Expression of Tumor-associated Aldehyde Dehydrogenase Gene in Rat Hepatoma Cell Lines

Kwang-huei Lin, Mark D. Brennan, and Ronald Lindahl

Department of Biology, The University of Alabama, Tuscaloosa, Alabama 35487

ABSTRACT

Significant changes in aldehyde dehydrogenase (ALDH) activity occur during rat hepatocarcinogenesis in vivo. To compare the structure and expression of the tumor aldehyde dehydrogenase gene in rat hepatoma cell lines and normal rat liver, several rat hepatoma cell lines, including HTC, H4-II-EC3, JM2, MCA-RH7777, and four lines established in this laboratory, have been examined for T-ALDH gene expression using a tumor ALDH complementary DNA.

Northern blot analysis of polyadenylate-containing RNA from log-phase cells and normal rat liver with T-ALDH complementary DNA indicates production of a single major 1.7-kilobase transcript in the high activity lines HTC, JM2, RLT-2M, RLT-3C, RLT-9F, and intermediate activity line RLT-5G. There is a direct correlation between expression of T-ALDH enzyme activity and the amount of 1.7-kilobase transcript. Nuclear protection experiments confirm that there is only one major T-ALDH transcript in the high activity lines. Thus, cell line differences in T-ALDH activity are reflected in the level of a single T-ALDH transcript.

Southern analysis was used to identify the T-ALDH gene in genomic DNA. The results indicate that no significant amplification or rearrangement of the T-ALDH gene has occurred in these hepatoma cell lines. DNA methylation has been proposed to play an important role in gene expression. Genomic DNA from HTC, JM2, MCA-RH7777, H4-II-EC3, RLT-2M, RLT-9F, RLT-3C, and RLT-5G were digested with MspI and HpaII to examine methylation patterns. A digestion pattern consistent with hypomethylation was detected only in DNA from the high T-ALDH activity cell lines HTC, JM2, RLT-2M, and RLT-9F. This suggests that constitutive expression of T-ALDH in the hepatoma cells is related to changes in DNA methylation patterns.

INTRODUCTION

Significant changes in ALDH activity occur during rat hepatocarcinogenesis in vivo. The phenotype is characterized by the appearance of cytosolic NAD(P)H-dependent, benzaldehyde oxidizing activity, the demonstration by gel electrophoresis of a new ALDH activity not found in normal liver, and characteristic histochemical localization of ALDH activity (1–5). Expression of the tumor ALDH (T-ALDH) phenotype is variable. In four distinct tumor induction protocols, the proportion of T-ALDH-positive neoplasms has ranged from 25 to 97% (2, 3, 5, 6). Additionally, among neoplasms induced by the same protocol, the T-ALDH activity may vary considerably (2, 3, 5, 6). However, expression of the tumor ALDH phenotype is limited to preneoplastic or neoplastic hepatocytes, and is not a general response to cell injury, death, or liver regeneration (1–3, 5, 6).

The development of an in vitro model system with the use of rat hepatoma cell lines has allowed us to study the regulation of T-ALDH activity in detail (7, 8). This in vitro system has provided the best evidence to date for the role of changes in gene expression in the genesis of the T-ALDH phenotype. Five of eight rat hepatoma cell lines maintained in our laboratory constitutively express the T-ALDH phenotype to varying degrees (7, 8). The tumor ALDH phenotype can also be enhanced in vitro by 3-methylcholanthrene or benzo(a)pyrene in many of these cell lines, or induced in two other lines which do not constitutively possess T-ALDH activity (7, 8). Both new transcription and translation are required for T-ALDH expression in these lines (7). Thus, depression of a gene(s) normally repressed in the liver is responsible for the expression of the T-ALDH phenotype.

To study the expression and regulation of the T-ALDH gene in rat primary hepatocellular carcinomas, hepatoma cell lines, and normal rat liver, we have cloned a cDNA encoding T-ALDH. The cDNA sequence is approximately 1.8 kilobases in length and apparently contains the entire aldehyde dehydrogenase coding sequence because this clone directs the synthesis of a functional T-ALDH molecule (9).

