-RNA was produced by *in vitro* transcription of (2J2-197)-pGEM using the RiboMAXTM Large Scale RNA Production System and T7 polymerase. (2J2-197)-RNA was subsequently treated with RQ1 RNase-free DNase for 10 min at 37°C, extracted using the MasterPure Complete DNA and RNA purification kit, and quantified.

Table 2. Primer sequences for gene amplification

Oligonucleotide	Sequence (5'→3')
CYP2J2 forward	GGACCCACCAAACCTCTCTTCAGCAA
CYP2J2 reverse	ATAAAGCAGAGCCCATCGCAGAGTTG
c-Fos forward	ATGTTCTCGGGCTTCAACGCAGA
c-Fos reverse	CAGTGACCGTGGGAATGAAGTTGG
c-Jun forward	CATGAGGAACCGCATTGCCGC
c-Jun reverse	TAGCATGAGTTGGCACCCACTG
β -actin forward	ACGGGGTACCCACACTGTGC
β -actin reverse	CTAGAAGCATTTGCGGTGGAC

RNA extraction and RT-PCR

Total RNA was extracted using the acid guanidinium thiocyanate/ phenol method of Chomczynski and Sacchi [18]. RNA samples were quantified by spectrophotometry and electrophoresed on a 1% denaturing agarose gel to confirm integrity. For quantification of CYP2J2 mRNA, various amounts (0.1–100 pg) of (2J2-197)-RNA were added to a fixed quantity (0.5 μ g) of RNA extracted from HepG2 cells that had been cultured as described above. Competitive RT-PCR for the quantification of CYP2J2 mRNA was performed with CYP2J2 forward and reverse primers (table 2). Following treatment with RQ1 RNasefree DNase for 10 min at 37°C, RT-PCR was performed in a thermal cycler (GeneAmp PCR system 2400; PerkinElmer) using the Qiagen OneStep RT-PCR kit. Optimized cycling conditions were: 30 min at 50°C, 15 min at 95°C and then 28 cycles of denaturation (20 s at 94°C), annealing (20 s at 55°C) and extension (30 s at 72°C), followed by a final extension for 10 min at 72°C. RT-negative and template-negative controls were included. PCR products were electrophoresed on 2.5% agarose gels in TBE buffer (90 mM Tris/HCl/90 mM boric acid/2.5 mM EDTA) containing 1 μ g/ μ l ethidium bromide, and visualized on a trans-illuminator (Gel Doc 2000; BioRad). The intensities of CYP2J2 competitor and target were determined densitometrically (Multi Analyst software; BioRad).

Semi-quantitative RT-PCR was performed by co-amplification of c-Fos or c-Jun with β -actin (control gene) using the primers shown in table 2. c-Fos-forward and -reverse primers corresponded to nt 4–26 and 173–196 respectively (193 bp product). c-Jun-forward and -reverse primers are on the basis of those reported previously [19], and correspond to nt 777–797 and 951–972 respectively (196 bp product). The β -actin-forward and -reverse primers were reported previously [20], and correspond to nt 511–531 and 1149–1169 respectively (659 bp product). RT-PCR was performed in a single tube using the Qiagen OneStep RT-PCR kit as described by the manufacturer with an initial DNase treatment

using RQ1 RNase-free DNase (Promega) for 10 min at 37°C. RT-PCRs were allowed to proceed as follows in a thermal cycler: 30 min at 50°C, 15 min at 95°C and then cycles of denaturation (20 s at 94°C), annealing (20 s at the temperatures specified below) and extension (30 s at 72°C), followed by a final extension for 10 min at 72°C. Conditions were optimized for annealing temperature, primer concentration and cycle number to ensure that reactions were within the exponential phase of amplification. For co-amplification of c-Fos and β -actin, annealing temperature was 58°C, primer concentrations were 1 μ M and 0.2 μ M for c-Fos and β -actin primers respectively, and 26 cycles of amplification were used. For c-Jun and β -actin co-amplification, the annealing temperature was 58°C, primer concentrations were 1 μ M and 0.2 μ M for c-Jun and β -actin primers respectively, and 25 cycles of amplification were used. RT-negative and template-negative controls were routinely included. PCR products were electrophoresed on 2% agarose gels in TBE buffer, as described above, containing 1 μ g/ μ l ethidium bromide, and were then visualized on a transilluminator. The intensities of the c-Fos and c-Jun products were determined densitometrically, and were normalized to the β -actin signal.

Transient transfections and luciferase and β -galactosidase assays

HepG2 cells were seeded at a density of 6×10^5 cells/well in six-well plates 24 h before transfection. Transfections were performed using the FuGENE 6 transfection reagent at a ratio of FuGENE 6:total DNA of 3:2. Briefly, cells were co-transfected with 1 μ g/well CYP2J2 promoter constructs, and 0.5 μ g/well pCMV- β -galactosidase expression plasmid to control for transfection efficiency. Expression plasmids encoding AP-1 proteins were added at 0.5 μ g/well. The plasmid DNA mixture was incubated with FuGENE 6 (diluted to 100 μ l with serum-free DMEM) for 20 min at room temperature and then added to each well. After 24 h, the medium was replaced with fresh DMEM, and the cells were incubated for a further 48 h before harvesting. Luciferase activity was measured in a scintillation counter (1900 TR; Packard) using the Steady-Glo Luciferase Assay System and β -galactosidase activity was assayed at 420 nm in a Cary 300 BioSpectrophotometer. All transfections were performed in duplicate, and each experiment was conducted on at least three separate occasions.

Isolation of total cell lysates and Western blotting

Following treatment, cells were treated with trypsin and washed twice with ice-cold PBS. Cells were pelleted by centrifugation for 3 min at 4°C at 15800 g and resuspended in 300 μ l of sample buffer [400 mM Tris/HCl/3% (w/v) SDS/10% (v/v) 2-mercaptoethanol/20% (v/v) glycerol/0.002% Bromophenol Blue]. The suspension was passed through a 23G needle 10 times and heated at 100°C for 5 min. After centrifugation for 3 min at 15800 g, the supernatant (total cellular lysate) was separated into aliquots and stored at -20°C. Equal volumes of protein samples were resolved by SDS/PAGE (10% gels) and transferred to nitrocellulose at 100 V for 1 h (25 mM Tris/192 mM glycine/20% methanol). Membranes were blocked for 1 h at room temperature in 5% (w/v) non-fat dried milk in Tris-buffered saline [50 mM Tris-HCl (pH 7.4)/200 mM NaCl/0.05% (v/v) Tween-20], and then incubated for 2 h at room temperature with primary antibodies diluted in blocking solution. Membranes were incubated with polyclonal anti-(rat CYP2J4) (16 μ g/ml), antic-Fos (Santa Cruz sc-7202; 0.75

$\mu\text{g/ml}$) or anti-c-Jun (sc-45; $0.4 \mu\text{g/ml}$), washed in Tris-buffered saline and incubated for 1 h at room temperature in secondary anti-(rabbit IgG) antibody conjugated with horseradish peroxidase. The secondary antibody was diluted in 5% blocking solution at 1:1000, 1:1500, and 1:2000 dilutions for CYP2J2, c-Fos and c-Jun blots respectively. After incubation with secondary antibody, the membranes were washed five times in Tris-buffered saline and visualized by enhanced chemiluminescence (Amersham Biosciences). Positive controls were included in Western blots as follows: CYP2J2, bacterial expressed CYP2J2 (*Escherichia coli* DH5 α cells); c-Jun, recombinant human c-Jun protein (Promega); and c-Fos, nuclear extract from phorbol-ester-treated Jurkat cells (Santa Cruz).

