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Enzymatic Conversion of Retinaldehyde to Retinoic Acid by Cloned Murine Cytosolic and Mitochondrial Aldehyde Dehydrogenases

MEI CHEN,[1] CHARLES ACHKAR, and LORRAINE J. GUDAS
Department of Pharmacology, Cornell University Medical College, New York, New York 10021
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SUMMARY

It has previously been reported that retinaldehyde can be converted to retinoic acid by cytosolic aldehyde dehydrogenase (AHD-2) in liver extracts [*Biochem. Pharmacol.* **42:** 1279–1285 (1991)]. To determine which enzyme(s) carried out this reaction in murine embryonic stem cells, two aldehyde dehydrogenases were cloned; the AHD-2 gene was cloned from a liver cDNA library, and a closely related gene, AHD-M1, was cloned from an embryonic F9 cell cDNA library by conserved oligonucleotide sequence screening. AHD-M1 contained an open reading frame of 1554 base pairs, which encoded 517 amino acids. The AHD-M1 gene encoded a protein with a putative amino acid sequence that was 94% and 97% identical to the mitochondrial aldehyde dehydrogenases of human and rat, respectively, and thus we have cloned the murine cDNA for this enzyme for the first time. The AHD-M1 cDNA was only 64% identical to AHD-2. Northern analysis showed that AHD-M1 mRNA was constitutively expressed in F9 and P19 embryonic teratocarcinoma stem cells and in AB1 embryonic stem cells. There was a 3–5-fold retinoic acid-associated increase in the amount of this mRNA during the differentiation of F9 cells into parietal endoderm. In contrast, we could not detect the expression of AHD-2 mRNA in AB1, P19, or F9 cells, even though the F9 cells could convert retinaldehyde to retinoic acid. When the AHD-M1 and AHD-2 cDNAs were inserted into the expression vector pSG5 and transfected into cultured COS cells, 3–5-fold and 100-fold increases, respectively, in the conversion of [^{3}H]retinaldehyde to [^{3}H]retinoic acid could be detected by high performance liquid chromatographic assay. We conclude that both enzymes are capable of converting retinaldehyde to retinoic acid in intact COS cells. AHD-2 is more active than AHD-M1 in this conversion, but AHD-2 is not the enzyme responsible for this conversion in F9 embryonic stem cells.

The vitamin A (retinol) derivative RA has profound effects on limb and nervous system morphogenesis in vertebrate embryos, as well as on epithelial cell differentiation. RA induces the differentiation of a number of cell lines, including P19 and F9 teratocarcinoma stem cells (1–4). In addition, RA and RA analogs can inhibit or reverse the processes of malignant transformation in some cell types and have been shown to be useful for the treatment of certain carcinomas, such as those of the head and neck, skin, cervix, breast, and prostate (5–10). RA is also used in the treatment of acute promyelocytic leukemia (5–10). The effects of RA are transduced via its binding to nuclear RA receptors (RARs) and retinoid X receptors (RXRs); in the presence of ligand, these transcription factors interact with specific regulatory elements in the promoter and/or regulatory regions of retinoid-responsive genes (11–14). RA initiates a cascade of events leading to the regulation of the transcription of many key genes such as homeobox genes that are required for embryonic pattern formation (15–18) and genes for extracellular matrix proteins that are needed for the differentiation of many cells (19–21).

Because retinoid-dependent processes such as stem cell differentiation are supported directly by RA (3, 4, 22, 23), a potent physiologically occurring metabolite of retinol (24, 25), the synthesis of RA is likely to be a key and closely regulated aspect of retinol metabolism. There is evidence that RA is formed enzymatically from retinol by a two-step oxidation process in which the generation of retinaldehyde from retinol is followed by conversion of retinaldehyde into RA (Fig. 1). Recently, the synthesis of RA from retinol was studied in the physiological range of retinol concentrations, and RA production was found in many cell types (26). The rate-determining step is thought

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[1] Current address: Department of Radiation and Cellular Oncology, University of Chicago, 5841 South Maryland Avenue, Box 442, Chicago, IL 60637.

