

DT-diaphorase activity in NSCLC and SCLC cell lines: a role for fos/jun regulation

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Summary To assess the potential differential lung tumour expression of NAD(P)H:quinone reductase (NQO1), the human (h) NQO1 promoter was characterized in gene transfer studies. A deletion panel of 5' flanking hNQO1 promoter constructs was made and tested in transient transfection assays in NSCLC and SCLC cell lines. The largest hNQO1 construct (–1539/+115) containing the antioxidant response element (ARE), exhibited robust levels of reporter activity in the NSCLC (H460, H520, and A549) cell lines and expression was over 12 to 77-fold higher than the minimal (–259/+115) promoter construct. In contrast, there was little difference in promoter activity between the largest and minimal promoter construct in the SCLC (H146, H82 and H187) cell lines. Deletion of the sites for NFκB and AP-2 and the XRE did not significantly affect hNQO1 promoter activity in either the NSCLC or SCLC cell lines. Robust promoter activity in NSCLC lines was mediated by a 359 bp segment of the proximal promoter that contained a canonical AP-1 binding site, TGACTCAG, within the ARE. Gel supershift assays with various specific Fos/Jun antibodies identified Fra1, Fra2 and Jun B binding activity in NSCLC cells to a promoter fragment (–477 to –438) spanning the AP-1 site, whereas SCLC do not appear to express functional Fra or Jun B. These results suggest a possible role for AP-1 activity in the differential expression of hNQO1 in NSCLC.

Keywords: NAD(P)H:quinone reductase; DT-diaphorase; fos/jun; NSCLC; SCLC

Many human tumors have elevated DT-diaphorase activities, such as liver (Beyer et al, 1987), colon (Schlager et al, 1990), lung (Malkinson et al, 1992) and breast (Berger et al, 1985). In the case of lung tumours, elevated NQO1 activity has recently been reported in non-small cell lung cancer (NSCLC) cell lines and primary tumours relative to SCLC and uninvolved lung (Schlager and Powis, 1990; Malkinson et al, 1992; Smitskamp-Wilms et al, 1995). This work validated NSCLC as a potential target against which anti-tumour drugs, which are efficiently bioactivated by NQO1, can be tested for their cytotoxicity. Biochemical studies have already demonstrated that NQO1 activity is induced by a wide range of chemicals including polycyclic aromatic hydrocarbons, azo dyes and phenolic antioxidants (Benson et al, 1980; DeLong et al, 1986; Joseph et al, 1994; Talalay and Prochaska, 1987; Talalay et al, 1988). Sequence analysis of the 5' flanking region of the human NQO1 gene shows the presence of an activator protein 2 (AP-2), antioxidant response element (ARE), also called the electrophile response element (EpRE), a xenobiotic response element (XRE) and a nuclear factor κB (NFκB)-like element within 900 bp of the transcriptional start site (Jaiswal, 1991). Interestingly, the ARE in the human NQO1 gene (–477 to –438) actually contains a centrally-located perfect AP-1 site, flanked by two imperfect AP-1 elements. In this study, we examined the mechanisms underlying the differential basal expression of hNQO1 in NSCLC versus SCLC. Initially, Jaiswal et al characterized the ARE in the hNQO1 gene in a hepatoma cell line and suggested that multiple AP-1 proteins bind to this DNA sequence

(Li and Jaiswal, 1992, 1994). In this report, we have performed functional assays to identify the *cis* DNA elements important in the transcriptional regulation of NQO1 in human lung tumour cells. Our data indicate that the ARE is important in regulating NQO1 promoter activity in NSCLC. We have also examined binding activity of Fos and Jun protein family members to the hNQO1 ARE and demonstrate Fra1, Fra2 and JunB binding activity only in NSCLC cell lines that constitutively express NQO1.

MATERIALS AND METHODS

Cell lines and cell culture

The human NSCLC cell lines (NCI-H460, NCI-H520, A549) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in modified Eagles medium (MEM) supplemented with 2 mM L-glutamine, 100 units ml^{–1} penicillin, 100 µg ml^{–1} streptomycin and 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO, USA). The human SCLC cell lines (NCI-H146, NCI-H82, NCI-H187) were also obtained from ATCC and were maintained in RPMI-1640 with the same supplements. PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) (Traver et al, 1997) was performed on these six cell lines to ensure wild-type or heterozygous NQO1 polymorphism status (wild-type: H460, A549, H146, H82; heterozygous: H520, H187).