Preparation of nuclear extracts

Nuclear extracts were prepared at 4°C from untransfected HepG2 cells or cells that had been transfected (24 h) with c-Jun expression plasmid ($0.5 \mu\text{g}/6 \times 10^5$ cells) as described by Schreiber et al. [21]. Nuclear extracts were also prepared from HepG2 cells subjected to normoxia (16 h at 21% O_2) or hypoxia (1% O_2). Cells were harvested, washed in 10 ml of PBS and pelleted for 15 s at 15800 g. The pellet was resuspended in 400 μl of buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.4 mM Na_3VO_4 , 1 mM NaF, 0.15 mM spermine, 0.5 mM spermidine, 2 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin and 1 $\mu\text{g/ml}$ leupeptin, and allowed to swell on ice for 15 min. After the addition of 0.6% (v/v) Nonidet P40, tubes were vortex-mixed and centrifuged at 15800 g for 30 s. Nuclear pellets were resuspended in 50 μl of buffer containing 20 mM Hepes, pH 7.9, 0.4M NaCl, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.4 mM Na_3VO_4 , 1mM NaF, 4 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, and 10 $\mu\text{g/ml}$ leupeptin. The tubes were rocked at 4°C for 15 min and then centrifuged for 5 min at 15800 g. The nuclear extract was separated into aliquots, frozen in liquid N_2 and stored at -80°C until used in electrophoretic mobility-shift assays (EMSAs). Protein was determined by the Lowry method using BSA as the standard [22].

EMSAs

Oligonucleotides used as probes or competitors in gel-shift assays are shown in table 1. Complementary oligonucleotides were annealed and end-labeled with [^{32}P]dCTP using the Megaprime DNA labeling system, and purified through ProbeQuantTM G-50 micro columns according to the manufacturer's instructions. A larger double-stranded probe consisting of 167 bp of the CYP2J2 promoter (nt -152 to +15) was used in some gel-shift assays. This fragment (2J2/167) was prepared by digestion of p2J2E with *NheI* and *BanII*, and was separated by electrophoresis on 2% agarose. The fragment was excised from agarose and purified (QIAquick kit). Binding reactions containing 10–50 fmol of the end-labeled probes and 5–30 μg of nuclear protein fractions (or 0.6 μg of c-Jun recombinant protein) were incubated for 20 min at room temperature and 10 min at 4°C in a buffer containing 50 mM NaCl, 10 mM Tris/HCl, pH 7.5, 1 mM MgCl_2 , 0.5mM EDTA, pH 8.0, 0.5 mM DTT, 4% glycerol, and 1 μg of poly(dI-dC), with the exception of reactions with c-Jun recombinant protein, which contained 0.2 μg of poly(dI-dC). Loading dye (2 μl) was added to the reactions, and protein-DNA complexes were resolved by electrophoresis with 5% PAGE in TBE buffer at 100 V for 1.5–2.5 h at 4°C . In competition experiments, 200-fold

excess unlabeled probe was included in binding reactions; the STAT5 consensus sequence from the β -casein promoter [23] was used as a negative control. In supershift experiments, nuclear protein was incubated with rabbit polyclonal c-Jun or c-Fos antibodies (2 μ g) for 1 h at 4°C before the binding reaction; an anti-ubiquitin antibody (Santa Cruz) was used as a negative control. Following electrophoresis, gels were dried and autoradiographed.

Statistical analysis

Results are expressed as means \pm S.E.M. throughout. Differences between experimental groups were detected using Student's *t* test. $P < 0.05$ was considered to be statistically significant. All data were derived at least in duplicate from at least three separate experiments.

Results

Isolation of the 5'-flanking region of the human CYP2J2 gene and identification of multiple potential AP-1 binding sites

Expression of the CYP2J2 gene in endothelial cells is impaired in a low-oxygen environment [8], but the underlying molecular mechanism is unclear. To address this point, we cloned and sequenced a 2.4 kb fragment of the 5'-flanking region of the human CYP2J2 gene from a human genomic library, corresponding to nt -2341 to +98 relative to the translation start site [24] (GenBank accession no. AF039089). This fragment contained the major transcription start site 26 bp upstream of the translation start site ([24]; Genbank accession no. AF272142). The Genomatix MatInspector Professional consensus sequence identification program [25] was used to identify potential transcription-factor binding sites within this 2.4 kb CYP2J2 upstream region. High- and low-stringency searching revealed a number of regions that resembled binding motifs for AP-1, as well as a potential CCAAT box and GC-rich regions, which bind the transcription factor Sp1 (fig. 1).

Regulation of human CYP2J2, c-Fos and c-Jun in HepG2 cells during hypoxia and reoxygenation

The regulation of CYP2J2 by the redox-responsive transcription factor AP-1 was investigated in HepG2 cells that were cultured in a low-oxygen environment. Preliminary studies found that the viability of HepG2 cells (as determined by the MTT assay) was slightly decreased (to 80% of normoxic control) following 16 h of hypoxia, and that viability was fully restored by 24 h of reoxygenation. In contrast, 40 h of hypoxia, which was not used in any of the present experiments, decreased viability to 40% of control. Thus cell viability was relatively unimpaired by the short-term hypoxia used in the present studies.

Using a competitive RT-PCR assay, CYP2J2 mRNA was down-regulated in hypoxic HepG2 cells (1% O₂; 16 h) to 25% of normoxic control [0.26 ± 0.02 as compared with 1.06 ± 0.07 pg/0.5 μ g of total RNA ($P < 0.0001$); fig. 2]; an effect similar to that reported previously in hypoxic endothelial cells [8]. In contrast, CYP2J2 mRNA levels in HepG2 cells were rapidly restored to control levels when the 16 h culture period at 1% O₂ was followed by a 30 min period of reoxygenation (fig. 3). Parallel measurement of c-Fos and c-Jun mRNA by

semi-quantitative RT-PCR indicated that both genes were upregulated several-fold in hypoxia (fig. 4A and 4B). The 30 min reoxygenation period restored c-Jun and c-Fos mRNA expression to control levels (fig. 4C).

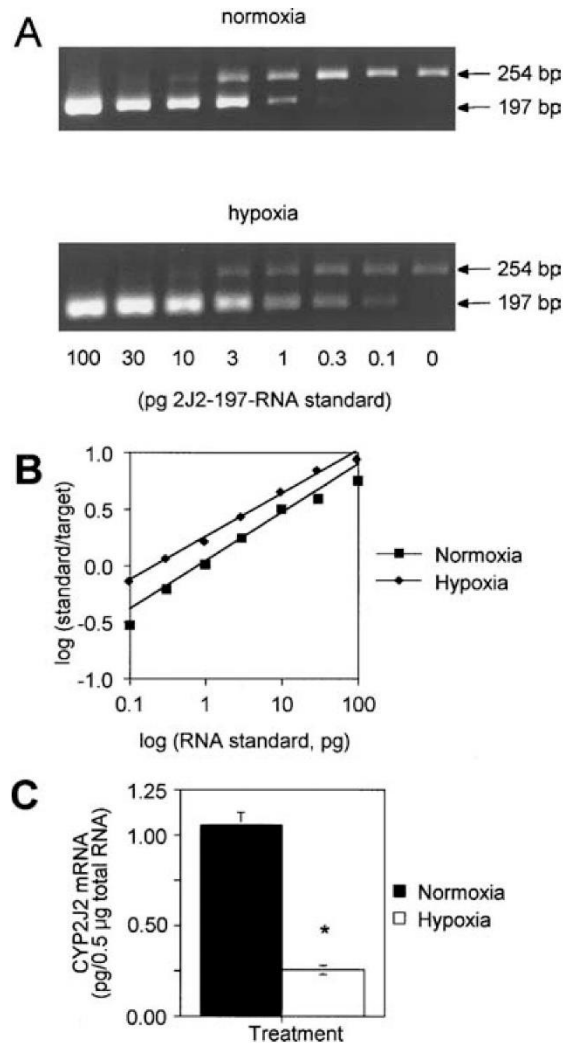


Figure 2. Competitive RT-PCR assay for CYP2J2 mRNA in hypoxic HepG2 cells (A) RNA was isolated from HepG2 cells that had been cultured for 16 h in normoxia (21% O₂; upper panel) or hypoxia (1% O₂; lower panel). Known quantities of (2J2-197)-RNA, prepared as described in the Experimental section, were added to 0.5 µg of cellular RNA, and competitive RT-PCR was performed. (B) Typical relationship between relative intensities for CYP2J2 target (254 bp) and 2J2-197 standard (197 bp) are shown. (C) Effect of hypoxia on CYP2J2 mRNA expression in HepG2 cells (means ± S.E.M., *n* = 3).