This study characterizes the molecular basis of the differential production of T-ALDH in our collection of rat hepatoma cell lines, whose ALDH activities represent the spectrum of activities seen in hepatic neoplasms. The results indicate that variations in T-ALDH phenotype are the results of altered expression of a single T-ALDH gene and that hypomethylation of this gene correlates with constitutive production of tumor aldehyde dehydrogenase.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from K. C. Biologicals, Inc. (Lenexa, KS). Restriction endonucleases EcoRI, PstI, MspI, HpaII, and HindIII were obtained from New England BioLabs, Inc. (Beverly, MA). Si nuclease and oligodeoxynucleases EcoKI, PstI, Mspl, Hpall, and HindIII were obtained from New England BioLabs, Inc. (Beverly, MA). Si nuclease and oligodeoxynucleases EcoKI, PstI, Mspl, Hpall, and HindIII were obtained from New England BioLabs, Inc. (Beverly, MA).

Normal rat livers were excised from adult Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), frozen in a dry ice/acetone bath, and stored at −80°C. Embryos (14-day) from pregnant Sprague-Dawley rats were removed and frozen in liquid nitrogen for RNA isolation.

Cells and Cell Culture. Rat hepatoma cell lines HTC, JM2, MCA-RH7777, H4-II-EC3, RLT-2M, RLT-3C, RLT-5G, and RLT-9F were maintained in monolayer culture as described previously (7, 8).

Determination of Aldehyde Dehydrogenase Activity. Aldehyde dehydrogenase activity was assayed at room temperature by monitoring the change in absorbance at 340 nm as caused by NADH and NADPH production during the oxidation of aldehyde substrate as described previously (10).

RNA Isolation and Blot Hybridization. For Northern analysis RNA was isolated from log-phase rat hepatoma cell lines and normal rat liver by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion (11). Preparation of poly(A*) RNA was accomplished by chromatography on oligodeoxynucleotide cellulose (12).

Received 10/9/87; revised 7/25/88; accepted 9/14/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a University of Alabama Graduate Council Research Fellowship to K-h. L., NIH Grant #21103 to R. L., and University of Alabama funds to M. D. B.

2 Submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Graduate School of The University of Alabama. Present address: Building 37, Room 3C20, NIH, Bethesda, MD 20892.

3 To whom requests for reprints should be addressed.

4 The abbreviations used are: ALDH, aldehyde dehydrogenase; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); T-ALDH, tumor aldehyde dehydrogenase; Hepatoma, MCA-RH7777, H4-II-EC3, RLT-2M, RLT-3C, RLT-5G, and RLT-9F were

7009
was ethanol precipitated and dried. The pellet was resuspended in H₂O and stored at −70°C.

Poly(A*) RNAs were denatured and subjected to electrophoresis on 1.2% agarose gels containing formaldehyde, as described previously (11). Filters were prehybridized, hybridized, and washed according to the method of Thomas (13). Plasmid probes were radiolabeled by nick translation in the presence of [γ-³²P]ATP (>3000 Ci/mmol), as described by Rigby et al. (14). Specific activities were 10⁶ to 10⁷ cpm/μg DNA. The final wash was in 1 x standard saline citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 60°C for 10 min, and filters were then fluorographed at −80°C by using Kodak XAR5 film and DuPont Cronex intensifying screens.

DNA Isolation and Filter Hybridization. High molecular weight, genomic DNA was isolated from tumor cell lines, rat embryo, and normal rat liver with the method described by Bingham et al. (15). Cells grown as monolayers were washed twice with ice-cold Tris-buffered saline (0.14 M NaCl, 5 mM KCl, 25 mM Tris-HCl, pH 7.4), and 0.05% triton X-100. Cells were detached with glass beads and harvested by centrifugation. The DNA preparation was dialyzed extensively against 10 mM Tris, pH 7.4, containing 1 mM EDTA.

After electrophoresis, DNA was transferred to nitrocellulose filters according to the method of Southern (16). Hybridization of the Southern blots was performed as described previously (17). The membranes were given a final wash in 0.5 x standard saline citrate at 65°C for 20 min. Washed filters were fluorographed as described above.