ABBREVIATIONS: RA, retinoic acid; AHD-2, murine cytosolic aldehyde dehydrogenase-2; AHD-M1, murine mitochondrial aldehyde dehydrogenase; ALDH-1, human homolog of AHD-2; ALDH-2, human homolog of AHD-M1; CT, 500 μM dibutyryl-cAMP and 500 μM theophylline; RACT, 500 μM dibutyryl-cAMP, 500 μM theophylline, and 1 μM retinoic acid; DME, Dulbecco's modified Eagle's medium; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; ORF, open reading frame; HPLC, high performance liquid chromatography; AHD-5, murine aldehyde dehydrogenase-5; AHD-7, murine aldehyde dehydrogenase-7.

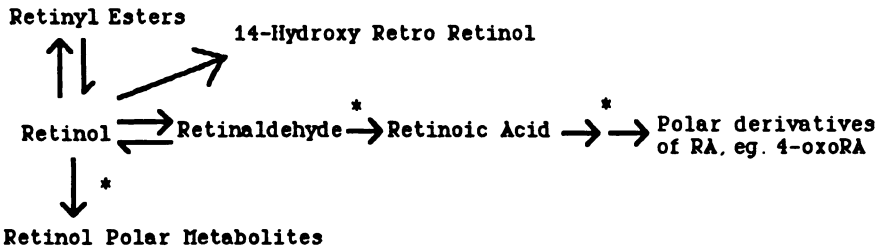

Fig. 1. General pathway of retinoid synthesis. Retinol can be converted to RA via a two-step oxidation in which retinol dehydrogenase produces retinaldehyde, which is then oxidized to RA. RA can be further oxidized to 4-keto- or hydroxy-RA.

*Steps increased by RA treatment of F9 teratocarcinoma stem cells which leads to their differentiation

to be the first one, the conversion of retinol into retinaldehyde (24). Although it has been suggested that the dehydrogenases involved in retinol and retinaldehyde metabolism are related to those involved in alcohol metabolism, detailed information is not available concerning the characteristics of the enzymes that are specifically involved in the synthesis of RA. Obstacles in such studies have included the conversion *in vitro* of free retinol and retinaldehyde by multiple cytosolic and microsomal dehydrogenases and oxidases (27–29). In addition, the rates of metabolism of retinol to RA are relatively low in many cell types, because RA is a quantitatively minor retinol metabolite and retinyl esters are often the major retinol metabolites (30) (Fig. 1).

Enzyme-catalyzed oxidation of retinaldehyde to RA has been observed in intact cells as well as in the extracts of many tissues from a variety of species. This reaction has been shown to be catalyzed by different classes of enzymes. These include NAD(P)-dependent aldehyde dehydrogenases, aldehyde oxidase, and xanthine oxidase (31–34). In addition, the cytochrome P450 enzymes 1A2 and 3A6 have been reported to be capable of converting retinaldehyde to RA *in vitro* (35). The question of which of these enzymes are the most important in the conversion of retinaldehyde to RA in intact cells has not been answered.

The aldehyde dehydrogenase family is composed of a number of enzymes that catalyze the oxidation of a wide range of aldehydes to the corresponding acids. They have similar molecular weights but differ in a range of characteristics, including subcellular distribution, charge, relative substrate preference, and inhibitor susceptibility. Mice express at least 13 alcohol dehydrogenase (EC 1.2.1.3) isozymes in a variety of tissues and subcellular compartments (36). (AHD is the name given to the gene family encoding the murine aldehyde dehydrogenases; the equivalent human enzymes are named ALDHs.) Some appear to be relatively tissue specific, whereas others are relatively broadly distributed. Most of these enzymes have been found in the mitochondria, endoplasmic reticulum, or cytosol of liver cell extracts. In many cases, developmental changes in the patterns of expression have also been observed. Some enzymes are substrate nonspecific; others are relatively substrate specific. Isoform AHD-2 has been shown to be the major NAD^+-dependent enzyme in the adult mouse liver capable of the synthesis of RA from retinaldehyde (37). An identical enzyme in dorsal retina has been reported to be able to generate RA from retinaldehyde *in vitro* and probably also *in vivo* (38, 39). Additionally, the AHD-2 gene product is important in the oxidation of benzaldehyde and aldophosphamide (36). ALDH-1, reportedly the human analog of AHD-2 (40), can catalyze the NAD^+-dependent oxidation of retinaldehyde to RA in liver (41). AHD-2 enzyme activity is present in a number of mouse tissues such as liver, lung, and testes, but it is apparently not expressed at detectable levels in mouse kidney, stomach, ovary, heart, and brain (42). The AHD-2 gene was recently cloned (43).