Construction of plasmids

The reporter plasmid vector, pGL₃ (Promega, Madison, WI, USA) contains a synthetic polyadenosine (poly-A) signal placed

Received 20 April 1998

Revised 6 August 1998

Accepted 7 August 1998

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upstream of the firefly luciferase gene and a SV40 late poly-A signal downstream of the reporter gene. A 1765 bp fragment (–1539 to +115) of the hNQO1 gene was isolated from a human placental cosmid library (gift from Dr NW Gibson). To generate 5' deletion constructs, convenient restriction enzyme sites were used to subclone the DNA fragments into the Sma I/Nco I site in pGL3.

Transient DNA transfections

Lipofection of plasmid DNA into the various human lung cells was performed as described (BRL, Gaithersburg, MD, USA). Briefly, each 35 mm plate received 1×10^6 cells, 1 μg of test plasmid, 1 μg of pCMV β gal (Clontech, Palo Alto, CA, USA) and 3 μl or 4 μl (NSCLC or SCLC respectively) of Lipofectamine (BRL, Gaithersburg, MD, USA). Cells were harvested after 24 h, lysed, and cell debris sedimented. For normalization of transfection efficiency, cells were transfected in parallel with the control plasmid, pSV40GL₃ (Promega, Madison, WI, USA). Cell extracts were then assayed for luciferase and β -galactosidase activities (Promega, Madison, WI, USA).

Nuclear extracts

High salt nuclear extracts were prepared using a modification of the method of Dignam et al (1983). Briefly, washed pelleted cells ($5\text{--}10 \times 10^8$) were suspended in two packed volumes of 10 mM Hepes, pH 7.9, 1.5 mM magnesium chloride, 60 mM potassium chloride and 0.5 mM DTT, and lysed by 15 strokes of an all glass Dounce homogenizer (B type pestle). The homogenate was centrifuged to pellet the nuclei. The crude nuclei were resuspended in 0.6 ml of 20 mM Hepes, pH 7.9, 25% glycerol, 500 mM potassium chloride, 0.5 mM DTT, 1.5 mM magnesium chloride, 0.2 mM EDTA, pH 8.0 and 0.1 mM EGTA, pH 8.0 and gently shaken for 30 min. After centrifugation, the supernatant was dialysed for 3 h and then frozen in aliquots at -70°C . The protein concentration in the nuclear extracts was determined using the Bradford protein assay (Bio Rad, Richmond, CA, USA).

Electrophoretic gel mobility shift assays

The double stranded oligomer used in the gel shift studies was synthesized as a complementary pair on an automated DNA synthesizer. The oligomer was end-labelled using T₄ polynucleotide kinase (BRL) and [32]γ-ATP (NEN, 3000 Ci mmol⁻¹) to a

Table 1 DT-diaphorase activity in cell lines from lung tumours

Human lung cell lines	DT-diaphorase activity (nmoles min ⁻¹ mg ⁻¹ protein)
NSCLC/NCI-H460	1782 ± 72
NSCLC/A549	2484 ± 52
NSCLC/NCI-H520	1122 ± 56
SCLC/NCI-H146	6 ± 0.6
SCLC/NCI-H82	3 ± 1.4
SCLC/NCI-H187	3 ± 1.0

Enzyme activities (mean \pm s.d.) were determined as described (Malkinson et al. 1992).

