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Aldehyde Dehydrogenases and Their Role in Carcinogenesis

Ronald Lindahl

Department of Biochemistry and Molecular Biology, University of South Dakota School of Medicine, Vermillion, South Dakota 57069

Referee: Dr. Henry Weiner, Dept. of Biochemistry, Purdue University, West Lafayette, IN 47907.

ABSTRACT: Aldehydes are highly reactive molecules that may have a variety of effects on biological systems. They can be generated from a virtually limitless number of endogenous and exogenous sources. Although some aldehyde-mediated effects such as vision are beneficial, many effects are deleterious, including cytotoxicity, mutagenicity, and carcinogenicity. A variety of enzymes have evolved to metabolize aldehydes to less reactive forms. Among the most effective pathways for aldehyde metabolism is their oxidation to carboxylic acids by aldehyde dehydrogenases (ALDHs).

ALDHs are a family of NADP-dependent enzymes with common structural and functional features that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes. Based on primary sequence analysis, three major classes of mammalian ALDHs — 1, 2, and 3 — have been identified. Classes 1 and 3 contain both constitutively expressed and inducible cytosolic forms. Class 2 consists of constitutive mitochondrial enzymes. Each class appears to oxidize a variety of substrates that may be derived either from endogenous sources such as amino acid, biogenic amine, or lipid metabolism or from exogenous sources, including aldehydes derived from xenobiotic metabolism.

Changes in ALDH activity have been observed during experimental liver and urinary bladder carcinogenesis and in a number of human tumors, including some liver, colon, and mammary cancers. Changes in ALDH define at least one population of preneoplastic cells having a high probability of progressing to overt neoplasms. The most common change is the appearance of class 3 ALDH dehydrogenase activity in tumors arising in tissues that normally do not express this form. The changes in enzyme activity occur early in tumorigenesis and are the result of permanent changes in ALDH gene expression.

This review discusses several aspects of ALDH expression during carcinogenesis. A brief introduction examines the variety of sources of aldehydes. This is followed by a discussion of the mammalian ALDHs. Because the ALDHs are a relatively understudied family of enzymes, this section presents what is currently known about the general structural and functional properties of the enzymes and the interrelationships of the various forms.

The remainder of the review discusses various aspects of the ALDHs in relation to tumorigenesis. The expression of ALDH during experimental carcinogenesis and what is known about the molecular mechanisms underlying those changes are discussed. This is followed by an extended discussion of the potential roles for ALDH in tumorigenesis. The role of ALDH in the metabolism of cyclophosphamidelike chemotherapeutic agents is described. This work suggests that modulation of ALDH activity may be an important determinant of the effectiveness of certain chemotherapeutic agents. The evidence that changes in ALDH are part of an adaptive response of preneoplastic and neoplastic cells to altered cell physiology or stress is then considered. Roles in the metabolism of aldehydes generated from lipid peroxidation and as part of the *Ah* gene-mediated response to xenobiotic exposure are both discussed. The data are consistent with a role for certain ALDHs in lipid aldehyde metabolism. Biochemical and genetic data also imply that changes in ALDH may be linked, in part, to cellular adaptation to oxidative stress.

Finally, a model of inducible ALDH gene regulation is proposed. The model incorporates current information about ALDH gene expression with the regulation of other genes known to be part of the adaptive responses occurring in neoplastic cells. The model suggests that regulation of class 1 and 3 ALDH gene activity may be complex, involving the tissue-specific ability to respond to a variety of physiological cues. The model also suggests several avenues for future research that should provide a clearer understanding of the regulation of this important gene family in response to a variety of factors.

KEY WORDS: aldehyde dehydrogenases, carcinogenesis, gene regulation.

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I. INTRODUCTION

Aldehydes are ubiquitous in the environment. For example, certain aldehydes such as formaldehyde, acetaldehyde, and acrolein are products of combustion and are present in smog and cigarette smoke.[1] In addition, many foods, especially fruits and vegetables, are sources of aldehydes, including a range of aliphatic and aromatic species[2] (Table 1). Aldehydes are responsible for the flavors and odors of foods and beverages. One particular aldehyde, malondialdehyde, is present in many foodstuffs, increasing in concentration in spoiled foods and in microwave-cooked red meats.

Aldehydes also function as communication molecules[2] (Table 2). They may act as information-transmitting molecules, either between species or within a species. Communication within a species is usually mediated by pheromones, which act to identify individuals or attract males and females for mating. Interspecies communication may be in the form of territory marking or defense. Plant-animal information exchange may also occur through aldehydes. The roles may be attractive, to assist in reproduction, or defensive, acting as natural pesticides.

The majority of aldehydes, however, are encountered as physiologically derived intermediates in the metabolism of other compounds. In this context both endogenous and exogenous sources are significant (Tables 3 and 4).[2] Endogenous sources include aldehydes arising from the metabolism of amino acids, biogenic amines, carbohydrates, vitamins, steroids, and lipids (Table 3). Of considerable recent interest in the context of the present review is the generation of aldehydes as the result of membrane lipid peroxidation.[3]

TABLE 2
Some Aldehydes That Act as Communication Molecules[a]

Propanal	Salicylaldehyde
Hexanal	Benzaldehyde
Hexenal	Crotonaldehyde
Octenal	Acrolein
Decanal	Citral

[a] Compiled from Reference 2.

Xenobiotics are the major exogenous source of aldehydes. The biotransformation of a large number of drugs and other xenobiotics generates aldehyde as intermediates or as products (Table 4). Some of the direct reactions that can produce aldehydes include oxidative deaminations and dealkylations, as well as the oxidation of primary alcohols. Another mechanism of particular relevance to the present discussion is ring hydroxylation adjacent to a heteroatom, followed by tautomerization. This process occurs during metabolism of the antitumor agent cyclophosphamide (CP).[14] Metabolic intermediates can produce aldehydes by hydroxylation or ester linkage hydrolysis.

Aldehydes are generally relatively long lived and highly reactive. Because aldehydes are long lived, they can diffuse or be transported from their site of generation to distant sites, for example, to another cell compartment, another cell, or even another tissue. The electrophilic nature of their carbonyl group is responsible for the high reactivity of aldehydes.

TABLE 1
Examples of Aldehydes Found in Foods[a]

Formaldehyde	Benzaldehyde	Malondialdehyde
Acetaldehyde	Phenylacetaldehyde	Acrolein
Propanal	4-Hydroxynonenal	Glyoxal
Hexanal	Crotonaldehyde	Methylglyoxal
Hexenal	Citral	
Octanal	C_{24-30} aldehydes	
Decanal		

[a] Compiled from Reference 2.

TABLE 3
Examples of Endogenously Produced Aldehydes[a]

Aldehyde	Source
Acetaldehyde	Threonine catabolism
γ-Aminobutyraldehyde	Putrescine catabolism
Betaine aldehyde	Choline catabolism
21-Dehydrocorticosteroids	Corticosteroid catabolism
3,4-Dihydroxyphenylacetaldehyde	Dopamine catabolism
Glutamic-γ-semialdehyde	Proline biosynthesis
Hexanal	Lipid peroxidation
5-Hydroxyindoleacetaldehyde	Serotonin catabolism
4-Hydroxynonenal	Lipid peroxidation
Malondialdehyde	Lipid peroxidation
Retinal	Vitamin A metabolism
Succinic semialdehyde	GABA shunt

[a] Compiled from References 2 and 3.

TABLE 4
Examples of Exogenous Sources of Aldehydes

Source	Aldehyde	Ref.
Combustion	Formaldehyde, acetaldehyde, acrolein	1, 2, 16, 18
Ethanol	Acetaldehyde	4
2-Butoxyethanol	Butoxyaldehyde	5
Diethylnitrosamine	Acetaldehyde	6
N-Butyl-*N*-(4-hydroxybutyl)nitrosamine	*N*-Butyl-*N*-(propyl-3-aldehyde)nitrosamine	7
Succinylcholine	Betaine aldehyde	8
Benzene	*trans, trans*-Muconaldehyde	9
Toluene	Benzaldehyde	10
Xylene	Tolualdehyde	11
Laetrile	Benzaldehyde	12
Codeine	Formaldehyde	13
Phenacetin	Acetaldehyde	13
Cyclophosphamide	Aldophosphamide	14
Nicotine	γ-3-Pyridyl-γ-methylaminobutyraldehyde	15

Most aldehydes exhibit significant biological effects, including cytotoxicity, mutagenicity, genotoxicity, and carcinogenicity.[1-3] Aldehydes cause their effects by reacting with cellular nucleophiles, including proteins and nucleic acids.[2] A variety of aldehyde-protein and aldehyde-nucleic acid adducts have been identified. Most physiologically relevant protein adducts are formed through protein sulfhydryl groups. The relevant nucleic acid adducts are those with nitrogen atoms of both purines and pyrimidines.

With respect to carcinogenesis, aldehydes can act either as carcinogens or as carcinostatic agents. Formaldehyde and acetaldehyde are respiratory tract carcinogens in rats and hamsters.[2,16] In addition, reactive aldehydes can be generated during the metabolism of other nonaldehyde carcinogens. For example, the metabolism of many nitrosamines and nitrosoureas produces aldehydes as intermediates. Diethylnitrosamine (DEN) bioactivation produces acetaldehyde.[5] The urinary bladder carcinogen *N*-butyl-(*N*-4-hydroxy-

butyl)-nitrosamine produces *N*-butyl-*N*-(propyl-3-aldehyde)-nitrosamine.[6] Acrolein (2-propenyl) is an intermediate in the metabolism of a variety of endogenous and exogenous compounds.[2,17] Acrolein is itself an active aldehyde or can be further metabolized to glycialdehyde, which is carcinogenic in both rats and mice.[18] The biotransformation of benzene, a known human carcinogen, produces *trans,trans*-muconaldehyde.[15,16] In many cases it is not known whether these aldehydes can function as ultimate carcinogens. However, the fact that they can form adducts with cellular macromolecules and are mutagenic and/or genotoxic suggests that they may play some role in carcinogenesis.

Because they can be cytotoxic, aldehydes may also be carcinostatic.[2,3,19] Except for certain aldehydes produced as the active metabolites of chemotherapeutic agents, however, interest in carcinostatic aldehydes per se has waned in recent years.[19] Early interest in aldehydes as carcinostatic agents focused on derivatives of pyridine- and isoquinoline-aldehydes.[20–22] These aldehydes were shown to be effective against a variety of transplantable mouse tumors. Similar results were reported for certain α-ketoaldehydes, especially glyoxal and methylglyoxal.[23,24] Most well studied recently have been the carcinostatic aldehydes produced by lipid peroxidation.[2,3] However, the short half-lives and severe side effects of these aldehydes have prevented their clinical usefulness. In light of the multiple effects of such aldehydes on processes related to cell growth and division,[25] perhaps a reassessment of the carcinostatic effects of aldehyde is needed.

Although aldehydes are highly reactive, not all of their interactions with biological systems should be considered deleterious. For example, retinoic acid, the oxidation product of retinal, is involved in embryonic differentiation, and retinal itself is required for vision.[26] Certain aldehydes generated by membrane lipid peroxidation may be chemotactic, recruiting cells to sites of injury or inflammation.[25] A variety of therapeutic agents produce aldehyde intermediates or reactive species. Aldophosphamide, an aldehyde metabolite of the antineoplastic agent CP, gives rise to phosphoramide mustard and acrolein, which in turn mediate the tumor cell-killing effects of this agent.[13,27] Similar mechanisms are involved in the therapeutic effects of other drugs. The cytotoxicity of aldehydes may, therefore, be used to advantage.

Reactions catalyzed by a number of different enzyme systems may produce aldehydes, including enzymes of intermediary metabolism, monoamine and diamine oxidases, a variety of esterases, and many of the phase I and phase II enzymes involved in xenobiotic metabolism. Because the number and substrate specificities of these enzymes are very large, the number of aldehydes that can be generated is virtually unlimited. Many of these enzyme systems are operative in the metabolism of normal cells. It is also apparent that a number of pathophysiological conditions, including cancer, alter the metabolic behavior of cells and tissues. Many of these alterations are reflected in the composition or activity of the enzymes cited above. The result may be the production of additional aldehydes not present in normal tissues or altered levels of normally produced aldehydes. How cells deal with the aldehydes they generate or encounter and the roles of the major aldehyde-oxidizing enzyme system, the ALDHs, in both normal and neoplastic cells are discussed in detail below.

II. ALDEHYDE-METABOLIZING ENZYMES

In mammals aldehydes can be metabolized by three different enzyme systems: aldehyde oxidase, the aldo-keto reductases, and the ALDHs (Figure 1). All three systems have widespread tissue distributions, and forms of each enzyme can be found in virtually all tissues. The three systems have broad and sometimes overlapping substrate preferences. However, each appears to have evolved different, if often poorly defined, physiological functions.

A. Aldehyde Oxidases

Aldehyde oxidase (EC 1.2.3.2, ALOX) is a cytosolic, molybdenum-containing flavoprotein. It is a large (mol wt 300,000) molecule composed of two apparently identical subunits. It can use molecular oxygen as the electron acceptor, gen-

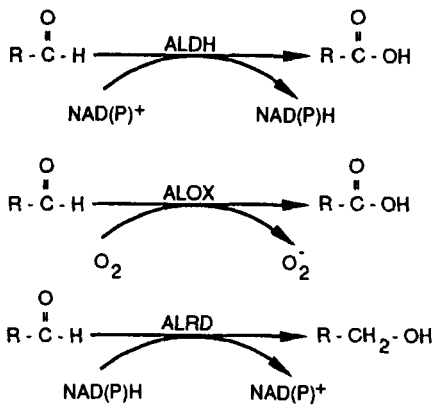

FIGURE 1. Reactions catalyzed by aldehyde-oxidizing enzyme systems.

erating the oxidized product, H_2O_2 and O_2^- (Figure 1). Although ALOX an oxidize certain aldehydes *in vitro,* the physiological function of the enzyme is believed to be the oxidation of purines, pyrimidines, and other nitrogen-containing heterocycles. For a review of ALOX, see Beedham.[28]

B. Aldo-Keto Reductases

Aldo-keto reductases (ALRD) are a family of enzymes that reduce a variety of aldehydes and ketones to their corresponding alcohols. They are small (mol wt 30,000–40,000), monomeric, cytosolic enzymes that use NADPH as coenzyme (Figure 1). Three families of ALRDs are known, and within each family, multiple forms exist. Aldehyde reductase (EC 1.1.1.2) catalyzes the reduction of a variety of aldehydes, especially uronic acids, some semialdehydes, and some ketones. Aldose reductase (EC 1.1.1.21) has a generally overlapping substrate specificity with aldehyde reductase, but prefers aldohexoses as substrates, producing the corresponding polyols. Aldehyde and aldose reductase are closely related structurally, and kinetic differences largely define their differing substrate specificities. Due to its preference for sugar aldehydes, aldose reductase has been implicated in the pathogenesis of several diabetic complications, including cataracts.[29] The carbonyl reductases catalyze the reduction of quinones, ketones, and aldehydes. This enzyme is more distantly related to the aldehyde and aldose reductases and appears to function preferentially in quinone, prostaglandin, and ketosteroid metabolism. Both aldehyde and carbonyl reductases also reduce a wide variety of xenobiotics. For reviews on ALRDs see Flynn[29] and Felsted and Bacher.[30]

C. ALDHs

ALDHs (EC 1.2.1.3) are also a family of related enzymes. They catalyze the irreversible oxidation of a wide variety of aldehydes to their corresponding acids (Figure 1). In contrast to the aldehyde reductases and ALOX, ALDHs are found in virtually every cellular compartment. Some forms of ALDH are constitutive, others are inducible, and both tetrameric and dimeric forms are known. Some forms display broad substrate specificity, oxidizing a variety of aliphatic and aromatic aldehydes, whereas other forms possess narrower substrate preferences. Kinetic studies have indicated that acetaldehyde derived from ethanol oxidation, medium-chain-length aliphatic aldehydes derived from membrane lipid peroxidation, and, perhaps, some aldehydes generated from neurotransmitter metabolism are potential physiological substrates for one or more ALDH forms. Whereas all ALDHs likely use NAD+ as coenzyme *in vivo,* some forms can utilize NADP+ *in vitro.*

In addition to the broad substrate ALDHs, a number of enzymes that use specific aldehydes as substrates are also known. Examples include glyceraldehyde-3-phosphate dehydrogenase,[31] betaine aldehyde dehydrogenase,[32] succinic semialdehyde dehydrogenase,[33] glutamic semialdehyde dehydrogenase,[34] and formaldehyde dehydrogenase.[35] The exact relationship of the broad-substrate ALDHs to these specific substrate enzymes is currently of considerable interest.[36,37] For example, formaldehyde dehydrogenase (EC 1.2.1.1) is a glutathione-dependent enzyme that is identical structurally and functionally to the class III alcohol dehydrogenase (EC 1.1.1.1).[28] However, both the betaine aldehyde and the glutamic semialdehyde dehydrogenases are more closely related to the broad-substrate ALDHs.[36]

The majority of aldehydes appear to be oxidized to their corresponding carboxylic acids.[27,38] This is primarily due to the fact that the oxidation reaction is irreversible, whereas the reductive reactions are generally reversible. Moreover, the catalytic constants (V_{max}/K_m) for ALDH-catalyzed aldehyde oxidations are generally much lower than those for reduction by aldehyde reductases or alcohol dehydrogenases.

D. Mammalian ALDHs

Historically, the naming of mammalian ALDHs has been based on one or more functional or physical properties of the particular enzyme being studied. Additionally, it has been common for each laboratory to develop its own terminology, leading to a large number of names for the various enzyme forms. Names include "high K_m" and "low K_m"; "mitochondrial" and "cytosolic"; "NAD-" and "NADP-dependent"; "E1" and "E2"; and "stomach-," "tumor-," "cornea-," and "2,3,7,8-tetrachlorodibenzo-*p*-dioxin-(TCDD-) inducible." The large number of different names for the same enzyme form has made the ALDH literature quite complex and often confusing. As noted below, only in the past 3 years has sufficient information on the structural relationships of the various ALDHs become available to develop a widely accepted classification scheme for the enzymes based on a single property, their primary sequences.

1. Tissue and Subcellular Distribution

ALDHs are ubiquitous in mammals, being detected at some level in all examined tissues. ALDHs also have a wide subcellular distribution, with one or more forms being identified in the mitochondrial, microsomal, peroxisomal, and/or cytosolic compartments of the cell. In humans, horses, sheep, and cattle, ALDHs are found predominantly in the mitochondria and cytosol.[39–47] In the rodents, ALDHs have been identified in virtually every subcellular compartment.[48–62] Within a tissue or cell, there appears to be a set of constitutively expressed ALDHs. Although there are exceptions, at least one of the mitochondrial and one of the cytosolic ALDHs are constitutive in a particular cell or tissue.