Elucidation of the mechanisms involved in the regulation of RA synthesis requires the identification of the enzymes involved in retinoid metabolism. We have undertaken experiments to clone some of the genes coding for these enzymes from F9 teratocarcinoma stem cells, because F9 stem cells serve as an attractive model system for studying the actions of RA. Previously, studies from Williams and Napoli (22) demonstrated that a small amount of retinol was converted to RA in undifferentiated F9 stem cells. More recent studies from our laboratory have shown that RA not only induces the differentiation of F9 stem cells into primitive endoderm in monolayer culture but also increases the metabolism of retinol to a variety of metabolites (see Fig. 1).[2] The conversion of retinaldehyde to RA also increases by approximately 3-fold after RA treatment of F9 cells.[3] We have now cloned two members of the aldehyde dehydrogenase gene family, one of which is the murine equivalent of the ALDH-2 gene. The mRNA encoding this enzyme, AHD-M1, is expressed in many embryonic teratocarcinoma and embryonic stem cell lines, such as F9, P19, and AB1 cells, and is induced 3–5-fold in F9 cells by RA treatment. Thus, AHD-M1 may be responsible for the conversion of retinaldehyde to RA in these embryonic cells. In contrast, we cannot detect the expression of AHD-2 transcripts in either F9 stem cells or F9 cells treated with RA.

Both the cytosolic and mitochondrial aldehyde dehydrogenases have been overexpressed in expression vectors containing the cDNA clones. Transfected COS cells were capable of conversion of ^{3}H-labeled retinaldehyde to RA *in vivo*, demonstrating that more than one cloned enzyme in this family can catalyze the oxidation of retinaldehyde to RA in intact cells. In embryonic cells, AHD-M1 and/or enzymes other than AHD-2 are most likely responsible for the conversion of retinaldehyde to RA.

Experimental Procedures

Materials. Restriction endonucleases and other enzymes were obtained from New England Biolabs. Radioactive reagents were obtained

[2] C. Achkar, unpublished observations.
[3] C. Achkar, unpublished observations.

from NEN. A random-primed DNA labeling kit was purchased from Boehringer Mannheim. A sequencing kit was purchased from United States Biochemicals. Nitrocellulose membrane filters (BA85) were from Schleicher and Schuell.

Cell culture. F9 teratocarcinoma stem cells and COS cells were grown in DME plus 10% calf serum and 200 μM glutamine; AB1 murine embryonic stem cells were grown on feeder cells in medium plus fetal calf serum as described above, and P19 teratocarcinoma stem cells were maintained in DME supplemented with 10% fetal calf serum (20). To analyze the levels of mRNA, cells were plated at 2×10^6/150- × 15-mm tissue culture dish and treated with fresh medium containing CT or RACT for the designated periods. Treatment of P19 cells with RA was carried out in monolayer culture; under these conditions the P19 cells differentiate into fibroblast-like cells.

cDNA library screening. A 30-base oligonucleotide was synthesized based on the most conserved regions of the aldehyde dehydrogenase gene family (44). This 30-nucleotide oligomer (5′-GTGACCCTGGAGCTTGGIGGA/GAAGAGCCCT-3′) was used to screen a cDNA library constructed from 8-hr RA-treated F9 cells. Phage were plated on *Escherichia coli* C600 and grown for 8 hr on LB plates. Nitrocellulose filter replicas from plates were treated according to a standard protocol, prehybridized in 6× SSC, 5× Denhardt's solution, 50 mM sodium phosphate, pH 7, 0.1% SDS, 20% formamide, 100 μg/ml sheared salmon sperm DNA, for 6 hr at 42°, and hybridized for 12–16 hr at 42° in the same solution containing the 5′-^{32}P-labeled oligonucleotide. Filters were then washed with 0.2× SSC/0.1% SDS at 42° for 1 hr. AHD-2 cDNA clones were obtained by screening a λgt11 mouse liver cDNA library (Clontech) using a 30-nucleotide oligomer (ATGTCTTCGCCTGCACAACCTCGAGTCCCT) based on the published AHD-2 clone sequence (43).

