Nucleic Acids Research, 1996, Vol. 24, No. 21 **4185–4191**

Characterization of the rat Class 3 aldehyde dehydrogenase gene promoter

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Received July 17, 1996 Revised and Accepted September 16, 1996

DDBJ/EMBL/GenBank accession no. L11282

ABSTRACT

The Class 3 aldehyde dehydrogenase gene (*ALDH-3*) is differentially expressed. Expression is either constitutive or xenobiotic inducible via an aromatic hydrocarbon (Ah) receptor-mediated pathway, depending upon the tissue. A series of studies were performed to examine the regulation of rat *ALDH-3* basal expression. DNase I footprint analysis identified four DNA regions within the proximal 1 kb of the 5′ flanking region of rat *ALDH-3* which interact with regulatory proteins. Reporter gene and gel mobility shift assays indicate that Sp1-like proteins interact with two proximal DNase I footprinted sites to confer strong promoter activity. Two distal DNase I footprinted sites are found within a region that inhibits rat *ALDH-3* promoter activity. This negative region is bound by NF1-like proteins and/or unique proteins. This 1 kb 5′ flanking region of rat *ALDH-3* may act constitutively in many cell types. In contrast with other Ah receptor regulated genes, no DNA elements or transcription factors acting within this region appear to be involved in regulating xenobiotic-inducible expression of rat *ALDH-3*.

INTRODUCTION

The mammalian aldehyde dehydrogenases (ALDH; Aldehyde NAD Oxidoreductase E.C.1.2.1.3) are a family of NAD(P)$^+$-dependent enzymes that catalyze the irreversible oxidation of a wide range of aldehydes to their corresponding carboxylic acids (1,2). Class 3 ALDHs include both a constitutively-expressed microsomal form and a xenobiotic-inducible cytosolic isoenzyme encoded by separate genes (1,2). The gene encoding the cytosolic Class 3 ALDH (*ALDH-3*) is also constitutively expressed in a number of normal tissues including GI tract and urinary bladder, and at the highest known level in the cornea of eye, but is not expressed in normal liver (1–4). However, this gene is activated in liver by a variety of xenobiotics including polycyclic aromatic hydrocarbons (PAH) such as 3-methylcholanthrene (3MC), and dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1,5–7). *ALDH-3* is also activated during neoplasia in a number of tissues and tumor-derived cell lines express variable levels of Class 3 ALDH (1,8,9). All data to date is consistent with *ALDH-3* expression being regulated at the level of transcription of a single 1.7 kb transcript in all tissues examined (10,11).

ALDH-3 has been cloned and characterized from both rat and human (12–13). Rat *ALDH-3* spans ~9 kb and contains 11 exons and 10 introns, but the first exon is non-coding (12). Reporter gene analysis of deletion mutations carrying various amounts of the *ALDH-3* 5′ flanking region reveals that ~5.5 kb of 5′ flanking region of the rat *ALDH-3* gene can be divided into a strong promoter region and several unique regions upstream of this promoter. The promoter region includes ~400 bp immediately proximal to the transcription start site. Approximately 600 bp upstream of the promoter is a region that inhibits promoter activity (14).

A single xenobiotic response element (XRE) near –3.0 kb appears to be responsible for the xenobiotic inducibility of rat *ALDH-3* (14). The *ALDH-3* gene appears to be a member of the 'Aromatic hydrocarbon (Ah) gene battery' (1,2). The Ah gene battery consists of at least six genes encoding phase I and II drug metabolizing enzymes. Of the Ah battery genes examined to date, all possess multiple *cis*-acting elements within the proximal 1 kb of their 5′ flanking regions. These may function to regulate xenobiotic induction via XREs and/or electrophile response elements (EpRE). The induction of *ALDH-3* expression by PAHs and dioxins requires the functional Ah receptor and Ah receptor nuclear translocator (Arnt) (15,16). However, the time course and dose-dependency of *ALDH-3* induction by TCDD are different from that of other Ah battery genes such as cytochrome P450 1A1 gene (*CYP1A1*) (7,12,17). Moreover, the tissue distribution of the Ah receptor does not correlate with the inducibility of *ALDH-3* expression by TCDD in all tissues (18). Therefore, the regulation of *ALDH-3* expression is likely to utilize both Ah-receptor-mediated and Ah-receptor-independent pathways.

Since *ALDH3* is considered a member of the Ah gene battery but differs in its response to xenobiotic inductions from other members of the battery, we have carried out a detailed analysis of the rat *ALDH3* promoter region.

