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HIGH LEVELS OF EXPRESSION OF THE NAD(P)H:QUINONE OXIDOREDUCTASE (NQO_1) GENE IN TUMOR CELLS COMPARED TO NORMAL CELLS OF THE SAME ORIGIN

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Abstract—NAD(P)H:quinone oxidoreductase (NQO_1) is a flavoprotein which catalyzes the two-electron reduction of quinones and azo-dyes and thus prevents the formation of free radicals and toxic oxygen metabolites that may be generated by the one-electron reductions catalyzed by cytochrome P450 reductase. Analysis of RNA indicated 20- to 50-fold higher levels of NQO_1 gene expression in the liver tumors and in the tissue surrounding the tumors of patients with hepatocarcinoma than in normal individuals. An approximately 50-fold higher level of NQO_1 mRNA was also observed in human hepatoblastoma (Hep-G2) cells than in normal liver. By deletion mutagenesis in the human NQO_1 gene promoter and subsequent transfection into hepatic and nonhepatic cell lines, a 1.42 kb DNA segment has been identified to contain cis-acting elements responsible for high levels of expression of the NQO_1 gene in tumor cells.

NAD(P)H:quinone oxidreductase (NQO_1)§ [1], previously known as DT diaphorase, is a flavoprotein (EC 1.6.99.2) that catalyzes reduction of different quinones, quinone imines and azo-dyes [2–6]. Several lines of evidence have indicated that NQO_1 may have a protective effect against carcinogenicity, mutagenicity and other toxicities caused by quinones and their metabolic precursors [7–12]. This protection would most likely result from the two-electron reduction catalyzed by NQO_1, which would compete with the formation of free radicals and toxic oxygen metabolites that may be generated as a result of a one-electron reduction catalyzed by enzymes such as NADPH–cytochrome P450 oxidoreductase (EC 1.6.2.4), NADPH–b_5 oxidoreductase (EC 1.6.2.2), and NADPH-ubiquinone oxidoreductase (EC 1.6.5.3). As a protective agent, NQO_1 has been shown to prevent the formation of highly mutagenic quinone metabolites [13], to detoxify benz[*a*]pyrene-3,6-quinone via a pathway that also involves glucuronide formation [14], and to reduce chromium (VI), thereby reverting its mutagenicity [15]. The capacity of many different chemicals of diverse structure to block carcinogenesis correlates with their capacity to induce NQO_1 [16]. Moreover, the covalent binding of phenolic metabolites to microsomal proteins can be decreased greatly by NQO_1 [17].

A possible role of NQO_1 in cellular protection has been inferred from studies on cancer cells. It is a well established fact that the activity of NQO_1 is higher in cancer cells than in normal cells. Increased levels of NQO_1 activity have been reported in rat, mouse and human hepatomas [18–20]. Very high levels of NQO_1 activity have also been observed in human colonic carcinomas [21] and in interstitial cell tumors of the rat testis [22]. Significant NQO_1 activity has also been measured in human breast carcinoma [23] and in human mammary tumor cells grown in culture [24]. Recently, Schlager and Powis [25] examined a large number of human tumors and showed elevated levels of NQO_1 in primary tumors from lung, liver, colon and breast compared to normal tissue. Increased NQO_1 activity is not only characteristic of established tumors, but has also been observed in persistent hepatocyte nodules suggesting a role of this enzyme in the early protection of the developing tumor [26, 27]. It has been shown that hypomethylation of DNA may be responsible for the increased expression of the NQO_1 gene in chemically induced persistent hepatocyte nodules [27]. A specific role for NQO_1 in cellular protection is also suggested by the demonstration of an increased cytotoxic effect exerted by menadione [8] as well as anthracycline anticancer drugs [28] upon treatment of the cells with the potent NQO_1 inhibitor dicumarol [3].

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§ The trivial names and abbreviations used are: NQO_1, the dioxin-inducible cytosolic form of NAD(P)H:quinone oxidoreductase (previously known as DT diaphorase, and also abbreviated by others as NMO1, NMOR, QR and QAO); Hep-G2, human hepatoblastoma cell line; Hepa-1, mouse hepatoma; MCF-7, human breast carcinoma; COS, monkey kidney COS cells; TCDD, 2,3,6,8-tetrachlorodibenzo-*p*-dioxin; CAT, chloramphenicol acetyl transferase; kb, kilobase pairs; and bp, base pairs.

