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STRUCTURE AND EXPRESSION OF THE CYTOSOLIC ALDEHYDE DEHYDROGENASE GENE IN CYCLOPHOSPHAMIDE-RESISTANT MURINE LEUKEMIA L1210 CELLS

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Abstract—These investigations were performed to clarify the molecular basis for the enhanced expression of cytosolic aldehyde dehydrogenase (ALDH-1) enzymatic activity in the cyclophosphamide-resistant L1210/CPA murine leukemia cell line, as compared to the parental L1210/O strain. Western immunoblot analysis was performed using a 15-fold greater quantity of cytosolic protein from the L1210/O as compared to the L1210/CPA cell line. Nevertheless, ALDH-1 immunoreactive protein could be detected only in the L1210/CPA cells. Northern analyses, performed using total cellular and polyadenylated RNA, again demonstrated ALDH-1-specific transcripts only in the L1210/CPA cell line. This transcript was identical in size to the ALDH-1 message expressed by normal murine hepatocytes. On Southern analysis, no evidence of gene amplification, gene rearrangement, or significant mutations of length was detected. These studies suggest that the ALDH-1 protein produced by the L1210/CPA cell line is structurally normal. Moreover, overexpression of the gene does not appear to have arisen as a result of an incremental process, such as gene amplification. Rather, a qualitative abnormality in the regulation of this gene appears to exist in the L1210/CPA cells, which distinguishes them from L1210/O cells and from normal murine lymphocytes.

Cancer chemotherapy is compromised most commonly by the intrinsic or acquired resistance of a tumor to the antineoplastic agents presently available. Accordingly, efforts to characterize the drug-resistant phenotype and to develop the means to negate it have been intense.

Among the best characterized mechanisms of antitumor drug resistance is the detoxification of cyclophosphamide and related oxazaphosphorines through the action of cytoplasmic aldehyde dehydrogenase (ALDH) [1–5]. Cyclophosphamide metabolism involves hydroxylation of the oxazaphosphorine ring at the 4-position; ring opening to yield the aliphatic aldehyde aldophosphamide; and a β-elimination reaction to release acrolein and phosphoramide mustard, the active moiety (Fig. 1) [2, 6, 7]. Alternatively, the intermediate metabolite 4-hydroxycyclophosphamide/aldophosphamide can be oxidized through the action of ALDH to yield the nontoxic acid, carboxyphosphamide. Although this reaction may be catalyzed by a minimum of three isozymes of aldehyde dehydrogenase, only the cytoplasmic form, ALDH-1, exhibits the tissue distribution and kinetic parameters in murine and human tissues consistent with a role in cyclophosphamide resistance [1, 5].

The murine leukemia L1210 cell line provides a convenient experimental system in which to investigate the genetic basis for specific cyclophosphamide resistance. Two closely related strains of L1210 cells have been isolated: the parental, cyclophosphamide-sensitive strain, L1210/O; and a cyclophosphamide-resistant derivative, L1210/CPA [8]. It has been shown that: (a) cyclophosphamide-resistant L1210/CPA cells exhibit a 200-fold higher level of ALDH enzymatic activity than the sensitive L1210/O cells; (b) resistance to cyclophosphamide may be reversed in the L1210/CPA cells by pretreatment with ALDH inhibitors; and (c) both strains of L1210 cells (L1210/O and L1210/CPA) are equally sensitive to phosphoramide mustard, the end product of the cyclophosphamide-activation pathway [1, 5]. Thus, anti-tumor drug resistance in this system appears to be due to the overexpression of the ALDH enzyme. However, the genetic basis for the differential expression of ALDH activity in these two cell lines remains unknown. The present studies represent an attempt to determine the molecular basis for the overexpression of cytoplasmic ALDH in the L1210/CPA murine leukemia cell line.

