Feedback Inhibition of the Retinaldehyde Dehydrogenase Gene ALDH1 by Retinoic Acid through Retinoic Acid Receptor α and CCAAT/Enhancer-binding Protein β*

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Aldehyde dehydrogenase 1 (ALDH1) plays a major role in the biosynthesis of retinoic acid (RA), a hormone required for several essential life processes. Recent evidence, using the aryl hydrocarbon receptor-null mouse, suggests that elevated hepatic RA down-regulates ALDH1 in a unique feedback pathway to control RA biosynthesis. To determine the mechanism of suppression of the ALDH1 gene by RA, transactivation studies were carried out in Hepa-1 mouse hepatoma cells. RA decreased expression of an ALDH1-CAT construct containing -2536 base pairs of DNA upstream of the transcription start site. Retinoic acid receptor α (RARα) transactivates the ALDH1 gene promoter through a complex with an RA response-like element (RARE) located at -91 to -175 bp, which bound to the RARα/retinoid X receptor β heterodimer. CCAAT/enhancer-binding protein (C/EBPβ) also transactivates the ALDH1 gene promoter through a CCAAT box located 3' and directly adjacent to the RARE, and the ALDH1 gene is down-regulated in C/EBPβ-null mouse liver. Exposure of Hepa-1 cells to RA results in a decrease in C/EBPβ mRNA levels; however, there was no difference in mRNA and protein levels between wild-type and AHR-null mouse liver. These data support a model in which the RARαs and C/EBPβ activate the ALDH1 gene promoter through the RARE and C/EBP response elements, and in Hepa-1 cells, high levels of RA inhibit this activation by decreasing cellular levels of C/EBPβ.

Retinoic acid is derived from vitamin A (retinol) and plays a critical role in mammalian development and cellular homeostasis (1). The biological activity of all-trans-retinoic acid (RA)1 is mediated by the nuclear receptors retinoic acid receptor (RAR) and retinoid X receptor (RXR) (2). The major biosynthetic pathway of retinoic acid from retinol is the irreversible oxidation of retinal to retinoic acid catalyzed by a cytosolic aldehyde dehydrogenase 1 (ALDH1).2 Aldehyde dehydrogenases are a family of enzymes that catalyze the oxidation of aldehydes to their carboxylic acid forms. They participate in the detoxification of acetaldehyde (3) and the metabolism of biogenic amines (4), corticosteroids (5), and retinoic acid (6). Recently, a critical role for this enzyme in mammalian development was established by the finding that retinaldehyde dehydrogenase-2 (Raldh2, also known as mouse ALDH1) homozygous null embryos fail to develop due to multiple abnormalities (7).

Studies using the aryl hydrocarbon receptor-null mouse model revealed a novel pathway of feedback control of RA synthesis (8). Due to lower cellular levels of an RA catabolism, these mice have elevated hepatic levels of RA and retinyl palmitate, the main storage depot of RA. Levels of the Raldh2 mRNA are also markedly lower in these animals as compared with wild-type mice, indicating that RA suppresses expression of the Raldh2 gene. In humans, the retinaldehyde dehydrogenase is designated ALDH1 (9).

In the present study, the molecular mechanisms by which RA down-regulates the ALDH1 gene expression were investigated. Transactivation studies revealed that RARα and the CCAAT/enhancer-binding protein β (C/EBPβ) control ALDH1 reporter gene expression, and positive activation by these factors is inhibited by RA. In Hepa-1 mouse hepatoma cells, the RA inhibitory effect is mediated by decreased cellular levels of C/EBPβ. However, this mechanism does not occur in the intact mouse liver.