For differential cDNA screening, duplicate filters were prehybridized and hybridized at 42° in 50% formamide, 5× SSC, 50 mM sodium phosphate, 5× Denhardt's solution, 0.1% SDS, 100 μg/ml sheared salmon sperm DNA, with cDNA probe synthesized from poly(A)$^+$ RNA purified from either CT- or RACT-treated (12 hr) F9 cells. Filters were washed in 0.2× SSC/0.1% SDS at 65° for 1 hr. Phage plaques showing any differential hybridization were picked and rescreened using the same cDNA probe. DNA was isolated from phage that still showed differential hybridization on a second screen.

Subcloning and DNA sequence analysis. The cDNA insert was released from λgt10 recombinant phage by *Eco*RI digestion and was subcloned into an *Eco*RI-linearized pSG5 eukaryotic expression vector. The double-stranded templates were sequenced by the dideoxy chain-termination method using T7 polymerase. The cDNA was sequenced bidirectionally using synthetic oligonucleotide primers based on previously determined sequences. Both the AHD-2 cDNA clone and the AHD-M1 cDNA clone were sequenced in both directions.

Isolation and analysis of RNA. Total RNA was extracted by using the guanidine isothiocyanate-$CsCl_2$ procedure and was subsequently enriched for poly(A)$^+$ RNA by oligo(dT)-cellulose chromatography as described (45). RNA was size-fractionated on 1% agarose gels containing 0.66 M formaldehyde and was transferred to nitrocellulose filters by blotting. RNA was attached to the filters by UV cross-linking, and the filters were prehybridized and hybridized at 42° with random primer-labeled inserts from cDNA clones. Prehybridization and hybridization solutions contained 50% formamide, 5× SSC, 50 mM sodium phosphate, 0.1% SDS, 5× Denhardt's solution, and 100 μg/ml sheared salmon sperm DNA. The final wash of the filters was carried out in 0.2× SSC/0.1% SDS for 1 hr at 65°.

Transfections. DNA transfections into COS cells were performed by the DEAE-dextran method, essentially as described (46). Twenty-four hours before transfection, 1×10^6 COS cells were plated in each 60-mm tissue culture dish. The following day, cells were washed with phosphate-buffered saline, incubated with 4 μg of DNA/DEAE-dextran mixture at 37° for 30 min, with occasional shaking, and then further incubated with DME containing 80 μM chloroquine for an additional 2.5 hr. After 2.5 hr, the medium was removed and cells were then treated with 10% dimethylsulfoxide in DME for 2.5 min. Finally, the medium was replaced with new DME containing 10% calf serum. F9 cells were transfected using the calcium phosphate method described previously (21, 47).

Synthesis of [^{3}H]retinaldehyde. Radioactive [^{3}H]retinaldehyde was synthesized from [^{3}H]retinol. Briefly, 250 μCi of [^{3}H]retinol (New England Nuclear) were incubated with 15 mg of MnO_2 and 10 ml of cold petroleum ether in a 37° shaking waterbath. After 10 min of incubation, the volume of the incubation mixture was reduced to 2 ml under argon and the mixture was then allowed to continue to incubate at 37° for another 5 min. The mixture was then extracted twice with 2 ml of ethanol, 5 ml of petroleum ether, and 2 ml of water. The upper petroleum ether layer was reduced to dryness under argon and dissolved in ethanol (48). The concentration was determined by using an absorbance spectrum and the purity of [H^3]retinal was verified by HPLC.

Metabolism of retinaldehyde to RA in cultured cells. Determination of retinaldehyde metabolism by intact cells was carried out essentially as described by Williams and Napoli (22). Because retinoids are light sensitive, all operations were carried out under subdued lighting. Retinaldehyde, in ethanol, was added to cultures so that the final concentration of vehicle was <0.1%. Radioactive [^{3}H]retinaldehyde for quantitative studies was added to cells at a final concentration of 50 nM. All cells were exposed to a total external concentration of 1 μM retinaldehyde for periods of time ranging from 0 to 7 hr.

Extraction and separation of retinoids. These procedures were performed according to the method of McLean *et al.* (49). Briefly, the incubation medium was collected and the monolayer of cells was washed twice with phosphate-buffered saline and then harvested by scraping. A control consisting of labeled retinaldehyde in medium without cells was always included during the incubation. Appropriate internal standards were added to the samples before extraction with butanol/acetonitrile (50:50, v/v), so that their HPLC elution profiles could be followed by measuring absorbance.

