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Regulation of Aldehyde Dehydrogenase Activity in Five Rat Hepatoma Cell Lines[1]

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ABSTRACT

Significant changes in aldehyde dehydrogenase (ALDH) activity occur during rat hepatocarcinogenesis *in vivo*. An NADP-dependent tumor ALDH isozyme has been studied extensively. To better understand the nature, origin, and importance of this tumor-associated phenotypic change, we have examined the ALDH activity of five well-established rat hepatoma cell lines, H4-II-EC3, HTC, McA-RH7777, JM1, and JM2. HTC, JM1, and JM2 express the tumor ALDH phenotype, as indicated by elevated NADP-dependent, benzaldehyde-oxidizing activity, the appearance of new isozymes by electrophoresis, and characteristic histochemical localization of ALDH activity *in situ*. The tumor ALDH phenotype is not detected in McA-RH7777 cells. H4-II-EC3 has intermediate tumor ALDH activity. Thus, the 5 cell lines provide a spectrum of tumor ALDH activities representative of the range of activities seen *in vivo*. Benzo(*a*)pyrene, 3-methylcholanthrene, and phenobarbital induce hepatic ALDH activity after treatment *in vivo*. The ability of these compounds to induce ALDH *in vitro* was assessed in H4-II-EC3, McA-RH7777, HTC, JM1, and JM2. Treatment of cell cultures for 72 hr with 3-methylcholanthrene (1.0 mM) increases the NADP-dependent ALDH activity in H4-II-EC3 and McA-RH7777 cell lines up to 34- and 11-fold, respectively. Treatment with benzo(*a*)pyrene (1.0 mM) also increases the NADP-dependent ALDH activity in both lines up to 17- and 48-fold, respectively. Treatment with 3-methylcholanthrene or benzo(*a*)pyrene increases ALDH activity 2-fold in HTC and JM2 but does not increase NADP-dependent ALDH activity in JM1. Only marginal increases in NADP-dependent ALDH are observed after phenobarbital treatment in 4 of 5 cell lines. The induction of ALDH is blocked by actinomycin D, α-amanitin, and cycloheximide. These studies support our hypothesis that changes in ALDH activity observed *in vivo* are due to mutational events occurring in initiated cells. It appears that rat hepatoma cell lines will provide an *in vitro* model for studying genetic regulation of the tumor ALDH.

INTRODUCTION

Work in our laboratory has shown that rat hepatomas induced by a number of chemical carcinogens have a unique ALDH[4] phenotype (1, 9, 10, 13). The tumor ALDH phenotype is characterized by increased total ALDH activity due to the appearance of several cytosolic isozymes not detectable in normal liver. The tumor isozymes preferentially oxidize aromatic aldehyde substrates using NADP as coenzyme. They also differ from the normal liver ALDH isozymes in a number of physical and functional properties (9, 14). *In vivo*, expression of the tumor ALDH phenotype is highly variable both between tumors and within a single tumor (28).[5]

In normal rat liver, ALDH activity is localized primarily to the mitochondrial and microsomal fractions, with little or no ALDH activity detectable in cytosol. At least 4 normal liver ALDH isozymes can be differentiated on the basis of substrate and coenzyme preference, substrate and coenzyme K_m, and sensitivity to inhibitors (12, 27). Normal liver ALDH is primarily NAD-dependent, and it oxidizes small aliphatic aldehydes.

In addition to these basal isozymes, several ALDHs can be induced in normal liver *in vivo* by various xenobiotics (5, 6), including 3-MC, BP, and PB. BP and 3-MC induce both NAD- and NADP-dependent ALDH in several strains of rats (26). PB induces a cytosolic NAD-dependent ALDH in certain genetically defined lines and certain strains of rats (4).

To date, our studies of ALDH and changes in the activity of this enzyme during hepatocarcinogenesis have been performed *in vivo* (1). Under such conditions, design and interpretation of experiments for the study of the regulation of hepatic ALDH activity are complex. For this reason, we proposed to establish an *in vitro* system, using stable, hepatoma-derived cell lines to study the genesis and regulation of hepatoma ALDH. Three well-established hepatoma cell lines, H4-II-EC3 (20), HTC (25), and McA-RH7777 (2), and 2 newly established lines, JM1 and JM2 (18), were selected. These lines have been derived from hepatocellular tumors induced by a variety of tumor-induction protocols and have been maintained *in vitro* for varying periods of time.