EXPERIMENTAL PROCEDURES

Materials—Mouse hepatoma-derived Hepa-1 cells were obtained from Daniel W. Nebert and characterized in an earlier report (10). All-trans-retinoic acid was obtained from Sigma. The ALDH1 gene promoter chloramphenicol acetyltransferase (CAT) constructs designed pCAT-2536, pCAT-673, pCAT-91, and pCAT-50 were provided by Akira Yoshida (City of Hope, Duarte, CA) and described previously (9). The deleted and mutated pCAT-91AC and pCAT-91mut1 vectors containing the deletion of CCAAT box and the mutation CCAAT→CCTCT, respectively, were generated by polymerase chain reaction-directed mutagenesis (Promega Biotech, Madison, WI) following the manufacturer’s recommendations. The Raldh2 cDNA was obtained from Gregg Duester (Burnham Institute, La Jolla, CA) (11). The SV40 promoter region prepared from pCAT-promoter vector (Promega Biotech, Madison, WI) was ligated with pUMSVOCAT, and the resultant pCAT-SV40 vector was used as an internal reference for CAT assay. The rat pMEX-C/EBPα and pMEX-C/EBPβ expression vectors were described earlier (12) and were provided by Peter F. Johnson (NCI-Frederick Cancer Research and Development Center, Frederick, MD). Ronald M. Evans (Howard Hughes Medical Institute, San Diego, CA) supplied the human RARα expression vector (13). RARα and RXRβ

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1 The abbreviations used are: RA, all-trans-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; ALDH1, human cytosolic retinaldehyde dehydrogenase 1; RARE, retinoic acid response element; PBS, phosphate-buffered saline; C/EBP, CCAAT/enhancer-binding protein; CP1, CCAAT protein 1; CP2, CCAAT protein 2; CTF, CCAAT transcription factor; AHR, aryl hydrocarbon receptor; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; bp, base pairs; Raldh2, retinaldehyde dehydrogenase-2.

2 This gene is formally named ALDH1A1 according to http://www.uchsc.edu/sp/sp/aldchbase/aldhcov.html.
antibodies were obtained from Affinity Bioreagents (Golden, CO), and the C/EBPβ antibody was from Santa Cruz Biotechnology (Santa Cruz CA). The aryl hydrocarbon receptor (AhR)-null (14) and C/EBPβ-null (15) mice were described previously.

**Transfection and CAT Assays**—Hepa-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) (Life Technologies, Inc.), and 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Transient transfection assays were carried out using the standard calcium phosphate co-precipitation technique (16). The cells were co-transfected with 6 µg of the test plasmid and 2 µg of pBSV-luciferase control plasmid (16). After 24 h, the treated cells were washed with phosphate-buffered saline and harvested by scraping and centrifugation, resuspended in 0.25% Tris- HCl, pH 7.8, 1 mM dithiothreitol, and lysed by freezing/thawing four times. Aliquots from the supernatant of the lysate were used for luciferase assays (17). The remainder of the supernatant was heated to 65 °C for 10 min to inactivate deacetylase and stored at −70 °C prior to determine the CAT activity. CAT assays were performed using the phase-extraction method (18) using [14C]-chloramphenicol as substrate, and CAT activity was normalized to luciferase activity. The CAT activity was determined by scanning the amount of 14C radioactivity in the thin layer chromatography plates using a Molecular Dynamics Storm PhosphorImager. Luciferase activity was measured using an Analytical Luminescence Laboratory Monolight luminoimeter.

**Electrophoretic Mobility Shift Assays (EMSA)***—Double-stranded oligonucleotides to the RARα-binding site (5′-CGAGTTACGGCAGGATGTACGC-3′) (19), the 5′ upstream human ALDH1 gene (91 to 59 region (5′-GCCTGATTTTGGTCACTCATCGTGTTTC-3′), −91 to −75 region (5′-GCCTGAGGGGTTCATCATC-3′), and an unrelated double-stranded oligonucleotide (5′-CTGAGGACACTGCCCAGCTCATGTGCGCT-3′) were end-labeled using the Klenow fragment of DNA polymerase I and [32P]dCTP (Amersham Pharmacia Biotech). Hepa-1 cell nuclear extracts (10 µg), prepared as described (20), were incubated for 30 min at 25 °C in a 20-µl reaction mixture (20 mM Tris- HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol, 80 mM KCl, 12% glycerol, 0.5 µg of poly[dIl-dc]), with 2 µl of the double-stranded oligonucleotide probe (50,000 cpm). The mixtures were subjected to electrophoresis in a 4% polyacrylamide gel at 4 °C. In competition assays, a large excess of unlabeled double-stranded oligonucleotide competitor was incubated together with the nuclear extract prior to adding the using the [32P]-labeled probe. The sequences of the CCAAT motif oligonucleotides were as follows: CCAAT protein 1 (CP1) (5′-AGACCTTGTAAAAAGACTAACAATTCTAACGAAAAC-3′), CCAAT protein 2 (CP2) (5′-GTTTTACTCGTGAGCAAGCAACACAGC-3′), CCAAT transcription factor (CTF) (5′-TACCTTTTTGGATTGAAGCCAATATGATAAT-3′), and C/EBP (5′-CTGAGGACACTGCCCAGCTCATGTGCGCT-3′) (21). For gel mobility supershifting assays, RARα and RXRβ antibodies were incubated with the probe-nuclear extract mixtures for additional 60 min prior to gel electrophoresis.