In addition to the constitutive ALDHs, cytosolic-inducible forms of ALDHs have been identified following exposure to certain xenobiotics, including drugs and carcinogens. Two are of particular interest. First, an ALDH can be induced in certain tissues by phenobarbital (PB).[63–65] Second, another form can be induced in certain normal tissues by some chemical carcinogens and by dioxins.[66–70] This second ALDH also appears in certain chemically induced and spontaneous tumors.[71–73] Interestingly, as discussed below, the ALDHs that appear in certain tissues only after an inductive stimulus can be the major constitutive ALDHs in different normal tissues.[74–80]

Liver has been considered to possess the highest ALDH activity, and for certain forms of the enzyme this is indeed the case. Several studies have identified multiple forms of ALDH in rat liver.[49–55,57,61,81] These forms have been differentiated largely on the basis of their kinetic properties. For the rat, mitochondrial ALDHs account for more than 50% of the total ALDH activity of the liver.[50–54,61,81,82–84] Two to three different isozymes can be differentiated on the basis of their substrate K_ms. One isozyme is located in the mitochondrial matrix and has a micromolar K_m for acetaldehyde. The other form(s) is found in the intermembranal space or, perhaps, in the outer membrane and has millimolar substrate K_ms for small aliphatic aldehydes. Microsomes can account for another 35% of the total rat liver ALDH activity.[54,55,61,81] Two microsomal ALDHs having near millimolar substrate K_ms for a variety of aliphatic and aromatic aldehydes have been identified. Although there are some strain differences, the cytosolic fraction of normal rat liver possesses $<10\%$ of the total ALDH activity.[52–54,57,61,81,85] An ALDH activity has also been identified in rat liver peroxisomes.[58,62,86]

An essentially similar ALDH subcellular distribution exists in mouse liver.[60,87–91] A variety of isozymic forms of ALDH have been identified in the mitochondria, microsomes, and cytosol. Mitochondria and microsomes generally possess ALDHs similar to those of the rat. The low-K_m

form is exclusively mitochondrial, and the high-K_m forms are microsomal and cytosolic. However, there appears to be a considerable amount of the constitutive cytosolic ALDH component in mouse liver. Inducible mouse cytosolic ALDHs are also known.[92–94]

Human liver also contains multiple ALDHs, distributed approximately equally between mitochondria and cytosol.[95–97] As for rat and mouse, forms with both high- and low-substrate K_ms are known. The low-K_m mitochondrial and high-K_m cytosolic forms account for the majority of the acetaldehyde-oxidizing capacity of human liver.[39,46] A human microsomal ALDH has also been identified.[98] Like rat and mouse liver ALDHs, different human ALDH forms may have very different substrate preferences.

In all examined species, tissues other than liver also possess significant amounts of ALDH.[49,56,88,90–103] In most cases the exact number and nature of ALDHs present have not been established. For example, brain and kidney possess significant ALDH activity, and the subcellular distribution and characteristics of the isozymes are generally similar to those of liver.[88,90,99,101,104–109]

However, other tissues, most notably cornea, lung, stomach, and urinary bladder, have a different subcellular distribution and activity profile from those of liver.[74,75,77–80,91,110] In these tissues the cytosol possesses a large amount of ALDH activity. In the cornea more than 90% of the total ALDH activity is cytosolic.[74,77,110] In stomach and urinary bladder, the contribution of the cytosol to total ALDH approaches 50%.[78,90] This difference is particularly striking in a species such as the rat, whose normal liver has virtually no cytosolic ALDH activity. Of even more interest is the fact that the major constitutive ALDHs in these tissues are the PB-inducible and/or tumor/dioxin-inducible ALDHs found in liver.[75–78,111,112] For these tissues and others, the contribution of the liver-like constitutive mitochondrial and/or cytosolic ALDHs to total ALDH activity may be significantly reduced.[95,96,113,114] For example, mouse and human hearts appear to lack the constitutive cytosolic ALDH.[90,96] However, this same form is the only ALDH detectable in human erythrocytes.[115]

The pre- and postnatal development of ALDH activity has been examined in several species.[116–121] Generally, ALDH increases during fetal and early postnatal development and reaches the adult tissue-specific activity pattern by 1 to 2 months of age. The increases may be especially rapid during the period from birth to weaning.[118]

In summary, the distribution of the three classes of ALDHs among mammalian tissues is complex. All tissues likely possess mitochondrial ALDH. However, depending on the tissue and species, they may also possess a constitutive cytosolic ALDH. In addition, for some tissues cytosolic ALDHs can also be induced under certain conditions.

2. Purification and Classification

ALDHs have been purified and characterized from a variety of mammalian tissues, including liver,[39–42,44,46,47,55,65,67,84,87,122,130] brain,[105,131–133] stomach,[134–136] cornea,[73,137] lens,[138] and testis.[139] A number of methods differing in detail have been employed. The usual approach is to prepare the appropriate subcellular fraction, solubilize with nonionic detergent as necessary, and fractionate the various activities by ion-exchange and/or affinity chromatography. Occasionally, isoelectric focusing methods have also been employed. Homogeneous or near homogeneous preparations of all the major forms of mammalian ALDH have been isolated by these methods.

In general, the constitutive mammalian ALDHs function as tetramers of approximately 220,000 to 250,000 mol wt. They are composed of identical subunits of approximately 55,000 mol wt, containing approximately 500 amino acids. The PB-inducible ALDH is a dimer of identical 55,000 mol wt subunits, each containing 501 amino acids. The tumor/dioxin-inducible ALDH also functions as a dimer. The identical monomers have a molecular weight of approximately 50,000 and contain 453 amino acids.

Until recently, it was not possible to establish the structural relationships among the various mammalian ALDHs, due to a lack of primary structural information on a sufficient number of enzymes from different sources. In 1988 the pri-

mary sequences were known for the human and horse mitochondrial and cytosolic pairs and the rat tumor/dioxin-inducible ALDH.[140–145] Even with this limited data, it was apparent that different classes of ALDHs could be identified, leading to the development of a standardized nomenclature for mammalian ALDHs based on the primary structure relationships of the various ALDHs[146] (Table 5). Class 1 is the cytosolic forms; class 2, the mitochondrial forms; and class 3, the tumor form. Since 1988 the primary structures of several additional mammalian ALDHs have been reported. Some of these sequences have been determined by protein-sequencing methods, some from their corresponding cDNAs, and others by a combination of these methods. Among them are a mouse cytosolic ALDH,[147] rat and bovine mitochondrial forms,[148] the rat PB-inducible form,[76] and second putative human mitochondrial enzyme.[149] A partial primary structure for the rat microsomal ALDH also exists[36] (Lindahl and Hempel, unpublished data).

Two other protein sequences, deduced from their cDNAs, have been published that contain within them either complete or almost complete ALDH sequences. These are rat liver 10-formyltetrahydrofolate dehydrogenase[150] and a human 56-kDa androgen-binding protein.[151] Two additional proteins originally isolated due to other functions have also been shown to be ALDHs. One has been identified as transparentin or major bovine corneal protein, BCP 54. This protein constitutes approximately 40% of total corneal soluble protein. It is a class 3 ALDH.[152] In addition, a protein first identified and purified as a positional marker in embryonic mouse retina has been shown to be a class 1 ALDH.[153]

In total, 13 complete or partial mammalian ALDH sequences are known. Based on overall alignments and positional identities, all can be placed into one of the three existing classes (Table 6). The human and horse cytosolic ALDHs, the 56-kDa androgen-binding protein, the rat PB-inducible ALDH, and the mouse retinal ALDH all share greater than 85% positional identity and are, therefore, class 1 enzymes. The rat formyltetrahydrofolate dehydrogenase also shows considerable identity to class 1 ALDH. All the mitochondrial ALDHs share greater than 95% identity and are class 2 enzymes. The tumor/dioxin-inducible ALDH, the rat constitutive microsomal ALDH, and BCP 54 are at least 77% identical and considered to be class 3 ALDHs. Among the classes, classes 1 and 3 contain both constitutive and inducible forms, whereas for class 2 only constitutive forms are known.

TABLE 5
Nomenclature of Mammalian ALDHs

Class 1 Aldehyde dehydrogenase *(ALDH1)*
Members of the class 1 ALDHs are the constitutive and inducible cytoplasmic ALDHs *human, horse,* beef, rat, mouse, sheep, and dog liver and the ALDH *inducible by PB*

Class 2 Aldehyde dehydrogenase *(ALDH2)*
Members of the class 2 ALDHs are the mitochondrial ALDHs isolated from *human,* horse, rat, beef, mouse, sheep, and dog liver

Class 3 Aldehyde dehydrogenase *(ALDH3)*
Members of the class 3 ALDHs are the rat liver *tumor-specific*/TCDD-inducible/3-MC-inducible cytoplasmic ALDHs. Also included in this class are the major constitutive or inducible ALDHs from rat liver microsomes and mammalian cornea, stomach, lung, and urinary bladder

Note: Sources identified in italics are considered the prototype enzyme for the class, based on primary structure determination. Other representatives are based on kinetic, physical, and immunological properties or subcellular distribution. See Reference 36 for details of the assignments of a particular form to a class.

3. Substrate Specificities

Given that the different classes of ALDH differ in tissue and subcellular distribution, it would not be surprising to find that the various ALDHs have different substrate preferences. This is indeed the case. However, for only a few ALDHs in a small number of tissues have putative physiological substrates been identified. In fact, it seems likely that a particular ALDH may be able to oxidize efficiently a number of aldehyde substrates, the exact substrate varying with the tissue and/or physiological situation. For example, the local concentrations of both potential substrates and enzyme within a subcellular compartment or region can have significant effects

TABLE 6
Known Mammalian ALDH Primary Sequences[a]

Enzyme	Source	Ref.
Class 1 constitutive	Human liver cytosol	140, 151, 154
	Horse liver cytosol	141
	Mouse liver cytosol	147
Class 1 inducible	Rat liver cytosol	76
	Mouse retina	153
Class 2 constitutive	Human liver mitochondria	142, 149, 154
	Horse liver mitochondria	143
	Rat liver mitochondria	148
	Beef liver mitochondria	148
Class 3 constitutive	Rat liver microsomes	Unpublished data
Class 3 inducible	Rat liver	75, 144
	Beef cornea	152

[a] See Reference 36 for details.

on oxidation rates. As for most enzymes, identification of potential physiological ALDH substrates has relied primarily on determination of K_m values. Generally for the ALDHs, substrate K_m values reflect substrate preferences identified by more precise catalytic efficiency measures such as V_{max}/K_m determinations. V_{max}/K_m values allow for local differences in enzyme concentration and/or activity and are especially useful when little is known about substrate or enzyme concentrations. However, when the subcellular levels of putative substrates or enzymes are known, K_m values, interpreted in the context of such information, can be useful in identifying potential physiological substrates. In this review, when K_m values are cited, they accurately reflect catalytic efficiency.

With respect to coenzyme specificity, all ALDHs have K_ms for NAD+ in the micromolar range.[41,42,46,55,57,123,124,133,134] Although some variability exists, generally class 1 ALDHs have K_ms of 1 to 10 μM, whereas the class 2 forms have K_ms in the 20- to 50-μM range. Class 3 ALDHs are the most variable, having K_ms for NAD+ ranging from approximately 5 μM for the enzyme from cornea to near 100 μM for the microsomal form.

Although NADP+ is a relatively good coenzyme for the class 3 ALDHs (K_m of 60 to 200 μM) and has been valuable as a marker for class 3 ALDH activity *in vitro* (see below), catalytic constants (i.e., V_{max}/K_m) indicate that even for the class 3 ALDHs, NAD+ is likely the preferred coenzyme *in vivo*. However, the use of NADP+ as coenzyme by the class 3 forms under certain physiological conditions should not be ruled out. The K_ms and catalytic constants for NADP+ for class 1 and 2 ALDHs are at least 10 to 100 times higher than those for NAD+, indicating that NAD+ functions as coenzyme *in vivo* for these forms.

As already noted, the spectrum of endogenous and exogenous aldehydes that are potential substrates for the ALDHs is vast (Tables 1 to 4). For most examined aldehydes, at least one ALDH can catalyze its oxidation with a K_m in the micromolar range. Small aliphatic aldehyde substrates, e.g., acetaldehyde, propionaldehyde, and malondialdehyde, are excellent substrates for both class 1 and class 2 constitutive enzymes.[42,46,49,52,123] The role of various ALDHs in the metabolism of acetaldehyde derived from ethanol is of considerable current interest. At the other extreme, large aldehyde-containing molecules, e.g., some corticosteroid aldehydes[155,156] and aldophosphamide derived from CP,[91] may also be substrates for certain class 1 or 2 forms. Biogenic aldehydes such as those derived from neurotransmitter metabolism[157,158] and 4-hydroxyalkenals from lipid peroxidation[159] are further examples of excellent substrates for various class 1 and/or 2 ALDHs.

The class 3 ALDHs also appear to have broad substrate specificities. For the prototypic class 3 ALDH (the liver-inducible and cornea and urinary bladder constitutive forms), there is good evidence that one physiological role for this particular enzyme is the oxidation of medium-chain (C6 to C9) aliphatic aldehydes derived from lipid peroxidation.[74,77,150] For normal tissues such as the cornea, the class 3 ALDH probably plays a key role in protecting the cornea from toxic effects of aldehydes generated by UV-induced lipid peroxidation.[74] Interestingly, malondialdehyde and 4-hydroxyalkenals generated by lipid peroxidation are not substrates for the class 3 ALDH. The microsomal class 3 ALDH appears to play a similar role in tissues where it is a major constitutive ALDH, such as the rat liver, which lacks constitutive class 3 ALDH activity. The class 3 forms also oxidize benzaldehydelike, but not phenylacetaldehydelike, aromatic aldehydes effectively.[161]

4. Molecular Biology and Evolution

Complete or partial complementary DNAs have been generated for members of each ALDH class (Table 6). The genomic regions encoding the human class 1 and 2 ALDHs have been isolated and characterized.[162,163] This information has been instrumental in establishing the relationships between the various forms and the development of the classification system. The data indicate that at least five different genes encode the major mammalian ALDHs (Table 6). The constitutive class 1 and 2 forms, the class 1 (PB-inducible), the class 3 microsomal, and the class 3 tumor/cornea enzyme all appear to be encoded by separate genes.

Amino acid sequences of the mammalian ALDHs can be aligned, based on cDNA and/or primary sequence data (Figure 2A). The lengths of the ALDH polypeptides range from 453 to 520 amino acids. All enzymes possess a common core of 430 amino acids, extending from residues 57 to 500 (using the class 1/2 numbering system [Figure 2A]). The class 3 forms have amino acid sequences extending several residues beyond the carboxyl terminus of the core sequence. The class 1 and 2 forms have 56 amino acid residue extensions to the N terminus of the core. The class 2 forms also possess N-terminal signal sequences.

The human class 1 and class 2 ALDH genes cover approximately 50 kb, and each consists of 13 coding exons separated by 12 introns (Figure 2B). The human class 1 ALDH gene is located on chromosome 9, and the class 2 gene is found on chromosome 12.[97,154,164] The major constitutive mouse class 1 ALDH gene *(Ahd-2)* maps to chromosome 19.[165] The location of the major mouse class 2 locus *(Ahd-5)* has not yet been determined.

Recently, a new ALDH gene has been identified from a human genomic library.[149] This gene, called *ALDH x*, appears to encode a mitochondrial ALDH distinct from any known ALDH. The gene is located on human chromosome 9 and may be transcriptionally active in liver and testis. The size of the putative transcript is much larger (3 kb) than any other ALDH mRNA. Nucleotide sequencing indicates the gene has a unique structure. It lacks introns in its coding region and has an intron (2.6 kb) in its 5′-untranslated region. The relationship of this enzyme to the other ALDHs remains to be established.

Even though the genomic organization of a class 3 ALDH is not yet available, some interesting observations regarding its putative organization and relationship to the class 1 and 2 genes have been noted.[36,145] The N terminus of the class 3 enzyme corresponds exactly to the beginning of exon 3 of the class 1 and 2 genes. Two of the gaps that are required for proper protein sequence alignment occur at exon boundaries. Additionally, the C-terminus extension of the class 3 protein appears to be the result of altering a stop codon to a coding codon. These observations suggest that exon addition, splice junction alterations, and stop codon migration have occurred during the evolution of the class 1 and 2 structures from an ancestor common to class 3.

The mouse homolog to the inducible class 3 ALDH *(Ahd-4)* maps to chromosome 11.[166] This study also provides genetic evidence for the identity of the stomach and cornea forms of class 3 ALDH. The human class 3 ALDH gene has been mapped to chromosome 17.[98] The major mouse constitutive class 3 ALDH *(Ahd-3)* has been identified but not mapped.[167]

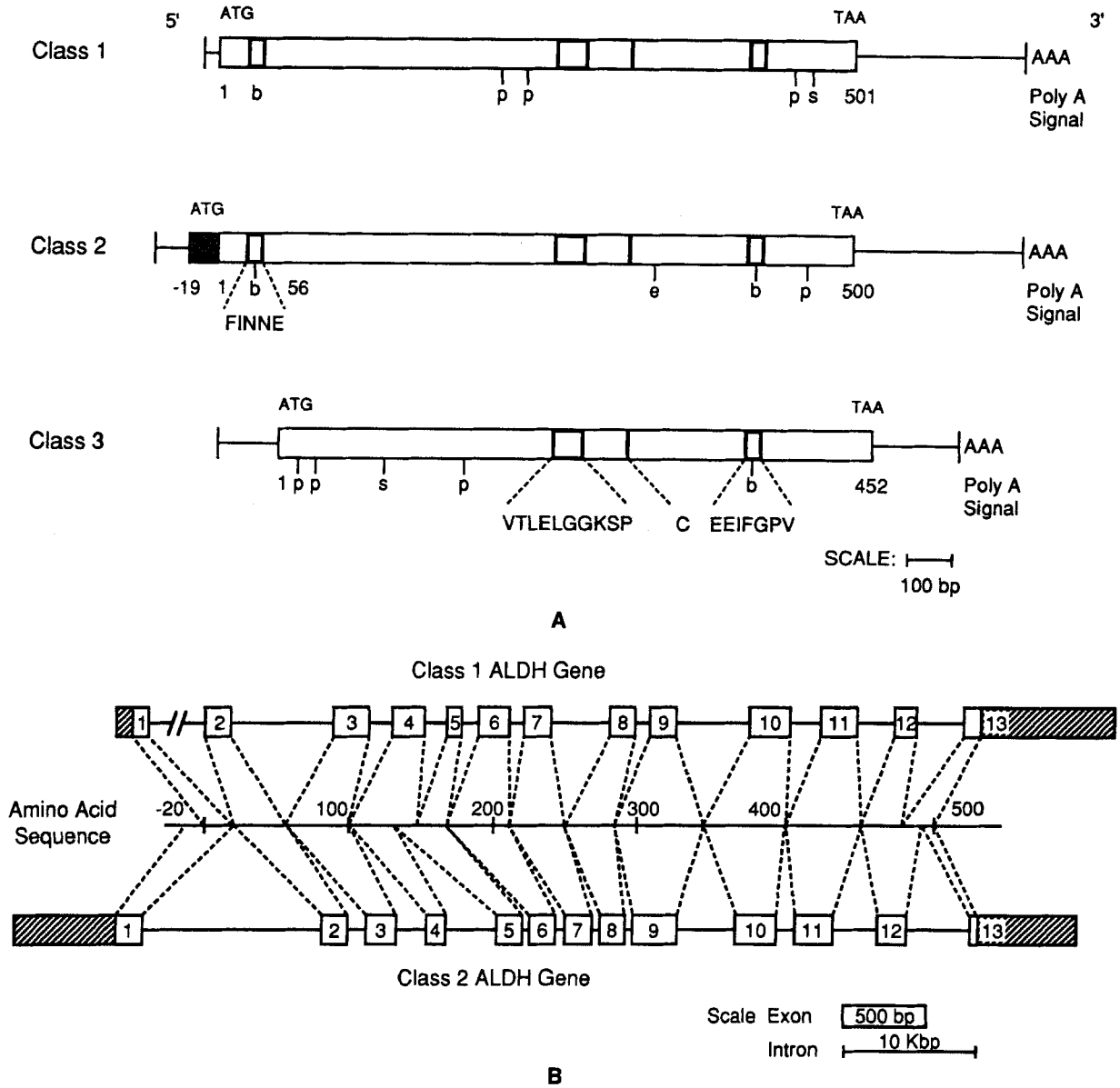

FIGURE 2. (A) Mammalian ALDH cDNAs. The alignment is based on the human, rat, and mouse class 1 sequences;[76,147,154] the human, rat, and bovine class 2 sequences;[148,154] and the rat tumor-specific and TCDD-inducible sequences.[75,144] Arabic numbers represent positions of key amino acid residues. The bold areas and corresponding one-letter sequences are regions of complete primary sequence identity between all known ALDHs. Lowercase letters represent restriction enzyme sites in rat cDNAs: b — Bgl II; e — Eco R1; p — Pst I; s — Sac I. Hatched area at the 5′ end of the class 2 cDNA encodes the mitochondrial signal peptide. (B) Genomic organization of human class 1 and class 2 ALDHs. Exons are boxed and dotted lines indicate alignment of amino acid sequences encoded by exons. 5′- and 3′-untranslated regions are indicated by shaded boxes. (Simplified from Reference 97 with permission.)