This paper describes the ALDH phenotype of these 5 cell lines and presents an initial analysis of the mechanisms underlying regulation of hepatic ALDH activity. The results reported also support our hypothesis (13) that the tumor ALDH phenotype is the result of transformation-associated mutational events occurring in initiated cells rather than alteration in liver metabolism due to the administration of a carcinogen.

MATERIALS AND METHODS

Chemicals. BP, 3-MC, PB, actinomycin D, cycloheximide, α-amanitin, dimethylsulfoxide, disulfiram, NAD, NADP, nitroblue tetrazolium, and phenazine methosulfate were from Sigma Chemical Company (St. Louis, MO). Aldehydes were obtained from Aldrich Chemical Company (Milwaukee, WI). Heat-inactivated fetal bovine serum was from Hyclone Company (Logan, UT; Lot 100415). DME medium was purchased from K. C.

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[4] The abbreviations used are: ALDH, aldehyde dehydrogenase [aldehyde:NAD(P) oxidoreductase, EC 1.2.1.3 and 1.2.1.5]; BP, benzo(*a*)pyrene; 3-MC, 3-methylcholanthrene; PB, phenobarbital; GGT, γ-glutamyl transpeptidase; DME, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.

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Culture Conditions for Rat Hepatoma Cell Lines. HTC cells were kindly provided by Dr. E. B. Thompson, National Cancer Institute. JM1 and JM2 were from Dr. G. Michalopoulos, Duke University Medical Center. The McA-RH7777 and H4-II-EC3 lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown routinely under an atmosphere of 5% CO_2 and 95% air as monolayers at 37° in 25-sq cm tissue culture flasks containing 5.0 ml of DME medium supplemented with 10% fetal bovine serum, 1% penicillin:streptomycin, 1% glutamine, and 1% nonessential amino acids. All lines have been maintained in culture for at least 30 generations in our laboratory.

To determine ALDH activity, cells (in DME medium:10% fetal bovine serum) at an early stationary phase were used. Cells were harvested by treatment with 0.025% trypsin (in phosphate-buffered 0.85% NaCl, pH 7.2) followed by centrifugation at 400 × *g*. Pelleted cells were overlayed with buffer and frozen at −80° for assay later.

Preparation of Cell Homogenates. After thawing, cells were prepared as 10% homogenates in 60 mM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol. Homogenates were made to 1% with Triton X-100 (final concentration, v/v) and incubated at 0° for 30 min. The solubilized homogenates were centrifugated at 48,000 × *g* for 30 min, and the resulting supernatants were drawn off for ALDH determinations.

Determination of ALDH Phenotype. ALDH activity was assayed at 25° by monitoring the change in A_{340} caused by NADH and NADPH production during the oxidation of aldehyde substrate in a modification of the assay described previously (8). Propionaldehyde and NAD were used to determine normal liver ALDH activity, and benzaldehyde and NADP were used to determine tumor ALDH activity. Protein concentration was determined by the method of Lowry *et al.* (15) with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis was performed, and gels were stained for ALDH activity as described (8).

For ALDH histochemistry, cells were cultured on 35-mm dishes, grown to confluency, and then stained for ALDH using either propionaldehyde-NAD or benzaldehyde-NADP (11). Occasionally, disulfiram (final concentration, 100 μM) was added to test its effects on ALDH activity (9). GGT activity was also determined in cell cultures. Cells were prepared as for ALDH histochemistry and stained for GGT according to the method of Rutenberg *et al.* (23).

Induction of ALDH Activity. Cells were grown to confluency and then deprived of serum for 12 to 24 hr before addition of the inducing agent. Known *in vivo* inducers of various ALDHs, including 3-MC, BP, and PB, were prepared in DMSO and prepared as 1000× stock solutions. Appropriate dilutions of inducer stock solutions were added to serum-free DME medium to give a final concentration range of 10 μM to 1 mM (final DMSO concentration in the medium, 0.1%). Some control cultures received 0.1% DMSO alone. Other control cultures were maintained in serum-free medium without supplementation. At designated intervals after addition of inducer, cells were harvested by trypsin treatment and frozen at −80° for ALDH activity determinations.

Effects of Actinomycin D, α-Amanitin, and Cycloheximide on ALDH Activity. To study the mechanism of ALDH activity induction, 3 metabolic inhibitors, actinomycin D, α-amanitin, and cycloheximide, were tested for the ability to block ALDH induction by 3-MC or BP. Actinomycin D and cycloheximide were dissolved in distilled water. α-Amanitin was dissolved in DMSO. ALDH activity was induced by 3-MC and BP as before, except that actinomycin D, α-amanitin, or cycloheximide was added to a final concentration of 0.5 μg/ml, 2 μg/ml, or 7 μg/ml, respectively. Inhibitors were added either simultaneously with inducers or 12 hr later. Cells were harvested 48 hr after addition of inducers and stored at −80° until assayed.