MATERIALS AND METHODS

Materials

HTC and MCA-RH7777 rat hepatoma cells were obtained from the American Type Culture Collection. Cell culture reagents, 3MC, Geneticin, poly(dI–dC) and acetyl CoA were purchased

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from Sigma. Protein assay reagents were from BioRad. T_4 polynucleotide kinase, various restriction enzymes and pCAT-basic vector were from Promega. [^{32}P]dCTP and [^{14}C]chloramphenicol were from Amersham. The double-stranded consensus sequence for AP1, mutant Sp1 sequence and antibodies against Sp1 and NF1 were purchased from Santa Cruz Biotechnology, Inc. PCR reagents were from Perkin Elmer Cetus. [^{32}P]ATP was from ICN. Quick-Sep Microcolumns were from ISOLABS. Gene Clean II was from BioExpress. TA cloning system was from Invitrogen. Qiagen-tip 500 columns were from Qiagen. Sprague–Dawley rats were from Sasco/Charles River Labs. TLC-plastic sheets silica gel 60 was from EM Separations. IBI Pustell sequence analysis software was employed in analyzing transcription factor binding sequences.

Cell culture

Rat hepatoma cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C.

Treatment of rats with 3-methylcholanthrene

3-Methylcholanthrene was suspended in corn oil at 20 mg/ml and administered intraperitoneally (three times over 24 h) to male Sprague–Dawley rats at 80 mg/kg total dose. Control rats were injected three times with corn oil. Rats were sacrificed by CO_2 asphyxiation 96 h post-injection and rat livers were removed, diced, frozen in liquid nitrogen, and stored at –75°C until needed for nuclear extract preparation.

Preparation of nuclear extracts

Nuclear extracts from Hela cells, 3MC-treated and control rat livers were prepared as described (19). Nuclear extracts from rat hepatoma cells (HTC and MCA-RH7777) were prepared as described (20). All nuclear extracts were aliquoted and frozen at –75°C until use. Protein concentration was determined by the BioRad Protein Assay.

Preparation of ^{32}P-labeled ALDH-3 basal promoter region PCR fragments and DNase I footprinting

Fragments of the proximal 1 kb of the *ALDH-3* 5′ flanking region were prepared by PCR (10; DDBJ/EMBL/GenBank accession no. L11282). The entire 1 kb region was spanned by six partially overlapping PCR products. The primers for the four fragments of interest in this study are: F1, 5′-ATGCTGCACCCATATTTG-3′, +4 to –122 bp; F2 5′-TGTGAGCTTCATGCCAAG-3′, –163 to –308 bp; F3 5′-GAGCCATGGTGTGTGCAG-3′, –748 to –892 bp; F4 5′-ATAACTGTAGACACGGGG-3′, –793 to –985 bp. One PCR primer from each pair was 5′-end-labeled with [^{32}P]ATP using T_4 polynucleotide kinase. PCR reactions were then performed in accordance with the manufacturer's instructions. Amplified fragments were visualized following agarose gel electrophoresis and purified from the gel using Quick-Sep Microcolumns. DNase I footprint analysis was performed as described (20).

Construction of ALDH-3 promoter-CAT reporter genes

5′ and 3′ end primers were selected to produce a series of deletion mutants of the proximal 1 kb 5′ flanking region of *ALDH-3* by PCR. The primers used are: P1, 3′-GAAACTCTCCGGAGAtCTGAGACGGTTCCG-5′, +113 to +142 bp; P2, 5′-GTCCGGATTATGCTGCAgCCATATTTGAAT-3′, –131 to –102 bp; P3, 5′-CCGCCTGCGTGACTGCAGCTTGCCCGATGT-3′, –392 to –363 bp; P4, 5′-CAGAGCCATGGTGcaTGCAGATTAAGGGAG-3′, –894 to –862 bp; P5, 5′-TTACCCATAACTGTcGACACGGGGGTGCCAG-3′, –991 to –962 bp. Each primer either possessed or was engineered (lower case bases) to contain a restriction enzyme site to facilitate subcloning. The amplified DNA fragments were visualized following electrophoresis and purified using Gene Clean II. The purified PCR DNA fragments were first cloned into the TA cloning vector:pCR-II™. The constructs were confirmed by releasing the inserts from the pCR-II™ vector by digestion with appropriate restriction enzymes. The digestion reaction was electrophoresed and the inserted PCR DNA fragment was again purified. The final purified PCR DNA fragments were cloned into pCAT-basic vectors. The constructs were purified for transfection using Qiagen-tip 500 columns.

Transfection and CAT assay

HTC and MCA-RH7777 cells grown on 60 mm dishes to 30–60% confluence were transfected with various *ALDH-3* promoter-CAT constructs by calcium phosphate precipitation (21). Transfectants were selected by Geneticin (G418) resistance. Medium was changed every 2 or 3 days until colonies appeared. Stably transfected cells were maintained in medium supplemented with G418.

Chloramphenicol acetyltransferase (CAT) activity was determined by thin layer chromatography (TLC) as described (22). The autoradiograms of TLC plates were analyzed by densitometry to estimate the CAT activities for each construct. Transfection efficiency was normalized to plasmid copy number according to Gillespie and coworkers (23).

Gel mobility shift assay

The DNA sequences of the oligonucleotides used in gel mobility shift assays are shown in Table 1. The single-stranded complementary oligonucleotides were synthesized and annealed to produce double-stranded oligonucleotides. Double-stranded oligonucleotides were end-labeled with [^{32}P]ATP, visualized following electrophoresis and purified from the gel using Quick-Sep Microcolumns.

