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AP-2-Mediated Regulation of Human NAD(P)H:Quinone Oxidoreductase$_1$ (NQO_1) Gene Expression

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ABSTRACT. NAD(P)H:quinone oxidoreductase$_1$ (NQO_1) is a flavoprotein that catalyzes two-electron reduction and detoxification of quinones. We have shown previously that twenty-four base pairs of the human Antioxidant Response Element (hARE) mediate basal and xenobiotic-induced expression of the NQO_1 gene [Li and Jaiswal, *J Biol Chem* **267:** 15097–15104, 1992]. In the present report, we have characterized a second *cis*-element, AP-2, at nucleotide position −157 of the human NQO_1 gene promoter that regulates basal and cAMP-induced transcription of the NQO_1 gene. The NQO_1 gene AP-2-mediated expression of the chloramphenicol acetyl transferase (CAT) gene and the binding of nuclear proteins to the AP-2 element were observed in HeLa (AP-2 positive) cells but not in human hepatoblastoma Hep-G2 (AP-2 deficient) cells, indicating the involvement of transcription factor AP-2 in the regulation of NQO_1 gene expression. Affinity purification of nuclear protein that binds to the NQO_1 gene AP-2 DNA element and western analysis revealed that AP-2 indeed binds to the NQO_1 gene AP-2 element and regulates its expression in HeLa cells. The involvement of AP-2 in the regulation of NQO_1 gene expression was confirmed by the observation that cDNA-derived AP-2 protein in Hep-G2 cells increased in the NQO_1 gene AP-2 but not mutant AP-2 mediated expression of CAT gene in Hep-G2 cells. BIOCHEM PHARMACOL 51;6:771–778, 1996.

KEY WORDS. quinone oxidoreductase; AP-2; transcription; cAMP induction

NQO_1† (or DT diaphorase) catalyzes the two-electron reduction of quinones and protects the cells from the adverse effects of semiquinones that are generated due to one-electron reduction of quinones catalyzed by cytochrome P450 reductase [1, 2]. The semiquinones are known to enter in the redox cycling to produce oxygen free radicals that cause oxidative stress leading to neoplasia [3, 4]. The expression of the NQO_1 gene is increased in tumor tissues as compared with normal tissues of similar origin and is induced in response to a variety of xenobiotics including polycyclic aromatic hydrocarbons, planar aromatic compounds, antioxidants, oxidants, and tumor promoters [5–12]. Deletion mapping and transfection studies have shown that twenty-four base pairs of the DNA segment present in the promoter region of the human NQO_1 gene designated as hARE mediate high basal and induced transcription of the NQO_1 gene [10, 13]. In addition to the hARE, the nucleotide sequence analysis of the human NQO_1 gene promoter indicates the presence of a perfect AP-2 binding consensus sequence [8]. The AP-2 binding site was characterized previously in the SV40 genome and in the promoter region of the hMTII gene [14, 15]. AP-2 protein is known to bind to the AP-2 element and to regulate the target genes in response to cAMP, 12-O-tetradecanoylphorbol-13-acetate (TPA), and retinoic acid [14, 16].

In the present report, we investigated the role of the AP-2 binding site and transcription factor AP-2 in the regulation of NQO_1 gene expression. The deletion mutagenesis in the NQO_1 gene promoter and transfection in mammalian cells indicated that the AP-2 binding site is required for basal and cAMP-induced expression of the NQO_1 gene in human cervical carcinoma (HeLa) cells. The AP-2-mediated basal expression and the cAMP response was absent in AP-2-deficient human hepatoblastoma (Hep-G2) cells, indicating the possible involvement of transcription factor AP-2 in the regulation of expression and cAMP induction of the NQO_1 gene. The role of AP-2 in the AP-2-mediated regulation of NQO_1 gene expression was also supported by the observation that the AP-2 binding site from NQO_1 gene generated a slow-moving complex with nuclear proteins from HeLa cells and bacterially expressed cDNA-derived recombinant AP-2 protein but not with nuclear extract from AP-2-deficient Hep-G2 cells. The NQO_1 gene AP-2 binding site was used to affinity purify the interacting nuclear protein from HeLa cell nuclear extract to positively determine the identity of nuclear protein that binds to NQO_1 gene AP-2 DNA element. This nuclear protein ran

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† *Abbreviations:* NQO_1, NAD(P)H:quinone oxidoreductase$_1$, also known as quinone reductase (QR), quinone:(acceptor) oxidoreductase (QAO), and DT diaphorase (EC 1.6.99.2); hMTII, human metallothionein II; hARE, Human Antioxidant Response Element; AP-2 element, binding site for AP-2 *trans*-acting protein; and cAMP, cyclic AMP.

as 50 kDa (approximately) protein on SDS–PAGE and cross-reacted with antibody against human transcription factor AP-2. These results clearly established that AP-2 protein binds with the AP-2 element and regulates the expression and cAMP induction of the NQO_1 gene. Further experiments using cDNA-derived human AP-2 protein in Hep-G2 cells positively regulated the NQO_1 gene AP-2-mediated expression of CAT gene, thus confirming the earlier conclusion that AP-2 indeed regulates the expression of the human NQO_1 gene. The possible role of AP-2 protein in the regulation of NQO_1 gene expression during differentiation and development is discussed.