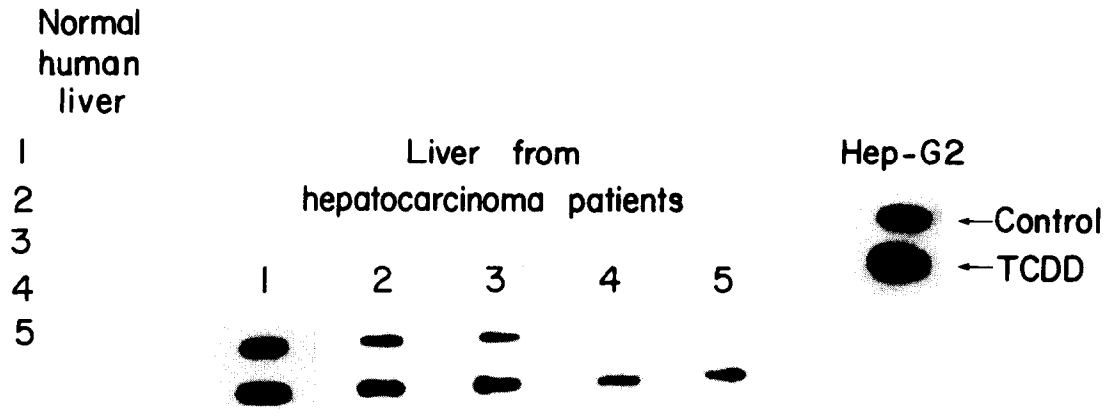

Fig. 1. Comparison of the levels of expression of the NAD(P)H:quinone oxidoreductase (NQO_1) gene in human hepatoblastoma (Hep-G2) cells and liver tumors and in tissue surrounding the tumors of hepatocarcinoma patients with the levels in livers of normal individuals. RNA was isolated from TCDD-treated and untreated Hep-G2 cells, livers of hepatocarcinoma patients, and normal individuals and analyzed by slot blotting (each slot containing 15 μg of respective RNA) and hybridization to human NQO_1 cDNA. In the case of patients with hepatocarcinomas, the RNA was isolated from the center of the tumors and tissue surrounding the tumors. In the lanes of liver from hepatocarcinoma patients, the upper and lower slots indicate the NQO_1 cDNA hybridizable RNA from the center of the tumor (top panel) and 6 cm from the center (lower panel), respectively. Hybridization of the same blot with a human β-actin probe after stripping off the counts from the filter did not reveal any significant differences between tissues from normal individuals and those of patients with hepatocarcinoma (data not shown) except for patients 4 and 5 whose blots showed approximately 4-fold less hybridization.

The mechanism responsible for the increased levels of NQO_1 activity in established tumors and tumor cell lines is unknown. Recently, it has been reported that the presence in an animal of a growing ascites hepatoma leads to a substantial (5-fold) increase in NQO_1 activity in the liver [29]. Moreover, the intraperitoneal injection of rats with hepatoma cytosol results in a rapid increase in liver cytosolic NQO_1 activity in a dose-dependent manner [29].

Recently, we cloned and sequenced the human NQO_1 gene and determined its exon–intron organization [30].* In the present report, we show that the NQO_1 gene is expressed at much higher levels in the livers of patients with hepatocarcinoma and human hepatoblastoma (Hep-G2) cells than in normal human liver. We have also attempted to elucidate the molecular mechanism responsible for higher expression of the NQO_1 gene in tumor cells. By deletion mutagenesis and transfection studies, we have been able to identify a 1.4 kb fragment of DNA in the upstream region of the NQO_1 gene responsible for high levels of expression of the NQO_1 gene in tumor cells.