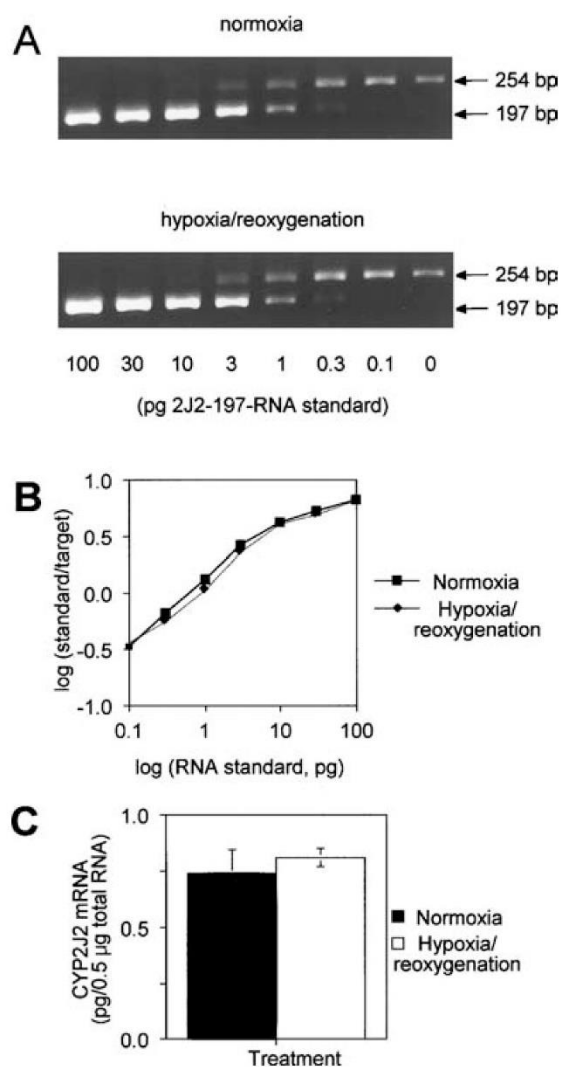


Figure 3. Effect of hypoxia-reoxygenation on CYP2J2 mRNA expression by competitive RT-PCR

(A) RNA was isolated from HepG2 cells that had been cultured for 16.5 h in normoxia (21% O₂; upper panel) or 16 h of hypoxia (1% O₂), followed by 0.5 h normoxia (lower panel). Competitive RT-PCR was performed as described in the legend to figure 2. (B) Typical relationship between relative intensities for CYP2J2 target (254 bp) and 2J2-197 standard (197 bp). (C) Effect of hypoxia-reoxygenation on CYP2J2 mRNA expression in HepG2 cells (means \pm S.E.M., $n = 3$).

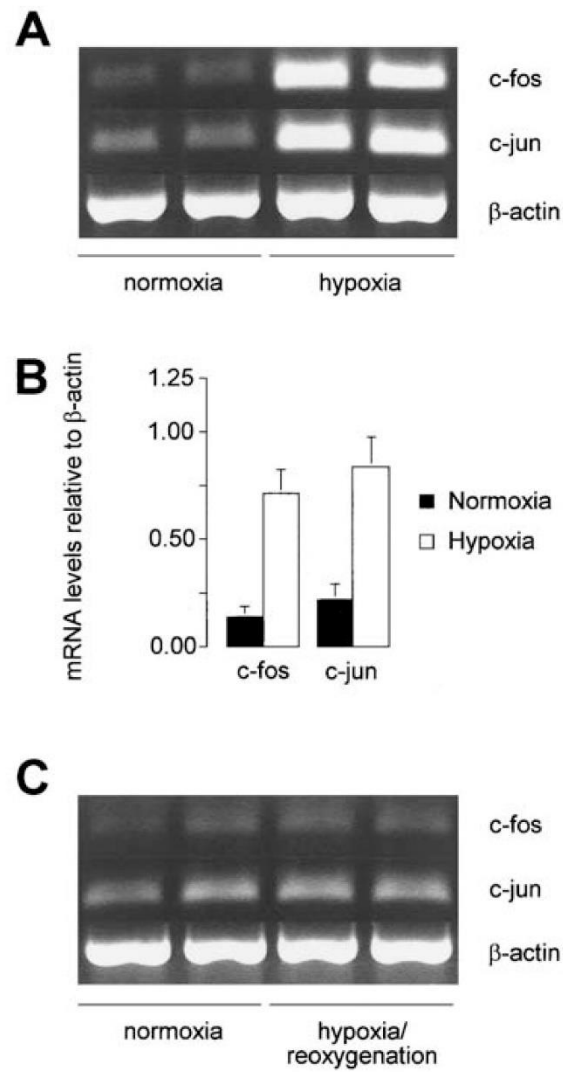


Figure 4. Effect of hypoxia and hypoxia-reoxygenation on c-Fos and c-Jun mRNA expression in HepG2 cells

(A) Amplification of c-Fos, c-Jun mRNA, and β -actin levels in HepG2 cells cultured for 16 h in normoxia (21% O_2) or hypoxia (1% O_2). (B) Densitometric analysis of semi-quantitative RT-PCR of relative c-Fos and c-Jun mRNA expression (means \pm S.E.M., $n = 3$ separate experiments). (C) Amplification of c-Fos, c-Jun mRNA, and β -actin levels in HepG2 cells cultured for 16.5 h in normoxia (21% O_2) or hypoxia (16 h at 1% O_2), followed by 30 min reoxygenation (21% O_2).

Results from the immunoblot analysis of total-cell lysates were consistent with mRNA measurements. Thus, compared with normoxia, hypoxia decreased CYP2J2-immunoreactive protein and increased c-Jun protein levels to approximately 3-fold those of the control; c-Fos protein was essentially undetectable in normoxic cell lysates, but was up-regulated strongly in hypoxic HepG2 cells (fig. 5A). Hypoxia-reoxygenation restored c-Fos and c-Jun protein expression to control levels, but CYP2J2 remained suppressed (fig. 5B).

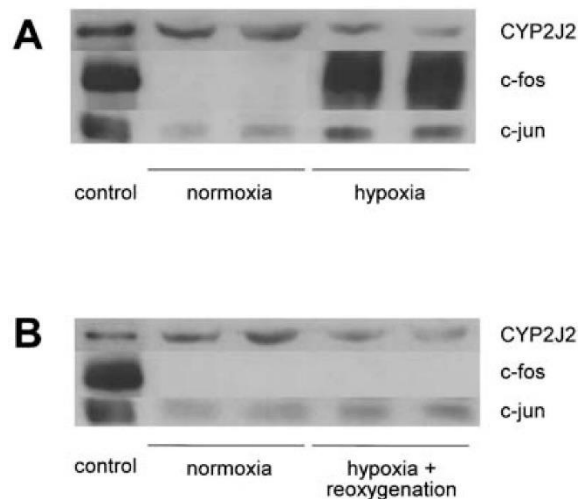


Figure 5. Effect of hypoxia and hypoxia-reoxygenation on CYP2J2, c-Fos and c-Jun protein in HepG2 cells

(A) Western blot analysis of CYP2J2, c-Fos and c-Jun expression in lysates from HepG2 cells cultured for 16 h in normoxia (21% O₂) or hypoxia (1% O₂). (B) CYP2J2, c-Fos and c-Jun protein in lysates from HepG2 cells cultured for 16.5 h in normoxia (21% O₂) or hypoxia (16 h at 1% O₂), followed by 30 min reoxygenation (21% O₂). Control lanes contain: CYP2J2, lysate from *E. coli* cells containing CYP2J2; c-Fos, lysate from phorbol-ester-treated Jurkat cells; and c-Jun, recombinant human c-Jun.