Gel mobility shift assays were performed as described (20). The DNA–protein complexes were subsequently separated on 5% polyacrylamide gels and visualized by autoradiography. In competition gel-shift and supershift experiments, a large molar excess of various unlabeled competitor oligonucleotides or antibodies to the DNA binding proteins of interest were added with the labeled oligonucleotide.

RESULTS

DNase I footprint analysis

Approximately 1 kb of DNA immediately upstream of the rat *ALDH-3* transcription initiation site functions as a strong basal promoter (14). To determine the DNA sequences involved in basal *ALDH-3* expression, nuclear extracts from different sources [HTC hepatoma cells (high constitutive Class 3 ALDH enzyme activity), MCA-RH7777 hepatoma cells (low Class 3 ALDH enzyme activity), normal rat liver (no Class 3 ALDH enzyme activity) and 3MC-treated rat liver (high induced Class 3 ALDH enzyme activity)] were used in DNase I footprint assays. Nuclear extracts from sources differing in *ALDH-3* expression produce

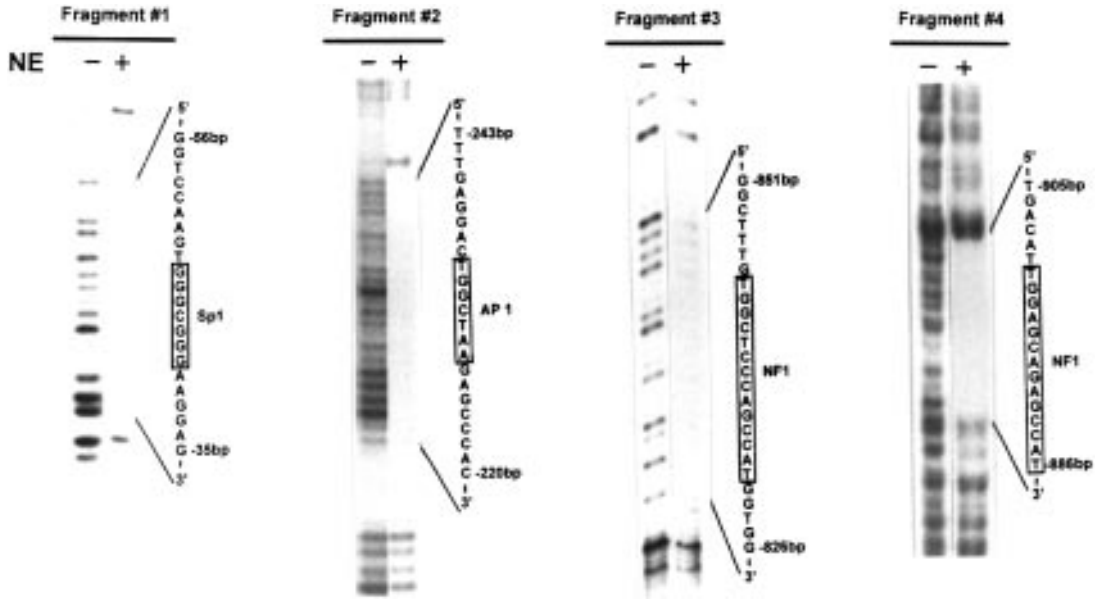

Figure 1. DNase I footprint analysis of the *ALDH-3* 5′ flanking region. Four ^{32}P-labeled PCR DNA fragments as indicated were digested by DNase I without (–) or with (+) nuclear extracts (NE) from HTC cells. The position and sequence of each footprinted region, derived from Maxam–Gilbert sequencing, is given on the right. Putative transcription factor binding sequences are boxed.

Table 1. Double-stranded oligonucleotides employed

NAME	SEQUENCE	SIZE
51/52	5′-AAGGACTCCAAGGTCCAAGTGGGCGGGAAGGAGGGTCCTTAAAT-3′ 3′-TTCCTGAGGTTCCAGGTTCACCCGCCTTCCTCCCAGGAATTTA-5′	44bp
21/22	5′-ACAGGCTCCTTTGAGGACTGGCTAAGAGCCCACACAAACTTTCA-3′ 3′-TGTCCGAAGAAAACTCCTGACCGATTCTCGGGTGTGTTTGAAAGT-5′	45bp
31/32	5′-GCAAGAGCACAGGCTTTGTGGCTCCCAGCCATGGTGGGGACTTGTCCT-3′ 3′-CGTTCTCGTGTCCGAAACACCGAGGGTCGGTACCACCCCTGAACAGGA-5′	48bp
41/42	5′-TGCTATGTGAGTGACATTGGAGCAGAGCCATGGTGTGTGCAG-3′ 3′-ACGATACACTCACTGTAACCTCGTCTCGGTACCACTCTCGTC-5′	42bp
51/52	5′-CTCCCAGCCATGGTGGGGACTT-3′ 3′-GAGGGTCGGTACCACCCCTGAA-5′	22bp
61/62	5′-CTCCCAGttATaaTGGGGACTT-3′ 3′-GAGGGTCaaTAttACCCCTGAA-5′	22bp
AP1	5′-CGCTGATGACTCAGCCGGAA-3′ 3′-GCGACTACTGAGTCGGCCTT-5′	21bp
HNF4	5′-AGGACTGCAAGGTCCATGTGGG-3′ 3′-TCCTGACGTTCCAGGTACACCC-5′	20bp
HNF5	5′-TCATCTGTTTGCAGCA-3′ 3′-AGTAGACAAACGTCGA-5′	16bp
NF1	5′-TATTTTGGATTGAAGCCAATATGA-3′ 3′-ATAAAACCTAACTTCGGTTATACT-5′	24bp
PuF	5′-CCTTCCCCACCCTCCCCACCCTCCCCAT-3′ 3′-GGAAGGGGTGGGAGGGTGGGGGTA-5′	28bp
SP1	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′	22bp
SP1-M	5′-ATTCGATCGGttCGGGGCGAGC-3′ 3′-TAAGCTAGCCaaGCCCCGCTCG-5′	22bp