MATERIALS AND METHODS

Materials. The cyclophosphamide-resistant L1210 cell line which was characterized in these experiments was derived from the strain developed by DeWys [8]. BDF1 mice, providing the normal murine lymphoid tissues which were used as controls, were obtained from Cumberland View Farms (Clinton, TN). Plasmid pUC13-ALDH-1 [9] was provided by Dr. Akira Yoshida. This plasmid contains a 1.6 kbp

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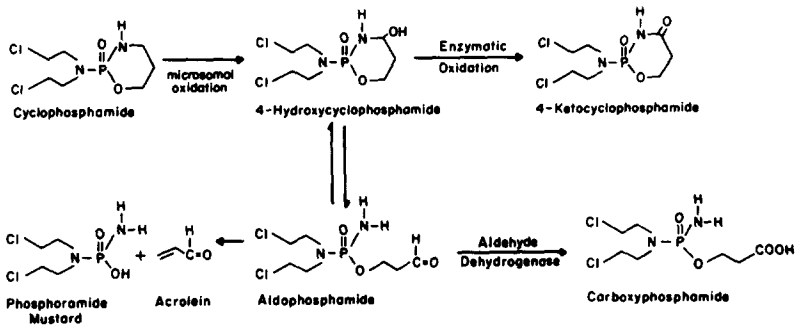

Fig. 1. Metabolic activation of cyclophosphamide. Cyclophosphamide must be activated by P450 microsomal enzymes in the liver in order to produce the active alkylating agent, phosphoramide mustard. Aldehyde dehydrogenase can oxidize aldophosphamide, an intermediate metabolite in this pathway, to yield the nontoxic acid, carboxyphosphamide.

fragment of human ALDH-1 cDNA, spanning the coding sequences for amino acids 161 through 500 of the protein, the 3'-polyadenylation signal, and an additional 538 bp of noncoding, 3'-flanking DNA. The ALDH-1 fragment was excised from the plasmid with Eco RI, and was subdivided into fragments of approximately 900, 560, and 160 bp with Pst. I. ^{125}I-Labeled Protein A and [^{32}P]dCTP were obtained from New England Nuclear Research Products (Boston, MA). Rabbit anti-mouse, ALDH-1 specific antibodies were prepared in our laboratory as previously reported [5].

Murine lymphoid tissues. Single cell suspensions were prepared from the thymus and spleen of a BDF1 mouse by passing the tissues through a stainless steel screen. The splenocytes were further purified by centrifuging the cell suspension at 200 *g* for 30 min on a Ficoll–Hypaque density gradient (specific gravity 1.077), and recovering the mononuclear cell layer. By morphologic examination, final preparations from both the thymus and spleen contained greater than 95% lymphocytes.

Southern analysis. Genomic DNA from normal BDF1 splenic and thymic lymphocytes, and from logarithmically growing L1210/O and L1210/CPA cells, was prepared according to the method of Blin and Stafford [10]. Twenty micrograms of genomic DNA from each sample was digested to completion with the indicated restriction endonuclease; electrophoresed through 0.8% agarose gels; transferred to nitrocellulose filters; and hybridized according to standard methods [11]. The filters were probed successively with ALDH-1 and β-actin cDNA fragments labeled with [^{32}P]dCTP according to the random primer method of Feinberg and Vogelstein [12]. Filters hybridized to ALDH-1 probes were washed successively in 2 × SSC with 0.1% sodium dodecyl sulfate (SDS) at room temperature (where 1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0); and in 0.1 × SSC with 0.1% SDS at 42°. Filters hybridized to the β-actin probe were washed in the same solutions at 65°. Hybridizing sequences were visualized by autoradiography using Kodak X-OMAT AR film. Equivalent loading of the gels, and relative gene copy number, were estimated by densitometric analysis of the autoradiographs using a Hoefer scanning transmission densitometer.

Northern analysis. Total cellular RNA was extracted in a guanidinium isothiocyanate buffer, and separated from DNA and proteins by cesium chloride gradient centrifugation [13]. Polyadenylated RNA was purified from total cellular RNA by affinity column chromatography, according to standard protocols [11]. The RNA was resolved by electrophoresis through 1.0% formaldehyde-containing agarose gels, and was transferred to nitrocellulose filters by capillary blotting. The filters were hybridized to ^{32}P-labeled cDNA probes, washed, and analyzed using conditions identical to those described above for DNA analysis.