System Gold (Beckman), an HPLC system, was used to separate the various retinoids. A 5-μm reverse phase C_{18} column (0.46 × 25 cm; Vydac), with a flow rate of 1.5 ml/min, was used. Retinoids were separated using a gradient from 0% acetonitrile in ammonium acetate (15 mM, pH 6.5) to 100% acetonitrile. Nonlabeled retinoids were detected at a wavelength of 360 nm. A radiochromatography detector (Packard A-500) was used to monitor and count labeled retinoids.

Results

Isolation and sequencing of the cDNA coding for AHD-M1. Studies from our laboratory have shown that RA not only induces the differentiation of F9 teratocarcinoma stem cells into primitive endoderm but also increases the metabolism of retinol and RA, presumably through the transcriptional induction of some of the enzymes involved in the metabolic pathways (Fig. 1). In addition, a 3-fold increase in the conversion of retinaldehyde to RA is seen in F9 cells after RA addition. Evidence from other laboratories has suggested the involvement of members of the aldehyde dehydrogenase gene family in this enzymatic step. We devised a strategy to isolate the genes involved in RA synthesis by first screening an F9 cDNA library using an oligonucleotide probe synthesized from the conserved region of the catalytic domain of aldehyde dehydrogenase. Our secondary screening involved the differential hybridization of the "oligonucleotide-positive" clones identified in the first screen to cDNA probes made from poly(A)$^+$ RNA from RA-treated F9 cells, as compared with F9 stem cells.

Approximately 200 positive clones were obtained in the original screening of 5×10^5 phage plaques with the 30-nucleotide oligomer. All of these positive clones were pooled and then subjected to a secondary differential hybridization screening using ^{32}P-labeled cDNA prepared from either 12-hr RACT-treated F9 cells or F9 stem cells treated with CT. One phage

clone that showed positive hybridization in both the oligonucleotide and differential screenings was obtained, and two additional rounds of plaque purification were carried out. The phage DNA was purified and the cDNA insert was released from λ phage vector by *Eco*RI restriction digestion, subcloned into *Eco*RI-digested β-Bluescript vector, and subjected to DNA sequencing analysis. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2. The cDNA insert contained an ORF that encoded a murine mitochondrial aldehyde dehydrogenase of 517 amino acids. The name AHD-M1 was assigned because there may be other, as yet uncloned, mitochondrial aldehyde dehydrogenases in mice. The ORF was flanked at the 5′ end by 65 base pairs of untranslated sequence and at the 3′ end by 280 base pairs of untranslated sequence.

Fig. 3. Effects of CT and RACT on the expression of AHD-M1. Total cellular RNA was isolated from F9 cells that had been treated for 12 hr with CT or RACT and was further fractionated into poly(A)$^+$ RNA. Northern analysis was carried out with 2 μg of poly(A)$^+$ RNA and the blot was hybridized to ^{32}P-labeled cDNA probes representing AHD-M1 (*top*) and β-actin (*bottom*). β-Actin mRNA was used as a control for the amount of RNA loaded per lane because this mRNA does not change after RACT addition (15). This experiment was performed three times. The RNA bands were quantitated by densitometry or Phosphorimaging.

Fig. 2. A, Nucleotide sequence and deduced amino acid sequence of AHD-M1. The nucleotide sequence depicted starts at the 5′ end of the sense strand and encodes the complete ORF beginning at the initiation codon at nucleotide 66 and ending at the termination codon at nucleotide 1617. The amino acid sequence is shown immediately below the nucleotide sequence. B, Homology between AHD-M1 (*upper line*) and AHD-2 (*lower line*). *Dashes between aligned amino acids*, exact match in sequence; *two dots*, conservative replacement. There was 65% identity between AHD-2 and AHD-M1. The less conserved region used for Northern oligomer probe synthesis is *boxed*.

In the 3′ noncoding region, a poly(A)$^+$ addition signal (AATAAA) was found. The deduced amino acid sequence of the AHD-M1 that we have cloned is very similar to those of the rat and human mitochondrial enzymes (50, 51). The mouse sequence is 97% and 94% identical to the rat and human sequences, respectively. Not unexpectedly, the mouse, human, and rat cytosolic aldehyde dehydrogenase sequences are considerably more divergent from the mouse mitochondrial sequence, with only 65%, 67%, and 64% similarities, respectively.