Although the human class 1 and 2 genes have been isolated, no data are yet available on the regulatory regions involved in ALDH gene expression. As discussed below, this is an area that should provide a wealth of information in the future regarding the differential expression of this gene family.

III. ALDHs AND TUMORIGENESIS

Carcinogenesis is generally considered to be a multistage process. The first step, termed initiation, is the result of changes occurring in cells following their interaction with a carcinogen. For most chemicals, carcinogenic forms result from

the metabolic transformation of the parent compound by phase I and II drug-metabolizing enzymes. Initiation involves the interaction of activated forms of the carcinogen with critical cellular macromolecules. For genotoxic chemical carcinogens, the critical molecule is DNA. Initiation events are considered to be irreversible and heritable. Initiated cells, therefore, represent a persistent cell population from which overt neoplasms can arise. They are generally termed putative preneoplastic or premalignant cells.

Promotion is the second stage of carcinogenesis. During promotion, initiated cells are further altered in such a manner that expression of the cancer phenotype results. Promotion is generally considered to be a continuous, epigenetic, and reversible process. Most promoting agents are tissue specific and selectively expand clonal populations of initiated cells. They may work by being cytotoxic to uninitiated cells or by being preferentially growth stimulatory to initiated cells. The final stage of carcinogenesis, in which further phenotypic changes in clonally expanding tumor cell populations occur, is called progression. Progression may include the conversion from a benign to a malignant tumor as well as changes that produce tumor phenotype heterogeneity.

Multistage models have been described for carcinogenesis in all major tissues, including skin, liver, breast, colon, pancreas, and urinary bladder. Using these models the events involved in initiation and promotion have been studied in considerable detail. From these studies one conclusion is that a number of preneoplastic or premalignant cell populations can be identified at the very early stages of carcinogenesis by a variety of biochemical and/or morphological changes undergone by these cell populations. Many of the changes involve physiological, cellular, and biochemical alterations that can confer on the cells a selective proliferative advantage over normal cells.[168]

For liver carcinogenesis, among the earliest identifiable changes is the appearance of focal populations of hepatocytes that differ histochemically from normal hepatocytes.[169] These altered hepatic foci (AHF) are believed to represent one population of initiated, putative premalignant cells. A substantial portion of these AHF subsequently disappear during the early stages of promotion. However, some foci continue to progress along the path toward the cancer phenotype. Whether these persistent focal populations themselves progress or a new cell population with a progressively neoplastic phenotype arises within these persistent foci is currently unknown.[169] The result, however, is a small population of larger premalignant lesions often referred to as preneoplastic nodules, premalignant hepatic nodules, or, simply, hepatocyte nodules. A small fraction of these hepatic nodules are believed to progress to malignant cancers. For the purposes of this review, the simple model of initiated hepatocyte → AHF → hepatocyte nodule → hepatocellular carcinoma is assumed. It should be noted, however, that other pathways to liver cell cancer, involving other liver cell types, may exist.[170]

In 1972 Feinstein and Cameron[71] reported that ALDH activity was elevated in rat hepatomas induced in Sprague-Dawley rats by the aromatic amine 2-acetylaminofluorene (2-AAF). Since that initial report, changes in ALDH occurring during carcinogenesis in a number of tissues have been reported. Although most work has involved rodent liver carcinogenesis,[72,171–185] alterations in ALDH activity have been demonstrated during rodent urinary bladder carcinogenesis,[112] in rodent and human leukemic cells,[186–188] in human colon[189] and liver[190] primary neoplasms, and in a human mammary adenocarcinoma cell line.[191]

Reports of additional examples of changes in ALDH activity during tumorigenesis continue to appear. However, work in the past several years has increasingly focused on the molecular mechanisms underlying changes in ALDH activity and on the physiological roles for ALDH in tumor cells. With respect to mechanisms, considerable evidence now exists that in those preneoplastic and neoplastic cells that exhibit changes in ALDH activity, altered transcriptional activity of an ALDH gene is responsible. To date, changes in class 1 and class 3 ALDH gene expression have been described.

As for possible functions of ALDHs during tumorigenesis, three areas are of current interest. Among the preferred substrates for class 3 ALDH are medium-chain-length aldehydes derived from lipid peroxidation. Therefore, several groups are currently focusing on the role of ALDH in the metabolism of these potentially cytotoxic mole-

cules in normal and tumor cells. There is also considerable evidence that differences in the ALDH activity of certain tumor cells may affect their sensitivity to antitumor drugs such as CP. In addition, class 3 ALDH gene appears to be a member of the aromatic hydrocarbon- [*Ah*-] responsive gene battery. In this context, there is much current interest in the expression of the class 3 ALDH gene in normal and abnormal cells in relation to xenobiotic-induced cellular damage, including oxidative stress. The interrelationships between these potential ALDH functions in tumorigenesis are also of interest.

In the remainder of this review, the "tumor ALDH phenotype" will be described, and the evidence for alterations in gene expression as the mechanism of its expression will be presented. Finally, the various hypotheses for the functional roles of tumor ALDH in both normal and neoplastic cells will be evaluated.

A. The Tumor ALDH Phenotype

Early characterization of the tumor ALDH phenotype that used whole tissue preparations indicated that tumor ALDH preferentially oxidized aromatic aldehydes, using NADP as coenzyme.[171] In contrast, normal liver ALDH was primarily NAD-dependent and oxidized small aliphatic aldehydes. The tumor ALDH also differed from normal liver ALDH in a number of physical properties. The electrophoretic mobility and isoelectric point of tumor ALDH were different from those of normal liver ALDH.[171] The tumor form also appeared to be more stable to heat, pH extremes, and guanidine·HCl and urea than was normal liver ALDH.[171]

Early studies of the generality of the tumor ALDH phenotype indicated that primary hepatic tumors induced in Sprague-Dawley, Buffalo, or Fischer rats by dietary 2-AAF or 4-dimethylaminoazobenzene, with or without PB promotion, invariably showed the tumor ALDH phenotype.[171] However, only 1 of 29 ethionine-induced liver tumors possessed this phenotype by the criteria then available. A series of Morris hepatomas of different growth rates, carried in Buffalo rats, uniformly lacked the tumor ALDH. In fact, these tumors had barely detectable ALDH activity. Spontaneous mouse liver tumors also lacked tumor ALDH, as did tumors appearing in other organs of rats bearing high-ALDH-activity, AAF-induced hepatic tumors. No correlation was observed between tumor histology and ALDH activity. The tumor ALDH phenotype was not due to reexpression of an embryonic or fetal liver ALDH.[116]

The tumor ALDH was purified from AAF-induced liver tumors.[72] On SDS-polyacrylamide gels, a single polypeptide of 53,000 kDa was seen. On nondenaturing polyacrylamide gel electrophoresis, a series of three to five isoforms with ALDH activity were resolved. Polyclonal antibodies against the purified tumor ALDH were generated. Subcellular distribution studies indicated that tumor ALDH was localized to the cytosol.[81] This study also confirmed that in normal rat liver, ALDH activity consists of at least three forms, localized largely to the mitochondrial and microsomal fractions.[52,54]

During this period two other ALDHs inducible in normal rat liver cytosol were described. One was the class 1 ALDH, inducible by PB in certain genetically defined strains.[63,64] The second form was the class 3 ALDH inducible by TCDD.[67,68] From the outset it was clear that the tumor ALDH was functionally and structurally distinct from the PB-inducible form.[73] In contrast, the tumor and TCDD-inducible ALDH, although clearly differing in the kinetics of induction, appeared to be identical. This conclusion was based on the then available criteria of substrate and coenzyme preference, electrophoretic properties, and immunochemistry. As already discussed and to be described in detail below, the TCDD-inducible and tumor ALDHs are identical proteins. They represent two different means of expression of the same class 3 ALDH gene.

As a result of these early studies, two hypotheses emerged regarding the origin of the tumor ALDH. Was the tumor ALDH phenotype the result of altered liver metabolism due directly to carcinogen and/or promoter exposure? Or was it the result of transformation-associated, stable genetic changes induced in cells affected by carcinogen? To address these questions, a series of studies were undertaken that examined the expression of the class 3 ALDH during the time

course of rat hepatocarcinogenesis, using a variety of tumor induction protocols[168,174–178] (Table 7).

The employed protocols were selected because they represented a variety of initiation and promotion schemes known to produce liver cancer in rodents.[192–195] The protocols employ a number of different initiators of differing genotoxicity coupled with a number of different mechanisms for selecting initiated cells and allowing their progression to overt neoplasms. For example, the resistant hepatocyte model uses cytotoxic levels of a genotoxic initiator, DEN, to initiate large numbers of hepatocytes, followed by a hepatotoxin such as 2-AAF or CCl_4 and partial hepatectomy to kill noninitiated hepatocytes and produce highly synchronous populations of initiated cells that progress through the early stages of tumorigenesis together.[196,197] The resistant hepatocyte model represents a protocol of high selective pressure. Initiated cells are allowed to proliferate under conditions in which normal hepatocyte growth is inhibited. The ethionine-choline deficiency protocol of Shinozuka et al.[198] combines dietary exposure to the nongenotoxic initiator ethionine with the highly efficient but very low selective pressure of a dietary choline deficiency. The dietary 2-AAF and AAF-PB[199] and the DEN injection into 1-d-old rats, followed by dietary PB promotion at weaning, protocols of Peraino and colleagues,[200] represent tumor induction regimens of intermediate selective pressure. The same is true for the partial hepatectomy- (PH-) DEN-PB protocol of Pitot et al.[201] These three protocols use genotoxic initiators in relatively low doses combined with the efficient, noncytotoxic, promoting activity of PB. For detailed reviews of the use of the protocols in elucidating mechanisms of chemical carcinogenesis, the reader is referred to the reviews of Emmelot and Scherer,[192] Pitot and Sirica,[193] Pitot,[169] Farber and Rubin,[168] and Farber.[194,195]

Examining the time course and localization of class 3 ALDH during hepatocarcinogenesis induced by these protocols indicated that the tumor ALDH phenotype is the result of transformation-associated, stable genetic changes induced in initiated cells. Every protocol produced

TABLE 7
Protocols Used to Study Expression of ALDH During Carcinogenesis

Protocol	Tissue	Initiator	Promoter	Comments	Ref.
Peraino et al.[199]	Liver	2-Acetylaminofluorene	PB	One month dietary AAF followed by continuous dietary PB	172
Peraino et al.[200]	Liver	Diethylnitrosamine 2-Acetylaminofluorene	PB PB	I.p. injection of initiator to 1-d-old rats followed by dietary PB at weaning	175
Solt and Farber[196,197]	Liver	Diethylnitrosamine	PH with 2-AAF or CCl_4	I.p. injection of necrogenic dose of DEN followed by exposure to hepatotoxin (2-AAF or CCl_4) and PH	176, 179, 183, 184
Shinozuka et al.[198]	Liver	Ethionine	Choline deficiency	Dietary ethionine exposure coupled with diet deficient in choline	177
Pitot et al.[201]	Liver	Diethylnitrosamine	PH and PB	Exposure to DEN following PH followed by dietary PB; many variations	178, 181, 182, 185
Epstein et al.[202]	Liver	2-Acetylaminofluorene	None	Repeated cycles of dietary 2-AAF; acts as complete carcinogen	179
Irving et al.[203]	Urinary bladder	*N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine	None	Long-term exposure to BBN in drinking water; acts as complete carcinogen	112

malignant neoplasms expressing class 3 ALDH (Table 8). However, each protocol provided unique insights regarding the mechanism of expression. The proportion of neoplasms expressing the phenotype was directly related to the selective pressure of the protocol. Ninety-six percent of the neoplasms generated by the resistant hepatocyte model expressed class 3 ALDH.[176] Only 25% of the carcinomas induced by the ethionine-choline deficient diet protocol were ALDH-positive.[177] The intermediate selective pressure protocols produced 50 to 60% ALDH-positive neoplasms.[172,174,175,178] Interestingly, the specific activity of the ALDH-positive tumors was not closely correlated with selective pressure, although the ethionine-choline-induced tumors were least active. Exposure to an initiating agent is absolutely required for expression of the tumor ALDH phenotype.

The usefulness of employing several different probes to examine the tumor ALDH phenotype was also clearly demonstrated during these studies. The use of total activity assays with different substrate and coenzyme combinations measured the relative contributions of class 1, 2, and 3 ALDH activity in late-stage neoplastic nodules and carcinomas, but it did not detect activity changes early in tumorigenesis. However, histochemical analysis identified significant and characteristic changes in ALDH as early as 1 month after initiation (the resistant hepatocyte model) in the form of class 3 ALDH-positive enzyme-altered foci[176–178] (Figure 3c). ALDH-positive neoplastic nodules were also first identified histochemically.[176–177] Other early histochemical changes were also noted. They included changes in ALDH distribution within a liver acinus from centrilobular to periportal[177,178] (Figure 3g) and the appearance of occasional foci that were devoid of any ALDH activity.[177] Histochemistry also identified carcinomas not found by gross examination.

Histochemistry also confirmed that the changes in ALDH activity occurring during hepatocarcinogenesis were limited to hepatocytes. Changes in other liver cell populations that occurred in some protocols were uniformly ALDH-negative. This included areas of fibrosis, cystic degeneration, and cholangiofibrosis, as well as neoplasms of other cell populations, such as cholangiocarcinomas.[177] The ALDH activity of individual hepatocellular carcinomas generated by a particular protocol was also quite variable.[172,174–178] Histochemical analysis also indi-

TABLE 8
Distribution of Tumor-Associated Class 3 ALDH Activity During Hepatocarcinogenesis

	Protocol[a]					
	Resistant hepatocyte	Dietary AAF/PB	Dietary AAF only	DEN/PB (1 d old)	PH/DEN/PB	Ethionine + choline deficiency
Percentage of class 3 ALDH-positive tumors[b]	96 (26/27)[c]	80 (19/24)	58 (7/12)	62 (13/21)	46 (6/13)	25 (7/28)
Class 3 ALDH activity of positive tumors	108[d]	327	201	274	73	32
Class 3 ALDH activity of all tumors examined	108	260	120	176	43	10
Class 3 ALDH activity of normal liver	5	9	8	13	5	6

[a] See Table 7 for descriptions.
[b] Tumors expressing tumor-associated ALDH phenotype by total activity assays, gel electrophoresis, or histochemistry.
[c] Number of ALDH-positive tumors in total number of examined tumors.
[d] Specific activity (milliinternational units per milligrams protein) (adapted from Reference 177).

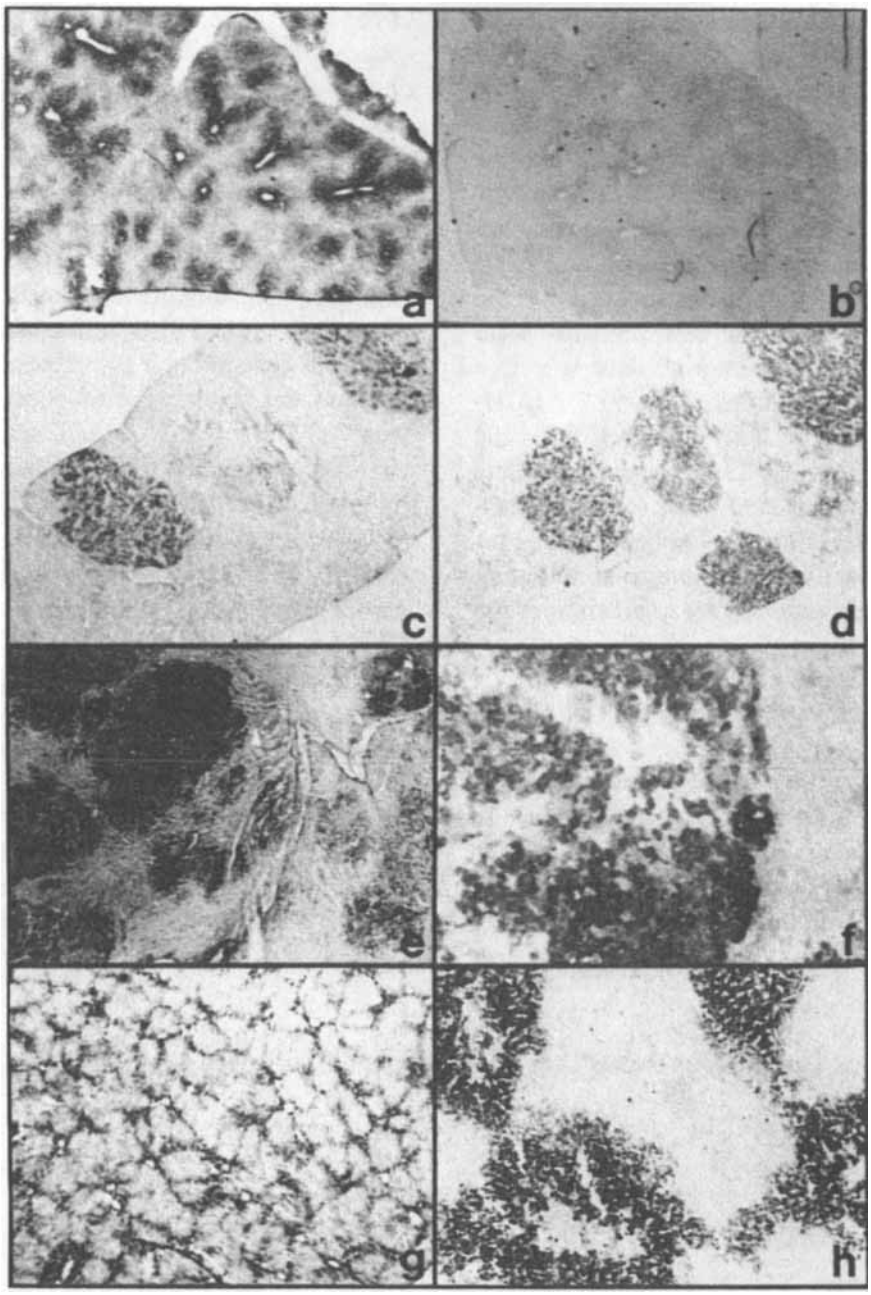

FIGURE 3. Histochemical localization of ALDHs during rat hepatocarcinogenesis: (a) distribution of class 1 and 2 ALDH in normal liver; (b) distribution of class 3 ALDH in normal liver; (c) class 3 ALDH-positive early hepatocyte nodules; (d) GGT-positive hepatocyte nodules in a tissue section serial to that of panel c; (e) class 3 ALDH activity in a hepatocellular carcinoma; (f) immunohistochemical localization of class 3 ALDH in a hepatocellular carcinoma, using anti-class 3 ALDH antibodies; (g) periportal distribution of class 1 ALDH in ethionine plus choline-deficient diet-treated rat liver; (h) periportal distribution of bound aldehyde (as determined by Schiff-positive staining) in CCl_4-treated rat liver. (Panels a to e and g are from the author's laboratory. Panel f was kindly provided by Dr. R. Richmond, Northern Kentucky University. Panel h is taken from Reference 267 with permission.)

cated that the ALDH activity within a single lesion could also be quite heterogeneous (Figure 3e, f).