**Western Blot Analysis**—Hepa-1 cells were harvested, lysed in 1 ml of phosphate-buffered saline containing 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, and 1 mM phenethylmethylsulfon fluoride and subjected to centrifugation at 1,500 × g for 5 min. Protein concentrations were determined by the BCA protein assay (Pierce), and 50 µg of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide. Proteins were transferred to Immobilon-P membranes (Millipore) and probed as recommended by the manufacturer. Rabbit polyclonal RARα antibody was used at 1:200 dilution in phosphate-buffered saline, 5% non-fat dry milk, and proteins interacting with antibodies were detected by an enhanced chemiluminescence blot detection system (Amersham Pharmacia Biotech).

**Northern Analysis**—Total RNA from Hepa-1 cell cultures or mouse liver was isolated by homogenizing cells in a guanidine/phenol solution (Biotex Laboratories, Houston, TX). Total RNA (20 µg) was subjected to electrophoresis in a 1% agarose, 2.2 M formaldehyde gel and transferred to GeneScreen Plus membranes in 20 × SSC (3 M NaCl, 30 mM sodium citrate, pH 7.0). The RNA was fixed to the membranes by baking at 80 °C in a 20 × SSC solution, and membranes were incubated at 42 °C with the [32P]-labeled probes. cDNAs were labeled by random priming with DNA polymerase I Klenow fragment using [32P]dCTP (Amersham Pharmacia Biotech). Labeled probes were added to the membranes at 2.0 × 10⁶ cpm/ml. Filters were washed in 0.1 × SSC and 0.5% sodium dodecyl sulfate, and the membranes were exposed to autoradiographic film overnight at −80 °C with aid of an Phosphor screen.

**RESULTS**

RARα Activates the ALDH1 Gene Promoter through a RARE—AhR-null mouse livers present high levels of RA together with decreased ALDH1 mRNA levels when compared with wild-type mouse livers, suggesting an involvement of RAR in ALDH1 gene regulation. To delineate the mechanism of retinoic acid action on human ALDH1 gene expression, a reporter plasmid containing 2536 bp of the human ALDH1 gene promoter sequence linked to a CAT gene (pCAT-2536) was used. The pCAT-2536 construct was transfected into Hepa-1 cells and treated with RA. RA at 0.01 µM inhibited about 50% of the activity and 70% at 1 µM (Fig. 1). These data indicate that RA is able to decrease activity of the ALDH1 gene promoter.

To assess whether the RA effects on ALDH1 gene regulation were mediated by the RAR, Hepa-1 cells were co-transfected with an RARα expression vector and the pCAT-2536 reporter construct. In the absence of exogenous RA, increased RARα levels resulted in an increase in the CAT activity to a maximum of 7-fold over the control with no exogenous RARα (Fig. 2). The amounts of transfected RARα expression vector correlated with an increase in RAR protein (Fig. 2). RARα was also detected in non-RARα-transfected cells. Addition of RA inhibited RARα-mediated transcription by approximately 50%. These data establish that RARα is responsible, in part, for controlling expression of the ALDH1 gene and that RA inhibits RARα-mediated transactivation.

To establish the location in the ALDH1 gene promoter of the RARα response element, deletion constructs were examined by co-transfection with the RARα expression vector. All reporter constructs were activated by RARα except for the pCAT-50 (Fig. 3). These data indicated that the putative ALDH1 RARE is located between −50 and −91 from the transcription start site. Nucleotide sequence analysis of the human (−39 to −107 bp) ALDH1 gene 5′-flanking region revealed that a sequence similar to the consensus RARE motif, AGTTCA, is located at −75/−81 bp (Fig. 4). This segment is a possible site through which RARα might exert ALDH1 transcriptional control. A CCAAT box and octamer-binding site were also found in this region.