RESULTS

The 5 cell lines examined possess a wide spectrum of ALDH activities (Table 1). This range is similar to that seen for primary tumors *in vivo*. HTC, JM1, and JM2 have a high constitutive tumor ALDH activity, as characterized by elevated NADP-dependent activity using benzaldehyde as substrate. H4-II-EC3 has intermediate tumor ALDH activity. McA-RH7777 does not possess detectable levels of the tumor ALDH activity. On a comparative basis, the NAD-dependent ALDH specific activity of McA-RH7777 is below the normal liver range.

Analytical gel electrophoresis indicates that the increased

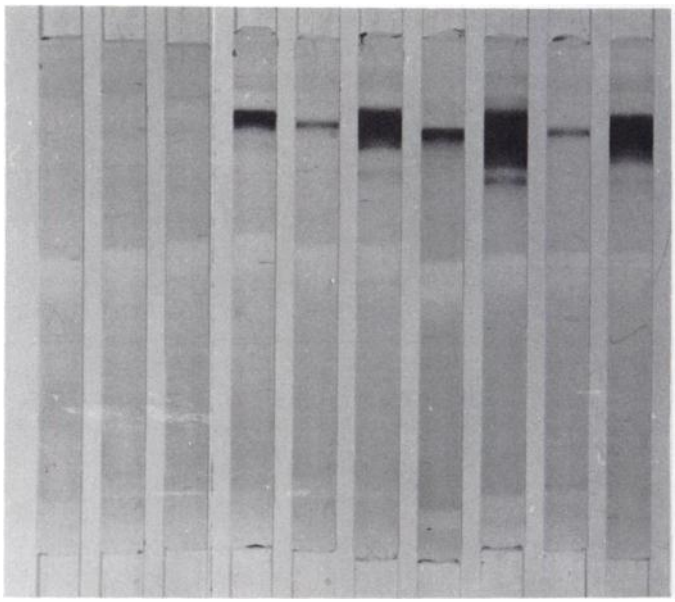

Fig. 1. Polyacrylamide gel electrophoresis of 5 rat hepatoma cell line ALDHs. Gels were electrophoresed at 2.5 mA/gel for 3 hr at 4° and stained for ALDH. *Lanes 1 and 2*, McA-RH7777; *Lanes 3 and 4*, H4-II-EC3; *Lanes 5 and 6*, HTC; *Lanes 7 and 8*, JM1; *Lanes 9 and 10*, JM2. *Odd-numbered gels* were stained with propionaldehyde and NAD. *Even-numbered gels* were stained with benzaldehyde and NADP. In the absence of substrate or coenzyme, no ALDH activity was observed in the gels.

Table 1

ALDH activity in 5 rat hepatoma cell lines

	Specific activity (mIU/mg of protein)[a]						
	Cell lines						
Substrate/coenzyme	HTC	JM1	JM2	H4-II-EC3	McA-RH7777	Primary rat hepatoma[b]	Normal rat liver[b]
Propionaldehyde-NAD	112.5	53.6	155.0	30.1	10.1	84.8 ± 14.6[c]	20.3 ± 0.4
Benzaldehyde-NADP	466.6	212.2	680.7	97.5	5.5	260.4 ± 60.2	7.2 ± 0.3

[a] Data for at least 2 determinations for cell lines. Ranges of activities were routinely within 10% of the mean.
[b] Induced by dietary 2-acetylaminofluorene/PB exposure (13).
[c] Average for 24 tumors and 51 normal livers, ±S.D.

NADP-dependent activity is due to new cytosolic ALDH isozymes appearing in HTC, JM1, JM2, and H4-II-EC3 (Fig. 1). The electrophoretic mobility of these new isozymes is identical to those of the tumor ALDH activity seen *in vivo*.

Histochemically, the 3 high-activity lines HTC, JM1 and JM2 stained intensely for ALDH using benzaldehyde and NADP (Fig. 2). The cell culture is heterogeneous in that not all cells stain, and staining is more intense in regions of cell aggregation compared to monolayer regions. The NADP-dependent ALDH activity of the 3 high-activity cell lines is very sensitive to disulfiram inhibition (data not shown). The cell lines (McA-RH7777 and H4-II-EC3) with lower enzyme activities do not stain histochemically for ALDH (Fig. 2).