RESULTS

The aldehyde dehydrogenase activities of the eight rat hepatoma cell lines used in this study were determined (Table 1). In culture, RLT-2M, RLT-3C, RLT-9F, JM2, and HTC express constitutively high NADP-dependent ALDH activity, the marker of the tumor ALDH phenotype. RLT-5G possesses intermediate tumor ALDH activity. H4-II-EC3 and McA-RH7777 do not possess significant NADP-dependent activity. Expression of T-ALDH activity in each cell line has been relatively constant for at least 1 year. Thus, each cell line has a unique level of T-ALDH activity, falling within the spectrum of T-ALDH activities seen in primary hepatocellular carcinomas in vivo.

To characterize the expression of T-ALDH in rat hepatoma cell lines, poly(A*) RNA from log-phase cells for the eight cell lines and normal rat liver was prepared and probed with radiolabeled pT-ALDH (Fig. 1), a plasmid carrying the pUC8 backbone and 1.8-kilobase T-ALDH cDNA insert isolated from an HTC cell cDNA library (9). This analysis detects 1.7-kilobase T-ALDH cDNA in all cell lines and normal liver. Poly(A*) RNAs (4 μg/lane) were electrophoresed, transferred to nitrocellulose, and probed with 32P-labeled pT-ALDH. The same blot was also hybridized with 32P-labeled actin cDNA to verify that approximately equal amounts of intact poly(A*) RNA were loaded in each lane. Lane 1, HTC; Lane 2, H4-II-EC3; Lane 3, JM2; Lane 4, McA-RH7777; Lane 5, normal rat liver; Lane 6, RLT-2M; Lane 7, RLT-3C; Lane 8, RLT-5G; Lane 9, RLT-9F. kb, kilobase.

**Table 1** Aldehyde dehydrogenase activity of eight rat hepatoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Propionaldehyde/ NAD</th>
<th>Benzoaldehyde/ NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLT-2M</td>
<td>151 ± 26*</td>
<td>349 ± 48</td>
</tr>
<tr>
<td>RLT-3C</td>
<td>65 ± 6</td>
<td>104 ± 12</td>
</tr>
<tr>
<td>RLT-5G</td>
<td>19 ± 2</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>RLT-9F</td>
<td>67 ± 4</td>
<td>149 ± 26</td>
</tr>
<tr>
<td>HTC</td>
<td>119 ± 10</td>
<td>265 ± 11</td>
</tr>
<tr>
<td>JM2</td>
<td>142 ± 23</td>
<td>300 ± 30</td>
</tr>
<tr>
<td>H4-II-EC3</td>
<td>8 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>McA-RH777</td>
<td>12 ± 4</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Normal rat liver</td>
<td>20 ± 1</td>
<td>7 ± 0</td>
</tr>
</tbody>
</table>

*Mean specific activity (μIU/mg of protein) ± SD. Activities were averaged from at least three determinations. All cell lines were in culture 4 days before enzyme activity determinations.

From Ref. 8.

Although transcripts of one apparent size were detected in each cell line, S₁ nuclease protection experiments were performed to determine whether the transcripts seen in the various lines were identical to one another and whether multiple T-ALDH transcripts were found within a line. An S₁ protection probe, pT-ALDH, containing the full length T-ALDH cDNA, was linearized with EcoRI. This was hybridized with total cellular RNA from various cell lines. A 1.7-kilobase protection fragment (corresponding to the size of T-ALDH cDNA insert) was present for each high activity line (data not shown). Although minor differences of ALDH sequence in the 5' or 3' noncoding regions of each line cannot be determined in this particular S₁ mapping experiments due to the large size of the probe used (9), the S₁ results do indicate that all internal sequences, which include the ALDH coding sequences, in each high activity line are identical. Thus, cell line differences in T-ALDH activity are reflected in the level of presumably identical 1.7-kilobase transcripts that share a common protein coding region.