MATERIALS AND METHODS

Chemicals and Reagents

Bacterially (*Escherichia coli*) expressed recombinant AP-2 protein was obtained from Promega (Catalogue No. E3050), Madison, WI. This product has now been replaced by a new product that includes AP-2 as a single protein and not as a recombinant protein with a molecular weight much higher than that of the AP-2 protein alone. HeLa and Hep-G2 cells were obtained from the American Type Culture Collection (ATCC), Bethesda, MD. The modifiers of cAMP levels (forskolin and Ro 20-1724) and other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.

Construction of Plasmids

The construction of plasmid $pNQO_1CAT1.55$ has been described previously [8]. This plasmid contained 1.55 kb of 5′ flanking region and 110 bp of the first exon attached to the chloramphenicol acetyl transferase gene. For construction of plasmids $pNQO_1CAT0.176$ and $pNQO_1CAT0.147$, we first made a plasmid $pNQO_1CAT0.130$. The plasmid $pNQO_1CAT0.130$ contained 130 bp of the 5′ flanking region and 110 bp of the first exon attached to the CAT gene. This plasmid was created from the original plasmid $pNQO_1CAT1.85$ by digestion with *Pst* I. The *Pst* I enzyme generated a deletion of 1720 bp from the 5′ end of the NQO_1 gene promoter. The remaining plasmid was self-ligated to make $pNQO_1CAT0.130$. To construct the plasmid $pNQO_1CAT0.176$, we amplified the region between −0.176 and −0.130 of the human NQO_1 gene promoter with *Hind* III and *Pst* I ends by polymerase chain reaction. This fragment of the DNA was subcloned in the plasmid $pNQO_1CAT0.130$ at the *Hind* III-*Pst* I site to generate plasmid $pNQO_1CAT0.176$. Similarly, the two strands of the oligonucleotides were synthesized corresponding to the region between −0.147 and −0.130 in the human NQO_1 gene promoter with *Hind* III and *Pst* I ends, annealed, kinased, and subcloned in the plasmid $pNQO_1CAT0.130$ at the *Hind* III-*Pst* I site to make the recombinant plasmid $pNQO_1CAT0.147$.

Both strands of the NQO_1 gene containing the AP-2 binding site (region between −162 and −143) were synthesized, annealed, kinased, and ligated to *Bam* HI linkers and cloned at the *Bam* HI site of the pBLCAT2 [17] to generate $pNQO_1$-2X AP-2-tk-CAT. Purine to pyrimidine and vice versa changes were incorporated to make mutant AP-2. Similar to AP-2, mutant AP-2 was cloned at the *Bam* HI site of the pBLCAT2 to generate $pNQO_1$-2X mutant AP-2-tk-CAT. These plasmids were sequenced to determine the orientations of the AP-2 elements cloned in the pBLCAT2 vector. The plasmids containing two AP-2 or mutant AP-2 arranged as direct repeats in 5′ → 3′ orientation with the CAT gene were selected for transfection and further studies. The nucleotide sequences of oligonucleotides containing AP-2 and mutant AP-2 binding sites were as follows. The normal and mutated AP-2 binding sites are shown in larger size letters.

Nucleotide sequence of normal AP-2:

5′-TTCATC**CCCCAGGCT**CCCTC-3′

Nucleotide sequence of mutant AP-2:

5′-TTCATC**AAAACTTAG**CCCTC-3′

Transient Transfection and Expression of NQO_1-CAT Plasmids

Ten micrograms of the NQO_1-CAT plasmids were co-transfected with 5 μg of the pRSV-β-galactosidase plasmid in human cervical carcinoma [HeLa (AP-2 positive)] and human hepatoblastoma [Hep-G2 (AP-2 deficient)] cells. These cells were cultured in monolayers by procedures as described earlier [10]. The pBLCAT3 and pBLCAT2 vector plasmids were used as controls. The transfected cells were treated with either DMSO (control) or modifiers of cAMP levels [forskolin (10 μM) and Ro 20-1724 (100 μM)] for 4 hr at 37°. The cells were harvested/homogenized by sonication in 0.2 M Tris buffer (pH 7.4), and analyzed for CAT gene expression by measuring CAT activity [18]. The CAT activities are presented as the means of three experiments in picomoles of [^{14}C]-chloramphenicol acetylated per minute per unit of β-galactosidase activity.