The DNase I footprinted regions and consensus transcription factor binding sites are in bold and underlined, respectively. The 10 bp DNA motif is underlined in 51/52 and 61/62. Lower case letters in 61/62 and Sp1-M represent the mutations from 51/52 and Sp1 wild-type, respectively.

identical results, therefore, only the results from footprinting nuclear extracts of HTC hepatoma cells are presented.

The first DNase I protected site spans the 5′ flanking region from –56 to –35 (fragment #1, Fig. 1). A consensus Sp1 site (GGGCGGG; –47 to –41) is found in this region. The second protected region extends from –243 to –220 (fragment #2, Fig. 1). A putative AP1 site (TGgCTAA; –234 to –228) resides in this region. The third and fourth protected regions span from –851 to –826 and –905 to –886 (fragments #3 and #4, Fig. 1). Both regions harbor similar putative NF1 sites (TGGA/CN_{5-6}GCCAT) at –844 to –831 and –899 to –886, respectively.

The region between –747 and –309 contains many putative transcription factor binding sequences, including cAMP response elements (CREs), a liver activator protein (LAP) site and xenobiotic response elements (XREs), etc. However, PCR fragments containing these sequences do not footprint (data not shown).

CAT reporter gene assays

To explore how the four DNase I footprinted regions function in *ALDH-3* gene expression, several *ALDH-3* promoter-CAT constructs were prepared and stably transfected into HTC and MCA-RH7777 cells (Fig. 2A). In both cell types, the construct containing the putative Sp1 binding site (–120 to +130; CI2; Fig. 2A) is expressed at levels somewhat higher than pSV2CAT (Fig. 2B). The construct containing both the putative Ap1 and the Sp1 binding sites (–380 to +130; CI3, Fig. 2A) shows stronger promoter activity than either the CAT construct containing the putative Sp1 binding site or pSV2CAT (Fig. 2B). This 250 bp promoter sequence was also found to drive CAT gene expression in a variety of other cell lines that have little or no Class 3 ALDH enzyme activity. Expression of the CAT gene driven by the *ALDH-3* promoter was slightly higher than pSV2CAT in H4IIEC3 rat hepatoma cells and BRL 3A buffalo rat liver endothelial cells;

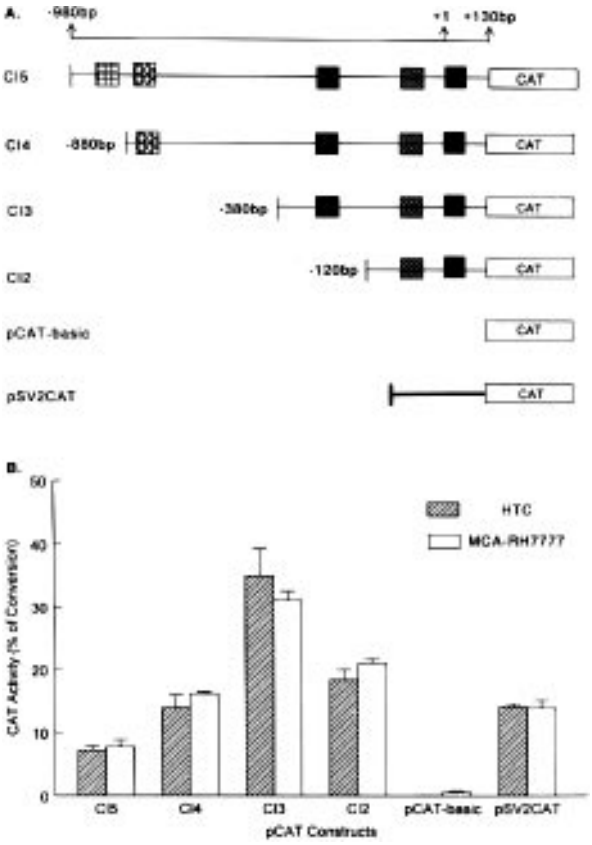

Figure 2. *ALDH-3* promoter-CAT reporter gene assay. (**A**) Construction of the *ALDH-3* promoter-CAT reporter genes. Various 5′-end deletions of the *ALDH-3* 1 kb 5′ flanking region (+130 to –980) were cloned into pCAT-basic. The black box represents the first exon of *ALDH-3* and other boxes represent four DNase I footprinted regions. pCAT-basic lacks any promoter sequence and pSV2CAT contains SV40 early promoter sequence. +1 represents the transcription initiation site. (**B**) Analysis of promoter activity of various DNase I footprinted regions. HTC and MCA-RH7777 cells were stably transfected with the indicated *ALDH-3* promoter-CAT constructs. The CAT activity was measured and normalized to the plasmid copy number. Bars represent the standard deviation from at least three independent experiments.