Cloning of AHD-2 cDNA. AHD-2 has been reported to be able to catalyze the conversion of retinaldehyde to RA *in vitro* (37). This gene was previously cloned from mouse (43). We attempted to isolate the gene encoding AHD-2 from an F9 cell library by using a 30-nucleotide oligomer (see Experimental Procedures), without success. We then isolated an AHD-2 cDNA clone from a mouse liver cDNA library, sequenced this cDNA, demonstrated by computer analysis that the sequence was identical to the previously cloned gene, and inserted the full length cDNA clone into pSG5 for expression studies. A comparison of the amino acid sequence deduced from our AHD-2 cDNA with that deduced from our AHD-M1 cDNA is shown in Fig. 2.

AHD-M1 and AHD-2 mRNA expression during F9, P19, and AB1 cell differentiation. Thirteen aldehyde dehydrogenases have been identified in mouse tissues. We do not know how many of these are expressed in F9 cells and what degree of amino acid sequence similarity they share. Thus, the expression of the AHD-M1 and AHD-2 genes was examined in several embryonic cell lines. To avoid cross-hybridization, an oligonucleotide probe made from a region less conserved among cytosolic and mitochondrial aldehyde dehydrogenases (Fig. 2B, *boxed region*) was used to analyze the expression of the AHD-M1 gene during the differentiation of F9 cells in response to RACT. As shown in Fig. 3, an approximately 2.2-kilobase mRNA that exhibits a 3–4-fold increase 12 hr after RACT addition, in comparison with CT, was detected. When Northern analysis was carried out using the AHD-M1 cDNA insert as a probe and the RNA blot was subjected to high stringency hybridization and washing conditions, a similar result was obtained; this result indicates that no cross-hybridization of the AHD-M1 probe with other dehydrogenases occurred.

In contrast to the expression of AHD-M1 mRNA in F9 stem cells that we observed, we could not detect hybridization to mRNA from F9 stem cells or RA-treated F9 cells when the AHD-2 cDNA was used as a probe, even after a longer exposure (1 week). By comparison, we can detect the expression of *Hox a-1* transcripts, which represent approximately 0.005% of the total poly(A)$^+$ RNA, after a 3–4-day exposure under similar conditions of hybridization and washing (15). This result (data not shown) demonstrates that the AHD-2 mRNA is not expressed or is expressed only at very low levels (below our limit of detection) in F9 stem cells and in F9 cells after RACT treatment.

We examined the expression of both the AHD-M1 and AHD-2 mRNAs in two other embryonic stem cell lines, P19 and AB1. Total RNAs were prepared from these cells after 24 or 48 hr of RACT or CT treatment. AHD-M1 mRNA was expressed in all three cell lines examined (Fig. 4). A slight increase in AHD-M1 expression occurred after 24 hr of RACT treatment, compared with CT treatment, in F9 cells (Fig. 4, *lanes 1* and *2*) and in AB1 cells (Fig. 4, *lanes 9* and *10*). Both F9 cells and AB1 cells differentiate into an epithelial cell type, parietal endoderm,

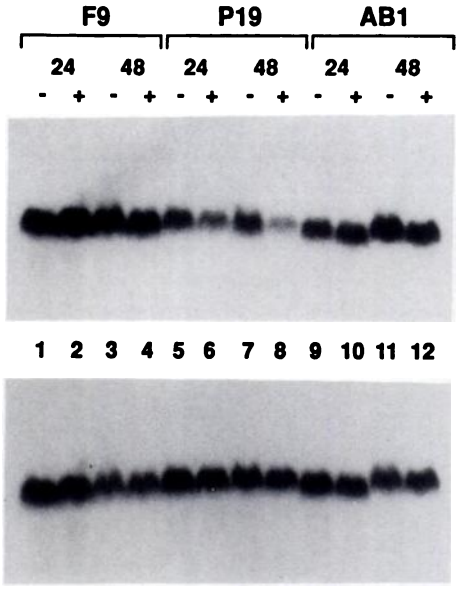

Fig. 4. Expression of the AHD-M1 gene in different embryonic stem cell lines and teratocarcinoma stem cell lines. Total cellular RNA was isolated from F9, P19, and AB1 cells that had been treated for 24 or 48 hr with CT (−) or RACT (+), and 10 μg of total RNA were then subjected to Northern analysis as described in Experimental Procedures. The blot was hybridized to AHD-M1 (*top*) and β-actin (*bottom*) cDNA probes and then exposed to X-ray film for 8–12 hr. *Lanes 1-4*, F9 cells; *lanes 5-8*, P19 cells; *lanes 9-12*, AB1 cells. *Lanes 1*, *3*, *5*, *7*, *9*, and *11*, control CT-treated cells. *Lanes 2*, *4*, *6*, *8*, *10*, and *12*, RACT-treated cells. *24* (above the Northern blot), 24-hr treatment with the drugs; *48*, 48-hr treatment with the drugs.