Table 2

Induction of NADP-dependent ALDH by 3-MC, BP, and PB in 5 rat hepatoma cell lines

Cell line	Specific activity (mIU/mg of protein)[a]			
	Control[b]	3-MC[c]	BP[c]	PB[c]
H4-II-EC3	69.0	2366.3 (34×)[d]	1198.3 (17×)	445.4
McA-RH7777	17.4	198.1 (11×)	837.7 (48×)	59.1 (3×)
HTC	1022.1	2155.4 (2×)	2590.2 (2.5×)	2042.0 (2×)
JM1	826.0	857.8 (1×)	1195.9 (1.4×)	460.1
JM2	949.8	1741.0 (2×)	1632.5 (2×)	1626.4 (2×)

[a] Data for at least 2 determinations. Ranges of activities were routinely within 10% of the mean.
[b] Control cultures received 0.1% DMSO in serum-free medium. Control specific activities are those at the time of maximal induction by appropriate inducer.
[c] Final concentration, 1 mM.
[d] Numbers in parentheses, multiple of induction, compared to appropriate control.

The relationship of ALDH phenotype to another known tumor biochemical marker, GGT, was examined in the 5 cell lines (Fig. 2). Histochemically, McA-RH7777 is moderately GGT-positive but ALDH-negative. HTC is moderately GGT-positive and strongly ALDH-positive. JM1 and JM2 are slightly GGT-positive but strongly ALDH-positive. H4-II-EC3 is negative for both ALDH and GGT. Consistent with these histochemical observations, Richards *et al.* (22) have reported that HTC and McA-RH7777 have moderately high GGT activity, while the GGT activity of H4-II-EC3 is very low. These results indicate that there is little correlation between ALDH activity and GGT activity.

The specific activities, electrophoretic profiles, and histochemical localization studies indicate that HTC, JM1, and JM2 are representative of ALDH-positive hepatocellular neoplasms seen *in vivo*. By the same criteria, McA-RH7777 is representative of an ALDH-negative neoplasm. The marginally elevated specific activities, the electrophoretic profiles, and negative staining by histochemistry indicate that H4-II-EC3 is representative of a marginally ALDH-positive neoplasm.

Known *in vivo* inducers of ALDH were tested for their ability to increase the ALDH activity in the 5 cell lines (Table 2). Treatment of cells with 3-MC (1.0 mM) increases the NADP-dependent ALDH activity in H4-II-EC3 and McA-RH7777 cell lines up to 34- and 11-fold, respectively. BP at 1 mM also