Western analysis. L1210 cells were washed three times in cold phosphate-buffered saline, and resuspended in 10 mL of freezing buffer to which protease inhibitors had been added (0.1 M sodium phosphate at pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 200 units/mL aprotinin, 50 μg/mL leupeptin). The cells were subjected to three cycles of freezing and thawing in a solid CO_2/methanol bath, and particulate matter was removed by selective centrifugation (10 min at 4000 *g*; then 45 min at 48,000 *g*). The supernatant was recovered and assayed for the presence of cytosolic ALDH-1 proteins by Western blot analysis. The ALDH-1 proteins were detected using affinity-purified rabbit anti-mouse antibodies, and visualized autoradiographically using ^{125}I-Protein A (0.1 μCi/mL), according to methods previously described [5].

RESULTS

Western immunoblot analysis was performed on cytosolic extracts prepared from logarithmically growing L1210/O and L1210/CPA cells, using a 1:200 dilution of the anti-ALDH-1 antibody previously described (Fig. 2). This antibody has been shown to be specific for the cytosolic isoform of ALDH, and to cross-react with ALDH-1 proteins derived from normal murine hepatocytes, from a murine hepatoma (1C1C7), and from two murine

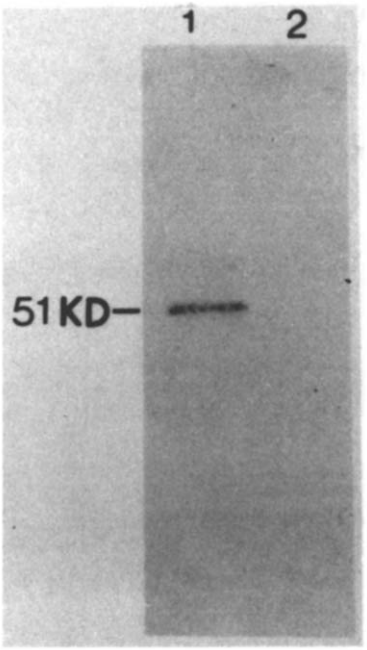

Fig. 2. Western immunoblot analysis of L1210/CPA and L1210/O cytosolic proteins, probed with an antibody specific for the cytosolic ALDH-1 isozyme. Lane 1 was loaded with 10 μg of protein from the L1210/CPA cell line; lane 2 contained 150 μg of L1210/O cytosolic proteins. The proteins were resolved by SDS–polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose filters, and probed with a monospecific, rabbit anti-mouse ALDH-1 antibody. The antibody was detected using ^{125}I-labeled Protein A, and visualized autoradiographically.

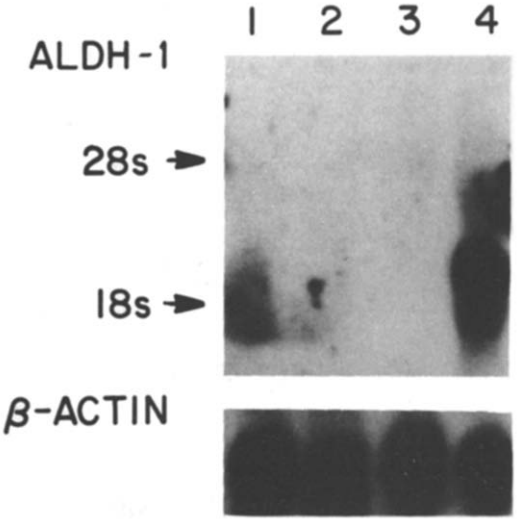

Fig. 3. Expression of the ALDH-1 gene in normal murine lymphocytes and in L1210 murine leukemia cells. Total cellular RNA was extracted from L1210/O and L1210/CPA cells, and from thymic and splenic lymphocytes isolated from a normal BDF1 mouse. Approximately 10 μg of RNA of each type was resolved electrophoretically on a formaldehyde-containing 1.0% agarose gel, and was transferred by capillary blotting to a nitrocellulose membrane. The filter was hybridized sequentially to ^{32}P-labeled ALDH-1 560 bp cDNA and β-actin probes, as described in the text. Lane 1: normal murine thymic lymphocytes; lane 2: normal murine splenocytes; lane 3: L1210/O cells; and lane 4: L1210/CPA cells.

hematopoietic cell lines (416B and P388/CPA) [5]. A 51 kDa band was detected readily in the L1210/CPA lane. The size and immunoreactivity of this band, as well as the kinetic characteristics of the purified enzyme which were reported previously [5, 14], suggest that the ALDH protein manufactured by L1210/CPA cells is structurally normal. However, despite loading a 15-fold greater quantity of cytosolic proteins in the L1210/O lane, no ALDH-1 immunoreactive species could be detected in these cells. These data suggest that the elevated level of ALDH activity characteristic of L1210/CPA cells can be attributed to the aberrant production of a normal ALDH-1 protein, rather than to a mutation which has enhanced the activity of the enzyme.