specific activity of 10–30 000 cpm and applied to a G-10 Sephadex column (Clontech, Palo Alto, CA, USA). The human NQO1 promoter fragments were the sense strand, 5'-AAATCGCAGT-CACAGTGACTCAGCAGAATCTGAGCCTAGG-3' annealed to the antisense strand, 5'-CCTAGGCTCAGATTCTGCTGAGT-CACTGTGACTGCGATT-3' and represented sequences from -477 to -438 and contained the ARE; the sense strand, 5'-GATTACAGGCGTGAGCACCG-3' annealed to the antisense strand 5'-CGGTGCTCACGCCTGTAATC-3' and represented sequences from -749 to -730 and contained the XRE; the sense strand, 5'-AACAAATTCGTCTCCACGGAGCATGTCT-3' annealed to the antisense strand 5'-AGACATGCTCCGTGGA-GACGAATTTGTT-3' and represented mid-promoter sequences (MPR) from -527 to -499. The sense strand, 5'-tcgaGGTA-ATTTGCATTTCTAA-3' was annealed to the antisense strand 5'-tcgaTTAGAAATGCAAATTACC-3' and represented sequences -537 to -554 plus TCGA overhangs and contained the octamer (Oct-1) motif region of the mouse immunoglobulin enhancer (Ephrussi et al, 1985). The sense strand, 5'-tcgaCTAGT-GATGAGTCAGCCGGATC-3' was annealed to the antisense strand 5'-tcgaGATCCGGCTGACTCATCTAG-3' and represented an artificial consensus AP-1 motif region (Shy et al, 1996) plus TCGA overhangs. Briefly, 3 µg of nuclear extract were incubated at 25°C for 30 min in binding buffer consisting of 20 mM Hepes, pH 7.9, 2.5 mM magnesium chloride, 1 mM DTT, 2 µg bovine serum albumin and 10% glycerol with 150 ng poly dIdC. Approximately 20 000 cpm of end-labelled DNA probe was added to the reaction and binding carried out at 25°C for an additional 30 min. The specific polyclonal antibodies were added to some of the samples after the addition of labelled DNA fragments and incubated for an additional 30 min at 25°C. Unlabelled fragments were added to some of the samples and pre-incubated for 30 min at 25°C prior to the addition of labelled fragments. Samples (20 µl) were subjected to electrophoresis on a 5% non-denaturing polyacrylamide gel (0.25× TBE) at 25°C and 20 mA for 1 h. Gels were dried and exposed to Hyperfilm (Amersham, Arlington Heights, IL, USA) at -70°C.

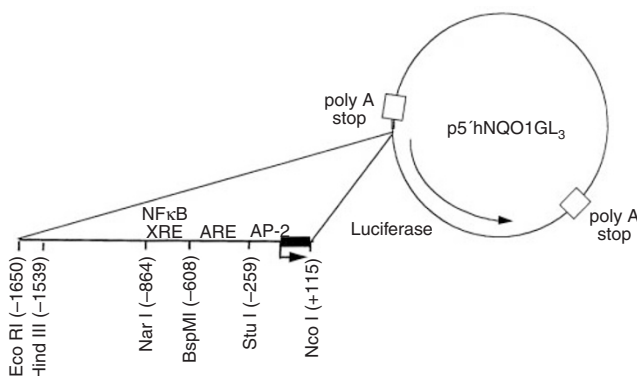


Figure 1 Human NQO1 promoter constructs. The 5' deletion constructs were prepared using convenient restriction enzyme sites to examine areas important for basal promoter activity. The 3' extent of all inserts was to the Nco I site at + 115. DNA fragments were inserted into the Sma I-Nco I site in pGL₃.

RESULTS

DT-diaphorase activity in cell lines derived from human lung tumours

The six representative cell lines listed in Table 1 have well characterized morphological and growth characteristics. The NSCLC cell lines were derived from large cell (H460) squamous (H520) and adenocarcinoma (A549) histological types and each had markedly elevated DT-diaphorase activity relative to the SCLC cells (H146, H82 and H187). There was also a good correlation between DT-diaphorase activity and hNQO1 mRNA content (Malkinson et al, 1992).

NSCLC exhibit greater hNQO1 promoter activity than SCLC

To identify and characterize potential regulatory regions within the hNQO1 promoter region, we constructed a series of hNQO1 promoter-LUC constructs with progressive deletions of the proximal and distal hNQO1 promoter starting from nucleotide -1539 (Figure 1). These constructs were transfected using lipofection into both NSCLC (see Figure 2A for H460 results) and SCLC (see Figure 2B using H146) cell lines and lysates were assayed for

luciferase expression. The results of multiple experiments, summarized in Figure 2C, demonstrate different patterns of promoter activity between NSCLC and SCLC cell lines.

The largest hNQO1 construct (1539/+115) exhibits robust luciferase expression in the H460, A549, and H520 cell lines (Figure 2C). Deletion from -1539 to -864 resulted in no significant change in luciferase activity and truncation to -608, removing the NFκB and XRE elements (see Figure 1), maintained this robust activity (Figure 2C). Further deletion to -259 decreased luciferase activity by 85–90%, to a range between fivefold (H460, H520) and 23-fold (H549) over the promoterless vector. Interestingly, this region between -608 and -259 contains the *cis* element, ARE, within which lies a perfect AP-1 flanked by two AP-1-like motifs. In contrast, in the H146, H82 and H187 cell lines, the hNQO1 promoter is much less transcriptionally active (Figure 2B, C). The data showed that deletion of the region containing the AP-1 site did not substantially affect the level of luciferase expression.