The distribution of class 3 ALDH-positive preneoplastic lesions relative to other markers of preneoplastic cells indicates that class 3 ALDH is an excellent marker for preneoplastic cell populations that have a high probability of progressing to malignant neoplasms.[174,178] In the resistant hepatocyte model, ALDH-positive foci represented

only 9% of the total foci identified as being positive for γ-glutamyl transpeptidase (GGT), the most widely used marker of preneoplastic cell populations in rodent hepatocarcinogenesis studies.[169] However, 96% of the hepatocellular carcinomas were positive for class 3 ALDH. Likewise, in the PH-DEN-PB protocol, fewer than 4% of the total enzyme-altered foci were ALDH-positive, but 60% of the hepatocellular carcinomas were ALDH-positive.[178] Even in the ethionine-choline deficiency protocol, fewer than 10% of the foci were ALDH-positive, whereas 25% of the carcinomas were class 3 ALDH-positive.[177]

Additional studies have confirmed and extended the results described above. Ritter and Ericksson[179] studied expression of the tumor ALDH phenotype during rat hepatocarcinogenesis by using both the resistant hepatocyte model and repeated cycles of dietary 2-AAF exposure. They found that hepatocyte nodules and hepatocellular carcinomas induced by both protocols possessed significantly elevated class 3 ALDH, characteristic of the tumor ALDH phenotype. They also suggested the presence of different subpopulations of putative preneoplastic cells, some of which possessed elevated class 3 ALDH activity. They interpreted the heterogeneity of ALDH activity in hepatocellular carcinomas as indicating that high ALDH activity in preneoplastic cell populations represents cells that are only one of many sources of hepatocellular carcinomas.

Canuto and colleagues[183,184] also reported significantly elevated class 3 ALDH activity in hepatocyte nodules and carcinomas induced by the resistant hepatocyte model. They reported a transient increase in class 3 ALDH activity in perinodular liver early in hepatocarcinogenesis. Interestingly, Canuto and colleagues observed that nodules and carcinomas obtained later in the process had higher class 3 ALDH activity than nodules or carcinomas appearing early in hepatocarcinogenesis.

Using a number of carcinogens, Richmond and colleagues[181,185,204] have employed histochemical and immunohistochemical methods to examine ALDH activity during both rat and mouse hepatocarcinogenesis. The majority (>80%) of rat and mouse carcinomas induced by DEN with or without promotion by PB expressed the tumor ALDH phenotype, as assessed by either histochemical methods or immunohistochemical techniques employing anti-class 3 ALDH antibodies. Considerable heterogeneity in ALDH activity both within and between neoplasms was observed. Virtually identical results were obtained for mouse liver neoplasms induced by ethylnitrosourea and dichloroacetic acid. For dichloroacetic acid-initiated tumors, Richmond et al. found that the proportion of ALDH-positive preneoplastic lesions increased as foci progressed to nodules, nodules to adenomas, and adenomas to carcinomas. This is consistent with the hypothesis that ALDH-positive preneoplastic cells represent one cell population having a high probability of progressing to carcinomas.

A quantitative histochemical method for assessing changes in ALDH during hepatocarcinogenesis has been developed.[180] Hepatocellular neoplasms induced by vinyl chloride or the resistant hepatocyte model possessed class 3 ALDH activity. Directly adjacent normal hepatocytes lacked class 3 activity. Interestingly, areas of cholangiofibrosis within one vinyl chloride-induced neoplasm also possessed elevated class 3 ALDH activity. This is in contrast to the areas of cholangiofibrosis found in ethionine-treated livers, which were completely devoid of ALDH activity.[177]

Pretlow et al.[182] examined AHF for changes in ALDH activity in rats given 5 weeks exposure to DEN in their drinking water. They found that ALDH was second only to GGT in identifying AHF. ALDH-altered foci represented about 12 to 18% of the foci detected as GGT-positive, depending on the method of tissue fixation.

Tumor ALDH activity has been reported in a human hepatocellular carcinoma.[190] This ALDH has been shown by a variety of methods, including amino acid sequencing, to be human class 3 ALDH. Thus, identical changes in ALDH occur in both rodent and human hepatocarcinogenesis.

Overall, the analysis of changes in ALDH during hepatocarcinogenesis, using a variety of tumor-induction protocols, indicates that

1. Expression of the tumor ALDH phenotype is due to initiator-induced, stable genetic changes occurring in a subpopulation of initiated cells.
2. The role of promotion in expression of the phenotype is to allow the preferential growth

of this subpopulation of cells, resulting in the ultimate neoplasm.

3. Early in the promotion process the normally repressed class 3 ALDH gene is activated.
4. Class 3 ALDH gene activation in a particular initiated cell population is dependent on whether or not the initiator has affected the class 3 ALDH gene.
5. The degree of selection pressure exerted by a particular promotion regimen, coupled with the extent of initiator-induced genetic damage (considerable with DEN and 2-AAF; little or none with ethionine) determines both the first appearance of class 3 ALDH activity and the number of ALDH-positive hepatocellular neoplasms ultimately produced.

Inasmuch as several of the hepatocarcinogenesis protocols employ PB as a promoter, and PB is known to induce a class 1 ALDH, changes in this particular ALDH during hepatocarcinogenesis have also been examined. Elevated hepatic class 1 ALDH activity has been detected in morphologically normal liver of some animals during the promotion phase of several protocols (Table 9).[174,175,177] This activity has been termed promotion-associated ALDH. It is identical to that induced by PB in genetically responsive strains of rats.[124] Several observations are of interest. First, the increase in class 1 ALDH activity is not localized to putative preneoplastic cell populations, but involves the entire liver. Second, PB exposure is not required for induction of class 1 ALDH during hepatocarcinogenesis. Seven of nine animals receiving ethionine followed by the choline-deficient diet possessed elevated class 1 activity during the first nine weeks of combined exposure. No animals receiving only ethionine or the choline-deficient diet expressed this change. After 9 weeks ALDH activities were normal in all groups. Third, in protocols that used PB as a promotor, elevated class 1 ALDH activity required exposure to both an initiator and PB for expression. For example, from two protocols a total of 34% (25/74) of animals receiving 2-AAF + PB and 21% of animals receiving DEN + PB (7/34) had promotion-associated ALDH activity.[174,175] In contrast, no animals receiving only AAF (0/40) and only 3% (1/33) receiving PB alone expressed this phenotype. Three percent (1/29) of the control animals also had elevated class 1 ALDH activity. Only 5% (2/36) of animals receiving PH, DEN, and PB had increased class 1 ALDH activity.[178] Interestingly, the protocol having the greatest selective pressure, the resistant hepatocyte model, did not induce the promotion-associated ALDH phenotype.[176] The appearance of class 1 ALDH suggests that additional changes in ALDH activity, independent of expression of class 3 ALDH, may also occur during hepatocarcinogenesis. While the promo-

TABLE 9
Distribution of Promotion-Associated Class 1 ALDH During Hepatocarcinogenesis

	Protocol[a]					
Enzyme activity	Resistant hepatocyte	Dietary AAF/PB	Dietary AAF only	DEN/PB (1 d old)	PH/DEN/PB	Ethionine + choline deficiency
Constitutive class 1 plus class 2 ALDH: specific activity[b]	30	25	25	31	31	26
Promotion-associated class 1 ALDH: specific activity	NA	70	NA	74	57	38
Percentage of animals expressing	0	34	0	21	14	20

[a] See Table 7 for descriptions.
[b] Milli international units per milligrams protein.

tion-associated phenotype must be due to some event or events occurring during promotion, it is not associated with a particular promoting agent, although the genetic background of the animal may be important.

With respect to changes in ALDH activity during tumorigenesis in other tissues, only three tissues have been examined — rat urinary bladder, human colon neoplasms, and a human mammary adenocarcinoma cell line. By total activity assays, human colon primary adenocarcinomas possess significantly more class 3 ALDH activity than does normal colonic mucosa.[189] Class 3 ALDH is also detectable histochemically in human primary colon neoplasms. Liver metastases of colon adenocarcinomas also possess very high levels of class 3 ALDH, compared with normal colon or with normal liver.

A subline (MCF-7/OAP) of the human breast adenocarcinoma cell line MCF-7/O possesses ALDH activity approximately 50-fold greater than that of the parental line.[191] The functional and physical properties of this ALDH indicate that it is also a class 3 ALDH.

Changes in ALDH occur during rat urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).[112] In contrast to liver, normal urinary bladder epithelium possesses significant class 3 ALDH activity. However, histochemical analysis clearly demonstrates changes in class 3 ALDH activity during bladder carcinogenesis before any changes are detectable by other methods. Bladder epithelial ALDH activity decreases dramatically after initiation, except in areas containing putative preneoplastic bladder cells, which then appear as ALDH-positive areas of hyperplasia. Thus focal populations of putative initiated cells, hyperplastic regions, and ALDH-positive neoplasms appear against an ALDH-negative background. The dramatic decrease in bladder ALDH in normal urothelium is consistent with the fact that the class 3 enzyme is the major ALDH in normal bladder.[78] Seventy percent of examined bladder papillomas and carcinomas possessed class 3 ALDH activity significantly greater than that found in normal bladder. Thus carcinogenesis in both urinary bladder and liver involve qualitatively and quantitatively similar changes in ALDH activity.

The work of Campbell et al.[112] also provides additional insights into both the mechanism of expression and the possible roles of ALDH during tumorigenesis. No change occurs in the liver ALDH phenotype during bladder carcinogenesis. This suggests that expression of the class 3 ALDH during bladder carcinogenesis is the result of bladder-specific changes in class 3 ALDH gene expression. As for liver, changes in bladder class 3 ALDH appear to be transformation associated, stable, and limited to putative premalignant and malignant cells of the target tissue.

Irving and colleagues[7,205] have demonstrated that BBN is metabolized by the liver to the proximate urinary bladder carcinogen, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN), via an aldehyde-containing intermediate, *N*-butyl-*N*-(3-formylpropyl)nitrosamine (BFPN). Further, BFPN, the aldehyde-containing intermediate, is a substrate for both class 1 and class 2, but not class 3, liver ALDHs.[112,205] Because BCPN is the BBN metabolite active in urinary bladder and because BCPN does not directly inhibit class 3 ALDH,[112] the decreases in normal urothelium ALDH activity occurring during bladder carcinogenesis must be due to BCPN-induced alterations in class 3 gene expression in the target cells.

B. Mechanisms of Tumor ALDH Expression

In vivo studies have provided a wealth of descriptive information and evidence that changes in ALDH gene expression occur during tumorigenesis. However, such studies are complex, are often difficult to interpret, and do not lend themselves to detailed analysis of the underlying mechanisms. For these reasons, Lindahl and colleagues established an *in vitro* system to study the genesis and regulation of changes in ALDH occurring during tumorigenesis. The system uses stable, hepatoma-derived cell lines and cultured normal hepatocytes to study the regulation of class 3 ALDH under a variety of experimental conditions.

Initial efforts demonstrated that both established and newly derived rat hepatoma cell lines possessed the same wide spectrum of class 3 ALDH activities that characterized the tumor ALDH phenotype *in vivo* (Table 10).[183,206–210] Established lines such as HTC, recently established lines such as JM1 and JM2, and newly developed

TABLE 10
Distribution of Class 3 ALDH Activity in Rat Hepatoma Cell Lines

Cell line	Specific activity (mIU/mg protein)	Ref.
JM2	681	206
HTC	467	206
RLT 2M	433	207
RLT 9F	317	207
JM1	212	206
RLT-3C	113	207
RLT-5G	100	207
H4-II-EC3	97	206
MH_1C_1	21	183
McA RH7777	5	206
Yoshida AH130	0.4	210
Primary hepatocytes	3	209
Normal liver	5	209

lines including RLT-2M and -9F, possess high constitutive class 3 ALDH activity. Lines H4-II-EC3 and McA-RH7777 do not constitutively express class 3 ALDH and have very low class 1 and 2 ALDH activities. RTL-3C and -5G possess intermediate class 3 ALDH activity. Primary cultures of normal rat hepatocytes maintain the normal liver ALDH distribution for several days *in vitro*.

The identification of cell lines with varying ALDH activities prompted a series of studies to examine the mechanism of expression of the tumor ALDH phenotype. Exposure of low-activity cell lines or normal hepatocytes to polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC) or benzo[*a*]pyrene (BAP) induces class 3 ALDH significantly.[206,207,209] The levels of induction are cell-line dependent, with EC3 cells and normal hepatocytes responding better to 3-MC and McA-RH7777 cells better to BAP. Cell lines having high constitutive class 3 ALDH activity, such as HTC, JM2, or RLT-2M, respond only slightly to PAHs. Class 3 ALDH activity in RLT-9F is not inducible by 3-MC or BAP. Methylcholanthrene also induces class 3 ALDH in both HepG2 human hepatoma cells and normal human hepatocytes in culture.[211,212]

The high constitutive levels of class 3 ALDH are reduced, and the induction by PAHs is inhibited by actinomycin D and cycloheximide, indicating new transcription and translation are required for both.[206] The increase in ALDH activity is transient, peaking at about 5 d and gradually returning to basal levels over several days. Second exposures to inducer result in additional increases in enzyme activity. These studies confirmed and extended earlier studies in intact animals, indicating that acute exposures to a variety of PAHs or dioxins reversibly induced class 3 ALDH activity in normal rat liver.[67–70]

A single 1.7-kb messenger RNA encodes the class 3 ALDH in both constitutively high and PAH-induced cell lines.[208,209] Both the class 3 ALDH mRNA and the polypeptide are long lived (approximately 24 and 90 h, respectively). No evidence for amplification or rearrangement of the class 3 ALDH gene, correlating with enzyme activity, has been identified in any cell line.[208] However, preliminary evidence suggests a correlation between hypomethylation of the class 3 ALDH gene and high constitutive class 3 enzyme activity.[208]

Similar to primary tumors identified *in vivo*, some heterogeneity in ALDH activity occurs in cell lines. Histochemically, not all cells in a population stain identically for enzyme activity. Generally, cells growing in clumps tend to have higher levels of activity than cells growing in monolayer regions. This is especially true for cell lines of low and intermediate ALDH activity.[206,207] The increased enzyme activity that occurs following induction is due to an increase in the proportion of cells expressing class 3 enzyme activity rather than to an increase in activity in cells already expressing class 3 ALDH.[206] Cells grown in serum-free medium have higher class 3 activity than comparable cells grown in serum-containing medium.[206] Interestingly, the *in vivo* passage of RLT-series cells through newborn rats resulted in significant decreases in class 3 ALDH activity. By 6 d *in vivo*, RLT-9F cells possessed 42% of their original activity, whereas RLT-3C cells retained only 11%.[207] These results suggest that ALDH activity *in vivo* may be modulated by as yet unidentified circulating factors.

Initiators of hepatocarcinogenesis that produce preneoplastic and neoplastic cells expressing class 3 ALDH *in vivo* have also been tested for their ability to induce ALDH in hepatoma cell lines or in normal hepatocytes in culture.[209]

Ethionine, 2-AAF, or DEN do not alter class 3 ALDH activity in hepatoma cell lines, cultured normal hepatocytes, or a normal liver epithelial cell line (clone 9 cells) following short-term exposure. Even an *in vivo* protocol designed to mimic an *in vivo* tumor induction protocol (5 d of 2-AAF or DEN followed by chronic exposure to PB for up to 4 weeks) does not induce class 3 ALDH activity in low-activity hepatoma cell lines or clone 9 cells. The inability of chemicals other than PAHs and related compounds to induce class 3 ALDH in normal hepatocytes has been confirmed. Ethionine 2-AAF, DEN, or methylnitrosourea, all of which produce ALDH-positive neoplasms, do not induce class 3 enzyme activity in normal liver following acute or short-term chronic exposures.[213,214]

These results imply the existence of two distinct groups of class 3 ALDH inducers. The first group includes initiators of hepatocarcinogenesis, such as 2-AAF, DEN, and ethionine, which significantly induce ALDH activity in preneoplastic and neoplastic cells *in vivo*. However, they cannot induce ALDH activity in hepatoma cell lines or in cultured normal hepatocytes *in vitro*. This induction is due to initiator-induced, permanent genetic changes occurring in the affected cells that are expressed as the tumor ALDH phenotype in their descendants. The second group includes various PAHs and dioxins, which significantly induce class 3 ALDH activity both *in vivo* and *in vitro*. Although also having a genetic component, the induction by PAHs occurs immediately following exposure and is transient. These observations further suggest that expression of the tumor ALDH gene during tumorigenesis requires events occurring during both initiation and promotion. As will be discussed below, the presence of two classes of class 3 ALDH inducers also implies the existence of at least two different regulatory mechanisms involved in class 3 ALDH gene expression.

Little work has been done *in vitro* with respect to studies of the promotion-associated ALDH. Marselos and colleagues[215,216] have shown that PB will induce class 1 ALDH activity in primary cultures of both human and rat hepatocytes. They have also reported that simultaneous exposure of human HepG2 hepatoma cells to PB and 3-MC results in a synergistic increase in class 3, but not class 1, ALDH activity.[212] Although direct evidence is lacking, this increase in class 3 enzyme activity has been ascribed to increased levels of a 3-MC metabolite that is the actual class 3 ALDH inducer caused by PB induction of 3-MC-metabolizing enzymes. Similar results have been reported in normal rat liver following simultaneous *in vivo* PB–3-MC treatment.[216] As noted above, sequential exposure of low-activity rat hepatoma cell lines or clone 9 cells to 2-AAF or DEN and then PB does not affect either class 1 or class 3 ALDH activity.[209]

Additional insights into the mechanism underlying changes in class 3 ALDH gene expression during tumorigenesis come from the studies of Pitot and colleagues.[75,76,217] These studies have examined the induction of class 3 ALDH *in vivo* and *in vitro* by TCDD. As will be described below, the effects of TCDD are mediated through the Ah receptor. Translocation of the receptor-ligand complex to the nucleus affects transcription of a certain subset of structural genes. This subset includes the class 3 ALDH gene and the cytochrome P450IA1 *(CYP1A1)* gene. Dunn et al.[75] demonstrated that the dose response and kinetics of induction of class 3 ALDH and P450IA1 in liver by TCDD were distinctly different. The maximal activity of ALDH occurred 8 d after TCDD exposure, compared with 12 h for P450IA1. Striking differences were also found in the ability of various organs to express class 3 ALDH and P450IA1 in response to TCDD. Tissues that were able to produce one protein were not necessarily able to produce the other coordinately. The ability to respond did not correlate with the reported tissue distribution and abundance of the Ah receptor. For example, while liver and lung expressed both the class 3 ALDH and the P4501A1 genes in response to TCDD, kidney and small intestine, which possess high levels of the Ah receptor, expressed only P450IA1. Differences were also noted in the dose response of various tissues. The dose response in colon favors P450IA1, whereas in stomach class 3 ALDH is expressed at lower TCDD levels. These data suggest that expression of specific genes in response to TCDD can be modulated in different ways. Moreover, regulatory pathways, in addition to the classically defined Ah receptor-mediated pathway, may be involved.