Differential activation of the CYP2J2 promoter in HepG2 cells by AP-1 proteins

The apparent association between the relative expression of CYP2J2 and AP-1 in hypoxia and hypoxia-reoxygenation prompted a more extensive molecular assessment. The functional importance of AP-1-like elements in the CYP2J2 promoter was evaluated by transient transfection analysis.

Co-transfection studies were performed in HepG2 cells with the luciferase reporter construct p2J2A (−2341/+98; fig. 6) and combinations of expression plasmids encoding members of the AP-1 complex. c-Jun strongly activated p2J2A (to approx. 7-fold that of the untransfected control), and the related proteins JunB and JunD elicited small increases in reporter activity. In contrast, as shown in figure 6, co-transfection of Fos family genes (*c-fos*, *fra-1*, and *fra-2*) did not stimulate the activity of p2J2A. Moreover, with the exception of the combination of JunB and Fra-1, Fos proteins abolished the activation of the CYP2J2 5'-flank/reporter constructs produced by Jun proteins.

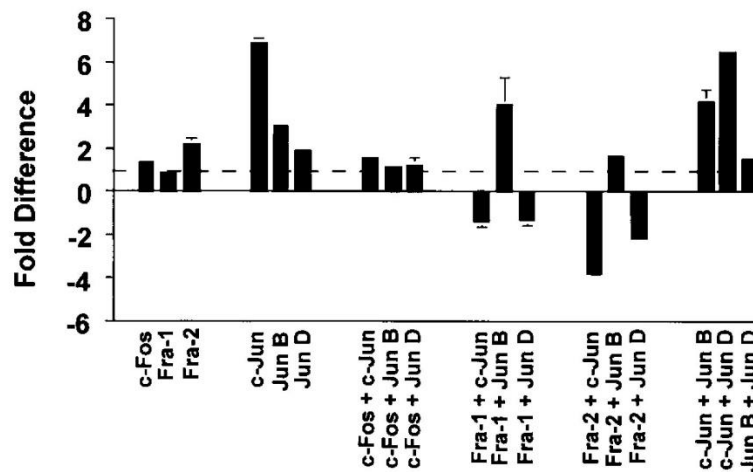


Figure 6. Activation of CYP2J2 by c-Jun

HepG2 cells were co-transfected with the CYP2J2-luciferase construct p2J2A (1 μ g/well) and combinations of expression plasmids encoding AP-1 proteins (0.5 μ g/well). A pCMV- β -galactosidase expression plasmid was included in each well to control for transfection efficiency (0.5 μ g/well). Luciferase activity was normalized to β -galactosidase activity; results shown are means \pm S.E.M. for at least three independent experiments.

Identification of a functional c-Jun binding element in the upstream region of CYP2J2

A series of deletion constructs was prepared by 5'-truncation of p2J2A and used in transfection studies to identify the c-Jun-responsive region of the CYP2J2 gene. Consistent with earlier results, p2J2A was strongly activated by c-Jun [to 8.2 ± 2.7 -fold of control ($P < 0.0001$); fig. 7]; c-Fos did not enhance reporter activity (1.2 ± 0.7 -fold of control) and abolished induction by c-Jun (1.1 ± 0.8 -fold of control). A similar pattern of transactivation was seen with the deletion constructs p2J2B–p2J2E, which all exhibited c-Jun responsiveness (the relative activity of construct p2J2E in the presence of c-Jun was 4.1 ± 0.6 -fold that of control; fig. 7). The role of two AP-1-like elements in CYP2J2 transactivation was tested. As shown in figure 8(A), p2J2E contained site A at –7 to +1 bp on the positive strand (CTGAGC CA) and site B at –56 to –63 bp on the negative strand (CGACGGTC).

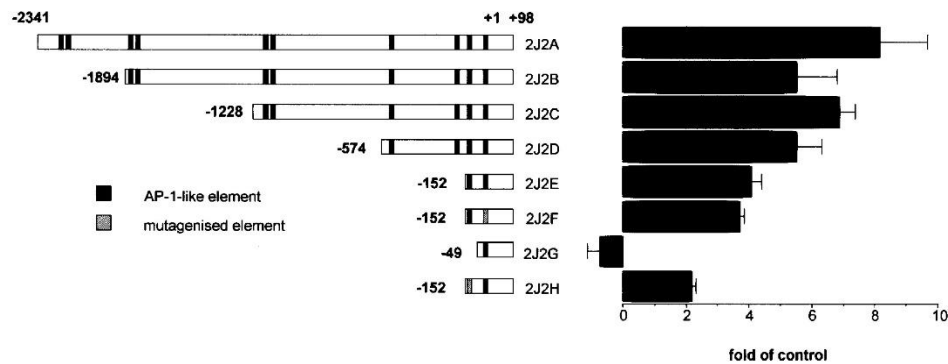


Figure 7. Location of a c-Jun-responsive region in the CYP2J2 gene

Identification of a c-Jun-responsive region in CYP2J2 using p2J2A and the deletion constructs p2J2B–p2J2E and p2J2G. The constructs p2J2F and p2J2H were prepared by mutagenesis of the AP-1-like elements at site A and site B respectively. HepG2 cells were co-transfected with reporter constructs, expression plasmids and a β -galactosidase expression plasmid, as described in the legend to figure 6. Luciferase activity was normalized to β -galactosidase activity; data are means \pm S.E.M. for at least three independent experiments.

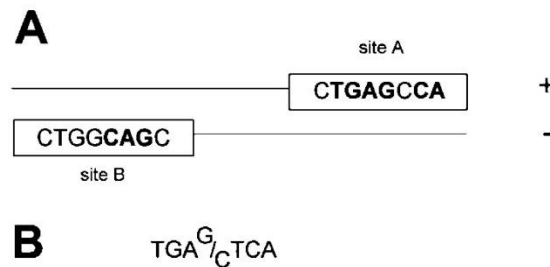


Figure 8. Sequences of the putative AP-1-like elements at sites A and B

(A) Sequences of sites A (positive strand) and site B (negative strand) and their relatedness to the AP-1 consensus sequence (B).

Site A more closely resembled the AP-1 consensus sequence TGA(G/C)TCA (fig. 8B). p2J2F was produced by mutagenesis of site A, but this construct retained c-Jun responsiveness when co-transfected into HepG2 cells (fig. 7). Although site B was detected only by low-stringency searching, construct p2J2G (–49/+98), which was prepared by 5' truncation of p2J2E and retained site A, was not inducible by c-Jun (fig. 7). To test further the apparent functional importance of site B, the AP-1-like sequence was mutagenized from the native sequence in p2J2E (p2J2H; fig. 7). The inducibility by c-Jun was retained in p2J2H but was markedly lower than that of p2J2E (2.2 ± 0.1 fold over control compared with 4.1 ± 0.6 -fold for p2J2E).