AP-1 is a component of ARE element-binding activity in lung cancer cells

To identify which proteins are bound to the ARE element, a gel shift mobility assay was employed to detect ARE-binding activity

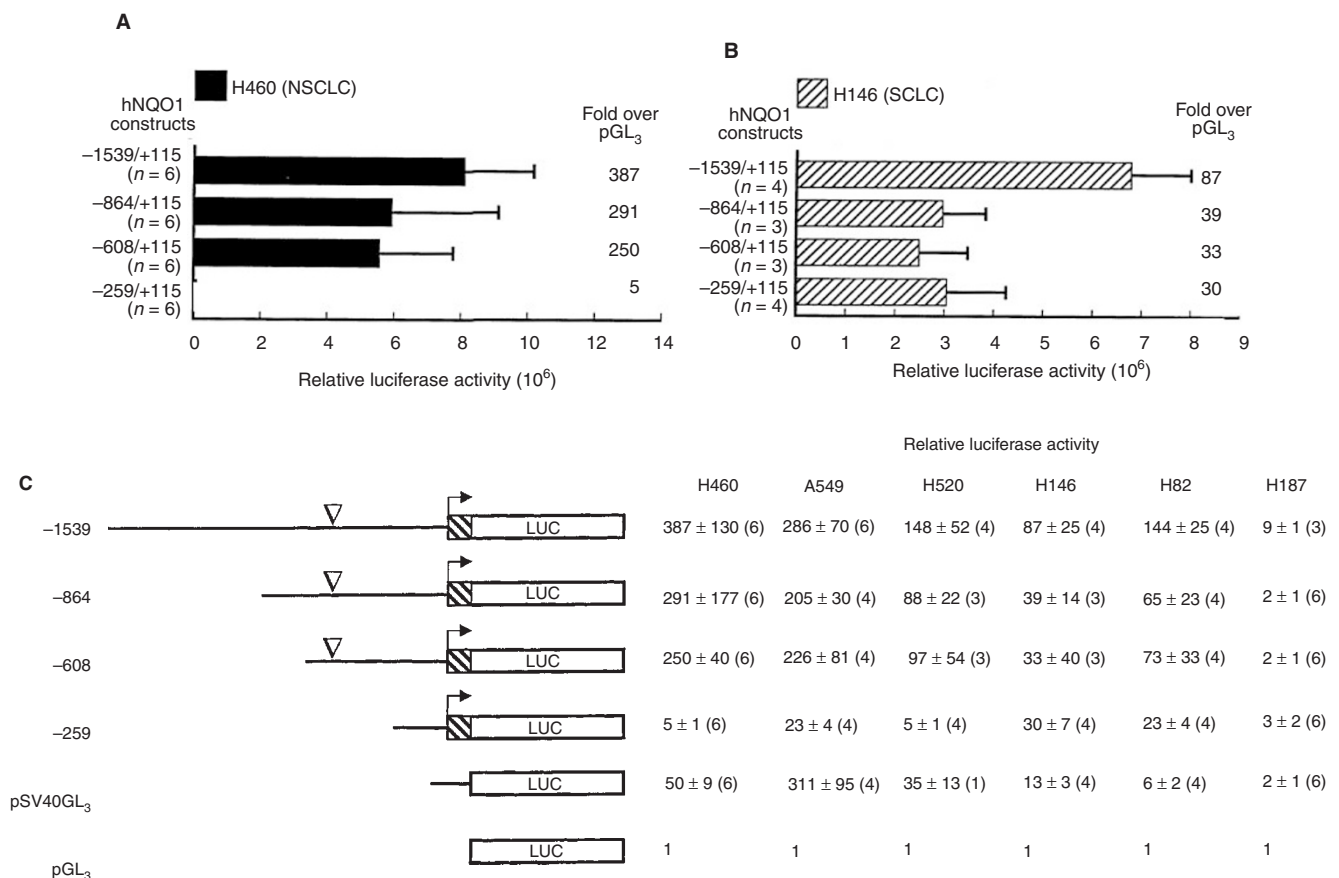


Figure 2 Basal hNQO1 promoter activity in three NSCLC and three SCLC cell lines. (A, B) Deletion constructs were prepared using convenient restriction sites. One µg of test plasmid and 1 µg of pCMVβgal were transfected into H460 cells (A) or H146 cells (B) using lipofection. After harvesting 16–18 h after transfection, lysates were assayed for luciferase and β-galactosidase activity. Data are expressed relative to 10⁶ light units of pSV40GL₃ transfected in parallel. N refers to the number of experiments performed with each construct. (C) The luciferase activity for each plasmid construct is determined relative to that of the promoterless vector, pGL₃. hNQO1 exon 1 is shown as a stippled box; values are shown plus or minus