EXPERIMENTAL PROCEDURES

Isolation and quantitation of RNA from livers of hepatocarcinoma patients and normal individuals. Total RNA was isolated from biopsies of human livers obtained from five normal individuals and five patients with hepatocarcinomas by the method of Chirgwin *et al.* [30]. In the case of patients with hepatocarcinomas, the RNA was isolated from the center of the tumor and 6 cm from the center (tissue surrounding the tumor). RNA was also isolated from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treated and untreated human Hep-G2 cells. Hep-G2 cells were grown and treated with TCDD as previously described [1]. RNA was analyzed by slot blotting carried out with a Schleicher & Schuell minifold II apparatus by the protocol recommended by the manufacturer. Each slot contained 15 μg of RNA. These blots were hybridized to two nick-translated probes: phNQO$_1$cDNA and pAC.H8-H, a human cardiac actin gene [31] used as a control. pAC.H8-H was a gift from Dr. Narayan Battula (National Cancer Institute, Bethesda, MD). The conditions of prehybridization, hybridization and washing of the slot blots were carried out as described [32]. The hybridized NQO_1 RNA bands after autoradiography (see Fig. 1) were analyzed by densitometry to estimate the fold increase in NQO_1 gene expression in tumors and tumor cells compared to normal cells.

Construction of NQO_1-CAT recombinant plasmids. The 1960 bp DNA segment was excised out by digesting the pUC18 plasmid carrying the 3 kb Pvu II fragment of the NQO_1 gene with Nco I. This 1960 bp Nco I fragment containing 1850 bp of the 5′-flanking region and 110 bp of the first exon (see Fig. 2) was treated first with mung bean nuclease followed by Klenow polymerase and cloned into the pBLCAT3 vector [33] after adding Bgl II linkers. This recombinant plasmid was called pNQO$_1$CAT-1.85. Two deletion mutants were made by digestion of pNQO$_1$CAT-1.85 plasmid with Hind III and Pst I restriction enzymes in separate experiments. The

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Hind III enzyme created a deletion of 307 bp from the 5′ end of the NQO_1 gene promoter and was named $pNQO_1CAT$-1.55. Similarly, the Pst I enzyme created a deletion of 1720 bp from the 5′ end of the NQO_1 promoter and was called $pNQO_1CAT$-0.13. The Hind III to Pst I fragment of DNA (region between -1850 and -130) of the NQO_1 gene promoter was attached to the basal thymidine kinase promoter (no enhancer) hooked to the CAT gene by cloning in the vector pBLCAT2 [33], a derivative of pBLCAT3, to test if this segment of DNA can confer its effect on a heterologous promoter. This recombinant plasmid was named pBLCAT2-NQO_1H-P.

Cell culture. The cells were grown as monolayer cultures at 37° in 90% air and 10% carbon dioxide in α-Minimal Essential Medium [for growing human hepatoblastoma (Hep-G2), human breast carcinoma (MCF-7) and mouse hepatoma (Hepa-1)] or Dulbecco's Modified Eagle's Medium [for growing monkey kidney COS cells] supplemented with 10% fetal bovine serum, penicillin (40 units/mL), streptomycin (40 μg/mL) and mycostatin (25 μg/mL). Cells were plated at a density of about 10^{5-6}/dish and grown to 70% confluency (about 6×10^6/dish) and used for transfection with recombinant plasmids.

Transfection and transient expression of NQO_1-CAT recombinant plasmids into hepatic and nonhepatic cells. The chimeric DNAs ($pNQO_1CAT$-1.85, -1.55, and -0.13) containing different lengths of NQO_1 gene promoter, as represented by numbers in kilobase pairs in each construct, attached to the bacterial chloramphenicol acetyl transferase (CAT) gene were transiently transfected into the hepatic (human Hep-G2 and mouse Hepa-1) and nonhepatic (human breast carcinoma MCF-7 and monkey kidney COS) cells by the calcium phosphate procedure [34]. The CAT gene expression was monitored by measuring the CAT enzyme activity [35]. The promoterless pBLCAT3 plasmid was used as the control. The RSV-βgal plasmid was co-transfected in each case to normalize the transfection efficiency by measuring the β-galactosidase activity [36].