To determine whether RARα binds to the RARE-like motif, EMSA was performed (Fig. 5). Addition of Hepa-1 nuclear extracts resulted in the formation of three DNA-protein complexes. This binding was saturated by excesses of unlabeled 81 bp (Fig. 4). This segment is a possible site through which RARα might exert ALDH1 transcriptional control. A CCAAT box and octamer-binding site were also found in this region.

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Regulation of the ALDH1 Gene

**FIG. 2.** Effect of RARs on the pCAT-2536 activity. Hepa-1 cells were co-transfected with pCAT-2536 and the RARα expression vector and treated for 48 h with 1 μM RA or Me2SO vehicle, and CAT activity was measured and normalized for transfection efficiency. Values shown are the mean ± S.D. of triplicate determinations. *p < 0.05 compared with 1st column; **p < 0.05 compared with 4th column.

**FIG. 3.** Effect of RARα on the activities of the ALDH1-pCAT deletion constructs. Deletion constructs containing different lengths of the human ALDH1 gene 5’-flanking region were co-transfected with the RARα expression vector (5 μg) into Hepa-1 cells. CAT activity was measured and normalized for transfection efficiency, and the fold induction was calculated as the ratio of CAT activity in RARα transfected cells to that in control cells (without the RARα expression vector). Values shown are the mean of triplicate determinations.

**FIG. 4.** Nucleotide sequence of human ALDH1 gene 5’-flanking region. CCAAT box and Oct regulatory elements are enclosed by boxes, and the RARE-like element is underlined.

**FIG. 5.** EMSA analysis of protein binding to the human −91/−59 ALDH1 gene promoter region. Nuclear extracts from Hepa-1 cells were incubated with radiolabeled −91/−59 alone (lane 2), −91/−59 plus a 100-fold molar excess of a nonspecific unlabeled competitor (NR-DNA) oligonucleotide (lane 3), −91/−59 plus a 10-, 20-, 50-, and 100-fold molar excess of unlabeled −91/−59 (lanes 4–7), or −91/−59 plus a 10-, 20-, 50-, and 100-fold molar excess of unlabeled consensus RARE (lanes 8–11). Lane 1 is radiolabeled probe alone. The arrows identify the shifted bands and the unbound radiolabeled probe.

**FIG. 6.** Binding of RAR to the human −91/−59 ALDH1 gene region. Nuclear extracts from Hepa-1 cells were incubated with radiolabeled consensus RARE (lanes 1–4) or radiolabeled −91/−59 (lanes 5–8) and then incubated with RARα antibody (lanes 3, 4, 7, and 8) for 60 min, and EMSA supershift analysis was performed. Lanes 1 and 5 are consensus RARE and −91/−59 radiolabeled probes, respectively, plus nuclear extract. Lanes 2 and 6 are incubations with a unrelated antibody (Ab) (Cyclin B, 8 μg). The arrows identify the shifted bands.

To compare the affinity of RARα for the human −91/−59 5’-flanking region with a consensus RARE, competitive inhibitions were performed (Fig. 5), and the relative intensity of complex 2 was determined (Fig. 6). RARE competed equivalently with the human −91/−59 oligonucleotide for binding to RARα/RXRβ, indicating that the human ALDH1 −91/−59 region is functionally similar to the consensus RARE.