and 4–5-fold higher than pSV2CAT in 10T1/2 mouse fibroblast cells and clone 9 rat epithelial cells (data not shown).

Sequential addition of the two putative NF1 binding sites causes repression of rat *ALDH-3* promoter activity (Fig. 2B). CAT activity is reduced ~2-fold (CI4) when the more proximal NF1 site is present and ~4-fold (CI5) when both NF1 sites are present. A similar result was also found in H4IIEC3 cells (14).

ALDH-3 promoter-CAT constructs lacking the *ALDH-3* transcription initiation site produce no CAT activity. *ALDH-3* promoter-CAT constructs containing the first exon of *ALDH-3*, but lacking the first 200 bp of intron 1 have CAT activity comparable with the CI3 construct. There was no effect on CAT activity of any *ALDH-3* promoter-CAT construct by treatment of cells with TCDD (data not shown).

Gel mobility shift assays

To identify DNA-binding proteins which interact with these *cis*-acting elements, a series of gel mobility shift assays were performed. Oligonucleotides (11/12, 21/22, 31/32, 41/42, Table 1) containing each of the four DNase I footprinted sites were used as probes in gel mobility shift assays. Nuclear extracts from HTC, MCA-RH7777 hepatoma cells, 3MC-treated and normal rat liver produced very similar gel mobility shift patterns, including competition and supershift assays, for each promoter region. Therefore, complete gel mobility shift assay data are presented for HTC cell nuclear extract plus the basic gel mobility shift patterns for nuclear extracts from MCA-RH7777 cells, 3MC-treated and normal rat liver.

Except for the protein–DNA complexes discussed in detail below, other complexes observed in gel mobility shift assays are considered to represent nonspecific binding or are not related to *ALDH-3* expression. They were either not competed by any of the oligonucleotides used in these studies or were present in nuclear extracts of cells or tissues regardless of their Class 3 ALDH enzyme activity (data not shown). Further, no DNA–protein complexes consistent with the large size (>300 kDa) and complexity of an Ah/Arnt-DNA interaction were observed in any *ALDH3* promoter region gel shift assay, including nuclear extracts from 3-MC treated liver.

Two closely migrating specific complexes are formed with the probe from –67 to –24 (11/12), which contains the putative Sp1 site (lanes 2 and 11–13, Fig. 3A). As expected, excess 11/12 and an Sp1 consensus sequence (22) strongly compete with this region (lanes 3 and 5). Neither oligonucleotides containing a mutant Sp1 binding site (Sp1-M) nor consensus binding sequences for PuF (24) or HNF4 (25) compete (lanes 6, 8 and 9). The Ap1 consensus oligonucleotide may be a weak competitor (lane 10). Surprisingly, the region from –253 to –209 (21/22) also effectively competes these two complexes (lane 4). Both specific complexes are supershifted by anti-SP1 antibody (lane 7, arrowhead, Fig. 3A). Thus, the proteins forming these two complexes with the region from –67 to –24 are either Sp1 or Sp1-related proteins.

The region from –253 to –209 (21/22) contains a putative AP1 binding site. One specific complex identical in mobility to the two complexes of the –67 to –24 region, is formed between this region and various nuclear extracts (lanes 2 and 11–13, Fig. 3B). This complex is competed by excess of 11/12 or Sp1 consensus oligonucleotide as well as itself (lanes 3–5). However it is not competed by a mutant Sp1, PuF, HNF5 or AP1 consensus sequences (lanes 6 and 8–10). This complex is also supershifted by anti-Sp1 antibody (lane 7). Thus, Sp1-like proteins also binds to the –253 to –209 region. However, the binding appears to be weaker than to the –67 to –24 region (Fig. 3B). When identical amounts of oligonucleotides 21/22 and 11/12 are used, 21/22 competed the two specific complexes less effectively than 11/12 (lanes 3 and 4, Fig. 3A). Another, faster migrating, specific complex was formed between the –253 and –209 region and nuclear extracts from hepatoma cells, but not whole liver. This complex is only competed by the oligonucleotide to this region.

The regions from –862 to –815 (31/32) and –916 to –875 (41/42) have qualitatively similar gel shift patterns with various nuclear extracts (Fig. 3C and D). Interestingly, one additional complex (C4) is observed in the region from –875 to –916 and the protein(s) forming C3 is absent in nuclear extracts of MCA-RH7777 cells. The three specific complexes (C1, C2, C3) common to both regions are competed by both oligonucleotides (lanes 3 and 4 in Fig. 3C and D). This suggests that the same or very similar proteins are binding to both regions with similar affinity.