Total cellular RNA from normal murine splenic and thymic lymphocytes, L1210/O cells, and L1210/CPA cells was purified and assayed by Northern hybridization for the expression of the ALDH-1 gene (Fig. 3). ALDH-1-specific transcripts were detected only in the RNA isolated from L1210/CPA cells. This transcript, which migrates, approximately, as a 1.4 kb band, was identical in size to the ALDH-1 message expressed by normal murine hepatocytes (data not shown). These results correlate with those of the Western blot, above, and support the judgment that the ALDH-1 protein produced by L1210/CPA cells is structurally normal.

Additional Northern blots were prepared using a 12-fold excess of L1210/O to L1210/CPA total cellular RNA, and polyadenylated RNA, respectively, in an effort to detect low levels of ALDH-1 expression by the parental L1210/O cell line (Fig. 4). Despite these attempts, ALDH-1-specific transcripts could not be demonstrated in the L1210/O cells. Anomalous expression of the ALDH-1 gene appears to be restricted to the L1210/CPA cell line and is not evident, even at trace levels, in the parental strain. Thus, the abnormality inherent in the L1210/CPA cell line would appear to represent a qualitative difference in gene regulation, rather than a progressive, or incremental process.

Genomic DNA from the L1210/O and L1210/CPA cell lines was digested to completion with Eco RI, and hybridized sequentially to ALDH-1 and β-actin probes (Fig. 5). The restriction patterns generated by the ALDH-1 genes from the two L1210 cell lines matched each other exactly. The relative intensities of the ALDH-1 and β-actin signals were equal in the two L1210 strains, thereby excluding gene amplification as the cause of drug-resistance in the L1210/CPA cell line.

The restriction patterns detected with the whole ALDH-1 probe were complex and difficult to interpret. Therefore, the ALDH-1 insert was subdivided into three fragments of approximately 900, 560, and 160 bp using sequential Eco RI and Pst I digestions. An additional Southern blot was

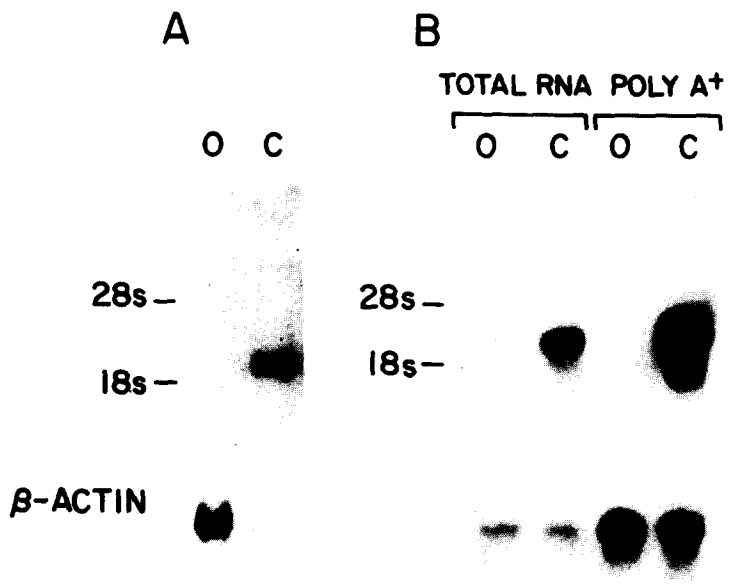

Fig. 4. ALDH-1 gene expression in L1210/O and L1210/CPA cells. (A) Northern analysis of total cellular RNA from L1210/O and L1210/CPA cells. Lane C: 3 μg of RNA from L1210/CPA cells; lane O: 30 μg of RNA from L1210/O cells. The RNA was size-fractionated electrophoretically, transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled ALDH-1 560 bp cDNA and β-actin probes, as described in the text. The lanes were deliberately loaded differentially in an attempt to demonstrate low levels of ALDH-1 expression in the parental, L1210/O cell line. (B) Northern analysis, using total cellular and polyadenylated RNA from L1210/O and L1210/CPA cells. Polyadenylated RNA was purified from total cellular RNA by affinity chromatography, using an oligo(dT)cellulose column. Approximately 15 μg of RNA was loaded in each lane. Lanes labeled O were loaded with RNA from L1210/O cells; lanes labeled C contained RNA from L1210/CPA cells. The filter was hybridized sequentially to ^{32}P-labeled ALDH-1 560 bp cDNA and β-actin probes.