C/EBPβ Activates the ALDH1 Gene Promoter—The CCAAT box found in the human ALDH1 gene promoter region could represent a binding site for a number of nuclear factors includ-
Regulation of the ALDH1 Gene

The effect of RA on C/EBP expression was determined in a competitive EMSA. Binding of the human ALDH1 gene promoter transactivation, Hepa-1 cells were co-transfected with a C/EBP expression vector pMEX-C/EBP and the pCAT-91 reporter construct. Transfection of the C/EBP expression vector resulted in an increase of the CAT activity that reflected the increased ratio of the vector to the reporter construct DNA (Fig. 10B). However, when constructs containing deleted and mutated CCAAT boxes (pCAT-91ΔC and pCAT-91mut1, respectively) were co-transfected with the pMEX-C/EBP expression vector, no changes in CAT activity were observed. Moreover, deletion and mutation of the CCAAT box resulted in a 5-fold decrease of CAT activity compared with that expressed by pCAT-91 (Fig. 10C), indicating that the binding of C/EBP is essential for basal promoter activity. A pMEX-C/EBP expression vector did not activate expression of pCAT-91 (data not shown). These data establish that C/EBP together with the RARα/RXR heterodimer is responsible in part for the expression of the ALDH1 gene.

To examine whether RA was able to alter binding of RARα/RXR heterodimer and/or C/EBP to −91 to −59 human ALDH1 gene promoter binding sites, Hepa-1 cells were treated with 1.0 μM RA, and the nuclear extracts were used for gel mobility shift analysis. Binding of the human −91 to −59 probe to complex 3 was significantly decreased using extracts from RA-treated cells compared with untreated cells (Fig. 11A). These data show that RA causes a decrease in C/EBP binding. However, addition of RA directly to the nuclear extracts of untreated cells did not alter formation of the complex thus ruling out a direct interaction of RA with C/EBP (data not shown).

Expression of C/EBP mRNA Is Down-regulated by RA—The effect of RA on C/EBP binding to ALDH1 gene CCAAT region could be due to a decrease in C/EBP transcription. To determine whether RA alters C/EBP expression, Hepa-1 cells were treated with RA, and mRNAs encoding ALDH1, C/EBPα, and C/EBPβ were analyzed by Northern blotting. C/EBPα, C/EBPβ, and ALDH1 mRNA levels were significantly reduced after RA treatment (Fig. 11B, lanes 2 and 3).

To determine whether levels of expression of C/EBPβ are altered in the AHR-null, mRNA was measured (Fig. 12). C/EBPβ mRNA levels were not different between wild-type and AHR-null mice. Levels of C/EBPβ protein were also not different between the two genotypes (data not shown). However, ALDH1 expression was markedly lower in livers of mice lack-
These data suggest that the ALDH1 gene is controlled by C/EBPβ in the mouse, but its regulation by RA in liver may be through a C/EBPβ-independent mechanism.

DISCUSSION

A feedback pathway to control levels of RA synthesis was uncovered using the AHR-null mouse (8). This mouse model has a defect in RA catabolism leading to elevated RA levels and decreased ALDH1 mRNA levels, suggesting that RA play an important role in ALDH1 gene regulation. The goal of these studies was to determine the mechanism by which RA controls ALDH1 gene expression.

Transactivation studies using the human ALDH1 gene promoter linked to a reporter vector revealed that RARα stimulates promoter activity in the absence of exogenous RA, indicating that RARα mediates ALDH1 gene expression. Although
no exogenous ligand was added, the endogenous RA probably activates RARα. Similar spontaneous activation was observed in other studies when RXR, RARα, and RARB expression vectors were used (22–24). By using deletion constructs of the ALDH1 gene 5’-flanking region, a putative RARE within the −91/−59 region of the human ALDH1 promoter was identified. This segment of DNA is highly conserved between the human, mouse, and rat ALDH1 gene promoters (9) and contains an octamer factor-binding site and CCAAT box. The RARE-like motif was located upstream and adjacent to the CCAAT box. EMSA confirmed that RARα/RXRB heterodimers bind to the human −91/−59 ALDH1 gene 5’-flanking region with similar affinity to the consensus RARE DR-5 type element. The 15 bp corresponding to the human −91/−75 region was identified as the minimal response element.

RAREs can be grouped into 3 categories, depending on the orientation of their AGTTCA half-site as follows: 1) direct repeats; 2) palindromes; and 3) complex elements with little or no obvious consensus structure. The RARE reported here seems to belong to the last group. Complex RAREs are typically only weakly responsive, and its activation requires RAR overexpression and generally shows lower affinity for the receptor in vitro (25–27). However, the affinity of the human −91/−59 ALDH1 gene promoter region to the RARα/RXRB appears to be similar to RARE/DR-5 element, which is one of the most potent sequences found in promoters of RARα-responsive genes (28).