Similar differential responses of the class 1 ALDH and two cytochrome P450 genes, *CYPIIB1* and *IIB2*, to PB exposure have also been described.[76] The PB induction of hepatic class 1 ALDH is known to be under genetic control.[63,64] Responsive *(R)* and nonresponsive *(r)* rat strains have been identified. The class 1 ALDH and *CYPIIB1* and *IIB2* mRNAs are not coordinately induced by PB in the *RR* and *rr* animals. Class 1 ALDH mRNA is detectable at low levels in untreated animals of both genotypes and is induced only in the *RR* genotype. Both *RR* and *rr* animals have variable low levels of both cytochromes. *CYPIIB1* is induced by PB in both *RR* and *rr* animals. However, *CYPIIB2* is strongly induced in *rr* animals, but its expression in *RR* animals treated with PB is completely repressed. Kidney and lung both possess significant basal levels of class 1 ALDH mRNA that are not increased by PB treatment. These results suggest that the gene defined by the *R* and *r* alleles is not the structural gene for the PB-inducible class 1 ALDH, but it may be a structural gene encoding a protein involved in the regulation of genes by PB and, perhaps, other xenobiotics. Thus the genes encoding both inducible ALDHs can be regulated by multiple mechanisms.

Studies of the molecular mechanisms underlying changes in class 1 and/or class 3 ALDHs during tumorigenesis indicate that new transcription and translation are required. Preliminary evidence also suggests that changes in DNA methylation accompany increased transcriptional activity. The identification of two classes of inducers of class 3 ALDH implies that multiple mechanisms exist for regulating class 3 ALDH gene expression. This hypothesis is also supported by (1) the differential response of the class 1 and class 3 ALDH genes and various phase I drug-metabolizing enzyme genes to TCDD or PB, and (2) data indicating that the PB-responsiveness locus *(R)* may not be the class 1 ALDH structural gene but a gene producing a class 1 ALDH gene regulatory protein. The integration of this information with that obtained from the tumor-induction protocols is discussed in the last section of this review.

IV. ROLES FOR ALDHs IN TUMORIGENESIS

That multiple forms of mammalian ALDH exist and that these forms have differing tissue and subcellular distributions, may be constitutive or inducible by a variety of agents, and possess differing substrate preferences imply that these enzymes may have multiple physiological functions. The role of certain of the constitutive ALDHs in the oxidation of acetaldehyde resulting from ethanol metabolism has received by far the most attention. Important roles for ALDHs in the oxidation of metabolites from neurotransmitter and biogenic amine metabolism have also been of interest, largely due to the alterations in neurotransmitters and amines that may accompany excess alcohol consumption.

Until recently, other potential physiological roles for the various ALDHs have been much more speculative. However, several factors have stimulated interest in identifying other roles for ALDHs in cellular processes. The recent reports that several previously purified but unidentified proteins are ALDHs[150–153] are certain to increase interest in the role of this enzyme family in hormone metabolism, vision, and developmental processes. The availability of the large number of protein and DNA sequences has stimulated interest in the evolution of this gene family and will also stimulate the search for physiological functions. Finally, the observation that the class 1 and class 3 liver-inducible ALDHs have constitutive counterparts in other normal tissues has sparked interest in identifying roles for these enzymes that are consistent with multiple modes of expression. With respect to carcinogenesis, these roles may include the metabolism of carcinogens or their metabolites; other xenobiotics and their metabolites, including antitumor agents; and/or aldehydes produced from alterations in metabolic pathways in preneoplastic or neoplastic cells. The remainder of this review will consider three potential physiological roles for ALDHs in relation to tumorigenesis.

A. Role of ALDH in Oxazaphosphorine Metabolism

Among the more interesting functions for ALDHs in relation to tumorigenesis is their role in the metabolism of certain chemotherapeutic agents, including oxazaphosphorine drugs such as CP. CP is currently the most widely used antineoplastic drug. It is also used extensively as an immunosuppressant both in diseases with an

autoimmune component and during bone marrow transplantation.[14] As an antineoplastic drug, CP is effective in treating a spectrum of malignancies, including leukemias and lymphomas; neuroblastoma; retinoblastoma; and carcinomas of the lung, ovary, breast, and cervix. It is also used in the treatment of certain forms of rheumatoid arthritis, multiple sclerosis, and lupus erythromatosus.

CP is a prodrug that requires biotransformation through a series of intermediates to produce the active forms (Figure 4). The ultimate physiologically cytotoxic metabolite is phosphoramide mustard, which acts as an alkylating agent, cross-linking DNA and rendering the target cells nonviable. The production of the intermediate metabolite 4-hydroxycyclophosphamide is catalyzed by hepatic mixed-function oxidases. 4-Hydroxycyclophosphamide exists in equilibrium with its open-ring tautomer, aldophosphamide. 4-Hydroxycyclophosphamide/aldophosphamide is the transport form of CP that reaches the target cells.[14,186] Within the target cells aldophosphamide undergoes beta elimination, either spontaneous or enzyme catalyzed, to produce phosphoramide mustard and acrolein (Figure 4).

Aldophosphamide has an alternative fate in target cells, the oxidation of the aldehyde group to produce carboxyphosphamide, which is not cytotoxic (Figure 4). Hill and colleagues[218] reported that a commercial preparation of yeast ALDH could catalyze the irreversible oxidation of aldophosphamide to carboxyphosphamide. At that time, however, they felt that ALOX was the

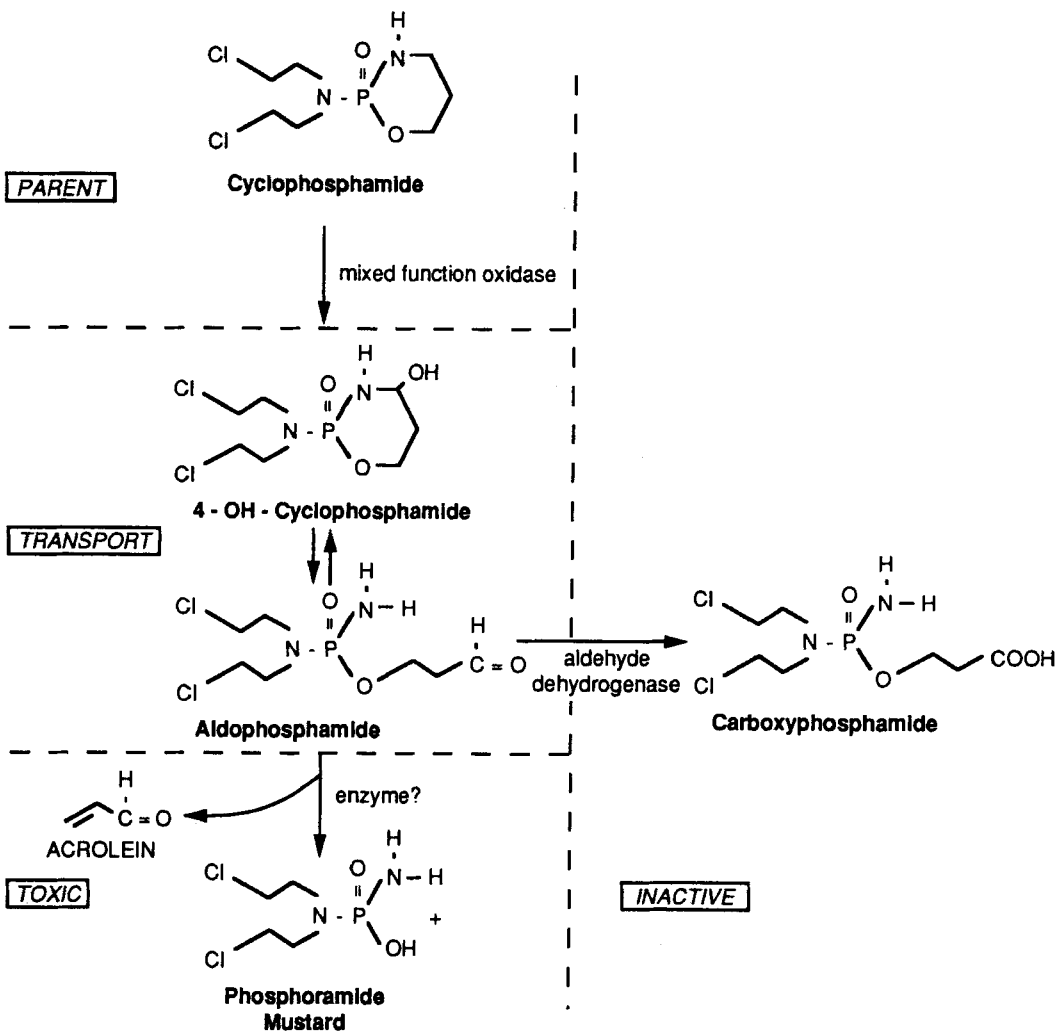

FIGURE 4. Metabolism of CP. (Adapted from Reference 14 with permission.)

major catalyst of this reaction. Subsequently, it was demonstrated convincingly that one or more ALDHs are primarily responsible for this oxidation,[91,104,186,219–222] although a minor role for ALOX in this reaction cannot be completely ruled out.

Because the oxidation of aldophosphamide to carboxyphosphamide is a detoxification reaction, the cytotoxicity of CP should be inversely correlated with target cell ALDH activity. Cells having higher activity of the relevant ALDHs should be more resistant to CP than cells lacking ALDH activity. The following observations support this hypothesis. First, the level of ALDH is the major determinant of the relative resistance to CP of bone marrow stem cells[187,188,223] and intestinal crypt cells.[224] Second, although ALDH is ubiquitously distributed among normal hematopoietic stem cells, many leukemia and lymphoma cell lines are virtually devoid of ALDH.[188] Third, increased ALDH activity is one means by which tumor cells acquire resistance to CP.[186,225,226] Fourth, inhibition of ALDH activity restores the sensitivity of resistant murine leukemia cell lines to CP and makes resistant hematopoietic stem cells sensitive to CP.[188,225]

Which ALDH(s) confers resistance to CP? Domeyer and Sladek[221] first reported that rat and mouse liver cytosolic ALDHs could convert aldophosphamide to carboxyphosphamide *in vitro*. Hilton and Russo[186,227] demonstrated that a cytosolic ALDH was responsible for conferring CP resistance to L1210 mouse leukemia cells *in vivo*. Most recently, Sladek and colleagues[91,222] examined the ability of several mouse and human ALDHs to oxidize aldophosphamide. From liver from both species, a constitutive class 1 ALDH is responsible for the vast majority of the aldophosphamide oxidation, although other ALDHs may make a minor contribution. For mouse stomach, another constitutive class 1 enzyme appears to be responsible for aldophosphamide oxidation. Hipkins et al.[228] reported no correlation between the PB-inducible ALDH activity and either CP metabolism *in vivo* or the chemotherapeutic effect of CP in tumor-bearing mice with or without PB pretreatment. A similar lack of correlation between inducible class 1 ALDH activity and the cytotoxic effects of CP in rat embryos has been reported recently.[229] Thus, it appears that a constitutive, but not the PB-inducible, class 1 ALDH plays a role in modulating CP cytotoxicity.

With respect to other ALDHs, it appears that class 3 ALDH does not play a role in CP cytotoxicity. No correlation was found between the class 3 ALDH activity and the CP sensitivity of eight rat hepatoma cell lines.[230] Sreerama and Sladek[191] have shown that a CP-resistant subline of MCF-7/O human breast adenocarcinoma cells (MCF-7/OAP) possesses very high class 3 ALDH activity. However, aldophosphamide is not a substrate for this enzyme in either hepatoma cell lines or in MCF-7/OAP cells. No evidence exists that class 2 ALDH plays a significant role in aldophosphamide oxidation.[91,222]

Modulation of target cell ALDH activity has been suggested as a means of changing the cytotoxicity of CP.[14] If the activity of the major aldophosphamide-oxidizing ALDH(s) could be increased in normal cells and/or decreased in tumor cells, the relative cytotoxicity and, therefore, therapeutic effectiveness of CP would be improved. Both strategies appear to have merit. However, the limited tissue specificity of inducible ALDHs and the lack of correlation between induction of class 1 or 3 ALDHs and changes in CP cytotoxicity in studies to date suggest that this approach will require additional work.

More promising is the use of inhibitors of ALDH to reduce the relevant ALDH activities of tumor cell populations. Many alcohol aversion therapies are based on the inhibition of liver ALDHs, which results in increased steady-state levels of acetaldehyde, which in turn causes the severe nausea and headaches that serve as deterrents to further alcohol consumption. Among these agents are disulfiram, cyanamide, and chloral hydrate. Disulfiram and cyanamide both inhibit the conversion of aldophosphamide to carboxyphosphamide *in vitro*.[221,231] Treatment of L1210/OAP and P388/CLA CP-resistant murine leukemia cells with cyanamide or disulfiram makes them sensitive to CP.[14,225,232] Similar effects have been reported *in vivo* when tumor-bearing animals have been treated with both CP and an ALDH inhibitor.[233,234] However, similar studies by other groups have reported no improvement in CP therapeutic potential *in vivo* following treatment with either disulfiram[235,236] or cyanamide.[231] Chloral hydrate has also recently been shown to poten-

tiate the cytotoxicity of CP *in vitro*[237] and *in vivo*.[233] One limitation of the ALDH induction/inhibition approach is the selectivity of both potential inducing agents or inhibitors for only certain members of the enzyme family. Finding inducers and/or inhibitors of the relevant ALDHs will require additional work.

Largely overlooked in studies of the role of ALDHs in CP metabolism and cytotoxicity is the acrolein generated whenever aldophosphamide is converted to phosphoramide mustard. Although it is generally accepted that phosphoramide mustard is the major physiologically relevant alkylating agent,[238] acrolein does contain an aldehyde group. Petersen and colleagues have shown that conjugation of acrolein to glutathione generates a form of acrolein that is a substrate for class 1 and 2, but not class 3, ALDHs. They have also demonstrated that acrolein proper is an excellent irreversible inhibitor (apparent Ki $< 1\ \mu M$) of class 1 and 2 ALDHs.[240,241] These results indicate that although acrolein may not be a directly cytotoxic CP metabolite, its effects on target cell relevant ALDHs may play a role in determining the extent of aldophosphamide oxidation to carboxyphosphamide. Acrolein-mediated inhibition of ALDH would increase the cytotoxicity of CP in cells that would appear to have sufficient relevant ALDH activity to be resistant to CP.

Several questions remain with respect to ALDH and antitumor drug metabolism. Which ALDH(s) plays a major role in the inactivation of oxazaphosphorines? Are different ALDH forms involved in different tissues? Identification of the relevant enzyme activities may allow ALDH activity to be used as a marker for tumors against which CP and related molecules would be effective. The development of inhibitors specific for ALDH forms involved in inactivation of CP-like compounds would also be possible. Cells resistant to CP can also acquire resistance to other chemotherapeutic agents. In some cases, changes in ALDH may be responsible.[14] What is the mechanism of this resistance? As noted above, the normal cell counterparts of some tumors possess the relevant ALDH, implying that in certain tumor cells the relevant ALDH activity may be repressed. What is the mechanism of this repression? Are occasional tumor cells emerging as CP-resistant because down-regulation of the relevant ALDH gene did not occur? If the mechanism of such down-regulation can be identified, can it be activated specifically in tumor cells to lower ALDH activity, making CP more effective? As the regulatory elements controlling expression of the relevant ALDH genes are identified, perhaps the ALDH activity of normal cells can be increased to protect them, leaving tumor cells relatively more susceptible to the cytotoxic effects of CP. Although of some interest in the past, the development of cytotoxic aldehydes with greater therapeutic indices may be possible in the near future, as more becomes known about the major enzyme systems involved in their metabolism.

B. ALDH and Lipid Peroxidation

One approach to identifying potential physiological roles for ALDHs is to determine whether aldehydes from various sources are substrates for one or more forms of the enzyme. In this way both xenobiotic metabolizing and endogenous pathways have been shown to provide substrates for oxidation by ALDHs (Tables 2 and 3). One major endogenous source of aldehydes is the oxidation of membrane lipids. It appears that ALDHs may play major roles in the oxidation of many of the highly reactive aldehydes produced by this process.

Membrane lipid peroxidation is a continuous process reflecting the constant state of cellular membrane synthesis and degradation. The process is initiated by reactive reduced oxygen species (ROS) (superoxide and OH radicals, H_2O_2, singlet oxygen). Such molecules are normally produced in small amounts from metabolic pathways. Membrane lipid peroxidation can also be significantly stimulated by a number of factors that induce cellular oxidative stress, including a variety of oxygen radicals generated from the metabolism of xenobiotics and exposure of cells to UV radiation or prooxidant substances such as ADP plus iron or ascorbate plus iron.[242] Thus lipid peroxidation can be nonenzymatically or enzymatically driven.

Biological membranes are particularly sensitive to peroxidative events because the polyunsaturated fatty acids contained in their phospholipids are readily attacked by ROS (Figure 5). The at-

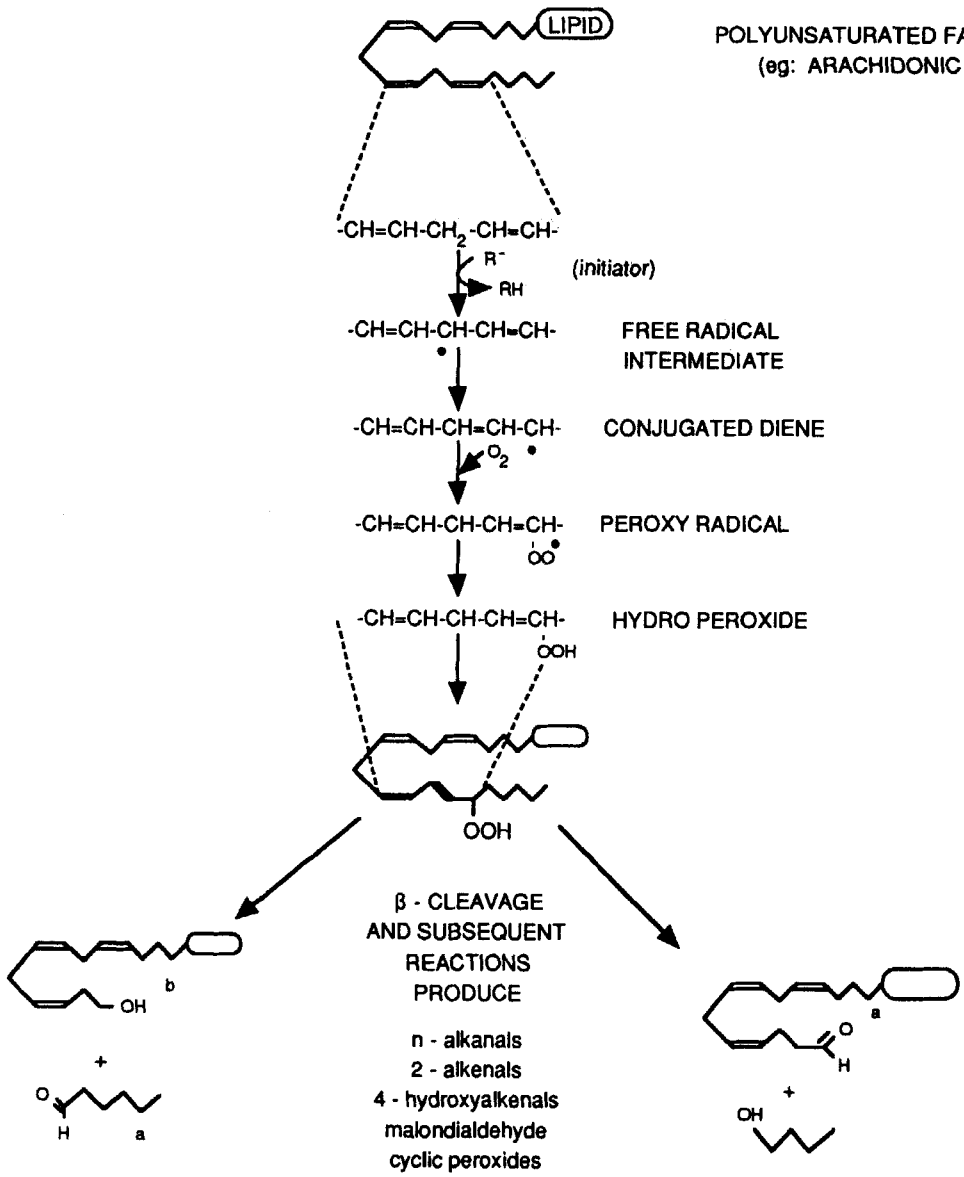

FIGURE 5. Peroxidation of arachidonic acid as an example of a free radical-induced lipid peroxidation pathway. Shown are some of the key intermediates and major types of products. Depending on the number of conjugated C-C double bonds in the fatty acid, a number of conjugated positional hydroperoxide isomers can be produced. For example, homolytic (β) cleavage of the 15-hydroperoxy isomer of arachidonic acid can produce a free aliphatic aldehyde (hexanal) and an aldehyde bound to the parent lipid. Peroxidation at other positions will produce a different spectrum of free aldehydes, some of which will still contain conjugated double bonds that become targets for subsequent peroxidation events. Additional reactions of secondary peroxidation products are the major sources of malondialdehyde and 4-hydroxyalkenals. See Reference 3 for a detailed review of the spectrum of possible reactions producing aldehydes. (Adapted in part from Reference 266 with permission.)

tack of ROS on polyunsaturated fatty acids results in initiation of a chain reaction-like series of events that progress more or less unchecked. Under normal physiological conditions, membrane lipid peroxidation does not usually result in untoward effects. However, in certain disease states, such as cancer or rheumatoid arthritis, or during exposure to radiation or xenobiotics, ROS-mediated lipid peroxidation may be a significant mediator of various pathophysiological changes.[243,244]

Linoleic, arachidonic, and docosahexaenoic acids are the major polyunsaturated fatty acids in mammalian biomembranes.[3] As such, they are the major targets for lipid peroxidation. The reactions involved in peroxidation can be very complex, and, depending on the fatty acid, a very large number of products can be generated (Figure 5). They include short-lived intermediates containing reactive radicals that catalyze further autooxidations, other unstable intermediates that spontaneously rearrange to form stable products, and relatively stable carbonyl-containing compounds. On a molar basis carbonyls may account for more than 50% of the stable products of lipid peroxidation.[245] Among the carbonyls, aldehydes account for >70% of the carbonyls generated from either linoleic or arachidonic acids. Thus aldehydes are major products of membrane peroxidation.