Binding of c-Jun to the human CYP2J2 promoter

The binding of c-Jun to an upstream region in CYP2J2 was evaluated in a series of EMSA studies. A 167 bp double-stranded fragment (2J2/167) corresponding to nt -152 to +15 of the CYP2J2 promoter was generated from p2J2E by digestion with *NheI* and *BanII*, and exhibited retarded complexes with nuclear protein fractions from untransfected HepG2 cells (fig. 9). Binding was competed by a 200-fold excess of unlabeled 2J2/167 probe, but not by a probe corresponding to the β -casein promoter STAT5 element (fig. 9A, lanes 3 and 4). Antibodies directed against c-Jun, but not ubiquitin, enhanced the apparent amount of a supershifted complex (lanes 5 and 6). The signal was more intense in nuclear extracts from c-Jun-transfected HepG2 cells (fig. 9B, lane 3), and was also strongly supershifted by an anti-c-Jun antibody (fig. 9B, lane 4). To confirm that c-Jun binds directly to CYP2J2, EMSAs were also performed using 2J2/167 and recombinant human c-Jun protein: a prominent shift was readily detected (fig. 9C, lane 2). This complex was competed successfully by excess unlabeled AP-1 consensus probe (lane 3).

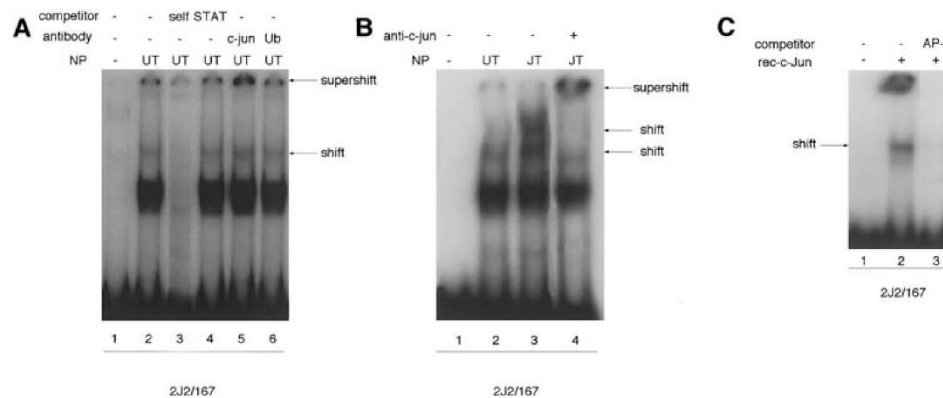


Figure 9. EMSA of the binding of c-Jun to the CYP2J2 promoter

The 32 P-labeled CYP2J2/167 double-stranded probe (bp -152 to +15 from the translation start site) was used in EMSA analysis with nuclear protein fractions (NP) from (A, B) untransfected (UT) and c-Jun-transfected (JT) HepG2 cells and with (C) recombinant c-Jun protein. Binding reactions allowed to proceed in the presence of antibodies to c-Jun and ubiquitin (Ub) are indicated. Reactions were also conducted in the presence of excess unlabeled CYP2J2/167 (self), STAT (STAT5 element from the β -casein promoter) and AP-1 consensus oligonucleotide. Retarded and supershifted complexes are indicated by arrows. The autoradiographs are representative of results from at least three separate experiments.

Further EMSA studies tested the interaction between c-Jun and the AP-1-like elements at sites A and B. Transfection analysis had eliminated a role for site A in c-Jun-dependent transactivation, despite results from EMSA analysis that indicated its capacity to interact with c-Jun protein (results not shown). EMSAs were performed using labeled probes containing the site B element [2J2-(-56/-63) probe; fig. 10]. The signal observed in nuclear fractions from untransfected HepG2 cells (fig. 10A, lane 2) was more intense in nuclear extracts

from c-Jun-transfected cells (fig. 10A, lane 3), and was competed by excess 2J2-(–56/–63) probe, but not the STAT5 element probe (lanes 4 and 5), thus confirming specificity. The signal was also block-shifted by an anti-c-Jun antibody, but not by anti-c-Fos or anti-ubiquitin antibodies (fig. 10A, lanes 6–8). Binding of c-Jun to the probe sequence was confirmed by the use of human recombinant c-Jun protein (fig. 10B, lane 2). Furthermore, mutagenesis of the AP-1-like sequence to produce 2J2-mt-(–56/–63) resulted in no interaction with recombinant c-Jun protein (fig. 10B, lane 3), and very weak interactions with nuclear extracts from untransfected and c-Jun-transfected HepG2 cells (results not shown).

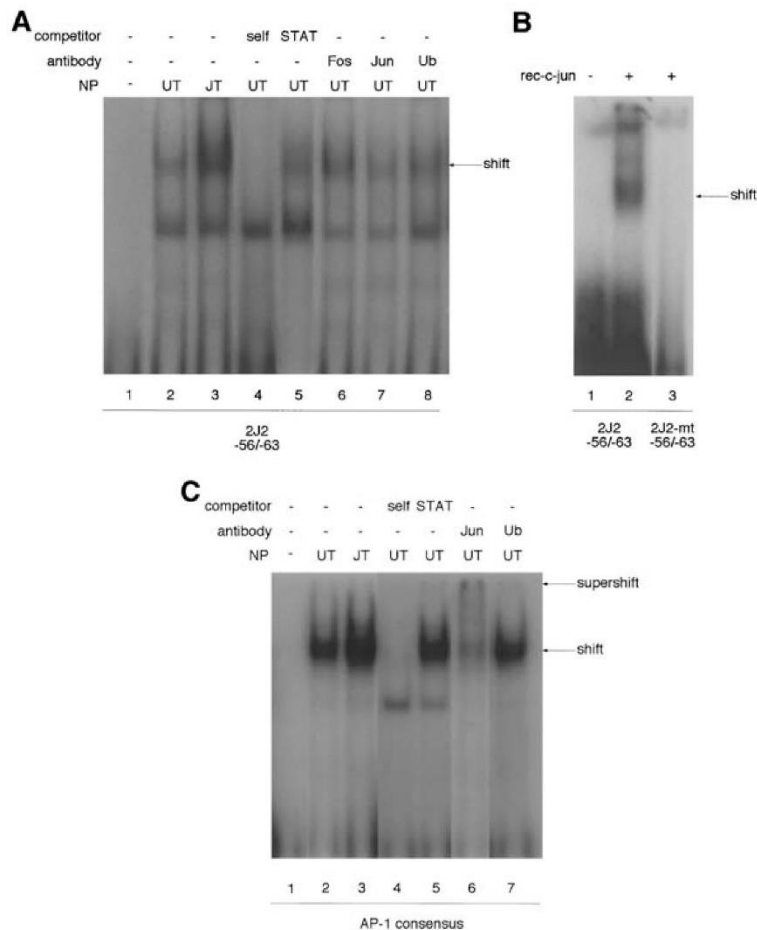


Figure 10. EMSA of the binding of c-Jun to an AP-1-like element in the CYP2J2 upstream region

A 32 P-labeled double-stranded probe corresponding to the AP-1-like element at –56 to –63 of the upstream region of CYP2J2 (2J2 –56/–63) and a mutant probe (2J2-mt –56/–63) were used in EMSA analysis with (A) nuclear protein fractions (NP) from untransfected (UT) and c-Jun-transfected (JT) HepG2 cells, and with (B) recombinant c-Jun protein. (C) A 32 P-labeled double-stranded AP-1 consensus probe was used in comparative binding studies in fractions from UT and JT HepG2 cells. Some reactions contained antibodies to c-Jun, c-

Fos, or ubiquitin (Ub), as indicated. Other reactions contained excess unlabeled CYP2J2(-56/-63) probe (self) or STAT probe (STAT5 element from the β -casein promoter). Retarded and supershifted complexes are indicated by arrows. The autoradiographs are representative of results from at least three separate experiments.