s.e.m.; → indicates the transcription start site; ▽ indicates the position of the ARE (antioxidant response element); (n) indicates the number of experiments performed with each construct

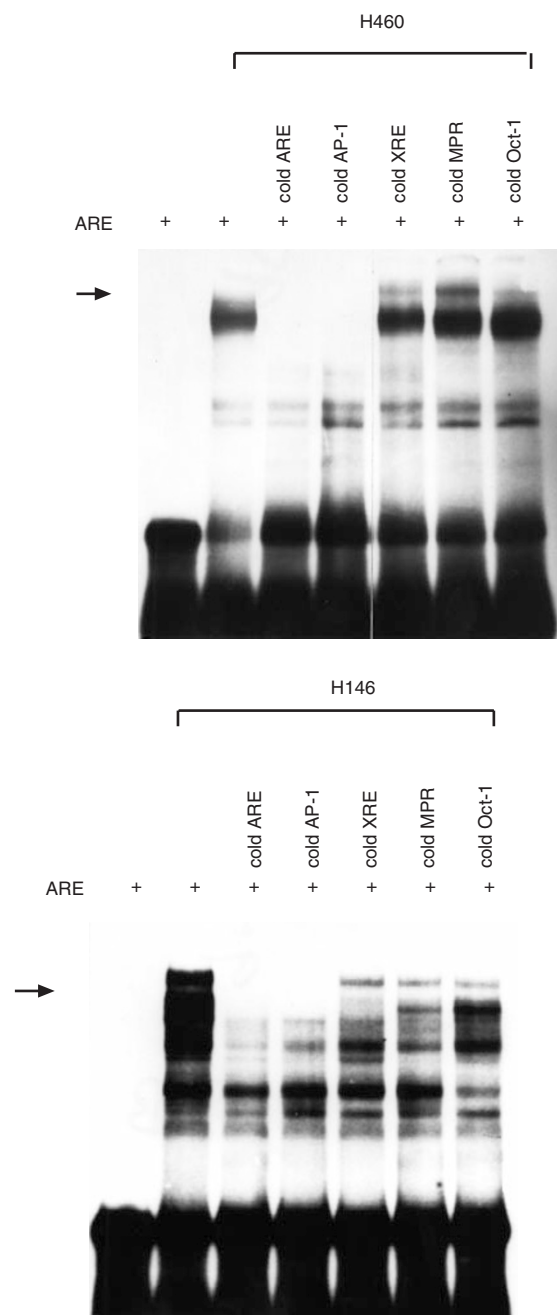


Figure 3 Gel mobility shift assay with cold competitor oligomers. 32 P-ARE oligomer was incubated with nuclear extracts from either H460 NSCLC (top panel) or H146 SCLC (bottom panel) in the presence of either 0 or 50-fold molar excess of cold ARE, AP-1, XRE, MPR or Oct-1. The incubations were then electrophoresed on nondenaturing gels. The arrow indicates the area of major binding activity

in nuclear extracts prepared from both NSCLC and SCLC with a 32 P-labelled synthetic oligonucleotide spanning -477 to -438 of the hNQO1 promoter. In nuclear extracts prepared from two representative cell lines, H460 and H146, we detected specific ARE-binding activity. This binding activity was abolished by the presence of a 50-fold excess of either unlabelled ARE or