Nuclear Extract Preparation and Band Shift Assays

The HeLa and Hep-G2 cells were grown in large volumes, and nuclear extracts were prepared by the procedure of Dignam *et al.* [19] as modified by Kadonaga [20]. Normal and mutated NQO_1 gene AP-2 binding sites were end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. Thirty thousand counts per minute of labeled AP-2 and mutant AP-2 were mixed with 4 μg of poly(dI · dC) · poly(dI · dC); 25 mM HEPES (K^+), pH 7.8; 12.5 mM $MgCl_2$; 1 mM dithiothreitol; 20% glycerol (v/v); 0.1% Nonidet P-40; and 0.1 M KCl and incubated at room temperature for 20 min. The incubation mixture was run on a 5% non-denaturing polyacrylamide gel, dried under vacuum, and autoradiographed. In several experiments, unlabeled AP-2 and mutant AP-2 were used as specific competitors.

Affinity Purification of AP-2 Protein

The HeLa cells were grown in suspension culture. The nuclear extracts from 40 mL of packed HeLa cells were prepared by the

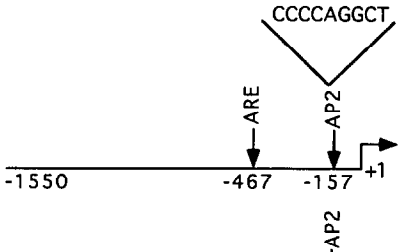
procedure of Dignam *et al.* [19] as modified by Kadonaga [20]. The final protein concentration was 7.3 μg/μL of binding buffer. The proteins that bound to the NQO_1 gene AP-2 binding site were affinity purified exactly by the method as described [20]. Two hundred and twenty micrograms of each strand of the normal NQO_1 gene AP-2 binding site (sequence of oligonucleotide as shwon above) were annealed, kinased, and self-ligated to obtain DNA fragments of 200–300 bp in length containing multiple AP-2 binding sites. This DNA was incubated with cyanogen bromide-activated Sepharose CL-2B to generate the AP-2 affinity matrix. The remaining reactive groups in cyanogen bromide-activated Sepharose CL-2B were blocked by its treatment with ethanolamine, and the affinity matrix was washed with 100 mL each of 0.01 M potassium phosphate buffer (pH 8.0), 1 M potassium phosphate buffer (pH 8.0), 1 M KCl, and water. The CL-2B-AP-2 affinity matrix was packed in a column and equilibrated with binding buffer [25 mM HEPES (K^+), pH 7.8; 12.5 mM $MgCl_2$; 1 mM dithiothreitol; 20% glycerol (v/v); 0.1% Nonidet P-40; 0.1 M KCl and 2.2 mg/mL of poly(dI · dC) · poly(dI · dC)]. HeLa cell nuclear extract, as prepared above, was passed through this affinity column for binding and retention of specific nuclear proteins that bind to the AP-2 binding site of the NQO_1 gene. The bound proteins were eluted with a step gradient of 0.2 to 0.6 M KCl in the binding buffer. All the fractions were analyzed for protein content, SDS–PAGE, and staining with Coomassie blue and band shift assays with the NQO_1 gene AP-2 binding site. Various fractions containing AP-2 binding activity as determined by band shift analysis were pooled and reloaded on a second NQO_1 gene AP-2 affinity column. The bound proteins were eluted as earlier. This procedure was repeated four times before purified AP-2 protein was obtained. To determine if the protein thus purified was AP-2, we ran an SDS–PAGE of denatured purified proteins from the fourth column, western blotted, and probed with antibodies directed against a peptide located at the C-terminus of human AP-2 (located after the DNA binding domain). AP-2 antibody was a gift from Dr. T. Williams (Yale University, New Haven, CT). The purified protein was also used in band shift assays to determine its specificity for NQO_1 gene AP-2 and its competition with hMTII gene AP-2.

Nuclear Run-On Transcription

Nuclear run-on assays were performed using nuclei isolated from forskolin (10 μM) and Ro 20-1724 (100 μM) treated HeLa cells by a procedure described previously [21]. Human β-actin was used as the control.

Co-transfection of Transcription Factor AP-2 Expression and NQO_1 Gene AP-2 Reporter Plasmids

The AP-2 expression plasmid SP(RSV)AP2 was obtained from Dr. T. Williams (Yale University). The construction of SP(RSV)AP2 plasmid has been described and was used successfully to express AP-2 protein in Hep-G2 cells to determine



		HeLa	Hep-G2
pNQO1 CAT1.55	Control	1239 ± 31	51 ± 06
	Forskolin	2302 ± 42	61 ± 03
	Ro20-1724	3597 ± 69	51 ± 09
pNQO1 CAT0.176	Control	51 ± 09	5 ± 01
	Forskolin	101 ± 11	5 ± 02
	Ro20-1724	153 ± 16	6 ± 02
pNQO1 CAT0.147	Control	28 ± 01	6 ± 01
	Forskolin	29 ± 06	6 ± 01
	Ro20-1724	33 ± 02	8 ± 02
pBLCAT3 (Vector alone)	Control	4 ± 01	5 ± 01
	Forskolin	5 ± 01	6 ± 02
	Ro20-1724	4 ± 01	3 ± 01