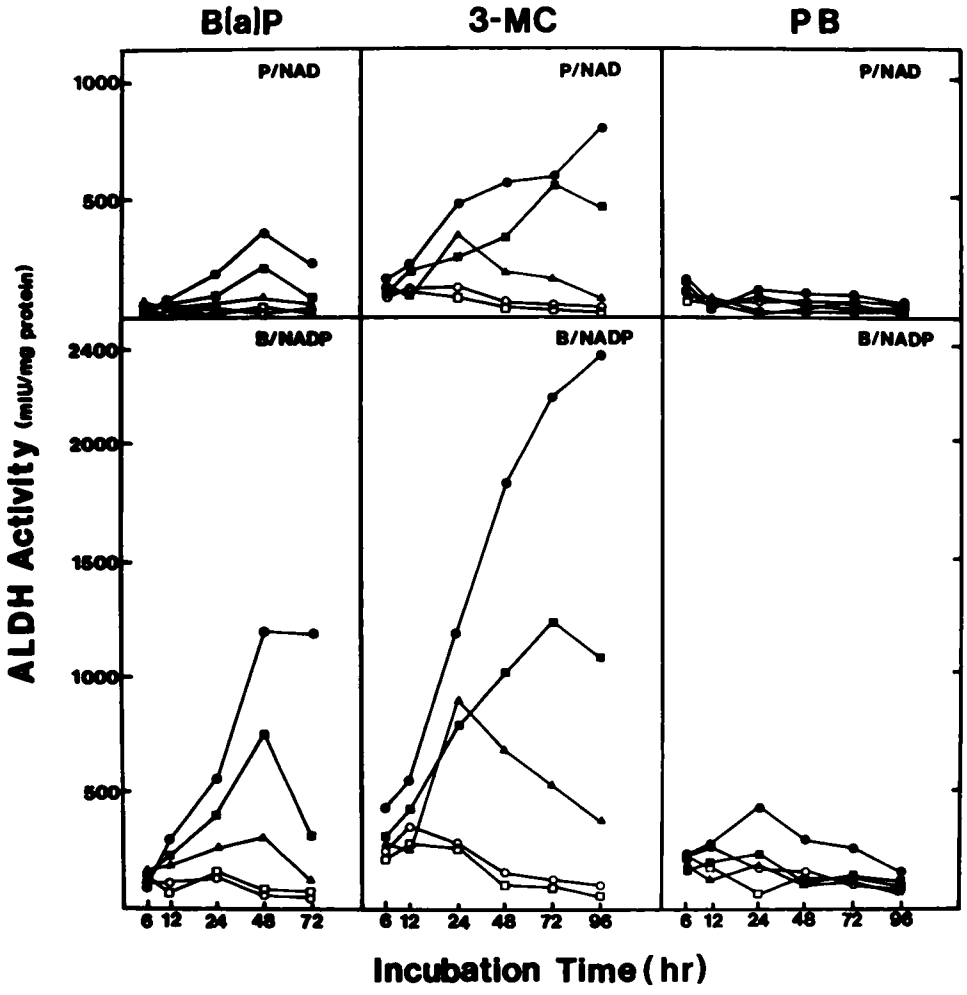

Chart 1. Kinetics and dose response of induction of ALDH in H4-II-EC3 by BP, 3-MC, and PB. Cells were treated as described in "Materials and Methods" and harvested by trypsin treatment at the indicated intervals, and ALDH activity was determined. ●, 1 mM; ■, 0.1 mM; ▲, 0.01 mM; ○, medium control; □, 0.1% DMSO control. Ranges of activities were routinely within 10% of the mean.

increases (up to 17- and 48-fold) the NADP-dependent ALDH activity in both lines. 3-MC and BP at 1 mM increase ALDH activity 2-fold in HTC and JM2 but do not increase NADP-dependent ALDH activity in JM1. Corresponding but only moderate increases in NAD-dependent ALDH activity are seen following 3-MC or BP-treatment of all lines.

Treatment of cells with PB (1.0 mM) causes only marginal increases in NADP-dependent ALDH activity in 4 of 5 cell lines and actually decreases the activity in JM1. Treatment with PB does not increase NAD-dependent ALDH activity in any line. Insulin and dexamethasone do not increase the NAD- or NADP-dependent ALDH activity of any cell line tested (data not shown).

The kinetics and dose-response of the increase of ALDH activity was tested in H4-II-EC3 following 3-MC, BP, and PB treatment (Chart 1). Increased ALDH activity can be detected as early as 6 hr after inducer addition. Maximal increases were observed at about 48 hr (BP, 1 mM), 96 hr (3-MC, 1 mM), or 24 hr (PB, 1 mM) after addition of the appropriate inducer. The increases in ALDH activity were dose dependent over the range of inducer concentrations tested (Chart 1). After maximal levels were reached, ALDH activity gradually declined. In general, similar changes in the time course of ALDH induction were also observed in HTC and McA-RH7777 following treatment with 3-MC and BP (Chart 2). The ALDH activity in the control groups of HTC slightly increased during incubation in serum-free medium.

To distinguish whether the increase in ALDH activity was due to an increase in the total number of cells, to increased ALDH activity in cells already expressing ALDH, or to induction of ALDH activity in ALDH-negative cells, H4-II-EC3 and McA-RH7777 were examined for ALDH histochemically after treatment with 3-MC (Fig. 2). By cell count, there was no increase in the total number of cells. However, the proportion of ALDH-positive cells increased after inducer addition in both lines, indicating the induction of ALDH activity in previously negative cells.

As noted earlier, after maximal induction by 3-MC or BP, ALDH activity gradually declined (Chart 1). To determine whether the decline in ALDH activity observed was due to regulation of the enzyme activity occurring in induced cells or to metabolism or inactivation of the inducer, the ability of preinduced H4-II-EC3 cells to respond to a second exposure to an inducer was tested (Chart 3). Previously induced cells repond to second inducer exposure with a second elevation (approximately 2-fold) of ALDH activity. While addition of fresh medium temporarily increases ALDH activity in uninduced controls, no such response is seen in previously uninduced cells receiving only fresh medium.