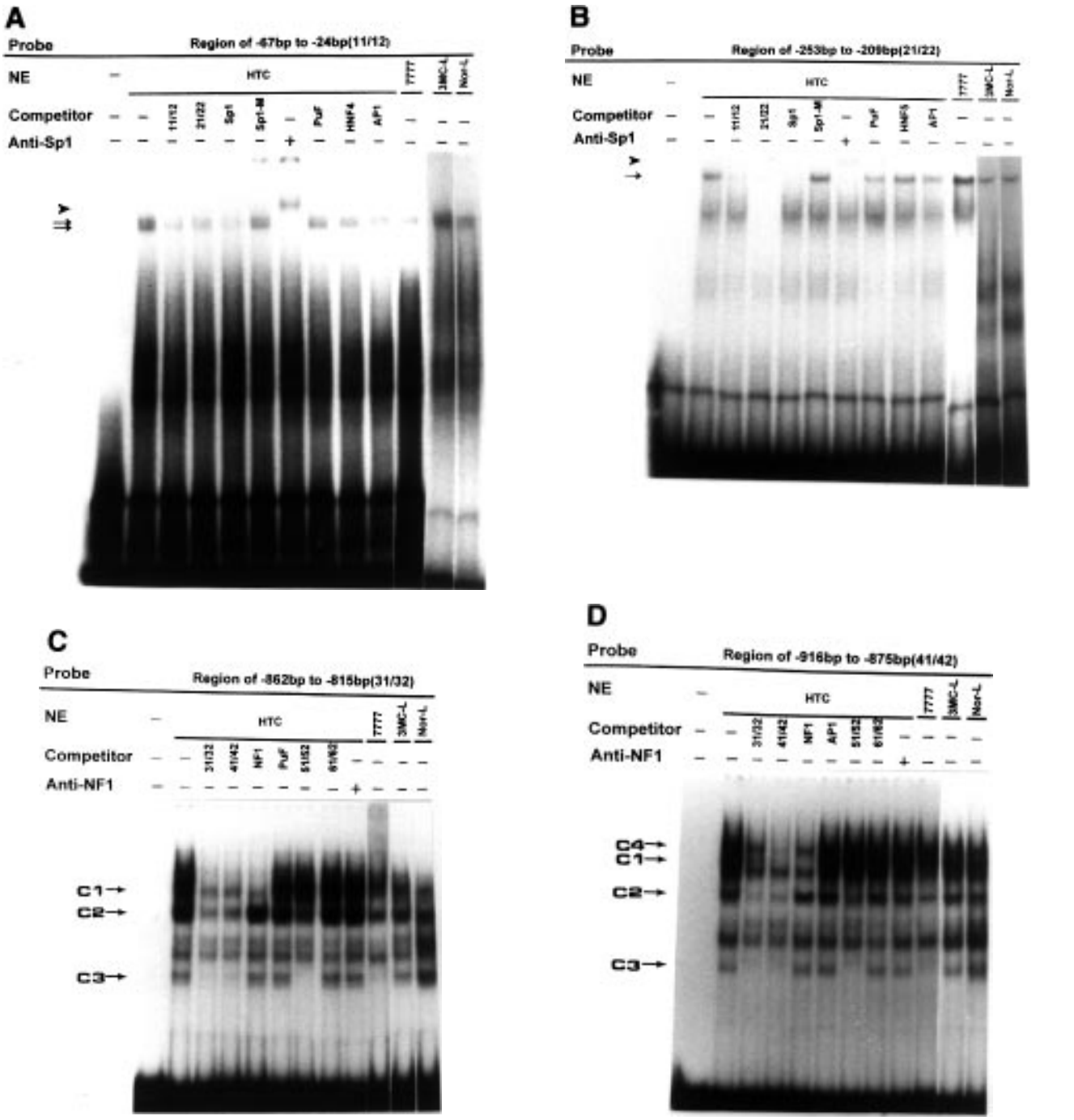

Figure 3. Gel mobility shift assays of various regions of the *ALDH-3* promoter. Labeled oligonucleotide probe corresponding to the various DNase I footprinted regions were incubated without or with nuclear extracts [20 µg plus 1 µg poly(dI–dC) on ice for 20 min] from HTC, MCA-RH7777 cells (7777), 3MC-treated (3MC-L) or normal rat liver (Nor-L) as indicated. Various unlabeled competitor oligonucleotides or antibodies were added to the reactions containing nuclear extracts from HTC cells as indicated. Specific complexes are indicated by the arrows and the supershifted complexes by the arrowheads. See Table 1 for the sequences of the various oligonucleotides. (**A**) The region from –67 to –24 (11/12); (**B**) the region from –253 to –209 (21/22); (**C**) the region from –862 to –815 (31/32); (**D**) the region from –916 to –875 (41/42).