FIG. 1. Deletion mutagenesis in the NQO_1 gene promoter and transient transfection in mammalian cells. The recombinant plasmids pNQO$_1$CAT1.55, 0.176, and 0.147 containing different lengths of the NQO_1 gene promoter and the vector control pBLCAT3 were transiently transfected in human cervical carcinoma (HeLa) and human hepatoblastoma (Hep-G2) cells. The RSV-β-galactosidase plasmid was co-transfected in each case to normalize the transfection efficiency. The transfected cells were treated with DMSO (control), forskolin (10 μM), and Ro 20-1724 (100 μM) for 4 hr at 37°. The cells were harvested, homogenized by sonication, and analyzed for CAT gene expression by measuring CAT activity. The CAT activities are represented as means ± SD of three independent transfection experiments and expressed as picomoles of [^{14}C]-chloramphenicol acetylated per minute per unit of β-galactosidase activity.

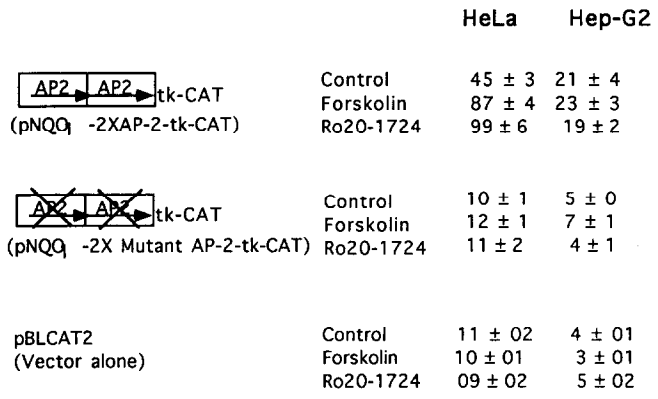


FIG. 2. NQO_1 gene AP-2-mediated expression and cAMP induction of the CAT gene. Two copies of human NQO_1 gene AP-2 were attached as direct repeats to the thymidine kinase promoter (basal promoter/no enhancer) hooked to the CAT gene to generate $pNQO_1$ 2XAP-2-tk-CAT and $pNQO_1$ 2X mutant AP-2-tk-CAT plasmids. pBLCAT2 plasmid was used as a control in the transfection experiments. Various plasmids were transfected in HeLa and Hep-G2 cells and treated with DMSO (control), forskolin and Ro 20-1724 for 4 hr at 37°. Forskolin and Ro 20-1724 are two agents that are known to increase the intracellular levels of cAMP. The RSV-β-galactosidase plasmid was co-transfected in each case to normalize the transfection efficiency. The transfected cells were harvested, homogenized by sonication, and analyzed for CAT gene expression by measuring CAT activity. The CAT activities are represented as means ± SD of three independent transfection experiments and expressed as picomoles of [^{14}C]chloramphenicol acetylated per minute per unit of β-galactosidase activity.

structure/function analysis of the DNA binding and transcription activation properties of AP-2 protein [22]. In the present study, 1, 2.5, and 5 μg of the expression vector alone or SP(RSV)AP2 plasmid containing cDNA encoding human AP-2 protein were co-transfected with 5 μg of reporter plasmids $pNQO_1$-2XAP-2-tk-CAT or $pNQO_1$-2X mutant AP-2-tk-CAT and 5 μg of control plasmid RSV-β-galactosidase in Hep-G2 cells and analyzed for CAT activity. The transfection and CAT assays were performed as described [13].

RESULTS AND DISCUSSION

NQO_1 is an interesting protein since it competes with cytochrome P450 reductase and protects the cells against redox cycling and oxidative stress due to exposure to quinones and their derivatives [1–4]. The NQO_1 gene is expressed ubiquitously in all human tissues [23]. However, the expression was greater in non-hepatic tissues than in hepatic tissues [23]. The expression of the NQO_1 gene is induced in response to all kinds of chemical carcinogens, drugs, chemoprotectors, peroxides, and ionizing radiations [11, 12]. Recently, a 24 bp of the DNA element was characterized in the promoter region of the NQO_1 gene designated as hARE, which is required for a major portion of the basal and induced expression of the NQO_1 gene [24]. hARE was found to contain two AP-1/AP-1-like elements and a "GC" box. hARE-like sequences have also been found in other detoxifying enzyme genes including glutathione S-transferase Ya and P subunit genes [25]. The products of proto-oncogenes Jun and other as yet unknown nuclear proteins bind to the hARE and may mediate signal transduction from xenobiotics and antioxidants to the hARE for increased expression of the NQO_1 and other detoxifying enzyme genes. A simultaneous activation of several gene products may be part of the cellular response to reduce the adverse effects of exposing compounds.