To determine whether the increase in ALDH activity is due to an enhanced biosynthesis of the enzyme, inhibitors of transcription and translation were examined for their effect on ALDH inducibility in H4-II-EC3 and HTC (Table 3). When added with inducer, actinomycin D and cycloheximide almost completely blocked induction of NADP-dependent ALDH activity by 3-MC in H4-II-EC3 (Table 3). Under the same conditions, actinomycin D and cycloheximide inhibited BP-induction of ALDH in H4-II-EC3 also. Similarly, α-amanitin significantly inhibited ALDH induction by either 3-MC or BP in H4-II-EC3. In HTC, all 3 inhibitors lowered ALDH to below basal levels when added simultaneously with 3-MC or BP (Table 3). Reductions in actinomycin D, α-amanitin, and cycloheximide inhibition were observed when H4-II-EC3 or HTC were treated with 3-MC or BP for 12 hr prior to inhibitor addition (Table 3). These observations indicate that new tran-

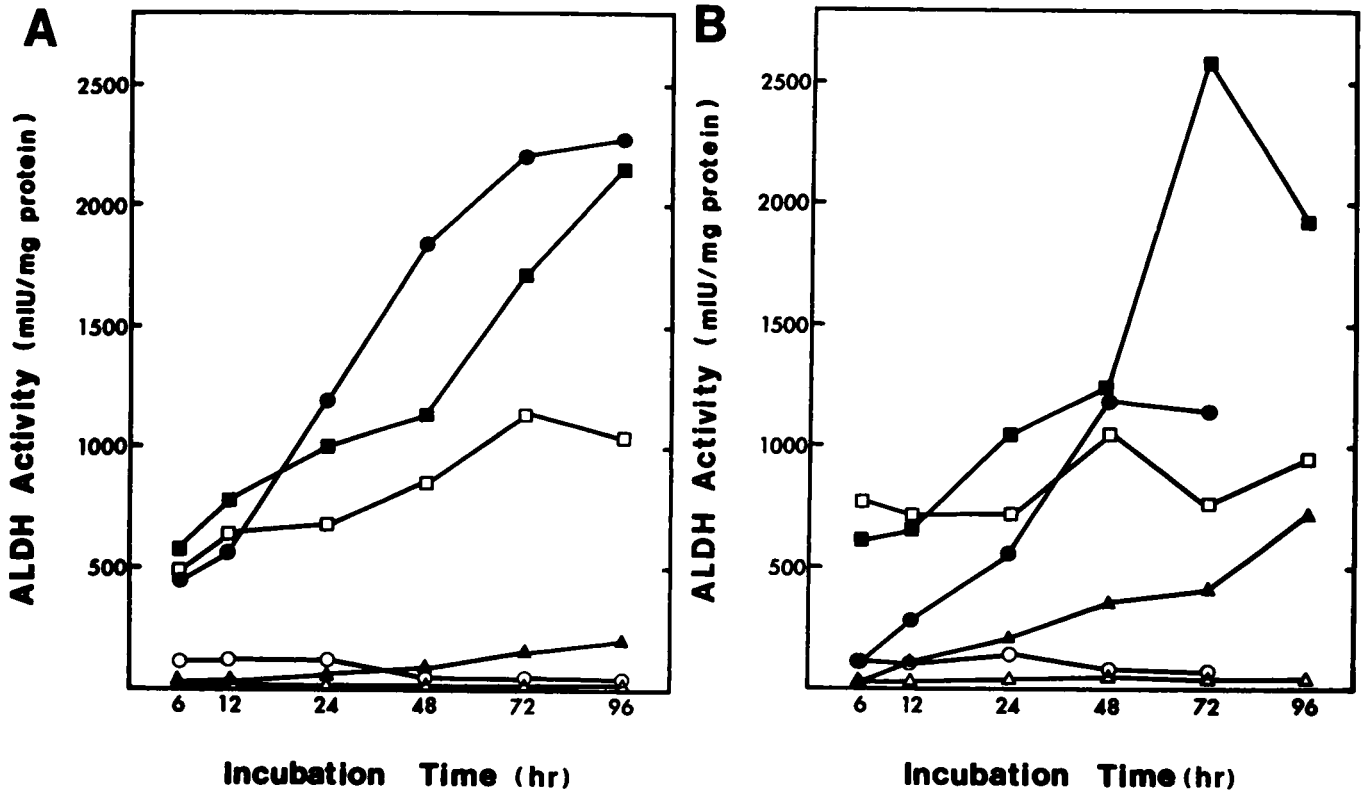

Chart 2. Kinetics of induction of NADP-dependent ALDH by 3-MC or BP in 3 representative cell lines, HTC, H4-II-EC3, and McA-RH7777. *A*, 1 mM 3-MC; *B*, 1 mM BP. Cells were treated as described in "Materials and Methods" and harvested by trypsin treatment at the indicated intervals, and ALDH activity was determined. ●, H4-II-EC3; ■, HTC; ▲, McA-RH7777; ○, H4-II-EC3 control; □, HTC control; △, McA-RH7777 control. Ranges of activities were routinely within 10% of the mean.