Since both regions contain putative NF1 binding sites, we probed various nuclear extracts with a labeled NF1 consensus sequence (26) to determine which complex(es) might contain NF1. One complex is formed using the consensus NF1 oligonucleotide probe (lanes 2 and 9–11, Fig. 4). This complex has the same mobility as C1 and is competed by 31/32, 41/42 and the NF1 consensus sequence (lanes 3–5 in Fig. 3C and D; lanes 3–5 in Fig. 4).

Hela cells are a rich source of various forms of NF1 (27) and were also used in gel mobility shift assays to confirm the NF1 complex identity (lanes 12–18, Fig. 4). A specific complex formed

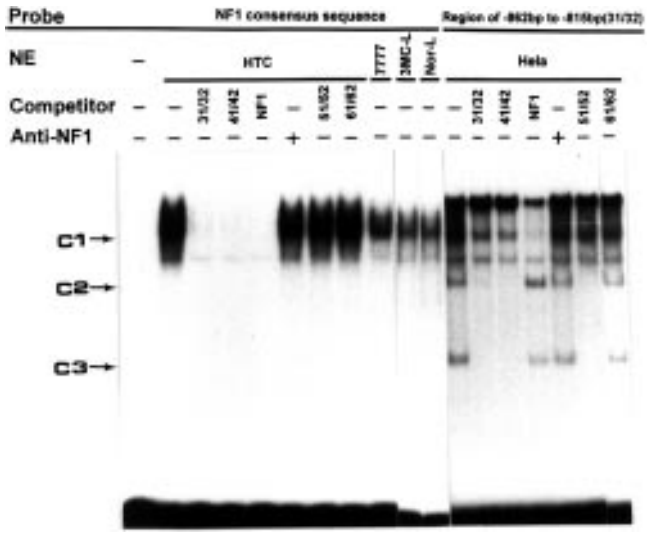

Figure 4. Gel mobility shift assay of the NF1 consensus sequence and the region from –862 to –815 of the *ALDH-3* promoter. Labeled NF1 consensus sequence was incubated without or with nuclear extracts (20 µg) from HTC, MCA-RH7777 cells (7777), 3MC-treated (3MC-L) and normal rat liver (Nor-L). Labeled oligonucleotide probe corresponding to the region from –862 to –815 (31/32) was incubated with nuclear extracts from HeLa cells as indicated. Various unlabeled competitor oligonucleotides or an anti-NF1 antibody were added as indicated to the reactions containing nuclear extracts from HTC and HeLa cells. Specific complexes are as indicated.

between Hela cell nuclear extracts and the region from –862 to –815 that is competed by both NF-1 regions of the *ALDH-3* promoter and by the NF1 consensus sequence (lanes 13–15, Fig. 4). These results suggest that the protein forming C1 with both regions is a member of NF1 family or a NF1-like protein.

Complexes 2 and 3 formed with the region from –862 to –815 and the region from –916 to –875 are also found in Hela cell nuclear extracts (compare lanes 2, 11 and 12 in Fig. 3C and D with lane 12 in Fig. 4). In addition to being competed by oligonucleotides to each region, these complexes are also competed by an oligonucleotide (51/52, Table 1; lane 7 in Fig. 3C and D; lane 17 in Fig. 4) which contains a 10 bp DNA motif (AGCCATGGTG) found in both regions (–836 to –827 and –891 to –882, respectively). These two specific complexes are not competed by a mutant form of this 10 bp DNA motif (61/62, Table 1; lane 8 in Fig. 3C and D; lane 18 in Fig. 4), nor by the NF1 consensus sequence (lane 5 in Fig. 3C and D; lane 15 in Fig. 4). Computer analysis indicates that the 10 bp DNA motif is not an obvious binding site for any known transcription factor. These results suggest that the protein(s) forming C2 and C3 with both regions are not NF1 and that other, unidentified, proteins bind to this unique 10 bp sequence within both regions. The additional complex C4 formed with the region from –916 to –875 is not competed by any consensus transcription factor binding sequence tested. This suggests that protein(s) forming this complex may also be novel.

It is not clear why commerically prepared anti-NF1 antibody does not supershift complex C1 (lane 9 in Fig. 3C and D; lanes 6 and 16 in Fig. 4). Interestingly, this same anti-NF1 antibody does not affect specific complexes formed between the –862 and –815 oligonucleotide and purified, recombinant NF1 protein (data not shown). It is possible that the quality of the particular lots of antibody used in these studies was less than optimal or that the antibodies do not recognize the particular NF-1 family member forming the C1 complex.

However, together the CAT assay and gel shift results indicate that NF1-like proteins and/or other proteins are functionally involved in binding both –862 to –815 and –916 to –875 of the *ALDH-3* 5′ flanking region and that these proteins act to inhibit *ALDH-3* basal expression. We are attempting to purify and characterize the additional protein(s) involved.