In the present report, we demonstrated that NQO_1 gene expression is also under the control of transcription factor AP-2. Nucleotide sequence analysis of 1850 bp of the NQO_1 gene promoter revealed the presence of a perfect consensus sequence for binding to the AP-2 protein at nucleotide position −157 (Fig. 1) [8]. To determine the role of the AP-2 binding site in the regulation of NQO_1 gene expression, we constructed NQO_1 gene promoter-CAT constructs in which different lengths of the NQO_1 gene promoter were attached to the CAT gene. These recombinant plasmids were transfected in HeLa and Hep-G2 cells, treated with either DMSO (control), forskolin or Ro 20-1724, and analyzed for CAT activity. Forskolin and Ro 20-1724 are two agents that cause accumulation of cAMP, thus increasing the intracellular levels of cAMP [14]. Therefore, forskolin- and Ro 20-1724-induced alterations in the gene expression will be referred to as cAMP-induced alterations in the gene expression. Various results on NQO_1-CAT transfections are shown in Figs. 1 and 2. The recombinant plasmid $pNQO_1$CAT1.55 containing 1.55 kb of the NQO_1 gene promoter directed high levels of CAT gene expression that was inducible by cAMP (forskolin and Ro 20-1724 treatment) (Fig. 1). Deletion of 1374 bp of the DNA from the 5′ end of the NQO_1 gene promoter created plasmid $pNQO_1$CAT0.176, that upon transfection in HeLa cells showed a low level of CAT gene expression that was induced

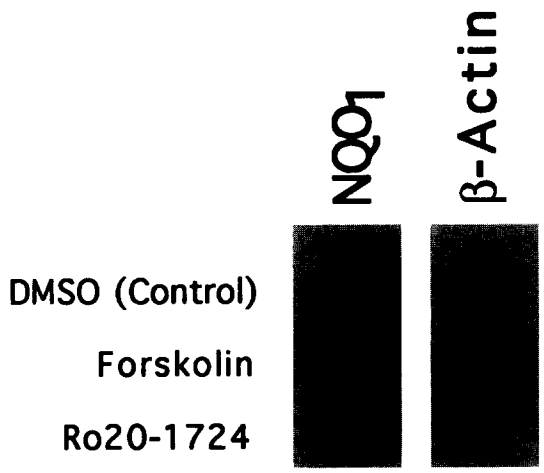


FIG. 3. Nuclear run-on assay. HeLa cells were treated with DMSO, forskolin (10 μM), and Ro 20-1724 (100 μM) for 4 hr at 37°, and the rate of transcription of the NQO_1 gene was determined as described in Materials and Methods. The intact nuclei were isolated, and nuclear RNA was prepared. Fifteen micrograms of the nuclear RNA was slot-blotted and hybridized to the NQO_1 and β-actin cDNA probes. The blot was washed and autoragiographed.