Analogous experiments were conducted with an AP-1 consensus probe (fig. 10C). The major shifted complex exhibited a very similar mobility in nuclear protein fractions to that seen with the 2J2(-56/-63) probe (fig. 10A). The signal in nuclear fractions from untransfected HepG2 cells (fig. 10C, lane 2), was increased in fractions from c-Jun-transfected cells (lane 3). Binding was competed by excess unlabeled AP-1 probe (lane 4), but not by an excess of the unlabeled STAT5 element probe (lane 5). Incubation with a c-Jun antibody, but not the antiubiquitin antibody, supershifted the complex (fig. 10C, lanes 6 and 7).

EMSA assays were also conducted in nuclear protein fractions from HepG2 cells cultured under hypoxic and normoxic conditions. The shift observed with the 2J2(-56/-63) probe in nuclear protein fractions from normoxic cells (fig. 11A, lane 2) was effectively competed by a 200-fold excess of the unlabeled probe (lane 3), but not by a similar excess of the probe corresponding to the STAT5 element from the β -casein upstream region (lane 4). The retarded complex was also block-shifted by an anti-c-Jun antibody (lane 6) but not by anti-c-Fos or antiubiquitin antibodies (lanes 5 and 7). In hypoxia, the intensity of the probe shift was markedly decreased (lane 8) and was apparently unaffected by either of the antibodies directed against c-Fos or c-Jun (fig. 11A, lanes 9 and 10). These results indicate that binding of c-Jun to the -56/-63 element was decreased in fractions from hypoxic cells.

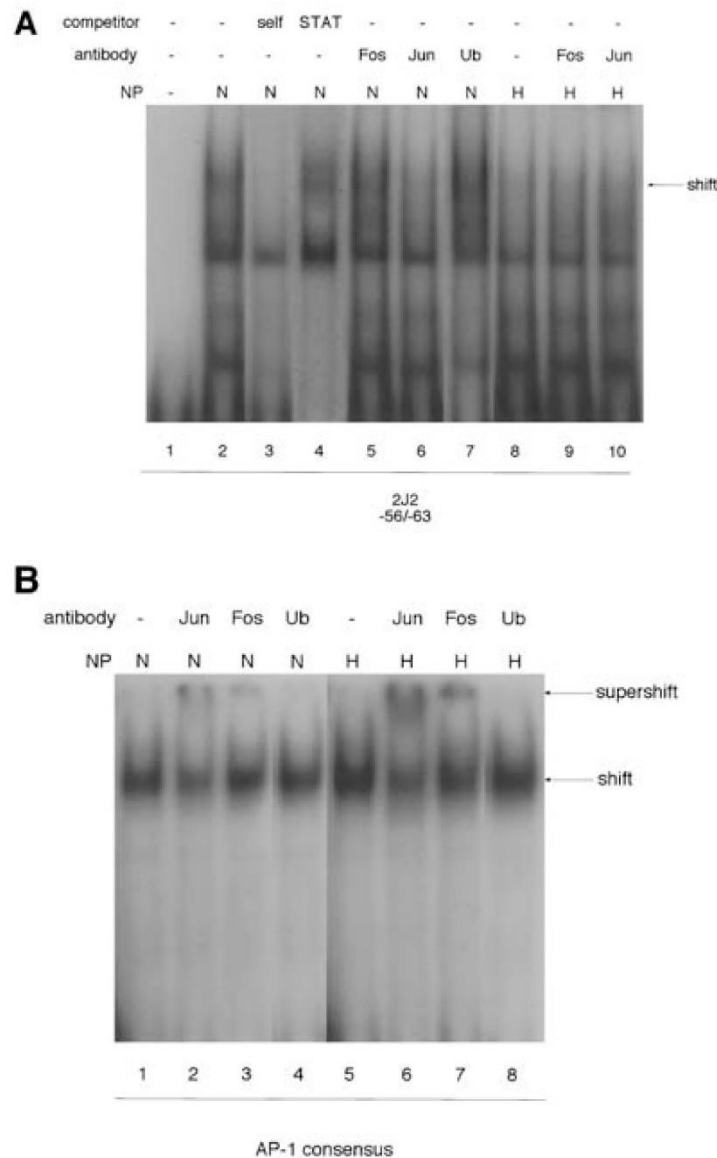


Figure 11. EMSA of the differential binding of c-Jun to the CYP2J2 AP-1-like element and an AP-1 consensus probe

32 P-labeled double-stranded probes corresponding to (A) the AP-1-like element at -56 to -63 of the upstream region of CYP2J2 (2J2 -56/-63) and (B) the AP-1 consensus sequence were used in EMSA analysis with nuclear protein fractions (NP) from HepG2 cells cultured for 16 h in normoxia (21% O₂; N) or hypoxia (1% O₂; H). Some reactions were performed in the presence of antibodies against c-Jun, c-Fos, or ubiquitin (Ub), or excess unlabeled 2J2- (-56/-63) probe (self) or STAT probe (STAT5 element from the β -casein promoter). Retarded and supershifted complexes are indicated by arrows. The autoradiographs are representative of results from at least three separate experiments.

Comparative studies evaluated the binding of the AP-1 consensus probe to fractions from differently cultured HepG2 cells (fig. 11B). In nuclear protein fractions from normoxic cells, the intensity of the retarded complex was diminished by an anti-c-Jun antibody (lane 2), but not by anti-c-Fos or anti-ubiquitin antibodies (lanes 3 and 4). In contrast with the results with the 2J2(-56/-63) probe, the intensity of the shift produced by the AP-1 consensus probe was more pronounced in nuclear protein fractions from hypoxic cells (lane 5); antibodies against c-Jun and c-Fos (lanes 6 and 7), but not ubiquitin (lane 8), supershifted the complex.

Discussion

The widespread distribution of CYP2J2 in tissues [2,4–6] and the diverse cellular effects of EETs suggest that this CYP is important in cellular physiology and pathophysiology. The present study implicates AP-1 proteins in the transcriptional regulation of *CYP2J2* and has identified a c-Jun-responsive region in the upstream region of the *CYP2J2* gene. Whereas c-Jun homodimers strongly activated *CYP2J2* expression, heterodimers formed between c-Fos and c-Jun were inactive. Thus the down-regulation of *CYP2J2* in hypoxia is associated with c-Fos induction. These results are compatible with the recent study of Yang et al. [8], in which CYP2J2 protein was decreased in vascular endothelial cells cultured under hypoxic conditions.

Several redox-responsive transcription factors are modulated in hypoxia, including hypoxia-inducible factor-1 ("HIF-1") [26,27], nuclear factor- κ B ("NF- κ B") [28] and AP-1 [13–15]. Owing to the presence of AP-1-like response elements within the 5'-flanking region of the *CYP2J2* gene, we investigated the possibility that AP-1 has a direct role in the transcriptional regulation of *CYP2J2* in hypoxia. Down-regulation of *CYP2J2* in hypoxic HepG2 cells was inversely related to increased expression of c-Jun and c-Fos mRNA and protein.

It is well established that c-Jun is expressed constitutively in cells and activates AP-1-dependent genes via homodimer formation [29–31]. AP-1-regulated genes are also activated in response to a range of stimuli that promote heterodimer formation between Jun and Fos proteins (e.g., c-Fos/c-Jun) [32]. Unlike c-Jun, c-Fos is either absent or expressed at only very low levels in resting cells but is rapidly up-regulated in response to a range of exogenous stimuli: this enables a change in the composition of AP-1 complexes from c-Jun homodimers to c-Jun/c-Fos heterodimers [29,31]. These reports are in accordance with the present results of the differential role of c-Jun and c-Fos in *CYP2J2* expression in HepG2 cells cultured in normoxia and hypoxia.