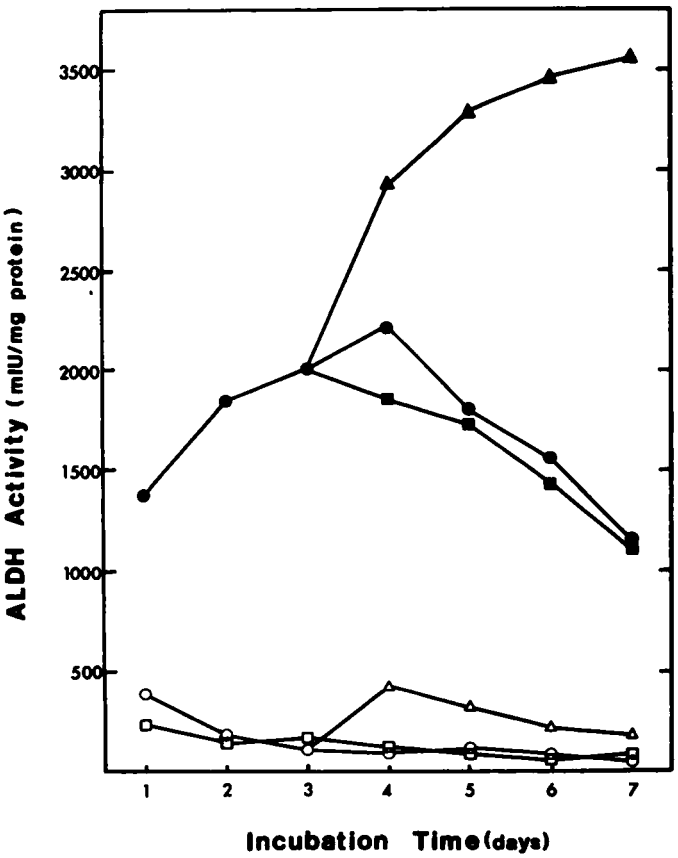

Chart 3. Response of preinduced H4-II-EC3 cells to a second inducer exposure. Seventy-two hr after treatment with 0.1 mM 3-MC (●), the response to a second exposure to 3-MC was tested by adding fresh medium and inducer (▲), changing the medium only (■), or making no changes in culture conditions (●). Control cultures were maintained in serum-free medium without supplementation (○), maintained in medium and 0.1% DMSO (□), or received fresh serum-free medium at 72 hr (△). Ranges of activities were routinely within 10% of the mean.

scription and translation are required not only for inducibility, but also to maintain high ALDH basal activity in HTC.

DISCUSSION

Four of the 5 rat hepatoma cell lines examined express the tumor ALDH phenotype. Each one has maintained its ALDH phenotype at relatively constant levels during approximately 2 years of culture (at least 30 passages) in our laboratory. The range of ALDH activities observed in these 5 cell lines is also representative of the heterogeneity in tumor-ALDH phenotype expression seen in chemically induced hepatocellular carcinomas *in vivo* (11, 13, 28).[5] The broad spectrum of activities in the various cell lines and their long-term stabilities *in vitro* support our hypothesis that the tumor ALDH phenotype is the result of transformation-associated mutational events occurring in initiated cells that are subsequently expressed as the tumor ALDH phenotype (13, 28).

The high basal activity lines HTC, JM1, and JM2 respond minimally to 3-MC and BP induction, while the low activity lines, McA-RH7777 and H4-II-EC3, each respond to induction with significant increases in NADP-dependent ALDH activity. Interestingly, H4-II-EC3 and McA-RH7777 respond differently to 3-MC and BP. H4-II-EC3 is mainly responsive to 3-MC, and McA-RH7777 responds preferentially to BP. Razzouk *et al.* (21) have reported that H4-II-EC3 and McA-RH7777 differ in their microsomal epoxide hydrolase activities, with McA-RH7777 possessing approximately 6 times more benzpyrene 4,5-oxide hydrolase activity than does H4-II-EC3. Novicki *et al.* (18) have reported that JM1 and JM2 are deficient in cytochrome P-450 and NADPH-cytochrome C reductase compared to normal liver. Miyake *et al.* (16) reported that McA-RH7777 had detectable but possibly abnormal cytochromes P-450, P-420, and b5. Whether the differences in inducibility of ALDH in the different cell lines are due to altered regulatory mechanisms at the level of the ALDH gene(s) or to cell line differences in ability to metabolize inducers is currently unknown.