RESULTS AND DISCUSSION

Comparison of the levels of expression of the NQO_1 gene in human hepatoblastoma (Hep-G2) cells and in livers of human hepatocarcinoma patients with the levels in livers of normal individuals. The analysis of RNA by slot-blot hybridization to human NQO_1 cDNA indicated 20- to 50-fold higher levels of NQO_1 mRNA content in the liver tumors and in the surrounding tissue of patients with hepatocarcinoma than in normal individuals (Fig. 1). Approximately 50-fold higher levels of NQO_1 mRNA were also observed in Hep-G2 cells than in normal human liver which was induced 3-fold by treatment with TCDD (100 nM for 3 days) (Fig. 1). Similar results were observed when we compared the amount of NQO_1 mRNA in mouse hepatoma (Hepa-1) cells with that of normal mouse liver; the nuclear run on experiments [37] using nuclei isolated from normal mouse liver and mouse hepatoma cells (Hepa-1) indicated that the increase in the expression of the NQO_1 in hepatoma cells was due to an increase in the transcription of the gene (data not shown). It should be noted that we compared the NQO_1 mRNA content of the hepatoma cells to that of the normal liver. This is because the liver is made up mostly of hepatocytes which will be expected to show low levels of NQO_1 gene expression. Our results as described above and those of others on NQO_1 activity measurements [18–25] clearly indicate that the NQO_1 gene is expressed at much higher levels in tumor and tumor cells than in normal cells and that these high levels of expression are due to an increase in the transcription of the NQO_1 gene. These findings raise the important issue of the mechanisms responsible for high levels of expression of the NQO_1 gene in tumor cells compared to normal

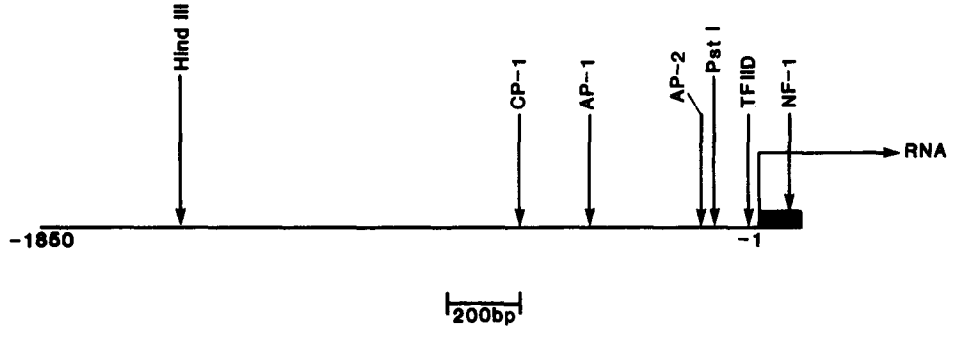

Fig. 2. NAD(P)H:quinone oxidoreductase (NQO_1) gene promoter showing potential binding sites for known transcription and trans-acting proteins. The nucleotide sequence of 1850 base pairs of the 5′ flanking region and the first exon (110 base pairs) of the NQO_1 gene was analyzed for the presence of potential binding sites of known transcription and trans-acting proteins. The potential binding sites for TFIID, CP-1, AP-1 and AP-2 proteins are shown. The TFIID is a transcription factor which recognizes the "TATA" box sequence and is known for its role in correct initiation of transcription. The AP-1 and AP-2 proteins are known to confer inducibility of the gene expression in response to 12-*O*-tetradecanoyal phorbol-13-acetate (TPA) [39], while the CP-1 protein causes an increase in the basal transcription of the gene [39]. The Hind III and Pst I restriction sites used to create 5′ deletions in the NQO_1 gene promoter are shown.