The literature clearly demonstrates the activation of AP-1 in hypoxia, but the effect of hypoxia followed by a period of reoxygenation on AP-1 activity is controversial. There are reports that AP-1 activity remains elevated following reoxygenation [33], while others have found that mRNAs corresponding to certain AP-1 subunits are increased in hypoxia and decreased by reoxygenation [34]. Similarly, AP-1 has been reported to be activated in ischaemia-reperfusion [35,36] or to be activated in ischaemia, normalized during the early reoxygenation phase, and then elevated by prolonged reoxygenation [37]. In the present study, c-Fos and c-Jun expression in HepG2 cells was not significantly altered from that of

the control by hypoxia-reoxygenation, consistent with rapid restitution of both the mRNA and the protein of these genes that control the acute-phase response to external stresses. Consistent with the apparent relationship to AP-1 subunit expression, CYP2J2 mRNA levels in HepG2 cells returned to control levels after hypoxia-reoxygenation, but CYP2J2 protein levels remained suppressed. This observation is consistent with the results of Yang et al. [8], who also demonstrated the down-regulation of CYP2J protein in hypoxia followed by 4 h reoxygenation. A number of studies have documented rapid increases in CYP mRNAs, but delayed synthesis of the corresponding proteins following *in vivo* exposure to foreign compounds [38,39]. Thus the time required for the restoration of CYP2J protein is likely to be longer than that required for normalization of the mRNA.

Transactivation of the CYP2J2 promoter by AP-1 proteins was examined in transient transfection studies. Activation by c-Jun and, to a lesser extent, JunB was observed. In contrast, c-Fos and the related proteins Fra-1 and Fra-2 abolished the pronounced induction of CYP2J2 promoter activity elicited by c-Jun alone. It is possible that Fra-1 and Fra-2, which are expressed in liver cells [40,41], may also contribute to CYP2J2 suppression in response to external stress stimuli. Taken together, these results suggest that c-Jun is involved in the maintenance of CYP2J2 protein levels in normoxic cells, and that down-regulation of CYP2J2 occurs in hypoxia largely because of c-Fos up-regulation. Indeed, in recent studies we have found that treatment of HepG2 cells with the nitric-oxide-releasing agent sodium nitroprusside also increases c-Fos expression and down-regulates CYP2J2 (V. Anggono, N. Y. Marden, and M. Murray, unpublished work). Thus external stimuli that up-regulate c-Fos may well exert a generalized down-regulatory effect on CYP2J2 expression in hepatocytes.

Antagonism of c-Jun-dependent gene activation by c-Fos has been reported previously. Kovacic-Milivojevic and Gardner [42] described the activation of the human atrial natriuretic peptide (ANP) promoter by c-Jun and its inhibition by overexpressed c-Fos. Thus it appears that the human ANP and CYP2J2 genes are regulated similarly by AP-1. Interestingly, this study also found that inhibition of c-Jun-dependent ANP promoter activity by c-Fos was cell-specific, with the inhibition by c-Fos being observed in atrial and ventricular cardiomyocytes but not in cardiac mesenchymal or GC cells [42]. It remains to be investigated whether CYP2J2 expression exhibits similar tissue-related regulation by c-Fos.

A region within 152 bp of the start of the CYP2J2 coding region was identified as being important for transactivation by c-Jun, and direct binding of c-Jun within this region was confirmed by EMSA. Of the two AP-1-like sites in this region of the gene, site A (−7 to +1 bp relative to the translation start site) was found from transient transfection studies not to contribute to c-Jun-mediated CYP2J2 activation, despite its capacity to bind c-Jun protein in EMSA analyses. Site B (−56 to −63 bp on the negative strand) was functional, and contributed to c-Jun-dependent activation of the CYP2J2 promoter. EMSA analysis corroborated these results and established that c-Jun bound directly to this element. Formation of c-Jun/c-Fos heterodimers diminished the binding of c-Jun to site B, as seen in nuclear-protein fractions from hypoxic HepG2 cells. In this regard, it is of interest that different combinations of leucine-zipper proteins have been shown to bind differently to AP-1 sites in genes. The promoter context is also important, because of potential steric effects of DNA-protein interactions on binding of c-Jun to site B. Considerations of this type may impede

the binding of c-Jun/c-Fos heterodimers to site B in hypoxia, and cause down-regulation of *CYP2J2*.

Although mutation of site B significantly inhibited activation by c-Jun, induction was not abolished completely. Thus another, as-yet-unidentified element may also contribute to the activation of the *CYP2J2* gene promoter by c-Jun. This additional element also lies between nt -152 to -50 but does not resemble the AP-1 consensus sequence and was not identified by sequence analysis. It is possible that c-Jun mediates this effect by binding to an atypical or cryptic AP-1 site, which differs considerably from the consensus sequence. Alternatively, c-Jun may modulate the expression of another regulatory protein that binds within the -152 to -50 region of *CYP2J2* to initiate transcription. c-Jun may enhance transcription of the *CYP2J2* gene by functional interaction with other transcription factors. In this regard, it is of considerable interest that c-Jun has been reported to activate transcription of target genes through functional interactions with Sp1 [43], PU.1 [44], GATA-2 [45], and GATA-4 [46]. We are currently evaluating these possibilities.

Note – The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank, and GSDB Nucleotide Sequence Databases under the accession number AF039089.

Acknowledgments – This work was supported by a grant from the Australian National Health and Medical Research Council, and equipment grants from The Wellcome Trust, UK, and the Clive and Vera Ramaciotti Foundations. The assistance of Stuart Purvis-Smith (Molecular and Cytogenetics Unit, Prince of Wales Hospital) in providing access to a hypoxic incubator is gratefully acknowledged. Gifts of expression plasmids from Dr. K. Imakawa and Dr. M. Karin and the anti-CYP2J4 antibody from Dr. Quin-Yu Zhang are also gratefully acknowledged.

References

1. Roman, R. J. (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol. Rev.* 82, 131–185.
2. Wu, S., Moomaw, C. R., Tomer, K. B., Falck, J. R., and Zeldin, D. C. (1996) Molecular cloning and expression of *CYP2J2*, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J. Biol. Chem.* 271, 3460–3468.
3. Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D. C., and Liao, J. K. (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 285, 1276–1279.
4. Zeldin, D. C., Foley, J., Ma, J., Boyle, J. E., Pascual, J. M. S., Moomaw, C. R., Tomer, K. B., Steenbergen, C., and Wu, S. (1996) *CYP2J* subfamily P450s in the lung: expression, localization, and potential functional significance. *Mol. Pharmacol.* 50, 1111–1117.
5. Zeldin, D. C., Foley, J., Boyle, J. E., Moomaw, C. R., Tomer, K. B., Parker, C., Steenbergen, C., and Wu, S. (1997) Predominant expression of an arachidonate epoxygenase in islets of Langerhans cells in human and rat pancreas. *Endocrinology* 138, 1338–1346.
6. Zeldin, D. C., Foley, J., Goldsworthy, S. M., Cook, M. E., Boyle, J. E., Ma, J., Moomaw, C. R., Tomer, K. B., Steenbergen, C., and Wu, S. (1997) *CYP2J* subfamily cytochrome P450s in the gastrointestinal tract: expression, localization, and potential functional significance. *Mol. Pharmacol.* 51, 931–943.