DISCUSSION

Our previous studies revealed that the 5′ flanking region of the rat aldehyde dehydrogenase 3 gene contains a proximal strong basal promoter region, a single xenobiotic (TCDD)-responsive region around –3 kb and two regions which repress promoter activity and the TCDD-response, respectively (14). The present results define four functional DNA regions within the proximal 1 kb of the *ALDH-3* 5′ flanking region. Two of these sites act as strong promoter elements while the other two appear to inhibit or limit promoter activity. Consistent with our previous results, no *cis*-acting elements or *trans*-acting factors regulating *ALDH-3* xenobiotic-induced or tissue-specific expression are present in this promoter region.

The two proximal sites that bind Sp1 with different affinities comprise the region that is required for strong *ALDH-3* promoter. It has been shown that Sp1 has a rather loose binding specificity to GC-rich sequences (25) and the region from –253 to –209 of *ALDH-3* does contain a cryptic Sp1 binding site (–235 ctGGCTAAG –227). Although identifying two Sp1-binding sites with considerably different binding affinities acting additively as the *ALDH-3* promoter was unexpected, it is not unknown. A similar result has been reported for the herpes simplex virus (HSV) thymidine kinase (TK) promoter (28–30). The HSV TK promoter contains two Sp1 binding sites with different affinities. As for rat *ALDH3*, disruption of this weaker Sp1 binding site strikingly reduces HSV TK promoter activity (30). Since no functional CAAT and/or TATA boxes have been identified in the rat *ALDH3* promoter, the two Sp1 sites likely serve as the major sites of transcription machinery alignment.

The two DNase I protected sites within the distal region of the proximal 1 kb portion of the *ALDH-3* 5′ flanking region contain similar NF1 binding sites (complex C1) and both act additively to inhibit *ALDH-3* promoter activity. NF1 is a family of at least six related DNA-binding proteins, all of which recognize a palindromic sequence or half-site sequence of the palindrome as a dimer (27,32,33). NF1 usually acts as a positive transcription factor (34,35), but it has been reported that NF1 binding sites in the promoter region of the 3-hydroxyl-3-methyl-glutaryl CoA reductase and the retinol-binding protein (RBP) genes act as negative transcriptional regulators (27,36).

Our gel mobility shift results indicate that the proteins forming C2 and C3 may also bind to these two promoter-distal DNase I protected sites at the unique 10 bp DNA motif (51/52). It is possible that binding of the proteins forming C2 or C3 to these sites may either alter the binding of NF1 or interfere with transactivation ability of NF1 when NF1 binds to these two sites. Additionally, the protein(s) forming the complex C4 with the more distal NF1 site may also function similarly to C2 or C3 to further modulate *ALDH-3* promoter activity.

Based on our structural and functional analysis, the following organization of the proximal 1 kb of the 5′ flanking region of rat

Figure 5. Functional organization of the proximal 5′ flanking region of the rat *ALDH-3* gene. Solid boxes represent two Sp1 binding sites. Open boxes represent the binding sites for NF-like protein and/or unidentified protein(s). Thick bar indicates the strong promoter region. Thin bar represents the inhibitory region. Transcription initiation site is indicated by an arrow. The first exon is indicated.

ALDH-3 is proposed (Fig. 5): two proximal regions binding Sp1 possess strong promoter activity, two distal regions binding NF1-like proteins and other, unique proteins inhibit the promoter activity. A similar promoter organization has been postulated for the mouse ALDH3 gene (31). Both putative Sp1 and NF1 sites in approximately the same relative positions have been identified by computer analysis. However, no functional analysis of the mouse *ALDH3* promoter has been reported.

Consistent with earlier reporter gene assays (14), no evidence for a significant role of any of the putative XREs identified by computer analysis was provided by the footprint, reporter gene or gel shift assays of the rat *ALDH3* promoter region from cells and tissues differing greatly in Class 3 ALDH activity. Further, the present extensive functional promoter analysis is consistent with our earlier observation that xenobiotics acting via EpREs, such as *t*-butylhydroquinone, are without effect in reporter gene assays (14).

Our results also confirm the importance of a thorough functional analysis of any putative *cis*-acting elements or *trans*-acting factors identified by other means. Of the ~15 *cis*-acting elements first identified by computer analysis of the rat *ALDH3* 5′ flanking region (10), only the Sp1 site at –35 bp was confirmed. The second Sp1 site was identified by sequence analysis as an Ap1 site/EpRE. The –826 bp NF1 site has been characterized as both Ap1 and XRE sites. The more distal NF1 site was either undescribed or an Ap1 site.

Thus, we conclude that the proximal region of the rat ALDH3 gene functions to regulate constitutive expression of *ALDH3* in a variety of tissues. This region is not significantly involved in either the xenobiotic-induced or tissue-specific expression of this gene. This is in striking contrast with the demonstrated or postulated functional importance of multiple XREs and EpREs found in the proximal 1 kb of other Ah battery genes, including mouse *CYP1A1*, *Gsta1*, *Nmo1* and *ALDH3* and human *CYP1A1* (40,41).

ACKNOWLEDGEMENTS

Submitted by Y. Q. Xie in partial fulfillment of the requirement for the M.A. degree, The University of South Dakota. Supported by NIH grant CA21103 to R.L. and NIH grants CA07175 and CA22484 to H.P.

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