in response to RA, so it is not surprising that they behave similarly with respect to the expression of AHD-M1 (4). In contrast, the expression of AHD-M1 was reduced 3–4-fold in P19 cells after 24 hr of RACT treatment, compared with CT treatment (Fig. 4, *lanes 5* and *6*), and the expression was decreased further after 48 hr (Fig. 4, *lanes 7* and *8*). As P19 cells treated with RA as monolayers differentiate into fibroblast-like cells, it might be expected that they would regulate the expression of the AHD-M1 gene differently from F9 or AB1 cells (4).

We could not detect the expression of AHD-2 mRNA in any of the three lines either before or after growth in the presence of RA or RACT. Our data indicate that AHD-2 is not the relevant enzyme involved in the oxidation of retinaldehyde to RA in these embryonic cells (data not shown).

Metabolism of [^{3}H]retinaldehyde in COS cells that overexpress AHD-M1 or AHD-2 cDNAs. To test the involvement of the mitochondrial and cytosolic aldehyde dehydrogenases in the conversion of retinaldehyde to RA, the cDNA coding regions for both enzymes were cloned into the eukaryotic expression vector pSG5 so that the expression of the inserted genes was placed under the control of the simian virus 40 early promoter (52). Both expression vectors were transfected into COS cells, which constitutively express the simian virus 40 large T antigen, by DEAE-dextran-mediated transfection, and total RNA was isolated after 24 hr. Northern blot analysis revealed that there was equivalent mRNA expression of the transfected AHD-M1 and AHD-2 genes in the COS cells (Fig. 5A, *lanes 2* and *6*, labeled M and C, respectively). Furthermore, there were no abundant cross-hybridizing enzyme mRNA species for these murine aldehyde dehydrogenases in COS cells.

Twenty-four hours after the transfections, the COS cells were cultured in the presence of 50 nM [^{3}H]retinaldehyde for