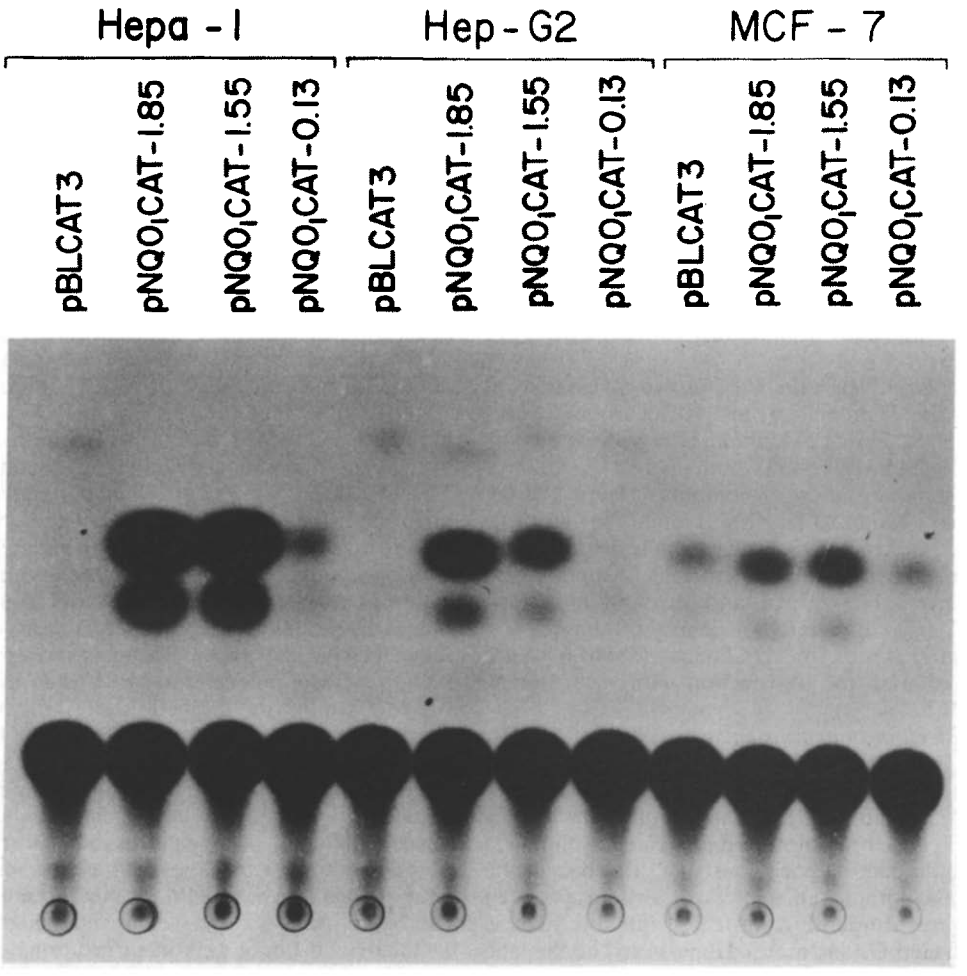

Fig. 3. Transfection and transient expression of NQO_1-CAT recombinant plasmids into various tumor cell types. The chimeric DNAs ($pNQO_1CAT$-1.85, -1.55, and -0.13) containing different lengths of NQO_1 gene promoter as represented by numbers in each construct attached to the bacterial chloramphenicol acetyl transferase (CAT) gene were transiently transfected into the hepatic (human Hep-G2 and mouse Hepa-1) and nonhepatic (human breast carcinoma MCF-7) cells by the calcium phosphate procedure [34]. In each case 10 μg of recombinant DNA was used for transfection. The CAT gene expression was monitored by measuring the CAT activity. The pBLCAT3 is a promoterless plasmid and was used as the control. RSV-βgal plasmid was used in each case to normalize the transfection efficiency.

Table 1. Transient expression of NQO_1-CAT plasmids

Name of construct	CAT activity* (pmol [^{14}C]chloramphenicol acetylated/min/mg protein)			
	Hep-G2	Hepa-1	MCF-7	COS-1
pBLCAT3	1.66	2.04	6.03	10.23
$pNQO_1CAT$-1.85	52.88	267.12	16.73	947.30
$pNQO_1CAT$-1.55	45.73	254.33	28.94	860.30
$pNQO_1CAT$-0.13	0.94	8.42	7.90	18.55
pBLCAT2	11.80	4.20	4.80	13.10
pBLCAT2-NQO_1H-P	139.80	170.80	21.90	134.80

* CAT = chloramphenicol acetyl transferase. Values represent the means of three independent transfections.

cells. In addition, our finding that normal liver tissue surrounding a liver tumor also has increased NQO_1 gene expression taken together with the report of Beyer *et al.* [29] on the induction of NQO_1 activity in rat liver by hepatoma cytosol make it important to consider the possibility that soluble factor(s) from tumor cells may carry the message for induction of NQO_1 gene expression in normal tissue surrounding the liver tumors and normal liver.