Although a number of assays for lipid peroxidation are available, the most widely used generally employ methods that are based on detection of the products of lipid peroxidation.[3,246,247] The most sensitive is the direct determination of lipid aldehydes by HPLC or GC-MS. The second method is the spectrophotometric measurement of lipid hydroperoxides based on their conjugated diene double-bond structures. The third and most widely used is the measurement of total malondialdehyde by a chromogenic assay with thiobarbituric acid. Although the thiobarbituric acid assay has many limitations and the results must be interpreted with extreme caution, its simplicity has made it the assay of choice for most routine determinations.

More than 200 different aldehydes have been identified as products of lipid peroxidation. However, only a small number are generated in relatively large amounts (Table 11),[245] including alkanals, 2-alkenals, 2,4-alkadienals, dialdehydes, and hydroxyalkenals. Although the quantity of the various produced aldehydes varies with the starting lipid hydroperoxide, three are generally considered to be major products of lipid peroxidation — malondialdehyde, hexanal, and 4-hydroxynonenal. Based on catalytic constants, K_m, and/or V_{max}/K_m determinations, considered in the context of substrate and enzyme concentrations as discussed above, at least one ALDH is involved in the oxidation of these lipid aldehydes.

Malondialdehyde is the major aldehyde produced from membrane lipid peroxidation, accounting for up to 70% of the total.[3] Early studies suggested that both liver mitochondrial and cytosolic fractions possessed ALDH capable of oxidizing malondialdehyde.[45,248–250] Subsequently, Hjelle and Petersen[251] identified two ALDHs in rat liver cytosol that effectively oxidize this aldehyde. One enzyme, which appears to be the constitutive class 1 ALDH, has a K_m for malondialdehyde of 16 μM. The other cytosolic ALDH, resembling the inducible class 1 form, has a K_m for malondialdehyde near 130 μM. Class 2 ALDH does not appear to use malondialdehyde as substrate (K_m of 7 mM). Malondialdehyde is also a very poor substrate for the class 3 enzyme (K_m of 5 mM).[160] These results suggest that at physiological malondialdehyde concentrations (1.5–3 μM), the constitutive class 1 ALDH would operate at about 25% of maximal velocity.

TABLE 11
Some Aldehydes Produced During Lipid Peroxidation[a]

Alkanals	Alkenals	4-Hydroxyalkenals	Others
Propanal	Acrolein	4-Hydroxynonenal	Malondialdehyde
Butanal	Pentenal	4-Hydroxyhexenal	2,4-Heptadienal
Pentanal	Hexenal	4-Hydroxy-2,5-nonedienal	2,4-Decadienal
Hexanal	Heptenal	4,5-Dihydroxydecenal	5-Hydroxyoctanal
Nonanal	Octenal		Butanone
	Nonenal		2-Pentanone
			3-Pentanone
			2-Octanone

[a] Compiled from References 2 and 3.

n-Hexanal is the major alkanal produced by the peroxidation of both linoleic (84%) and arachadonic (70%) acids.[3,252] Hexanal can account for approximately 15% of the total aldehyde production from lipid peroxidation. Hexanal and other medium-chain-length (C6–C11) alkanals are excellent substrates for the class 3 ALDHs, either the constitutive microsomal or the inducible form.[160,239,253] Among the examined substrates, the C8 to C11 alkanals (K_ms near 1 μM) were much better substrates than hexanal (K_m of 40 μM). These studies also indicated that the unsaturated (alkenal) forms of these aldehydes were good ALDH substrates. No systematic analysis of the ability of class 1 and 2 ALDHs to oxidize these aldehydes has been carried out. However, hexenal is an excellent substrate for a class 2 enzyme (K_m of 2 μM), and a class 1 ALDH also appears to be able to oxidize this aldehyde (K_m of 30 μM).[159]

4-Hydroxynonenal is the major hydroxyalkenal produced from lipid peroxidation. It may account for up to 5% of total aldehyde production. It is very strongly lipophilic and so cannot diffuse far from its site of production. In fact, it is estimated that the concentration of 4-hydroxynonenal may reach millimolar levels within microsomal membranes.[245,252–254] Class 2 ALDH is the major 4-hydroxynonenal-oxidizing enzyme *in vitro* (K_m of 17 μM).[159] Based on either K_ms or catalytic constraints, neither pure class 1 nor pure class 3 ALDH can oxidize 4-hydroxynonenal to an appreciable extent.[159,160,254] However, it has been demonstrated that ALDHs from microsomal and cytosolic fractions of normal rat liver, rat neoplastic nodules, and hepatomas can oxidize 4-hydroxynonenal.[183,184,255] 4-Hydroxyhexenal has been reported to be a substrate for both class 1 and class 2 ALDHs.[256] As noted, substrate preferences for various ALDHs may be influenced by local (subcellular) substrate concentrations. Therefore, an assessment of the actual ability of any particular form to oxidize any particular aldehyde must consider the kinetic parameters in light of the local substrate and enzyme concentrations, and it may be that class 1 and/or 3 ALDHs may oxidize 4-hydroxyal bends *in vivo*.

Consistent with the reactivity of aldehydes, the products of lipid peroxidation have been shown to have a variety of effects in biological systems. In general, the net result of the interaction of aldehydes with biologically relevant molecules results in cytotoxicity. Although short chain alkanals such as formaldehyde and acetaldehyde can be very active, longer chain alkanals such as octanal and decanal are not highly reactive in biological systems under physiological conditions. For alkanals the major interaction appears to be with sulfhydryl and amino groups, either in low-molecular-weight molecules such as glutathione or in proteins and nucleic acids. Generally, the result is cross-linking and inhibition of function.[2]

In contrast, alkenals and hydroxyalkenals are highly reactive and interact with biologically relevant molecules under physiological conditions and concentrations.[2,245] As for alkanals, the primary targets are sulfhydryl and amino groups. However, because the alkenals and hydroxyalkenals are much more reactive than alkanals, their effects on biological systems are more pronounced. Hydroxyalkenals are the most reactive, and their adducts are much more stable than those formed by alkenals.[245] Therefore, most interest has centered on the effects of hydroxyalkenals such as 4-hydroxynonenal on biological processes. A wide variety of effects have been observed at physiological (nanomolar-micromolar) aldehyde concentrations.

The alkylating properties of hydroxyalkenals are the basis of most of their effects. Although conjugation to glutathione may be a detoxifying mechanism for 4-hydroxynonenal,[242] eventual depletion of glutathione stores under conditions of high lipid peroxidation leads to its cytotoxicity. Inhibition of a wide variety of enzymes has been reported, including those of glycolysis and gluconeogenesis, the mixed-function oxidases, and the DNA polymerases.[242] Both inhibitory and stimulatory effects of 4-hydroxynonenal on adenyl cyclase have been reported.[242,257] Stimulation of guanylate cyclase and phospholipases A2 and C have also been reported.[25,258–260] 4-Hydroxynonenal is genotoxic, being mutagenic in bacterial and mammalian systems[260,261] and increasing the frequency of sister-chromatid exchanges and other chromosome aberrations in rat hepatocytes.[262] Hydroxyalkenals also inhibit cell proliferation, which may be due to direct effects on the enzymes of DNA replication and/or

repair[263] or to other effects on progression through the cell cycle.[25] Hydroxyalkenals are also chemotactic for neutrophils at very low concentrations and may play a role in the inflammatory response.[264,265] The interaction of 4-hydroxynonenal with biologically relevant molecules is so widespread that virtually all cellular systems and pathways are affected.

The relationship of lipid peroxidation to carcinogenesis and any role played by ALDHs in the process is currently unclear. Whether lipid peroxidation plays a causal role in the initiation or promotion process or is a result of other events occurring during initiation or promotion has not been examined in a systematic way.[25,243] Good circumstantial evidence exists that oxygen radicals may be the causative agents in initiation and/or promotion events in several multistage models of carcinogenesis.[266] It seems most likely that the direct effects of these radicals on DNA and proteins are most important in this context, rather than their ability to initiate membrane lipid peroxidation. However, a direct role for free radical-induced nuclear lipid peroxidation in initiation of hepatocarcinogenesis has been proposed in one model.[288]

Given the spectrum of effects on cellular processes that have been reported for peroxidation-derived aldehydes, it may be that one or more of these play some role at some stage of tumorigenesis.[25,243] Of particular interest in this respect are the direct cytotoxicity of many of these aldehydes, their effects on various second messenger systems and cell proliferation, and their chemotactic activities. Therefore, the ability of normal, preneoplastic and/or neoplastic cells to metabolize these aldehydes could be one determinant in modulating their effects.

Several studies have shown that lipid peroxidation is decreased in hepatomas when compared with normal liver. Early studies focused on rat hepatoma cell lines (Yoshida AH 130; Ehrlich ELD) carried in ascites form or in Morris hepatomas.[268–270] It was suggested that the extent of the decrease in lipid peroxidation of these tumor cells was correlated with their degree of dedifferentiation. Primary hepatocellular tumors, induced by ethionine, dimethylaminoazobenzene, or DEN, as well as preneoplastic nodules induced by ethionine or DEN, also have lowered levels of lipid peroxidation.[271,273] Tumor cells have either lowered levels of basal lipid peroxidation or normal levels of basal lipid peroxidation, but oxidative stress on these cells cannot induce lipid peroxidation further. Increased lipid peroxidation in human colorectal tumors, based on the thiobarbiturate assay, has been reported.[295]

Appearing at the same time were reports that hepatoma cell lines exhibiting reduced lipid peroxidation had levels of total ALDH activity considerably below that of normal liver.[210,275–277] Sessa and colleagues[275–277] were interested in establishing if the antitumor activity of various aldehydes was related to low ALDH activity of tumor cells in a manner somewhat analogous to that discussed earlier for CP. However, these studies are often cited in support of the concept that reduced production of lipid aldehydes, rather than an increase in their removal, is the basis of the reduced lipid peroxidation measured in tumor cells.

Based on observations with the liver tumor systems, it has become generally accepted that tumor cells from any source possess decreased lipid peroxidative capacity. Decreased lipid peroxidation results in reduced generation of potentially cytotoxic aldehydes. In turn, this means that tumor cells are less susceptible to the cell proliferation-inhibiting effects of these aldehydes. The net result is that a potential control of cell division is lost, tumor cells gain a proliferative advantage, and a neoplasm results.[25]

Four reasons are usually cited as responsible for low tumor lipid peroxidation capacity.[25,243] First, enzymatically induced lipid peroxidation is reduced as a result of the low levels of monooxygenase enzyme systems in tumor cells. Neoplastic and preneoplastic cells from many sources, including liver, have significantly reduced levels of phase I drug metabolizing enzymes, including cytochromes P-450 and mixed-function oxygenases.[195] Initiation of lipid peroxidation would be reduced in tumor cells because they cannot generate oxygen radicals, especially following oxidative stress.

Second, the lipid composition of tumor cell membranes may be significantly different from that of normal cells. In preneoplastic and neoplastic liver cells, the amounts of polyunsaturated fatty acids, most notably, arachidonic and do-

cosahexaenoic acids, decrease, and they are replaced by less unsaturated fatty acids (oleic acid) and saturated fatty acids.[271,273] Because the levels of substrates for lipid peroxidation are decreased, the levels of lipid peroxidation would correspondingly decline. Along with the relative increase in saturated fatty acids in tumor cell membranes is an increase in their cholesterol content. Thus, a third reason for reduced lipid peroxidation is an increase in tumor cell membrane rigidity. This may reduce the accessibility of oxygen to potential substrates.

A fourth reason often cited for decreased tumor lipid peroxidation is an increase in cellular antioxidant levels. Levels of lipophilic antioxidants such as vitamins A, C, and E are increased in tumor cells.[278,279] Such increases may serve to protect membrane lipids from peroxidative damage. Interestingly, the protective effects of antioxidants do not appear to include reactive oxygen species-scavenging enzymes such as superoxide dismutase or catalase, as their levels in tumor cells are generally lower than those of corresponding normal tissues.[266]

Until recently, it has been assumed that the levels of lipid aldehydes measured in cells accurately reflect their level of production, rather than representing steady-state levels resulting from the balance of their production and metabolism. Only when it was demonstrated by workers interested in lipid peroxidation that class 3 ALDH can oxidize 4-hydroxynonenal was it recognized that removal of lipid aldehydes by various pathways may significantly affect their steady-state levels.[183,184,280]

The relative roles played by the various pathways of lipid aldehyde metabolism are subjects of considerable uncertainty, partly due to the fact that different analytical techniques and different experimental systems have been used. It is generally agreed that malondialdehyde is oxidized by class 1 and 2 ALDHs (see above). The contribution of the two enzymes to malondialdehyde metabolism appears to be dependent on the subcellular site of generation and the local aldehyde concentration. Although the kinetic properties favor oxidation by class 1 ALDH under most conditions, higher local subcellular aldehyde concentrations could make the class 2 enzyme a significant contributor to malondialdehyde oxidation.

The metabolism of longer chain alkanals, alkenals, and hydroxyalkenals appears to be more complex. Conjugation of a variety of lipid aldehydes to glutathione by glutathione-*S*-transferases was first shown by Boyland and Chasseaud.[281] Subsequently, both spontaneous and enzyme-catalyzed conjugation of 4-hydroxyalkenals to glutathione was shown to be a potentially major pathway for the removal of these molecules from cells.[282,283]

Esterbauer and colleagues reported that 4-hydroxynonenal can be reduced to its corresponding alcohol *in vitro* by rat hepatocyte cytosolic fractions.[284] The reaction was NADH-dependent and was inhibited by pyrazole. This led Esterbauer et al. to conclude that the reduction is catalyzed by an alcohol dehydrogenase. They further suggested that the alcohol dehydrogenase-mediated reduction of hydroxyalkenals was the major fate of these compounds in liver. However, in liver the NAD/NADH ratio strongly favors the oxidized coenzyme and there is significant aldehyde reductase activity. It has been demonstrated that under these conditions the role of alcohol dehydrogenase as an aldehyde-reducing enzyme is of little relevance *in vivo*.[285,286]

The most thorough study of the relative roles of oxidative, reductive, and conjugative pathways in hydroxyalkenal metabolism is the recent work of Canuto and colleagues.[183,184,280] Their studies have examined the metabolism of 4-hydroxynonenal in normal liver and cultured hepatocytes, in preneoplastic and neoplastic liver, and in rat hepatoma cell lines. Three major conclusions emerge from these studies.

First, the relative roles of the various hydroxyalkenal removal systems vary in different tissues (Table 12). Based on the distribution of 4-hydroxynonenal-metabolizing enzymes, in normal liver glutathione-*S*-transferase-mediated conjugation to glutathione is the major pathway for hydroxyalkenal metabolism. Reduction by alcohol dehydrogenases may also be significant in normal hepatocytes. ALDHs and reductases may play only a minor role. However, two different hepatoma cell lines show very different distributions of hydroxyalkenal-metabolizing en-

TABLE 12
4-Hydroxynonenal-Metabolizing Enzyme Activities of Rat Hepatocytes and Hepatoma Cell Lines[a]

Cell	ALDH		Alcohol dehydrogenase (reduction reaction)	Aldehyde reductase	Gluthathione-*S*-transferase
	Classes 1 and 2	Class 3			
Hepatocytes	8[b]	2	58	8	280
MH_1C_1	10	4	2	6	171
HTC	28	45	28	26	30

[a] Adapted from Reference 183.
[b] Specific activity (milliinternational units per milligrams protein) with 4-hydroxynonenal as substrate.

zymes. In one line, MH_1C_1, glutathione conjugation appears to be the major mechanism for hydroxynonenal removal. Oxidative metabolism contributes more in these cells than it contributes in normal liver, but the activity appears to be largely mediated by class 1 ALDH. Reductive metabolism is almost nonexistent in MH_1C_1 cells. In contrast, in HTC cells oxidation via class 3 ALDHs appears to be the major pathway of removal, with conjugation being much reduced compared with either normal hepatocytes or MH_1C_1 cells. Reductive metabolism in HTC cells is only slightly different from that in normal liver, due primarily to an increase in aldehyde reductase-mediated metabolism. Thus not only is hydroxyalkenal metabolism different between normal and neoplastic cells, but within neoplastic cells the relative contribution of various pathways is different.

Changes in the metabolic fate of lipid aldehydes during tumorigenesis are detectable in preneoplastic cell populations several months before the appearance of overt neoplasms.[183,184] Further, in contrast to the results with hepatoma cell lines, the changes in preneoplastic and neoplastic cell hydroxyalkenal metabolism are more uniform. Relatively, glutathione-*S*-transferase-mediated conjugation and oxidative metabolism of 4-hydroxynonenal are increased in both cell populations compared with those of normal liver. Aldehyde reductase-mediated, but not alcohol dehydrogenase-mediated, reduction is also significantly elevated in neoplastic cells. Thus a second conclusion of the work of Canuto and colleagues is that during hepatocarcinogenesis an overall net increase in hydroxyalkenal removal occurs, beginning during preneoplasia and continuing in overt neoplasms.