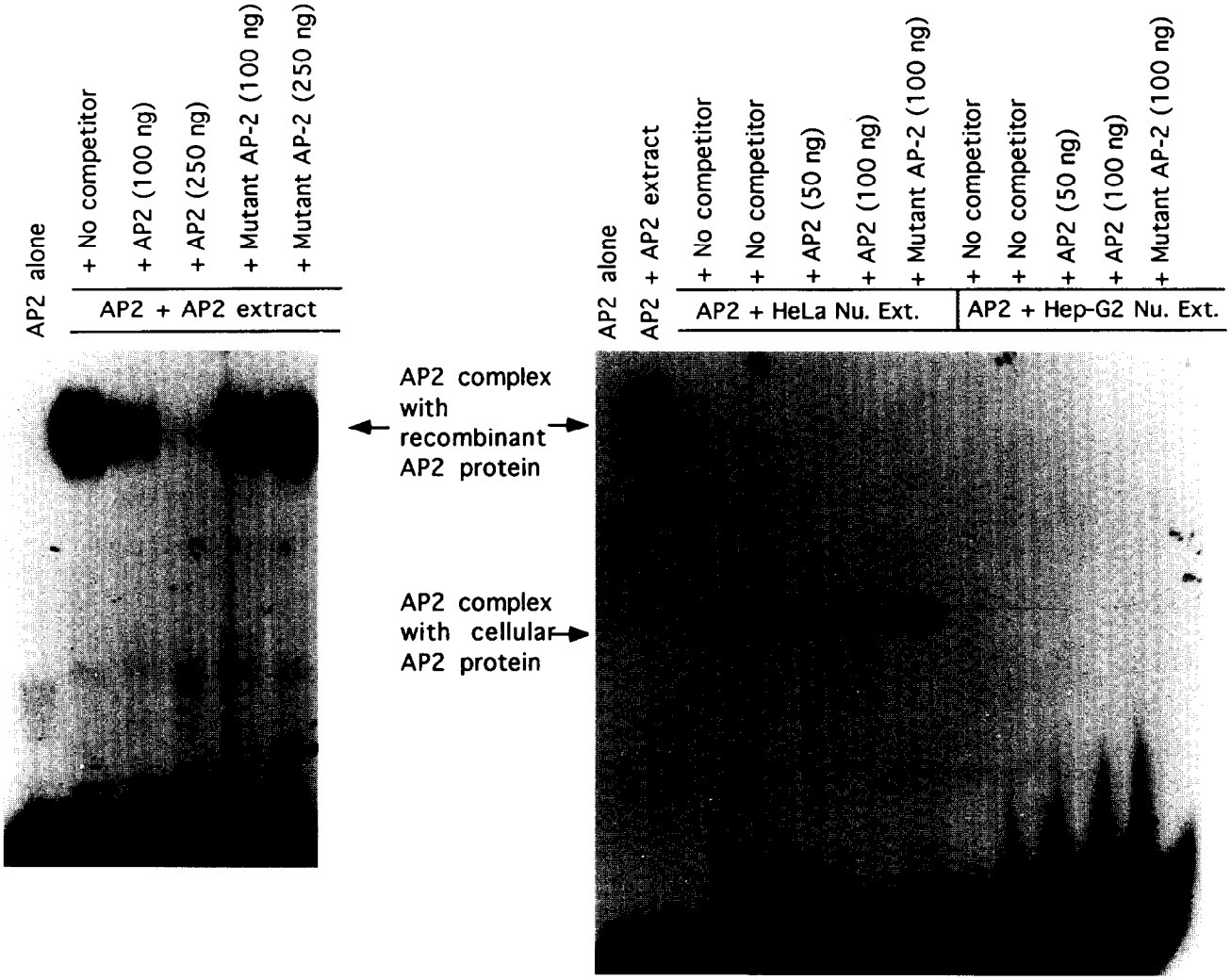


FIG. 4. Band shift assays with the NQO_1 gene AP-2 binding site. Twenty base pairs of the NQO_1 gene promoter (region between −162 and −143) containing AP-2 binding site was end-labeled with [γ-^{32}P]ATP. Thirty thousand counts per minute of the AP-2 oligonucleotide was incubated with 15 μg of HeLa or Hep-G2 nuclear extract proteins at room temperature for 20 min by a procedure as described under Materials and Methods. The incubation mixture was run on a 5% non-denaturing polyacrylamide gel, dried under vacuum, and autoradiographed. Unlabeled AP-2 and mutant AP-2 oligonucleotide were used as competitors.

by cAMP (forskolin and Ro 20-1724) (Fig. 1). The loss in the high basal expression was expected upon deletion of 1374 bp because it contained hARE, which is known to mediate a major portion of the basal expression of NQO_1 gene in tumor cells [10]. The cAMP-induced expression of the NQO_1 gene remained unaffected by this deletion. In similar experiments, transfection of HeLa cells with $pNQO_1CAT0.147$ containing 147 bp of the NQO_1 gene promoter resulted in loss of basal expression and cAMP induction (Fig. 1). Therefore, the DNA fragment between −176 and −147 containing the AP-2 binding element was required for a part of the basal expression and induced expression of the NQO_1 gene in response to cAMP. Both the strands of the 20 bp of the oligonucleotide containing AP-2 binding site and of the human NQO_1 gene promoter (region between nucleotides −0.162 and −0.153) were synthesized, annealed, and cloned in pBLCAT2 to generate plasmid $pNQO_1$-2XAP-2-tk-CAT. In this plasmid, two copies of the NQO_1 gene AP-2 binding sites were attached to the thymidine kinase promoter hooked to the CAT gene (Fig. 2). This plasmid upon transfection in HeLa cells produced CAT activity that was inducible by modifiers of cAMP levels (forskolin and Ro 20-1724). The mutations in the AP-2 binding site resulted in a loss of induction in response to cAMP (Fig. 2). It may be noteworthy that NQO_1 gene AP-2 binding site-mediated cAMP-induced expression of the CAT gene was not observed in Hep-G2 cells which lack the transcription factor AP-2 (Figs. 1 and 2) [14]. The comparison of results obtained with various NQO_1-CAT constructs in HeLa and Hep-G2 cells and the knowledge that Hep-G2 cells lack the transcription factor AP-2, and thus lack stimulation by cAMP, strongly suggested the involvement of AP-2 protein in AP-2 binding site-mediated expression and induction of the NQO_1 gene expression. These experiments also raised the question of induction of endogenous NQO_1 gene expression in response to