In the current study, exogenous RA inhibits ALDH1 promoter expression and blocks RARα stimulatory effects, suggesting that RARα may activate the gene at low cellular levels of RA, whereas RA inhibitory effects occur at higher concentrations. In this regard, RA has been shown to inhibit the expression of certain genes by antagonizing the enhancer interaction of the transcription factor activator protein 1 (29). In the case of the ALDH1 gene, however, no apparent activator protein 1 site was found in the 5’ promoter region. It was also reported that retinoids inhibit gene expression by antagonizing the enhancer action of C/EBPβ (30). In fact, C/EBPβ was found to be a positive regulator of the ALDH1 gene promoter, and its response element (CCAAAT box) is located downstream of the RARE. The deletion and mutation analysis demonstrated that the CCAAT box is critical for the ALDH1 promoter activity. The role of C/EBPβ in regulation of the ALDH1 gene in the context of the animal was suggested by the fact that levels of ALDH1 mRNA were markedly lower in C/EBPβ-null mice as compared with wild-type animals.

The possibility that the inhibitory effects of RA on ALDH1 gene transcription occur through interference with C/EBPβ (31, 32) was investigated using EMSA with Hepa-1 cell nuclear extracts treated with RA. The results demonstrated that C/EBPβ binding to the CCAAT box element decreased, whereas no change of RARα binding to the RARE was observed. Therefore, it seems that high levels of RA inhibit ALDH1 gene expression by antagonizing C/EBPβ action. However, the decrease of C/EBPβ binding appears not to be as a result of direct interference of binding to the CCAAT box since addition of RA directly to the nuclear extracts of untreated cells did not alter the complex formation (data not shown). Rather, RA appears to down-regulate C/EBPβ expression thus offering a potential mechanism for the RA inhibitory effects. Indeed, it was reported that RA regulates C/EBPα and C/EBPβ during liver development (33), blocks adipogenesis by inhibiting C/EBPβ-mediated transcription, and blocks the expression of the C/EBPα gene (34). Regulation of C/EBPβ gene expression by RA could be through interleukin 6, a positive C/EBPβ transcription factor, since its activity is blocked by RA (35). Although a RARE has not been reported to be associated with the C/EBPβ promoter region, the data reported herein opens the possibility of a direct regulation of C/EBPβ transcription by RA. Alternatively, down-regulation of C/EBPα may affect the C/EBPβ activity since it has been shown that C/EBPβ is under the control of C/EBPα at the post-translational level (36).

The current study was initiated because of the earlier finding that ALDH1 was expressed at low levels in the livers of AHR mice having an abnormally high RA concentration (8). These data suggested the intriguing possibility that RA feedback inhibits its synthesis by decreasing expression of ALDH1. In the Hepa-1 cells used in this study, exogenous RA decreases trans-activation of the ALDH1 promoter and also decreases C/EBPβ mRNA levels. Although these data would suggest that RA directly or indirectly affects C/EBPβ expression in cultured cells, another mechanism for reduction of ALDH1 in liver may be more important since C/EBPβ mRNA and protein are not significantly decreased in the livers of AHR-null mice.

In conclusion, the present study shows a negative feedback control of ALDH1 gene expression by RA. In cultured hepatoma cells, the inhibitory affect of RA appears to be through direct or indirect down-regulation of C/EBPβ, a factor that acts as a positive regulation ALDH1 gene transcription. This positive regulation by C/EBPβ was also indicated by analysis of the C/EBPβ-null mice. In hepatoma cells, RARα/RXRB acts positively on the ALDH1 gene at basal cellular levels of RA. Thus it remains a possibility that in the liver of AHR-null mice, RARα inhibits expression of the ALDH1 gene in the presence of high RA concentration by a mechanism distinct from involvement of C/EBPβ or through a post-translational modification of C/EBPβ. In any case, the ALDH1 regulatory region of the human, mouse, and rat ALDH1 gene promoters all have the RARE and CCAAT box regions suggesting conservation in the control of this gene by RARα and C/EBPβ and strongly indicate an important feedback control of RA synthesis mediated by RARα.

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