A final conclusion is that the assessment of the lipid peroxidation status of a particular tissue cannot be based only on determination of total lipid aldehyde levels. Steady-state levels of these molecules are the result of both their production and their removal, and changes in the rates of removal alone can significantly affect these levels. Therefore, altered levels of lipid aldehydes in a cell or tissue do not necessarily mean altered levels of production, but they may be accounted for by an alteration in removal by any number of pathways. Moreover, at least liver cells possess an array of defense mechanisms against the accumulation of cytotoxic aldehydes.

There is additional indirect evidence for a significant role for ALDHs in modulating the effects of lipid peroxidation. Much involves correlative changes in lipid peroxidation and ALDH activity under different conditions. The time course of hepatic lipid peroxidation and class 3 ALDH increases following TCDD treatment are virtually identical.[75,287,288] Both lipid peroxidation and class 3 ALDH gradually increase and peak at 8 d following acute TCDD exposure. In contrast, both aryl hydrocarbon hydroxylase and cytochrome P450IA1 activities peak within approximately 48 h of onset of TCDD exposure.[75] The declines in all these activities are gradual over a period of several days to weeks. The maximal induction of lipid peroxidation by other halogenated hydrocarbons (CCl_4, hexachlorobenzene, hexachlorocyclohexane) occurs within 24

h, with a correspondingly rapid decay. No increase in ALDH activity occurs during this period. In mice TCDD-induced lipid peroxidation appears to be genetically linked to the *Ah* locus.[287] TCDD-sensitive (C57BL/6J) mice that possess high-affinity TCDD receptors undergo lipid peroxidation at much lower TCDD doses (50-fold) than do TCDD-resistant (DBA/2J) mice that do not possess the Ah receptor, implying that increased lipid peroxidation may be part of the response to xenobiotic exposure that is mediated by the *Ah* locus.

Another interesting correlation between lipid peroxidation and ALDH activity occurs during hepatocarcinogenesis employing ethionine and dietary choline deficiency. As already noted, 2 months of dietary ethionine exposure significantly reduced hepatic lipid peroxidation and produced neoplastic cell populations having reduced lipid peroxidation.[271] An early effect of dietary choline deficiency is induction of lipid peroxidation.[271,290,291] Also as noted, a significant increase in class 1 ALDH activity occurs early in ethionine-choline-deficiency-induced hepatocarcinogenesis.[177] Histochemically, the distribution of promotion-associated ALDH activity is identical to that of bound aldehydes detectable histochemically by the periodic acid-Schiff reaction, following CCl_4-induced hepatic lipid peroxidation (Figure 6).[267,292] Although the exact mechanism by which choline deficiency causes initiating and/or promoting activity is currently unclear, it is apparent that changes in ALDH activity occur during this process.[267,293,294]

Coupled with the kinetic data indicating that aldehydes produced by lipid peroxidation are good substrates for one or more ALDHs, very good evidence now exists that changes in ALDH can accompany cellular lipid peroxidation. What is not known at present is the exact role or roles that changes in ALDH may play. Not all agents that affect lipid peroxidation also affect ALDH activity, and changes in ALDH may be either directly or inversely correlated with changes in lipid peroxidation capacity. Moreover, changes in two different ALDH classes appear to occur independently. Whether the changes in ALDH are related to protecting a certain population of cells from the cytotoxic effects of lipid aldehydes or are part of a more generalized response of the cell to lipid peroxidation-induced stress is also currently not known.

More systematic studies are required to establish the roles of ALDHs in lipid peroxidation and the relation of the two to tumorigenesis. Such experiments should employ measures of lipid peroxidation independent of lipid aldehyde concentration in order to establish levels of lipid peroxidation. Both *in vivo* and *in vitro* studies are needed. *In vivo* work should concentrate on a thorough direct analysis of changes in both lipid peroxidation and ALDH activity during tumorigenesis by using a variety of tumor-induction protocols. *In vitro* studies should examine effects of altering the lipid peroxidation levels of cells on ALDH activity and vice versa. For these studies, advantage should be taken of cells and cell lines differing in their lipid peroxidizing and/or ALDH activities. For example, do inducers of lipid peroxidation induce ALDHs? Are cells with differing ALDH activities differentially sensitive to lipid aldehydes?

A detailed analysis should also be done of the lipid peroxidation capacity of tissues that possess high constitutive levels of class 3 ALDH, such as cornea, lung, stomach, and urinary bladder. It is interesting that either these tissues are directly exposed to high levels of molecular oxygen (cornea and lung) or they are tissues in which aldehydes, including those produced from lipid peroxidation, may be resident for extended periods of time. For the lung, stomach, and urinary bladder, class 3 ALDH activity is localized to cells lining the surface of the organ that are in direct contact with the environment and directly exposed to aldehyde substrates. Both the cornea and lung are likely to be continuously producing lipid aldehydes, due to reactive oxygen species-mediated peroxidative processes.

C. Xenobiotic Metabolism and ALDHs

Both class 1 and class 3 ALDHs are inducible by xenobiotics, suggesting that ALDHs may play a role in the response of cells to exposure to foreign compounds. However, the role(s) played by various ALDHs in the xenobiotic response

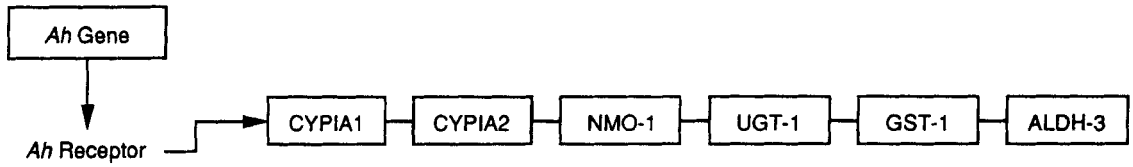

FIGURE 6. Members of the [*Ah*] gene battery. The *Ah* locus produces the *Ah* receptor, which then activates the individual members of the battery. The phase I and II genes are not genetically linked. (Modified from Reference 295 with permission.)

cannot be simple. For example, many of the xenobiotics that induce class 1 (PB) or class 3 sically considered conjugation enzymes because they link a phase I-activated substrate to various small endogenous molecules, resulting in the formation of very hydrophilic, easily excretable products. Like phase I enzymes, phase II enzymes may have endogenous substrates that can arise from a variety of metabolic pathways independent of phase I reactions. For example, glutathione-*S*-transferases and uridine 5′-diphosphate (UDP-) glucuronyl transferases have both xenobiotic and endogenously generated substrates. This metabolic scheme usually, but not always, results in the production of inactivated (TCDD, 2-AAF, 3-MC) ALDH do not contain or produce aldehydes during their metabolism. Conversely, CP does not induce an ALDH but rather an aldehyde-containing metabolite that is an ALDH substrate. These observations, along with others discussed below, imply that induction of ALDHs following xenobiotic exposure may be part of an overall adaptive response of the cell to stress.

The metabolism of foreign compounds has been divided into two stages. Phase I enzymes, primarily the cytochromes P-450, are responsible for the activation of a foreign compound by the addition of an oxygen atom to a relatively inert molecule. The cytochrome P-450-catalyzed addition of oxygen creates a much more reactive metabolite that is a substrate for any number of enzymes that can further metabolize the activated molecule. Cytochromes P-450 not only function in the activation of xenobiotics, but also play key roles in the synthesis and degradation of a number of endogenous substrates, including fatty acids, prostaglandins, biogenic amines, and steroids. Numerous recent reviews of various aspects of cytochromes P-450 have been published.[295–298]

The second stage reactions are catalyzed by a diverse group of enzymes, collectively known as phase II enzymes. Phase II enzymes are clasforms of the xenobiotic that are less harmful than the parent molecule. However, phase I metabolism of a xenobiotic may result in forms that are more toxic, mutagenic, and/or carcinogenic than the parent molecule, if they do not undergo subsequent phase II reactions. Thus xenobiotic metabolism may be a route of detoxification or a route of carcinogenesis, depending on the exact circumstances. Moreover, phase II reactions may produce molecules more toxic, mutagenic, or carcinogenic than the products of phase I metabolism.

One model of the integration of phase I and phase II enzymes for metabolism of certain xenobiotics is the [*Ah*] gene battery.[295,299] The [*Ah*] gene battery consists of at least six genes (Figure 6). Two genes encode the phase I enzymes — cytochromes P450IA1 *(CYPIA1)* and P450IA2 *(CYPIA2)* — and four encode phase II enzymes, including NAD(P)H:menadione oxidoreductase *(Nmo-1)*, UDP-glucuronyltransferase *(Ugt-1)*, glutathione-*S*-transferase (*Gst-1*, encoding the Ya and/or Yb subunits), and class 3 ALDH *(ALDH-3)*. This gene battery is activated via a series of receptor-mediated events by any one of several planar aromatic compounds, including 3-MC, BAP, β-naphthoflavone, or, particularly, TCDD. Classically, activation by planar aromatic compounds resulted in the induction of aromatic hydrocarbon hydroxylase (AHH) activity, cytochrome P450IA1. The receptor mediating this response was, therefore, designated the Ah receptor.

Activation of the [*Ah*] gene battery is believed to be generally similar to steroid hormone-mediated gene activation. It requires binding of the Ah ligand to a cytosolic Ah receptor, fol-

lowed by translocation of the ligand-receptor complex from the cytosol to the nucleus. This is followed by binding of the inducer-receptor complex to chromatin, resulting in transcriptional activation of some or all of the genes in the battery.[295,296,299–301] Like the steroid hormone receptors, evidence exists for a role in heat shock proteins in modulating the DNA-binding ability of Ah receptor ligand complexes.[302] However, unlike steroid hormones, nuclear translocation of Ah receptor-ligand complexes requires active participation of an additional protein, the *arnt* gene product.[303]

Inclusion of a particular gene in the [*Ah*] battery was originally based on the determination that expression of the gene is under control of the Ah receptor.[295] Genetic analysis assigned the *CYPIA1* and *IA2*,[304,305] *Nmo-1*,[306] *Ugt-1*,[307] and *Gst-1*[308] genes to the battery. The *ALDH-3* gene was tentatively included because it is inducible by TCDD and AHs.[295] As will be discussed below, it has subsequently been shown that class 3 ALDH gene expression can be regulated by Ah receptor-mediated events in some situations.

Among the genes of the [*Ah*] battery, regulation of *CYPIA1* has been most thoroughly studied.[296,300,301] The development of AHH-induction mutant murine hepatoma lines defective in various steps of the Ah receptor-mediated pathway has been very useful in studying *CYPIA1* expression. Three different classes of mutants have been identified.[309–311] One group, designated A or P1-, is heterogeneous and includes a variety of defects in the *CYPIA1* structural gene.[312] Group B mutants have defects in the Ah receptor and are designated receptorless, *r*-.[313] The third group, C, are defective in the cytosol-to-nucleus translocation of the ligand-receptor complex. They are designated nuclear-translocation defective, *arnt*-.[303,329]

Also very useful in the study of *CYPIA1* gene regulation has been the construction and expression of hybrid genes possessing putative *CYPIA1* upstream regulatory regions ligated to a heterologous reporter gene such as the bacterial chloramphenicol acetyl transferase *(cat)* gene.[314–318] Transfection and expression of these hybrid genes in wild type and AHH mutant cell lines have identified at least three DNA domains upstream of the *CYPIA1* coding region that respond to TCDD in an Ah receptor-dependent manner (Figure 7A). The first region is approximately 200 bp 5′ to the cap site, and it functions as a classic promoter. The second region is some distance (approximately 1 kb) from the *CYPIA1* cap site, and it possesses all the properties of an enhancer. It functions at a distance, independent of its orientation, to activate transcription of its target gene. This region, called the Ah response domain, is modular, possessing multiple copies of a sequence called a drug regulatory element (DRE) or xenobiotic response element (XRE). The core of an XRE is the highly conserved seven-base-pair sequence 5′-TNGCGTG-3′. XRE sequences share considerable homology with glucocorticoid response elements.[319] Gel retardation assays suggest that XRE sequences may be binding sites for Ah receptor-ligand complexes.[320–322] In addition, a third positive regulatory element even more distal to the Ah response domain may also exist.[323] However, this region has not been studied in detail.

Several studies also provide evidence for a region more proximal to the *CYPIA1* coding region (approximately −700 bp) that acts as a negative *cis*-acting controlling element (Figure 7A).[314,315,317,324] This region does not contain XRE sequences and appears to be the binding site for a *trans*-acting factor distinct from receptor-ligand complexes. Using various mutant cell lines and *CYPIA1* deletion hybrid gene constructs, this factor has been shown to act as a repressor of constitutive (ligand-independent) expression of *CYPIA1*.[315,324] Furthermore, activation of this repressor protein requires the presence of an active P450IA1 enzyme. Thus low constitutive levels of *CYPIA1* expression are maintained by a negative autoregulatory loop in which P450IA1 either directly activates the repressor or, through its enzymatic activity, produces a small effector molecule that activates the repressor.

Regulation of some phase II members of the [*Ah*] gene battery involves mechanisms similar to those described for *CYPIA1*. Use of AHH mutant cell lines provides evidence for a common mechanism of TCDD-mediated induction of both phase I and phase II genes. Single copies of XREs that confer positive regulation by planar aromatics are found within 1 kb upstream of the rat *Nmo-1* and *Gst-1* genes (Figure 7B).[324,325]

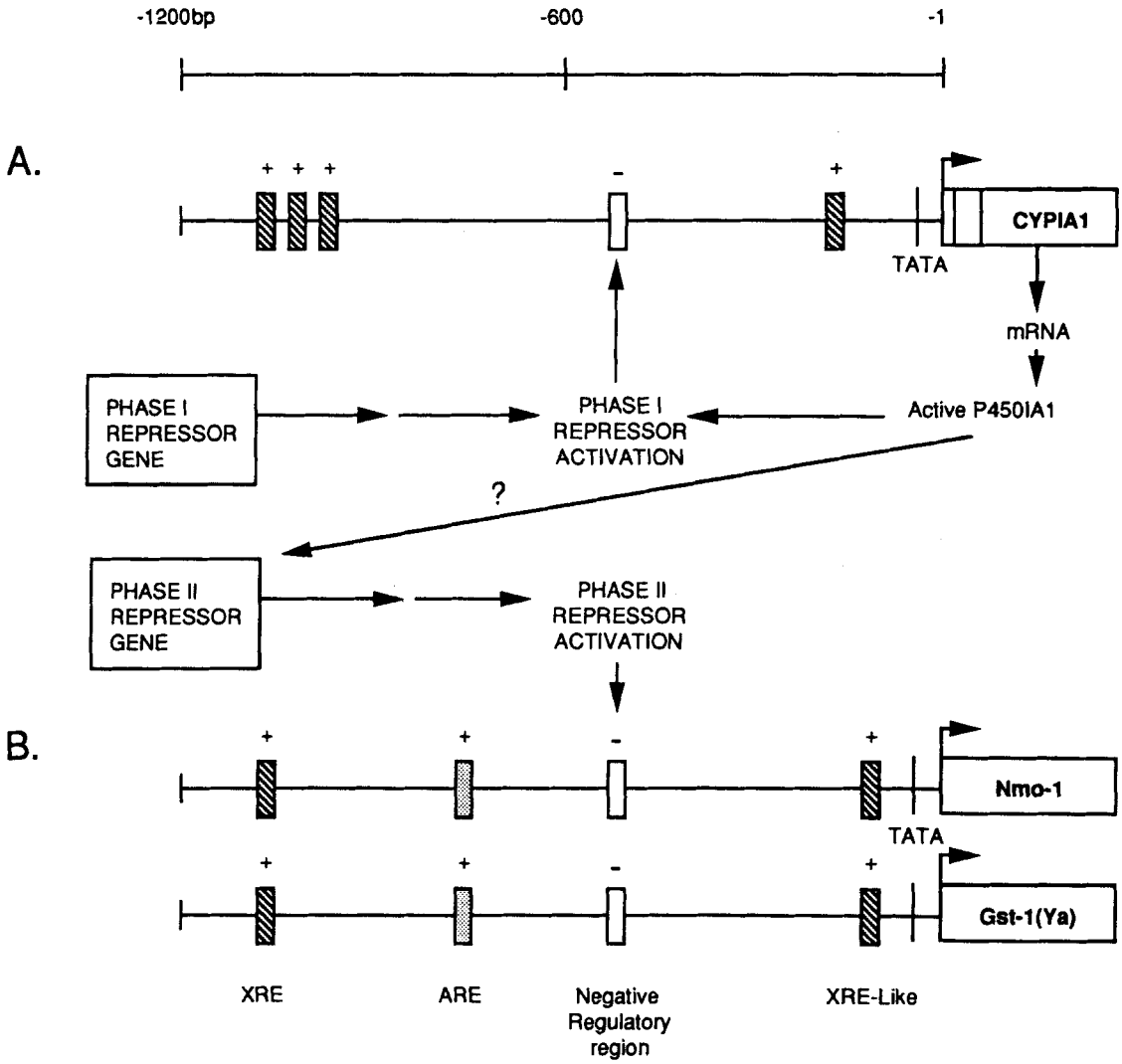

FIGURE 7. Schematic representation of the regulatory elements found upstream of the mammalian phase I and II members of the [*Ah*] gene battery. The exact location of the individual elements varies between species, but the relative order is believed to be that indicated. (A) The *CYPIA1* gene; (B) the *Nmo-1* and *Gst-1 (Ya)* genes. It is not currently known whether the phase I and II negative regulatory regions are similar or identical. See text for additional details. (Taken in part from Reference 299 with permission.)

Evidence also exists for negative regulatory regions upstream of the *Nmo-1*, *Ugt-1*, and *ALDH-3*, but not the *Gst-1*, genes (Figure 7B).[299,324] Phase II gene negative regulation also appears to be complex. Based on studies with AHH-defective cell lines, it was initially postulated that the same *trans*-acting factor was responsible for both phase I and phase II gene repression.[323] However, more recent studies with radiation-induced chromosomal deletion mouse lines provide evidence that two distinct negative *trans*-acting factors may be acting independently to regulate phase I and phase II constitutive gene expression.[299,326] In addition to the factor involved in autoregulation of phase I genes, the second negative *trans*-acting factor, encoded by a separate gene, represses constitutive expression of the *Nmo-1*, *Ugt-1*, and *ALDH-3* genes. Moreover, from studies using P450IA1-deficient mutant hepatoma cells, it appears that activation of the phase II gene *trans*-acting factor requires the presence of functional phase I enzyme activity.

Further evidence for regulation of phase II gene expression independent of Ah receptor-me-

diated mechanisms comes from the identification of a third type of regulatory element controlling certain phase II genes. This element, called an antioxidant responsive element (ARE)[324,325] or electrophile responsive element,[327] has been identified upstream of the *Nmo-1* and *Gst-1* genes (Figure 7B). The ARE core sequence, 5′-puGTGACNNNGC-3′, shares no identity with XRE core sequences. The ARE contains distinct but overlapping sequences that are responsible for both basal and inducible regulation of their genes. The *trans*-acting factors that bind to AREs have not been identified, but gel retardation assays indicate that at least one protein interacting with the ARE is constitutively expressed.[324] The ARE is responsive to metabolites of planar aromatic compounds, the products of phase I enzymes, as well as to a wide variety of compounds that contain or can be metabolized to compounds that contain electrophilic sites.[328,329] Included in the latter group are phenolic antioxidants, isothiocyanates, thiocarbamates, certain carboxylic acid esters, and hydrogen peroxide. AREs have, therefore, been postulated to be a mechanism by which cells may respond to oxidative stress.[329]

The identification of negative regulatory regions and AREs upstream of certain phase II members of the [*Ah*] gene battery is consistent with the concept that both Ah receptor-mediated and Ah receptor-independent mechanisms of regulation of these genes need to be postulated to explain the diversity of their expression. Talalay and colleagues have proposed that two types of inducers of phase I and II enzymes exist.[328,330] Monofunctional inducers are the electrophilic antioxidants. They activate phase II genes through AREs independent of Ah receptors or active P450IA1 or IA2 protein. Bifunctional inducers are the planar Ahs. They can act via the classic Ah receptor pathway through XREs to activate phase I and II genes. Bifunctional inducers can also activate phase II enzymes via AREs, following their conversion to electrophilic metabolites through phase I enzymes. Combined with the presence of negative regulatory elements that can modulate constitutive expression of phase II genes independent of inducer-mediated or phase I gene activation,[299,323] it is clear that multiple pathways exist for the control of the expression of phase II drug-metabolizing enzymes.