Southern analysis was used to identify the T-ALDH gene in genomic DNA. The 1750-base pair pT-ALDH cDNA hybridized to one major 21-kilobase EcoRI fragment in DNA prepared from six rat hepatoma cell lines, normal rat liver, and rat embryo. An additional 5-kilobase EcoRI fragment was observed in rat embryo DNA (data not shown). Hybridization patterns were more complex in HindIII or PstI-digested genomic DNAs (Fig. 2). At least six PstI and five HindIII sites were present within genomic T-ALDH DNA. Based on the three PstI and two HindIII sites in the pT-ALDH cDNA and on our Southern analyses, we estimate that there are at least four introns within T-ALDH genomic DNA. In addition, the size of the genomic region hybridizing to the pT-ALDH cDNA is between approximately 3.8 and 9.3 kilobases. The hybridization intensity of HindIII or PstI fragments from cell lines was as intense as
Fig. 2. Hybridization of tumor-aldehyde dehydrogenase cDNA with genomic DNAs. Genomic DNAs were combined with 10 ng bacteriophage λ DNA prior to digestion in either PstI (A) or HindIII (B). Following digestion, DNAs were electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed with radiolabeled pT-ALDH. After appropriate exposures were obtained, the nitrocellulose filters were reprobed with radiolabeled bacteriophage λ DNA. This allowed monitoring of digestion, provided size standards, and controlled for recovery and transfer of genomic DNA. Lane 1, RLT-2M (10 μg); Lane 2, RLT-9F (10 μg); lane 3, total embryo (10 μg); Lane 4, normal liver (10 μg); Lane 5, HTc (10 μg); Lane 6, H4-II-EC3 (10 μg); Lane 7, JM2 (10 μg); Lane 8, McA-RH7777 (4 μg). Positions of DNA molecular weight markers in kilobase pairs are shown on ordinate. The apparent size difference of all hybridizing fragments between Lanes 7 and 8 in B is an artifact of the gel run as evidenced by a similar shift in fragment sizes for the bacteriophage λ DNA that was included as an internal control (not shown).

To determine if changes in methylation patterns correlate with expression of T-ALDH, genomic DNA from HTC, JM2, H4-II-EC3, McA-RH7777, RLT-2M, RLT-9F, normal rat liver, and rat embryo were digested with the restriction enzymes Mspl and HpaII (Fig. 3). The Mspl digestion patterns of DNAs from all cell lines and normal liver were similar. For embryonic DNA, two additional major low molecular weight Mspl fragments (0.75 and 0.56 kilobase) were observed. Differences in digestion patterns were observed with the methylation-sensitive, isoschizomeric enzyme, HpaII. Patterns consistent with hypomethylation were detected in the four high T-ALDH activity lines, HTC, JM2, RLT-2M, and RLT-9F (Fig. 3). Interestingly, the hypomethylation patterns differed in these four lines. Whether these differences are related to the expression of T-ALDH activity is currently unknown. No low molecular weight HpaII fragments were detected in the low T-ALDH activity lines McA-RH7777 and H4-II-EC3 or in normal rat liver.

DISCUSSION

The development of an in vitro model has provided the best system to date for studying the expression of tumor aldehyde dehydrogenase gene (7, 8). The eight rat hepatoma cell lines maintained in our laboratory possess a broad spectrum of T-ALDH activity in culture. For all lines having the T-ALDH phenotype, expression occurs via a 1.7-kilobase T-ALDH transcript. Moreover, the presence of 1.7-kilobase T-ALDH mRNA correlates well with expression of T-ALDH phenotype. This result is consistent with our earlier observations that new transcription and translation are required both for T-ALDH induction by xenobiotics and to maintain high constitutive T-ALDH activity in these lines (7). There is also generally good agreement between the amount of ALDH activity and the level of 1.7-kilobase transcript. For RLT-5G, a cell line with a weak T-ALDH phenotype, the relative amount of 1.7-kilobase transcript is about 5-fold lower than expected based upon its enzyme activity. The molecular basis of this discrepancy is being investigated.