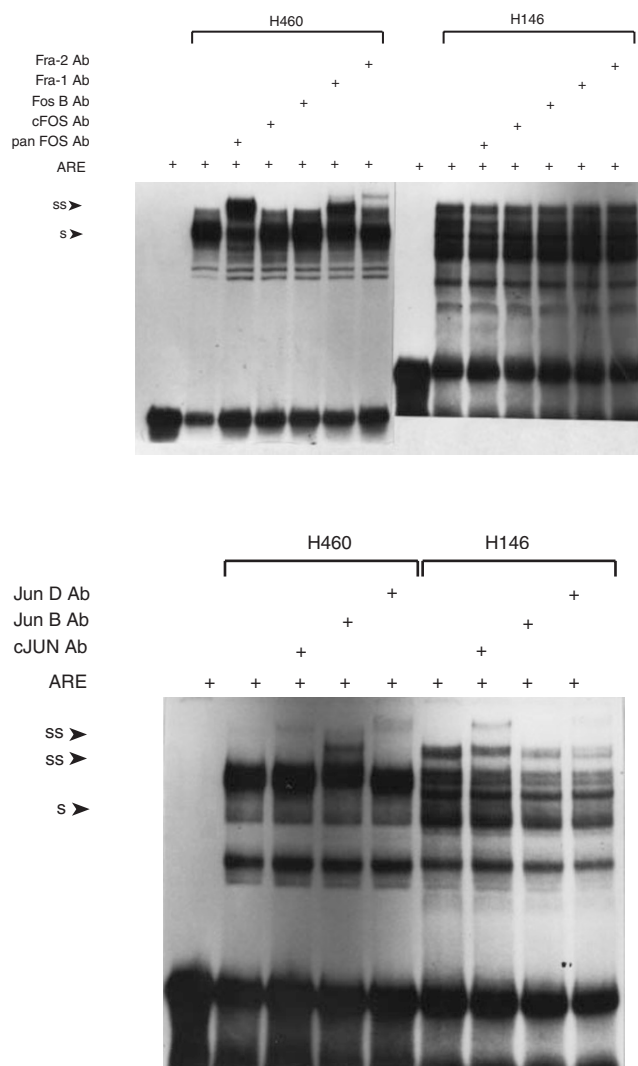


Figure 4 Gel mobility shift assay with various specific AP-1 antibodies. 32 P-ARE oligomer was incubated with nuclear extracts from either H460 NSCLC (left panel) or H146 SCLC (right panel) followed by the addition of specific antibodies as indicated. The positions of the DNA-protein shifted complexes (s) and the supershifted complexes (ss) are indicated by the arrows

unlabelled AP-1 consensus oligomer, suggesting that the same binding proteins are likely to interact with both ARE and AP-1 elements. In contrast, the unlabelled oligomers XRE, MPR, and Oct-1 were unable to compete any of the specific complexes (Figure 3). Nuclear extracts prepared from the remaining four cell lines gave similar results (data not shown).

Differential binding of AP-1 proteins to the hNQO1 ARE fragment

To characterize further the protein complex binding to the AP-1-binding site within the ARE region of the hNQO1 gene, nuclear extracts from NSCLC or SCLC were incubated with antibodies against panFos, cFos, FosB, Fra1, Fra2 (Figure 4, top panel), and cJun, JunB, JunD (Figure 4, bottom panel) prior to detection of

DNA-protein interactions by gel mobility shift assay. The antibodies against panFos, Fra1, Fra2, cJun, JunB and JunD induced a supershift of the labelled DNA probe incubated with nuclear extracts from H460 (Figure 4), A549 and H520 (data not shown) lung cells, indicating that their respective antigens participate in the formation of the complex bound to this *cis* element. In H146 nuclear extracts (Figure 4; H82 and H187 data not shown), however, only antibodies against cJun and JunD induced a supershift with this same labelled DNA probe. Antibodies to cFos and FosB did not produce supershifts in H460 (Figure 4, left panel). Supershifts using pan Fos, cFos, FosB, Fra1, Fra2, and JunB were not observed using SCLC nuclear extracts (Figure 4). All gel shift experiments were performed multiple times (at least five) and produced similar results.

DISCUSSION

Although many studies of hNQO1 regulation have been reported, these have been predominantly in liver systems (Jaiswal et al, 1988; Li and Jaiswal, 1992). The molecular mechanisms underlying increased NQO1 gene expression and activity in human NSCLC are unknown. To examine NSCLC-specific expression of NQO1, we transiently transfected a panel of deletion constructs containing the hNQO1 promoter and the luciferase reporter gene into both NSCLC and SCLC human cell lines. Deletion analysis of the largest hNQO1 promoter region demonstrated that a 359 bp proximal region (−608 to −259), which contains the ARE, was critical in mediating basal expression in NSCLC. Deletion of the sites for NFκB and AP-2 and the XRE did not significantly affect hNQO1 promoter activity in either the NSCLC or SCLC cell lines.