hybridized sequentially to the first two of these ALDH-1 fragments (Fig. 6). (The 160 bp fragment contains only non-coding, 3′-flanking DNA, and was not relevant.) The restriction patterns generated by the two L1210 cell lines, using each of these ALDH-1 probes, corresponded exactly. Thus, no evidence of a gene rearrangement or other significant mutation of length was detected. Although these investigations cannot rule out point mutations or microdeletions in the ALDH-1 gene, they indicate that neither gene amplification, rearrangements of significant length, nor mutations affecting the structural sequences of the gene are responsible for overexpression of ALDH-1 in the cyclophosphamide-resistant L1210/CPA cell line.

DISCUSSION

Among the genetic changes that have been demonstrated to promote anti-tumor drug resistance, gene amplification has received the most attention [15–17]. Amplification of specific genes, with overexpression of their protein products has been implicated in the resistance of certain tumors to methotrexate [16, 18–22], *N*-(phosphonoacetyl)-L-aspartate (PALA) [23, 24], deoxycorfomycin [25], fluorodeoxyuridine (FUDR) [26], and cisplatin [27]; and appears to be responsible for certain cases of pleiotropic drug resistance [28, 29]. Mutations which alter the structure or biological activity of proteins necessary for the activity of a particular drug are also well documented [16, 17]. Mutations of this type explain certain cases of resistance to methotrexate [19, 30, 31], steroid hormones [32, 33], and vinca alkaloids [34]. Gene deletions, which preclude the expression of critical proteins, may be considered in this category. Lastly, it has been demonstrated that drug resistance may occur as a result of genes induced by the cytotoxic agent itself, without recourse to somatic mutations. It has been shown, for example, that *cis*-regulatory sequences in the 5′-flanking region of the *mdr*1 gene respond to vincristine, cholchicine, and daunomycin [35]; and that expression of this gene can be induced *in vivo* by xenobiotic agents [36]. In KB carcinoma cells studied sequentially during colchicine selection, elevated expression of the *mdr*1 gene often precedes gene amplification [37].

The genetic basis for drug resistance in the L1210/CPA cell line appears to be distinct from these mechanisms. Cyclophosphamide resistance in these cells results from the high level of aldehyde dehydrogenase enzymatic activity in their cytoplasm [1, 3, 4]. The size and immunoreactivity of the ALDH-1 protein, the length of the ALDH-1 mRNA transcript, and the restriction pattern produced by the ALDH-1 gene on Southern analysis indicate that the enzyme produced by the L1210/CPA cell line is structurally normal. Moreover, aberrant ALDH-1 expression persists in these cells as a stable trait, in

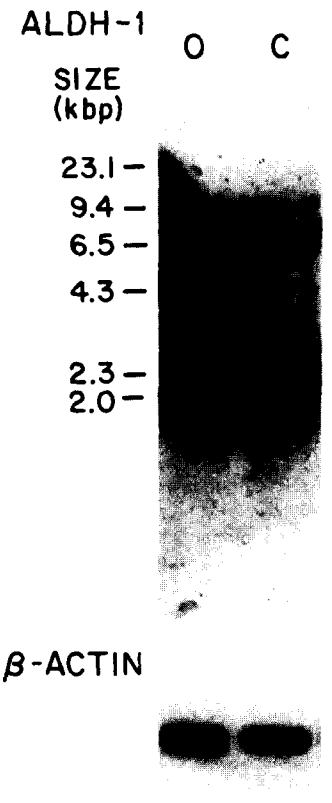

Fig. 5. Southern analysis of the ALDH-1 gene in L1210/O and L1210/CPA cells. Twenty micrograms of genomic DNA from L1210/O and L1210/CPA cells was digested to completion with restriction endonuclease Eco RI, resolved on a 0.8% agarose gel, and transferred to a nitrocellulose hybridization membrane. The filter was hybridized sequentially to ^{32}P-labeled ALDH-1 (1600 bp) and β-actin cDNA probes; and the hybridized sequences were visualized autoradiographically. Lane O: DNA from L1210/O cells; lane C: DNA from L1210/CPA cells. Densitometric analysis of the autoradiographs, normalized to equivalent β-actin signals in each lane, was consistent with equal numbers of ALDH-1 genes in the two L1210 cell lines.