Interestingly, T-ALDH cDNA does not show detectable cross-hybridization with mRNAs for any of the normal liver ALDH isozymes. This implies that the tumor and normal liver ALDH isozymes do not have a high degree of sequence identity, and is consistent with our earlier observations (9). S1 nuclease protection experiments indicate that T-ALDH RNA sequences from all high activity cell lines are fully com-
plementary (at least in the ALDH coding regions) to the pT-ALDH cDNA insert. The length of noncoding regions of the T-ALDH cDNA in 5’ and 3’ ends are 173 and 248 base pairs, respectively (9). In addition, we estimate that the resolution of our S1 experiments is about ±100 (SD) base pairs because the entire 1.7-kilobase T-ALDH cDNA was used as a protection probe. Thus, the T-ALDH transcripts in lines which constitutively express T-ALDH do not differ from one another in their internal coding sequences and clearly arise from a common structural gene.

Southern analyses indicate that no significant amplification or rearrangement of the T-ALDH gene occurs in the eight cell lines examined. Although the detailed structure of the T-ALDH gene in rat hepatoma cell lines is still unknown, based on Southern analysis and the nucleotide sequence of pT-ALDH cDNA, the genomic DNA encoding this gene contains at least four introns, and the size of this gene is estimated to be between 3.8 and 9.3 kilobases.

The observation that the DNA in hepatoma cell lines with constitutively high T-ALDH activity is hypomethylated relative to the DNA of cell lines with low T-ALDH activity is consistent with the hypothesis that DNA hypomethylation plays an important role in mammalian gene expression (18, 19). The degree of hypomethylation is not only consistent with T-ALDH activity of a cell line, but is also consistent with the observation that only a fraction of cells of a particular cell line express T-ALDH as judged by histochemistry (7, 8). We have recently examined the methylation status of the T-ALDH gene during hepatocarcinogenesis induced by the resistant hepatocyte model (20). The results indicate that hypomethylation of the T-ALDH gene occurs concomitantly with the first appearance of T-ALDH activity in enzyme-altered foci at approximately day 28 of the protocol (2). Moreover, an excellent correlation exists between neoplasms which express T-ALDH and the methylation status of the T-ALDH gene in these neoplasms.

It seems unlikely that the methylation pattern differences observed are characteristic of the genome of each cell line, rather than related to T-ALDH expression. Schulz et al. (21) have demonstrated that relative to normal liver, DNA from HTC and McA-RH7777 cells is hypomethylated and DNA from H4-II-EC3 cells is overmethylated. HTC cells constitutively express T-ALDH, but neither RH7777 or EC3 cells express T-ALDH. Therefore, there is no correlation between total genome methylation status and T-ALDH expression.

We have previously demonstrated (7) that benz(a)pyrene and 3-methylcholanthrene are able to increase T-ALDH activity up to 40-fold in rat hepatoma cell lines that do not constitutively express the T-ALDH gene. We are currently evaluating the methylation patterns of the T-ALDH gene in our cell lines after treatment with benzo(a)pyrene, 3-methylcholanthrene, or the DNA-demethylating agent, 5-azacytidine. Two observations we cannot explain at present are the appearance of an extra 5-kilobase EcoRI fragment in total rat embryo DNA and the apparent hypomethylation of the T-ALDH gene in embryo DNA. We have previously demonstrated that T-ALDH is not produced by whole rat embryos or embryonic rat liver (10). This suggests that hypomethylation is perhaps required, but not sufficient, for T-ALDH gene expression.

In summary, expression of the T-ALDH gene in rat hepatoma cell lines occurs via a single 1.7-kilobase transcript. The transcript is encoded by a 3.8- to 9.3-kilobase genomic DNA segment containing at least four introns. Production of T-ALDH RNA correlates with hypomethylation of the T-ALDH gene. Genomic Southern analysis provides no evidence that DNA amplification or rearrangement has occurred in these cell lines compared to normal rat liver or embryonic DNA.

ACKNOWLEDGMENTS

We wish to thank Dr. Snorri S. Thorgeirsson for providing facilities for Northern analysis.

REFERENCES


D. E. Jones et al., manuscript in preparation.
Expression of Tumor-associated Aldehyde Dehydrogenase Gene in Rat Hepatoma Cell Lines

Kwang-huei Lin, Mark D. Brennan and Ronald Lindahl


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/24_Part_1/7009

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.