Sequence analysis of the 5′ flanking region of the NQO_1 gene. The nucleotide sequence analysis of 1960 base pairs of the DNA fragment from the NQO_1 gene containing 1860 base pairs of the 5′ flanking region and 110 base pairs of the first exon showed the presence of potential binding sites for several transcription and trans-acting proteins (Fig. 2). The "TATA" box, known for binding to transcription factor TFIID and its role in the correct initiation of transcription [38], was found located in the −37 to −32 bp region upstream from the start site of transcription of the NQO_1 gene. The analysis also showed the presence of potential binding sites for CP-1 (position −649), AP-1 (position −462), AP-2 (position −156) and NF-1 (position +82) proteins with respect to the cap site. The CCAAT box sequence to which CP-1 protein binds is found in many different eukaryotic gene promoters and has been shown to increase the rate of transcription

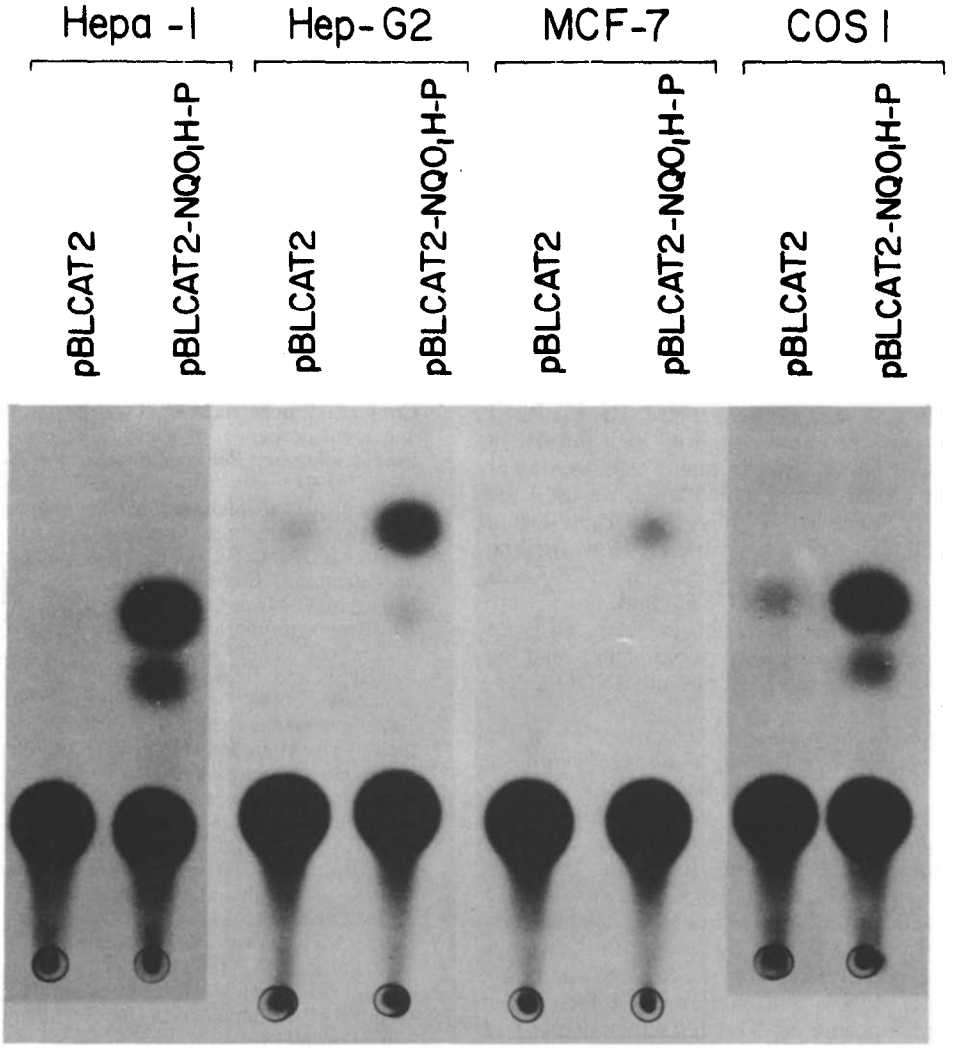

Fig. 4. Assignment of high levels of expression of the NAD(P)H:quinone oxidoreductase (NQO_1) gene in tumor cells to a 1.42 kb Hind III-Pst I fragment of DNA in the upstream region of the NQO_1 gene. The 1.42 kb segment of the NQO_1 gene promoter containing the cis-acting DNA elements responsible for high levels of expression of NQO_1 in tumor cells (as determined by comparing the CAT activity expressed from $pNQO_1CAT$-1.55 and $pNQO_1CAT$-0.13 plasmids in Fig. 3) was attached to the basal thymidine kinase promoter (no enhancer) hooked to the CAT gene by cloning in the vector pBLCAT2, a derivative of pBLCAT3, to test if this segment of DNA can confer its effect on a heterologous promoter. This NQO_1-tk-CAT recombinant plasmid was named pBLCAT2-NQO_1H-P. The pBLCAT2 was used as control plasmid. These plasmids (10 μg in each case) were transiently transfected into various cell types by the calcium phosphate procedure. The CAT gene expression was monitored by measuring the CAT activity. RSV-βgal plasmid was used in each case to normalize the efficiency of transfection.