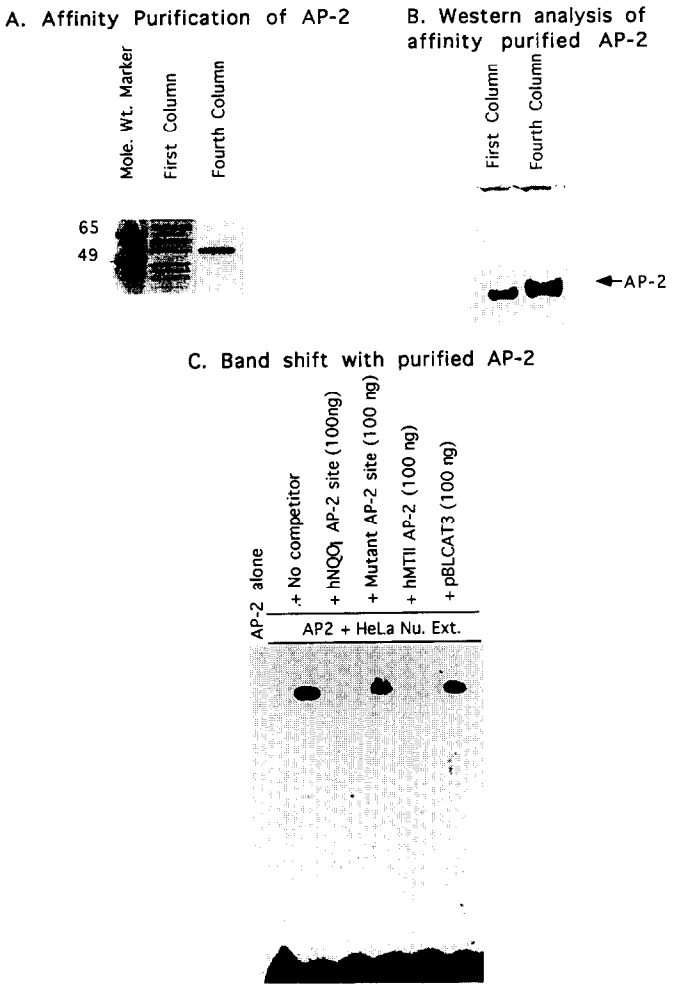


FIG. 5. Affinity purification and identification of nuclear protein that binds to the NQO_1 gene AP-2 binding site. The human NQO_1 gene AP-2 NQO_1 site-Sepharose CL2B affinity columns were used to purify the proteins that bind to the AP-2 element in the NQO_1 gene. Purified protein was obtained after the fourth passing of the HeLa cell nuclear extract through the affinity column. The purified protein that binds to the NQO_1 gene AP-2 was analyzed by (A) SDS–PAGE and Coomassie blue staining, (B) western blotting of the SDS–PAGE, and (C) band shift and competition assays. The protein eluted from the first (5 μg) and fourth (0.5 μg) columns was denatured by trichloroacetic acid precipitation and SDS treatment and run on the 10% SDS–PAGE. The first gel was stained with Coomassie blue. The second gel was western blotted and probed with antibodies against human transcription factor AP-2. For band shift assays, 0.2 mg of the purified protein was incubated with ^{32}P-labeled AP-2 oligonucleotides in the absence and presence of unlabeled competitors and separated on a 5% non-denaturing gel, dried under vacuum, and autoradiographed.

forskolin and Ro 20-1724. The nuclear run-on experiments, as shown in Fig. 3, clearly indicated induced transcription of the NQO_1 gene in response to treatment of HeLa cells with forskolin.

Further experiments using band shift assays and affinity purification of proteins clearly established the involvement of AP-2 protein in the NQO_1 gene AP-2 element-mediated expression of human NQO_1 gene (Figs. 4 and 5). A bacterially expressed and purified recombinant AP-2 protein (Promega) bound with high affinity to the NQO_1 gene AP-2 binding site (Fig. 4). This binding was competed specifically by the unlabeled AP-2 binding site but not by the mutated AP-2 binding site (Fig. 4). This clearly indicated that AP-2 transcription factor indeed recognized and bound to the NQO_1 gene AP-2 binding site. In addition, AP-2 binding activity was also observed in band shift assays with NQO_1 gene AP-2 binding site and HeLa cell nuclear extract (Fig. 4). The NQO_1 gene AP-2 nuclear complex observed with HeLa cell nuclear extract was much faster moving than that with bacterially expressed AP-2 protein (Fig. 4). This was expected because bacterially expressed AP-2 protein is a recombinant protein that is much larger in size than normal AP-2 protein in HeLa cells. The NQO_1 gene AP-2 binding complex in HeLa cells was com-