7. Campbell, W. B., and Harder, D. R. (1999) Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ. Res.* 84, 484–488.
8. Yang, B., Graham, L., Dikalov, S., Mason, R. P., Falck, J. R., Liao, J. K., and Zeldin, D. C. (2001) Overexpression of cytochrome P450 CYP2J2 protects against hypoxia-reoxygenation injury in cultured bovine aortic endothelial cells. *Mol. Pharmacol.* 60, 310–320.
9. Chen, J.-K., Capdevila, J., and Harris, R. C. (2001) Cytochrome P450 epoxygenase metabolism of arachidonic acid inhibits apoptosis. *Mol. Cell. Biol.* 21, 6322–6331.
10. Angel, P., and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* 1072, 129–157.
11. Kerppola, T. K., and Curran, T. (1993) Selective DNA bending by a variety of bZIP proteins. *Mol. Cell. Biol.* 13, 5479–5489.
12. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* 49, 729–739.
13. Lee, Y. J., and Corry, P. M. (1999) Hypoxia-induced bFGF gene expression is mediated through the JNK signal transduction pathway. *Mol. Cell. Biochem.* 202, 1–8.
14. Müller, J. M., Krauss, B., Kaltschmidt, C., Baeuerle, P. A., and Rupec, R. A. (1997) Hypoxia induces *c-fos* transcription via a mitogen-activated protein kinase-dependent pathway. *J. Biol. Chem.* 272, 23435–23439.
15. Bandyopadhyay, R. S., Phelan, M., and Faller, D. V. (1995) Hypoxia induces AP-1-regulated genes and AP-1 transcription factor binding in human endothelial and other cell types. *Biochim. Biophys. Acta* 1264, 72–78.
16. Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* 270, 16483–16486.
17. Devary, Y., Gottlieb, R. A., Lau, L. F., and Karin, M. (1991) Rapid and preferential activation of the *c-jun* gene during the mammalian UV response. *Mol. Cell. Biol.* 11, 2804–2811.
18. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
19. Chung, H.-O., Kato, T., and Kato, Y. (1996) Molecular cloning of *c-jun* and *c-fos* cDNAs from porcine anterior pituitary and their involvement in gonadotropin-releasing hormone stimulation. *Mol. Cell. Endocrinol.* 119, 75–82.
20. Lederer, E. B., Bastani, K., and Goebel, F.-D. (1996) A new quantitative RT-PCR assay for the vasoconstrictor endothelin. *Res. Exp. Med.* 196, 77–86.
21. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. *Nucleic Acids Res.* 17, 6419.
22. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randell, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–270.
23. Gebert, C. A., Park, S. H., and Waxman, D. J. (1997) Regulation of STAT5b activation by the temporal pattern of growth hormone stimulation. *Mol. Endocrinol.* 11, 400–414.
24. King, L. M., Ma, J., Srettabunjong, S., Graves, J., Bradbury, J. A., Li, L., Spiecker, M., Liao, J. K., Mohrenweiser, H., and Zeldin, D. C. (2002) Cloning of CYP2J2 gene and identification of functional polymorphisms. *Mol. Pharmacol.* 61, 840–852.

25. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* 23, 4878–4884.
26. Semenza, G. L. (1998) Hypoxia-inducible factor 1: master regulator of O₂ homeostasis. *Curr. Opin. Genet. Dev.* 8, 588–594.
27. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Acad. Sci. U.S.A.* 92, 5510–5514.
28. Koong, A. C., Chen, E. Y., and Giaccia, A. J. (1994) Hypoxia causes the activation of nuclear factor κ B through the phosphorylation of I κ B α on tyrosine residues. *Cancer Res.* 54, 1425–1430.
29. Minet, E., Michel, G., Mottet, D., Piret, J.-P., Barbieux, A., Raes, M., and Michiels, C. (2001) c-Jun gene induction and AP-1 activity is regulated by a JNK-dependent pathway in hypoxic HepG2 cells. *Exp. Cell Res.* 265, 114–124.
30. Lafyatis, R., Kim, S.-J., Angel, P., Roberts, A. B., Sporn, M. B., Karin, M., and Wilder, R. L. (1990) Interleukin-1 stimulates and all-*trans*-retinoic acid inhibits collagenase gene expression through its 5' Activator Protein-1-binding site. *Mol. Endocrinol.* 4, 973–980.
31. Mishra, R. R., Adhikary, G., Simonson, M. S., Cherniack, N. S., and Prabhakar, N. R. (1998) Role of *c-fos* in hypoxia-induced AP-1 *cis*-element activity and tyrosine hydroxylase gene expression. *Mol. Brain Res.* 59, 74–83.
32. Angel, P. E., and Herrlich, P. A. (1994) *The FOS and JUN families of transcription factors*. CRC Press, Boca Raton, FL.
33. Rupec, R. A., and Baeuerle, P. A. (1995) The genomic response of tumour cells to hypoxia and reoxygenation. Differential activation of transcription factors AP-1 and NF- κ B. *Eur. J. Biochem.* 234, 632–640.
34. Yao, K.-S., Xanthoudakis, S., Curran, T. and O'Dwyer, P. J. (1994) Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. *Mol. Cell. Biol.* 14, 5997–6003.
35. Morooka, H., Bonventre, J. V., Pombo, C. M., Kyriakis, J. M., and Force, T. (1995) Ischemia and reperfusion enhance ATF-2 and c-Jun binding to cAMP response elements and to an AP-1 binding site from the *c-jun* promoter. *J. Biol. Chem.* 270, 30084–30092.
36. Brand, T., Sharma, H. S., Fleischmann, K. E., Duncker, D. J., McFalls, E. O., Verdouw, P. D., and Schaper, W. (1992) Proto-oncogene expression in porcine myocardium subjected to ischemia and reperfusion. *Circ. Res.* 71, 1351–1360.
37. Tacchini, L., Radice, L., and Bernelli-Zazzera, A. (1999) Differential activation of some transcription factors during rat liver ischemia, reperfusion, and heat shock. *J. Cell Physiol.* 180, 255–262.
38. Morville, A. L., Thomas, P., Levin, W., Reik, L., Ryan, D. E., Raphael, C., and Adesnik, M. (1983) The accumulation of distinct mRNAs for the immunochemically related cytochromes P-450c and P-450d in rat liver following 3-methylcholanthrene treatment. *J. Biol. Chem.* 258, 3901–3906.
39. Iba, M. M., Alam, J., Touchard, C., Thomas, P. E., Ghosal, A., and Fung, J. (1999) Coordinate up-regulation of CYP1A1 and heme oxygenase-1 (HO-1) expression and modulation of δ -aminolevulinic acid synthase and tryptophan pyrrolase activities in pyridine-treated rats. *Biochem. Pharmacol.* 58, 723–734.
40. Shih, H., Pickwell, G. V., and Quattrochi, L. C. (2000) Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer. *Arch. Biochem. Biophys.* 373, 287–294.

41. Wild, A. C., Gipp, J. J., and Mulcahy, R. T. (1998) Overlapping antioxidant response element and PMA response element sequences mediate basal and β -naphthoflavone-induced expression of the human γ -glutamylcysteine synthetase catalytic subunit gene. *Biochem. J.* 332, 373–381.
42. Kovacic-Milivojevic, B., and Gardner, D. G. (1992) Divergent regulation of the human atrial natriuretic peptide gene by *c-jun* and *c-fos*. *Mol. Cell. Biol.* 12, 292–301.
43. Kardassis, D., Papakosta, P., Pardali, K., and Moustakas, A. (1999) c-Jun transactivates the promoter of the human p21^{WAF1/Cip1} gene by acting as a superactivator of the ubiquitous transcription factor Sp1. *J. Biol. Chem.* 274, 29572–29581.
44. Behre, G., Whitmarsh, A. J., Coghlan, M. P., Hoang, T., Carpenter, C. L., Zhang, D.-E., Davis, R. J., and Tenen, D. G. (1999) c-Jun is a JNK-independent coactivator of the PU.1 transcription factor. *J. Biol. Chem.* 274, 4939–4946.
45. Kawana, M., Lee, M.-E., Quertermous, E. E., and Quertermous, T. (1995) Cooperative interaction of GATA-2 and AP-1 regulates transcription of the endothelin-1 gene. *Mol. Cell. Biol.* 15, 4225–4231.
46. Herzig, T. C., Jobe, S. M., Aoki, H., Molkenstin, J. D., Cowley, Jr., A. W., Izumo, S., and Markham, B. E. (1997) Angiotensin II type_{1a} receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. *Proc. Natl Acad. Sci. U.S.A.* 94, 7543–7548.