of the gene in which they are present. The AP-1 protein has been shown to form heterodimer with c-*fos* gene product through leucine zipper motifs when cells are treated with TPA (12-*O*-tetradecanoyal phorbol-13-acetate) and to increase the transcription of the gene [39]. The AP-2 site confers TPA and cAMP inducibility [39]. The NF-1 protein recognizes the sequence "GCCAAT" and is antigenically unrelated to multisubunit CCAAT-binding CP-1 [39].

Transfection and transient expression of NQO_1-CAT recombinant plasmids into hepatic and nonhepatic cells. The promoterless plasmid pBLCAT3 expressed a very low level of CAT activity in both the hepatic cell lines (Hep-G2 and Hepa-1), as expected. When we used $pNQO_1CAT$-1.85 plasmid for transfection, the CAT activity was 32- and 131-fold higher compared to the promoterless plasmid pBLCAT3 in Hep-G2 and Hepa-1 cells, respectively (Fig. 3 and Table 1). The plasmid $pNQO_1CAT$-1.55 showed more or less similar increases in CAT activity as observed with plasmid $pNQO_1CAT$-1.85, indicating that the 307 bp in the 5′ end of the NQO_1 promoter is not needed for increased expression of NQO_1 gene in tumor cells. However, transfection with plasmid $pNQO_1CAT$-0.13 which contained only 130 bp of the 5′ proximal promoter segment produced no increased CAT activity over the promoterless plasmid. This indicates that cis-acting DNA elements present in the region between −1.55 and −0.13 (Hind III to Pst I fragment) are essential for the high levels of expression of NQO_1 gene in tumor cells. A similar pattern of results was obtained when we used the nonhepatic cell line MCF-7 (Fig. 3 and Table 1) and monkey kidney COS cells (Table 1). However, the amounts of CAT activities expressed in MCF-7 cells from plasmids $pNQO_1CAT$-1.85 and -1.55 were much less than their expression in other cell types (Table 1), but still were 3- to 5-fold higher than the CAT activity expressed from plasmid $pNQO_1CAT$-0.13.

Mediation of the high levels of expression of the NQO_1 gene in tumor cells by sequences within the 1.42 kb Hind III-Pst I fragment of DNA in the upstream region of the NQO_1 gene. The Hind III to Pst I fragment of DNA containing the cis-acting DNA elements responsible for high levels of expression of NQO_1 gene in tumor cells was attached to the basal thymidine kinase promoter (no enhancer) hooked to the CAT gene by cloning in the vector pBLCAT2, a derivative of pBLCAT3, to test if this segment of DNA can confer its effect on a heterologous promoter. This recombinant plasmid upon transfection into Hep-G2 and Hepa-1 cells showed 14- and 41-fold higher levels of CAT activity, respectively, compared to the control plasmid pBLCAT2 (Fig. 4 and Table 1), clearly indicating that the 1.42 kb Hind III-Pst I fragment indeed contains cis-acting DNA elements responsible for high levels of expression of the NQO_1 gene in tumor cells. Similar results were obtained when we used nonhepatic MCF-7 cells (human breast carcinoma) and monkey kidney COS cells (Fig. 4 and Table 1) for transfection.

The nucleotide sequence analysis of the 1.42 kb (Hind III-PstI) fragment of the NQO_1 gene promoter previously identified to contain cis-acting elements for high levels of expression in tumor cells showed the presence of consensus binding sites for CP-1, AP-1, and AP-2 proteins (Fig. 2). Whether proteins actually bind to the above-mentioned binding sites and affect the NQO_1 gene expression still remains to be determined. It is also possible that unknown binding sites for as yet unidentified regulatory proteins may be responsible for the high levels of expression of NQO_1 gene in tumor cells compared to normal cells of their origin. Further deletions, and linker scanning mutations are being created in the human NQO_1 gene promoter to identify the cis- and trans-acting elements responsible for higher expression of the NQO_1 gene in tumor cells than in normal cells.

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