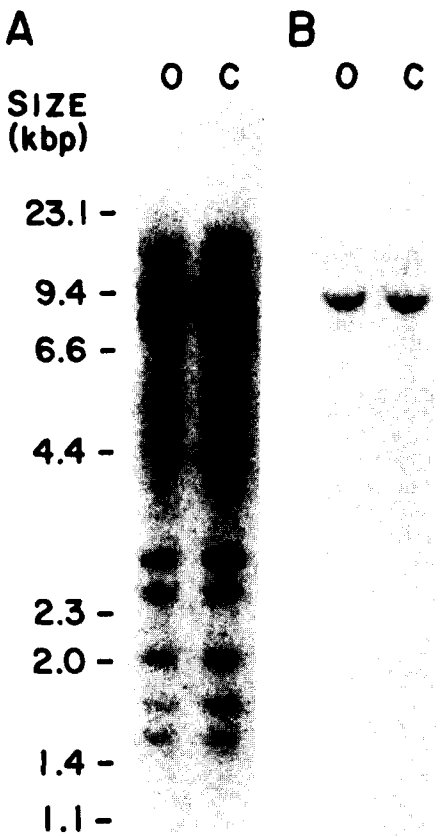

Fig. 6. Southern analysis of the ALDH-1 gene in L1210/O and L1210/CPA cells, probed with fragments of the ALDH-1 1600 bp insert. This Southern blot was prepared in a manner identical to the one shown in Fig. 5. Twenty micrograms of DNA from each of the two L1210 cell lines were digested with Eco RI, electrophoresed through a 0.8% agarose gel, and were transferred by capillary blotting to a nitrocellulose hybridization membrane. Panel A demonstrates the ALDH-1 fragments detected with the ALDH-1 900 bp cDNA probe; panel B shows the same filter hybridized to the ALDH-1 560 bp fragment. Lane O: DNA from L1210/O cells; lane C: DNA from L1210/CPA cells.

the absence of cyclophosphamide exposure. In this respect, ALDH-1 expression in the L1210/CPA cell line is different from many other drug-resistance genes, which are induced transiently in response to the chemotherapeutic agent, but which are down-regulated when the drug is withdrawn. Lastly, no evidence of gene amplification or gene rearrangement was discovered to explain the striking disparity in ALDH-1 expression between the parental and cyclophosphamide-resistant clones.

Cyclophosphamide resistance in the L1210/CPA cell line appears to be due to the elevated expression of a normal structural gene, in the absence of gene amplification, rearrangements, or significant mutations of length. Other mutations must be considered, therefore, which could influence the expression of this gene, but which would not have been detected by the investigations reported here. These include point mutations or microdeletions within the 5′- or 3′-*cis*-regulatory sequences of the gene, major rearrangements which place a structurally intact ALDH-1 gene in proximity to an alien sequence, and/or mutations of unrelated genes involved in the control of gene expression. Alternatively, epigenetic alterations in the ALDH-1 gene may be responsible for enhancing ALDH-1 gene expression. Lin and coworkers [38] have demonstrated hypomethylation of the ALDH-1 gene in four of six rat hepatoma cell lines which constitutively express the enzyme. Since the methylation of specific bases within or adjacent to structural genes has been associated with the inhibition of gene transcription [39–41], hypomethylation of the gene may be responsible for enhancing ALDH-1 expression in the L1210/CPA cell line. Lastly, post-transcriptional mechanisms may play a critical role in the regulation of this gene.

Abnormalities affecting RNA processing or stability also must be considered. Additional studies will be necessary to determine the fundamental mechanisms responsible for aberrant ALDH-1 gene regulation in the L1210/CPA cell line.

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