Which of these regulatory mechanisms might be operating in the expression of the class 3 ALDH gene? Conceptually, a broad-substrate, inducible enzyme such as class 3 ALDH is a likely candidate to be encoded by a gene that is subject to multiple forms of regulation. Expression may be an adaptive response to an altered cellular physiological environment, such as that likely to occur during chemically induced carcinogenesis. Expression may also be in response to acute stresses such as xenobiotic exposure or oxidative stress. The cell must deal not only with the direct effects of the stress but also with a multitude of potential indirect effects caused by such stresses. Constitutive expression of the same gene may be expected in those cells that are continuously, rather than intermittently, producing substrates for the product it encodes.

Considerable evidence exists that hepatic class 3 ALDH activity in whole animals or in liver cells in culture is inducible by planar AHs, including BAP, 3-MC, and TCDD.[67,70,73,75,206,209,214,217] Induction involves increased transcription and translation of the *ALDH-3* gene and mRNA.[75,206,209,217,297] Studies with AHH mutant cells confirm that the induction process requires functional Ah receptors and the translocation of the ligand-receptor complexes to the nucleus.[217,299] Thus the class 3 ALDH should clearly be considered to be a member of the [*Ah*] gene battery.

Consistent with the involvement of an Ah pathway-linked negative *trans*-acting factor in class 3 ALDH gene expression are observations that constitutive class 3 ALDH activity is increased approximately twofold in hepatoma cells grown in or transferred to serum-free medium.[206] Also in support of a negative *trans*-acting factor is the observation that the class 3 ALDH activity of hepatoma cell lines decreases significantly when the cells are carried subcutaneously in newborn rats, when compared with identical cells maintained *in vitro*.[207]

However, considerable evidence also exists that expression of the class 3 ALDH gene can be regulated by mechanisms independent of the Ah receptor. First, even though the class 3 ALDH gene is normally repressed in liver, it is constitutively expressed at very different levels in several normal mammalian tissues, including

stomach, urinary bladder, and, especially, cornea.[75,77,78] Second, the inducibility of the *ALDH-3* gene by TCDD does not correlate well with the tissue distribution of the Ah receptor. In the rat Ah receptor, levels are high in the thymus, kidney, and intestine,[331,332] but the *ALDH-3* gene is not inducible in those tissues.[75] *CYPIA1* is inducible by TCDD in the same tissues. These observations indicate that a mechanism must exist that allows constitutively high levels of *ALDH-3* expression in certain tissues. They also indicate that Ah receptor-mediated gene activation does not have to involve all members of the [*Ah*] gene battery.

Moreover, the time course of TCDD induction of the class 3 ALDH gene in liver is distinctly different from that of either other phase II genes *(Nmo-1, Gst-1)* or the phase I gene *(CYPIA1)*. For the latter three genes, transcription rates are maximal within the first 16 h following TCDD exposure.[75,333,334] In contrast, transcription of the *ALDH-3* gene does not begin until 24 to 48 h following exposure and is not maximal until several days later.[75,209] Although this observation is consistent with some form of negative regulation of phase II genes via an Ah receptor-mediated process, it indicates that not all genes respond identically to the same regulatory signals.

Other observations are also consistent with multiple forms of class 3 ALDH gene regulation. In contrast to the planar AHs the hepatocarcinogenic initiators used in the tumor induction protocols do not induce class 3 ALDH acutely and transiently either in cells in culture or in whole animals. Rather, they cause permanent changes in *ALDH-3* expression in liver and bladder putative preneoplastic and neoplastic cells. These changes are expressed as part of the AHF or as hepatocyte nodule phenotypes.[169–171,195] Also part of these phenotypes are permanent increases in *Nmo-1, Gst-1,* and *Ugt-1* expression in preneoplastic cell populations, while *CYPIA1* expression is repressed. These observations imply that changes in class 3 ALDH gene expression occurring during carcinogenesis are not due to xenobiotic exposure per se, but are part of an overall constitutive adaptive response of a certain population of cells to initiator exposure.[169,195] The response is independent temporally and spatially from any known Ah receptor-mediated response, suggesting that changes in class 3 ALDH gene expression may involve mutational events in Ah receptor-independent genes that are involved in *ALDH-3* gene regulation. One candidate is the gene encoding the phase II-associated negative *trans*-acting factor.

Also germane is the fact that the two groups of class 3 ALDH inducers — the PAHs and the hepatocarcinogenic initiators — may be compared conceptually to the bifunctional and monofunctional inducers described by Talalay and colleagues.[328,330] The bifunctional inducers can act via the Ah receptor, whereas the monofunctional inducers work independent of this mechanism.

Although it seems likely that the class 3 ALDH gene can be activated as part of constitutive adaptive response to stress during carcinogenesis, it appears that many other factors affect the ultimate response. Expression of the class 3 ALDH gene is variable both between different tumor-induction protocols and between individual tumors in a single protocol. It is interesting to note, however, that the consistency of response within a protocol is correlated with the degree of selective pressure exerted on putative preneoplastic cells by the initiating and promoting regimen (Tables 7 and 8). The greater the selective pressure, the greater the proportion of individuals possessing changes in class 3 ALDH activity. This is consistent with the idea that changes in *ALDH-3* expression during carcinogenesis may be related in some manner to adaptation to cellular stress. It is also interesting in this context that dietary choline deficiency with or without initiator (ethionine) exposure does not induce class 3 ALDH activity.[177] However, dietary choline deficiency does increase activity of two other phase II enzymes, *Nmo-1* and *Gst-1*[335] and the promotion-associated expression of the inducible class 1 ALDH.[177]

What of the regulation of expression of the class 1 ALDH gene in relation to xenobiotic metabolism and carcinogenesis? There is no evidence that expression of the class 1 ALDH gene involves Ah receptor-mediated mechanisms. The *ALDH-1* gene is not inducible by compounds that are known to utilize Ah receptor-mediated pathways. As discussed earlier, good evidence also exists that the induction of phase I and phase II

enzymes in response to PB need not be coordinated.[76] *CYPIIB1* expression is increased equally by PB in rats showing differential induction of class 1 ALDH in response to PB exposure. *CYPIIB2* expression is increased by PB only in the strain not responsive to PB class 1 ALDH induction. Differences also exist in the constitutive levels of expression of the three genes. Expression of the *Gst-Ya* gene in response to PB is also distinctly different from *ALDH-1* gene expression. Low constitutive and equal induction by PB in ALDH-responsive and -nonresponsive rat strains occurs at this locus. Thus the genetic evidence suggests that a regulatory gene exists that controls the PB-inducibility of the class 1 ALDH gene independent of other phase II or phase I genes.

Nucleotide sequences of the region immediately upstream of both the rat and the mouse class 1 ALDH gene are known.[76,147] Within the first 275 base pairs 5′ to the transcription initiation site, no sequences similar to the XRE or ARE core sequences are identifiable. However, a short repeated pyrimidine-purine sequence, 5′-CACACAC-3′ for rat and 5′-CACACACAC-3′ for mouse, is found approximately 265 bp upstream from the cap site. This same sequence has been found in the same upstream region of the *CYPIIB1* and *CYPIIB2* genes.[336] For the cytochrome genes, the length of the CACACA repeat is inversely related to the basal level of expression of the gene. The *CYPIIB2* gene possesses a 10 bp repeat and is expressed constitutively at higher levels than the *CYPIIB1* gene, which has a 38 bp long repeat.[76,296,336] The *Gst-Ya* gene does not possess a CACACA repeat in its immediate 5′ upstream region.[337] However, two short TGTGTG repeats are present in the −850 and −1100 bp regions.

The *CYPIIB2* gene possesses an exact consensus XRE core sequence approximately 10 bp 5′ (-275) of the CACACA repeat. It also possesses a near-consensus ARE core at approximately 130 bp upstream of the cap site. The sequence is 5′-GTGtCNNNGC-3′. In the *CYPIIB1* gene, the XRE sequence is missing due to base substitution and deletion, but the ARE sequence is present. These results suggest that the regulation of genes inducible by PB may also be subject to multiple forms of regulation and that, like the [*Ah*] gene battery, individual genes inducible by PB may differ in their regulatory mechanisms.

V. ALDH GENE REGULATION

The foregoing discussions suggest models for the regulation of both class 1 and class 3 ALDH gene expression. Class 3 ALDH gene regulation must include both Ah receptor-mediated and Ah receptor-independent mechanisms. Figure 8 presents a proposed organization of the region upstream of the class 3 ALDH gene. The region includes *cis*-acting elements for Ah receptor-ligand complex binding (XREs), a binding site for monofunctional inducers (AREs), and sites for interaction of negative *trans*-acting factors.

For the *ALDH-3* gene, an XRE confers Ah receptor-mediated inducibility. A single XRE is consistent with the organization of two other phase II genes, *Nmo-1* and *Gst-1*. The presence of an XRE-mediated autoregulatory loop may explain the differing kinetics of class 3 ALDH and phase I and other phase II enzyme inductions following TCDD exposure.

The presence of an ARE is based on analogy to *Nmo-1* and *Gst-1*. An ARE-like sequence could explain the delayed but permanent expression of class 3 ALDH during carcinogenesis. If initiated cells are mounting an adaptive response to stress, ARE-mediated induction of *ALDH-3* may be included. Alternatively, initiator-induced changes in the gene producing the *trans*-acting factor that interacts with the ARE may result in a molecule that interacts permanently with the ARE, resulting in continuous expression of *ALDH-3*. Either of these alternatives would be consistent with the observation that the overall expression of *ALDH-3* correlates with the selective pressure that a particular tumor induction protocol exerts on initiated cells.

The presence of an ARE-like element may also explain the constitutive expression of the class 3 ALDH gene in other tissues. Tissues constitutively expressing *ALDH-3*, such as the bladder, cornea, lung, and stomach, are under continuous oxidative stress. Thus constitutive expression in these tissues may be due to continuous activation of an ARE-like sequence, and the presence of an ARE-like element could ex-

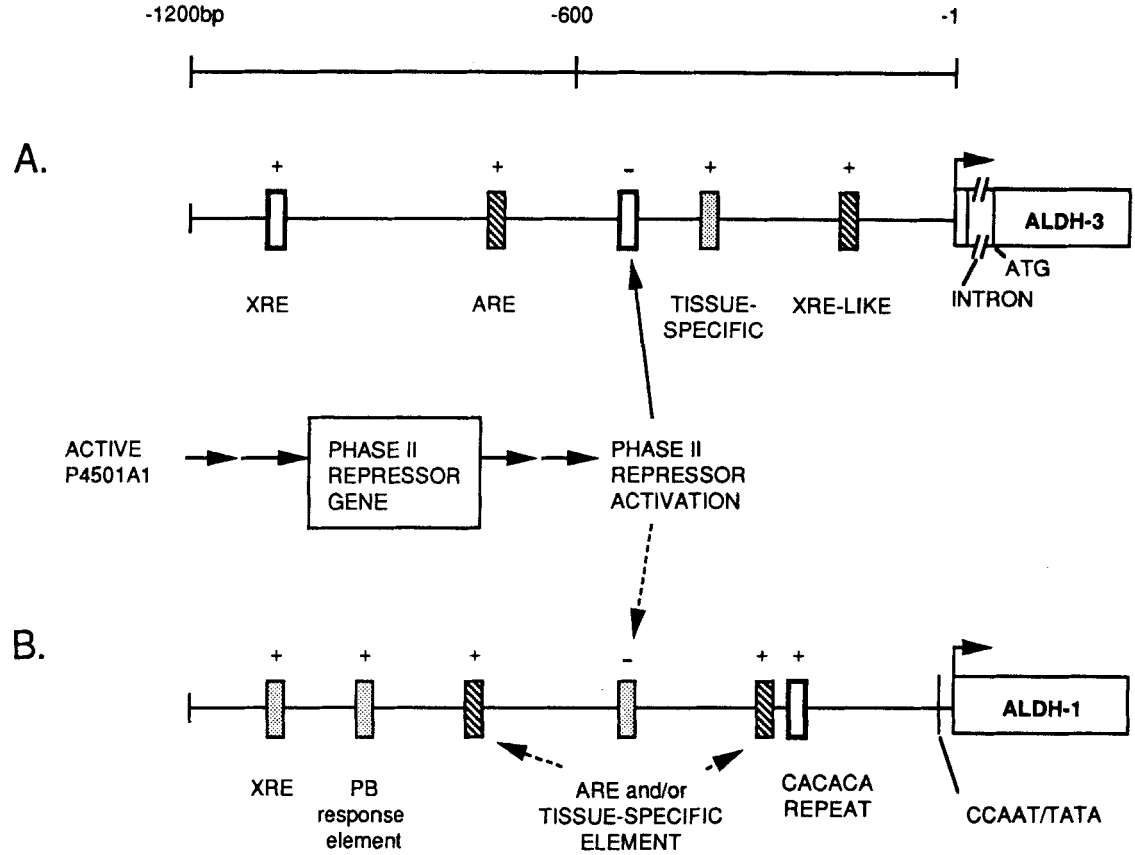

FIGURE 8. Proposed organization of class 1 and class 3 ALDH gene regulatory regions. The models include regulatory elements known to be present, based on direct sequencing or genetic data (bold); expected elements, based on experimental data (hatched); and elements whose presence is predicted, based on analogy to other genes showing similar expression patterns (dotted); (A) the *ALDH-3* gene; (B) the *ALDH-1* gene.

plain both inducible and constitutive expression of the class 3 ALDH gene.

The existence of a negative *cis*-acting element would explain repression of the class 3 ALDH gene in certain tissues, as well as its constitutive tissue-specific expression in others. High constitutive expression in cornea, for example, could be the result of the absence of a functional negative *trans*-acting factor, due either to repression of its gene or to the fact that corneal cells do not possess an additional molecule required for this factor to be functional. Alternatively, sequence elements responsible for positive tissue-specific constitutive expression, AREs, and/or true tissue-specific elements may also be present in lieu of or in addition to a negative *cis*-acting element. Both Ah receptor-dependent and Ah receptor-independent, constitutive and inducible gene expression can be explained by this organization.

Sequencing of the genomic region of the *ALDH-3* gene immediately upstream of the translation start site suggests the presence of a relatively large intron (>1.8 kb) in the 5′-untranslated region of this gene (Asman and Lindahl, unpublished data). Therefore, at least one additional exon is required to produce the functional 5′-untranslated region of the class 3 ALDH mRNA. Primer extension studies suggest this additional exon is >150 bp long. A similar 5′-untranslated exon has been found in the human mitochondrial *ALDH x* gene.[147]

As less is known about the organization of PB-inducible genes,[296] we cannot propose with certainty the nature of all of the regulatory elements upstream of the class 1 ALDH gene. How-

ever, because some upstream sequence is known for this gene, the presence of certain elements is established, for example, the CACACA repeat (Figure 8B). Genetic evidence also suggests the existence of a gene encoding a protein that specifically activates the class 1 ALDH gene in response to PB. Thus binding sites for a positive *trans*-acting factor can be predicted. No ARE-like sequence exists in the first 250 bp upstream. The fact that another PB-inducible phase II gene, *Gst-Ya,* does not possess a pyrimidine-purine repeat at −200 bp but has an ARE farther upstream (approximately −800 bp) suggests that an ARE may be located 5′ of the *ALDH-1* coding region. Because *ALDH-1* is both constitutively expressed and inducible, we can also propose that negative *cis*-acting and/or positive tissue-specific *cis*-acting elements are present. However, to be consistent with the proposed *ALDH-3* gene model, the presence of negative *cis*-acting elements is predicted. Although no data exist pro or con, an XRE element may exist upstream of the *ALDH-1* gene. This could be predicted on the basis that the *CYPIIB2* gene, which also possesses a short CACACA repeat, has an XRE core sequence immediately 5′ to it. The presence of an XRE, or, especially, an ARE may explain the induction of class 1 ALDH during the promotion phase of hepatocarcinogenesis by combined initiator-promoter exposure, if such a response were considered to be stress related.

Several experiments are suggested by the proposed organization of the class 1 and 3 ALDH genes. Determination of the complete nucleotide sequence upstream of the *ALDH-1* and *ALDH-3* genes would confirm the presence of any particular element. Should a particular sequence be identified, the construction of hybrid reporter genes and their expression under various conditions in transfected cells would confirm that these elements may be functioning in the proposed manner. Finally, induction of endogenous class 1 or class 3 ALDH activity by various inducers known to act through one or more of these elements would confirm that these elements function *in vivo*. The presence of AREs could be confirmed by testing the ability of various antioxidants known to work via these elements to increase endogenous class 1 or class 3 ALDH or hybrid reporter gene activity in hepatoma cell lines or hepatocytes in culture. Especially interesting in this context would be agents known to induce lipid peroxidation, as one would predict some association between *ALDH-3* expression, an ARE, and such inducers, if class 3 ALDH plays a role in lipid aldehyde oxidation.

The down-regulation of class 1 ALDH genes, which is postulated to occur as certain tumor cells develop sensitivity to CP-like chemotherapeutic agents, may be explained by the regulatory organization proposed above. If normal cells maintain a certain constitutive level of the CP-relevant ALDH via positive and/or negative regulatory elements, loss of a positive *trans*-acting factor, expression of a negative *trans*-acting factor, or changes in the *cis*-acting elements themselves could result in repression of the ALDH gene(s) involved in CP metabolism. When more is known about the mechanisms involved in acquiring CP sensitivity and resistance, such as when during tumorigenesis cells become sensitive and when and in what proportion cells acquire resistance, it may be possible to determine which of these alternatives is most likely. As regulatory elements are identified, ALDH gene transfection experiments similar to those described for the study of the role of ALDH in lipid peroxidation should be possible, which will also help define the mechanisms involved in modulation of ALDH activity as it relates to chemotherapy.

VI. CONCLUSIONS

Studies of the tumorigenesis-related expression of ALDH has provided a wealth of new information. For the ALDHs per se, such studies have contributed significantly toward establishing that the ALDHs are an enzyme family consisting of several distinct members encoded by at least six and perhaps many more different genes. They have also provided much information about the functional roles for many members of the family and created a framework by which to begin establishing the relationships between the various members.

With respect to differential gene expression, the ALDHs represent an excellent model system to study the tissue-specific regulation of gene expression. This enzyme system may be unique,

in that it consists of constitutive and inducible forms in both normal and neoplastic cells. The ALDH gene family offers excellent opportunities for formulating hypotheses and testing models of differential gene expression.

For tumorigenesis the ALDHs provide an excellent system for studying the biochemical changes that accompany the neoplastic transformation. Although not as well studied as the more commonly used biochemical markers of the transformed state, changes in ALDH gene expression hold great promise for studying the molecular mechanisms by which initiated cells gain a selective advantage over their normal counterparts. Because multiple mechanisms of ALDH gene regulation appear to exist, this gene family also has great promise for identifying those events and/or factors in the neoplastic transformation that alter gene expression in a tumor-specific manner.

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