Gel mobility shift experiments demonstrated the specific binding of nuclear proteins to an oligomer containing the human NQO1 ARE. Competition for binding to radiolabelled ARE was observed with an oligomer containing an AP-1 site, but not with oligomers containing the human NQO1 XRE, MPR or Oct-1 oligomers. Antibody supershift experiments using the broad-spectrum anti-Fos (antibody that reacts with all known Fos family members) strongly suggest the participation of Fos family proteins in the formation of the complexes that bind the ARE only in nuclear extracts from NSCLC. A more detailed supershift analysis using antibodies specific for each family member suggests that Fra1, Fra2 and JunB are the AP-1 members found in NSCLC but not in SCLC.

As early response genes, the Jun/Fos family is tightly regulated during cellular differentiation (Karin et al, 1997) and rapidly induced following growth factor stimulation (Coussens et al, 1994) or in proliferation processes (Angel and Karin, 1991). AP-1 activity has been associated with the regulation of many genes (Busslinger and Bergers, 1994) including the positive regulation of many phase II drug metabolizing genes *in vitro* (Diccianni et al, 1992; Friling et al, 1992; Prestera et al, 1993).

The patterns of expression of individual Fos family members overlap but are not identical (Cohen and Curran, 1988; Angel and Karin, 1991). Fra1 and Fra2 are both expressed at significant levels in cycling cells, in contrast to cFos and FosB (Kovary and Bravo, 1992). Fra1 has been identified in keratinocytes (Welter et al, 1995) and cardiac tissue (Milivojevic and Gardner, 1995). Fra1 is a unique member of the Fos gene family which is also under positive control by AP-1 activity. It is capable of binding different Jun proteins in a manner similar to that of other members

of the Fos family (Ryseck and Bravo, 1991). Unlike cFos, Fra1 lacks a transactivation domain (Suzuki et al, 1991; Wisdom and Verma, 1993) yet possesses oncogenic potential (Bergers et al, 1995). Even though Fra1 lacks a transactivation domain, it could, in theory, limit the Jun protein pool by competing for dimerization partners. Fra1 has recently been shown to be a negative regulator of antioxidant-mediated AP-1 activity in HeLa cells (Yoshioka et al, 1995) and hNQO1 promoter activity in HepG2 human liver cells (Venugopal and Jaiswal, 1996). However, the effects of Fra1 can also be dependent on cellular content. Expression studies with the human atrial natriuretic factor (ANF) gene revealed divergent regulation by Fra1 in rat cardiocytes (Milivojevic and Gardner, 1992). Interestingly, overexpressing Fra1 in atrial myocytes mimicked the suppressant effects of cFos, while in the ventricular myocyte, Fra1 behaved as a typical transcriptional activator of ANF promoter activity (Milivojevic and Gardner, 1995). As to JunB, the presence of JunB-binding activity in NSCLC cell lines is in agreement with its possible role in NQO1 expression since JunB is an efficient activator of promoters containing multimerized AP-1 sites (Chiu et al, 1989). Only the ARE in the human NQO1 gene contains a centrally-located perfect AP-1 site flanked by two imperfect AP-1 elements (Jaiswal et al, 1988). Additional studies to confirm AP-1's role in positive regulation of hNQO1 expression in lung cancer cells, will require co-transfection experiments in SCLC with vectors expressing the various absent AP-1 members. Given the potential for temporal differences in the activation of the individual AP-1 members and the stabilities of their protein products, such diversity of expression may represent novel mechanisms controlling the transcription of hNQO1 during lung cancer pathogenesis.

In summary, our results indicate that the ARE is critical to basal NQO1 expression in lung tumours (NSCLC) expressing high levels of DT-diaphorase. This initial investigation shows that Fra1, Fra2 and JunB binding activities are not detected in SCLC and thus suggests that SCLC lack the *trans*-activating factors of the AP-1 family that mediate differential NQO1 expression in lung tissue.

ACKNOWLEDGEMENTS

This work was supported by grant CA51210 from the National Institute of Health.

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