Deitrich *et al.* (5) reported that 3-MC increased hepatic NAD-dependent cytosolic ALDH activity approximately 20-fold in Long-Evans rats. Törrönen *et al.* (26) demonstrated that both multiple BP and multiple 3-MC exposures increased NADP-dependent hepatic ALDH activity of Wistar rats approximately 30-fold. Nakanishi *et al.* (17) also examined the effect of 3-MC on ALDH activity in livers of Wistar, Sprague-Dawley, and Long-Evans rats. Their results suggested both inter- and intrastrain variability in ALDH inducibility.

Perin *et al.* (19) and Sessa *et al.* (24) have reported low NAD-dependent ALDH activity in Yoshida AH 130 hepatoma as well as the non-liver-derived Sarcoma 180 and Ehrlich carcinoma lines. Canuto *et al.* (3) also examined Yoshida hepatoma AH-130 and reported decreased NAD(P)-dependent ALDH activity. While these observations are consistent with the variability in ALDH activity in the cell lines used in this study, the suggestion of Perin *et al.* (19) that diminution of ALDH activity may be related to the neoplastic transformation must be reexamined.

That new transcription and translation are required for induction confirms derepression of gene(s) for ALDH not detectable

Table 3

Effect of actinomycin D, α-amanitin, and cycloheximide on induction of NADP-dependent ALDH

Treatment	Specific activity (mIU/mg of protein)[a]			
	H4-II-EC3		HTC	
Experiment 1				
Control[c]	171.2		814.3	
3-MC (1 mM)[d]	2281.7		1024.8	
BP (1 mM)	980.8		1011.6	
	0 hr[b]	12 hr[b]	0 hr	12 hr
3-MC + actinomycin D	435.0	1280.1	660.2	764.2
3-MC + α-amanitin	ND[e]	ND	494.1	624.0
3-MC + cycloheximide	362.2	600.7	265.4	529.1
BP + actinomycin D	560.4	964.0	199.9	838.5
BP + α-amanitin	ND	ND	376.2	752.3
BP + cycloheximide	365.7	747.7	280.9	394.6
Experiment 2				
Control	85.1			
3-MC	3135.2			
BP	2987.7			
	0 hr	12 hr		
3-MC + α-amanitin	1340.1	2279.3		
BP + α-amanitin	1157.2	2524.4		

[a] Data for at least 2 determinations. Ranges of activities were routinely within 10% of the mean.
[b] Actinomycin D, α-amanitin, and cycloheximide were added simultaneously with inducers (0 hr) or 12 hr later (12 hr). Cells were harvested 48 hr after addition of inducer.
[c] Control cultures received 0.1% DMSO in serum-free medium.
[d] Cells were prepared and treated with 1 mM 3-MC or BP as described in "Materials and Methods."
[e] ND, not determined.

in normal rat liver as the mechanism underlying expression of the tumor ALDH phenotype. The minimal response to inducers in the high basal activity lines HTC, JM1, and JM2, coupled with the ability of transcription and translation inhibitors to reduce ALDH activity of HTC to below basal levels, further indicates that, in these lines the gene(s) encoding the NADP-dependent ALDH has been permanently derepressed in a large portion of the cell population. Although it is impossible to determine with certainty, all of the available data are consistent with this derepression occurring during the genesis of primary tumors from which these lines are derived (up to 18 years ago in the case of HTC).

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Fig. 2. ALDH and GGT activity *in situ* for 5 rat hepatoma cell lines. *A*, ALDH, benzaldehyde-NADP, HTC; *B*, ALDH, benzaldehyde-NADP, JM1; *C*, ALDH, benzaldehyde-NADP, JM2; *D*, GGT, HTC; *E*, GGT, JM1; *F*, GGT, JM2; *G*, ALDH, benzaldehyde-NADP, H4-II-EC3; *H*, ALDH, benzaldehyde-NADP, H4-II-EC3 induced by 1 mM 3-MC, 72 hr; *I*, GGT, H4-II-EC3; *J*, ALDH, benzaldehyde-NADP, McA-RH7777; *K*, ALDH, benzaldehyde-NADP, McA-RH7777 induced by 1 mM 3-MC, 72 hr; *L*, GGT, McA-RH7777. × 125.