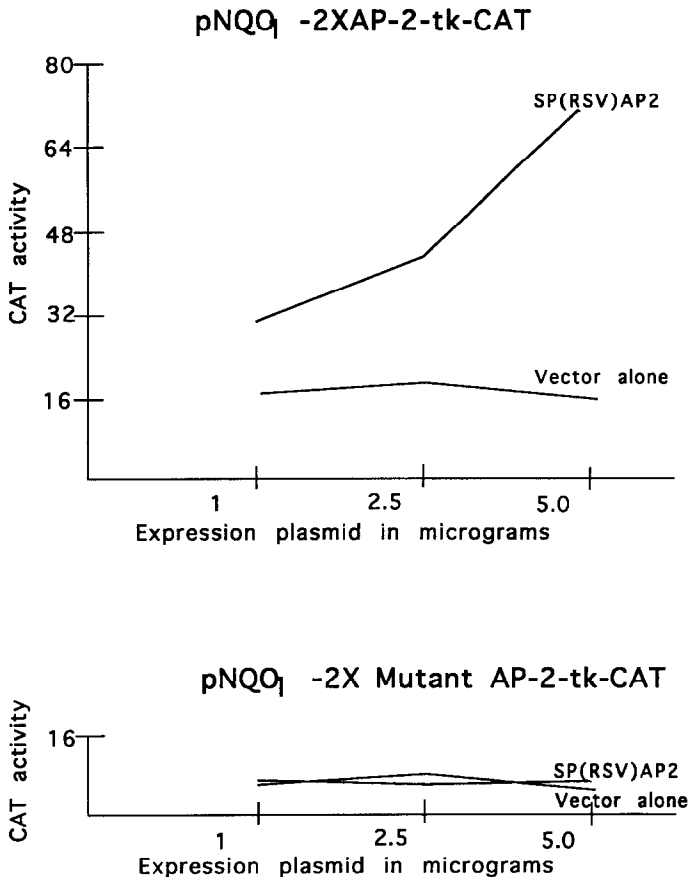


FIG. 6. Effect of cDNA-derived human AP-2 protein on the expression of NQO_1 gene AP-2-mediated CAT gene expression. The expression vector alone or plasmid SP(RSV)AP2 expressing human AP-2 protein was co-transfected with reporter plasmid pNQO$_1$-2XAP-2-tk-CAT or pNQO$_1$-2X mutant AP-2-tk-CAT and transfection control plasmid RSV-β-galactosidase. After 48 hr of transfection, the cells were scraped, homogenized, and analyzed for β-galactosidase and CAT activities. The CAT activities are represented as means ± SD of three independent transfection experiments. CAT activity is expressed as picomoles of [^{14}C]chloramphenicol acetylated per minute per unit of β-galactosidase activity.

peted specifically by unlabeled AP-2 oligonucleotide but not by mutant AP-2 oligonucleotide. As compared with HeLa cells, no binding activity was detected in Hep-G2 cells, which are deficient in AP-2 protein (Fig. 4). Therefore, various band shift experiments, as shown in Fig. 4, clearly established that AP-2 protein from HeLa cells binds to the AP-2 binding site and mediates NQO_1 gene expression and cAMP induction. The binding of AP-2 transcription factor to the NQO_1 gene AP-2 element was further confirmed by affinity purification of HeLa cell nuclear proteins that bind to the NQO_1 gene AP-2 binding site (Fig. 5). These nuclear proteins were purified by passing the HeLa cell nuclear extract on four NQO_1 gene AP-2 affinity columns in a sequence. The fourth column yielded purified protein that bound to the NQO_1 gene AP-2 binding site with high affinity. SDS–PAGE analysis of the purified protein revealed the size of the protein to be approximately 50 kDa (Fig. 5). The purified protein cross-reacted with antibodies against human AP-2 protein (Fig. 5). The binding of the purified protein with NQO_1 gene AP-2 element was competed by unlabeled AP-2 from NQO_1 (self) and hMTII gene but not by mutated AP-2 (Fig. 5). These results confirmed the identity of the purified protein as AP-2.

The role of AP-2 in the regulation of NQO_1 gene expression was also evident by the observation that cDNA-derived transcription factor AP-2 in Hep-G2 cells positively regulated the NQO_1 gene AP-2 but not mutant AP-2-mediated expression of CAT gene (Fig. 6). The increase in the AP-2-mediated expression was dependent on the concentration of plasmid SP(RSV)AP2 expressing cDNA encoded human AP-2 protein (Fig. 6). The above results combined with earlier observations confirmed that transcription factor AP-2 indeed regulates the expression of the NQO_1 gene.

AP-2 is known to bind DNA as a homodimer [26]. The expression of AP-2 is reported to be regulated during differentiation of a teratocarcinoma cell line, which indicated that AP-2 may be involved in the control of developmentally regulated gene expression [27]. In mouse embryos, a novel pattern of the expression of AP-2 was reported [27]. A majority of the expression of AP-2 was in neural crest cells and their major derivatives, including cranial and spinal sensory ganglia and facial mesenchyme. In addition, AP-2 was also found expressed in ectoderm and a longitudinal column of the spinal cord and hindbrain that is contacted by neural crest-derived sensory ganglia. AP-2 also was expressed in limb bud mesenchyme and in meso-metanephric regions. The expression pattern of the AP-2 has suggested a role for AP-2 in regulating the transcription of genes involved in the morphogenesis of the peripheral nervous system, face, limbs, skin, and nephric tissues [27]. In the present report, we have shown conclusively that NQO_1 gene expression is regulated by transcription factor AP-2. This raises important questions regarding NQO_1 gene expression and regulation by AP-2 in neural crest cells and their role during differentiation and development.

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