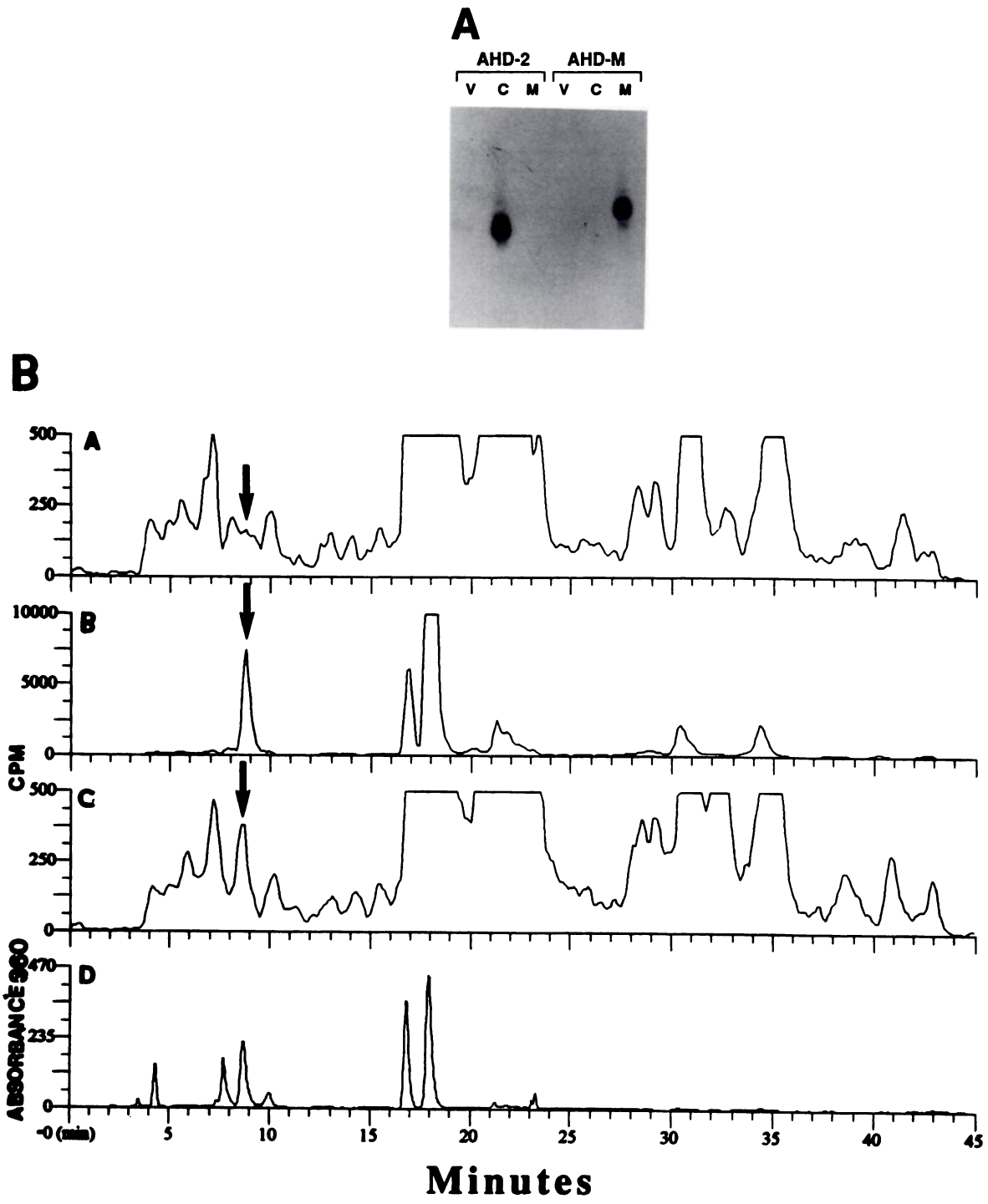

Fig. 5. A, Expression of AHD-M1 and AHD-2 mRNA in cDNA transfected cells. Total cellular RNA was isolated from COS cells transfected with either pSG5-AHD-M1 (*M*), pSG5-AHD-2 (*C*), or the psG5 vector alone (*V*). After a 24-hr transfection, 10 μg of RNA were fractionated, blotted, and hybridized to either the AHD-M1 or AHD-2 cDNA insert as probe. Exposure time of the autoradiogram was 1 hr. *Lanes 1-3*, RNA was hybridized to the AHD-2 cDNA probe; *lanes 4-6*, RNA was hybridized to the AHD-M1 cDNA. B, HPLC analysis of the production of [^{3}H]RA from [^{3}H]retinaldehyde in COS cells. Twenty-four hours after COS cells were either mock transfected or transfected with the pSG5-AHD-M1 or pSG5-AHD-2 expression vector, the cells were cultured in the presence of 50 nM [^{3}H]retinaldehyde for various times. Cells and medium were then harvested, retinoids were extracted, and the retinoids were separated by HPLC as described in Experimental Procedures. Several time points, including 1, 3, and 7 hr, were measured, and results were similar at all three time points; only results from the 7-hr time point are shown. This experiment was performed twice with very similar results. The *axes* are [^{3}H]retinoids versus elution time (in minutes). A, Mock-transfected COS cells 7 hr after addition of [^{3}H] retinaldehyde. *Arrow*, all-*trans*-RA peak. B, COS cells transfected with pSG5-AHD-2 as described and then treated with [^{3}H]retinaldehyde for 7 hr. *Arrow*, all-*trans*-RA peak. Note the difference in scale. C, COS cells transfected with pSG5-AHD-M1 as described and then treated with [^{3}H] retinaldehyde for 7 hr. *Arrow*, all-*trans*-RA peak. D, Nonradioactive standards (included in each run), as follows (from *left* to *right*): 4-oxo-RA, 13-*cis*-RA, all-*trans*-RA, 13-*cis*-retinol, and all-*trans*-retinol.

various times. Cells were then harvested, retinoids were extracted, and the retinoids were separated by HPLC (Fig. 5B). Compared with the "mock"-transfected COS cell control, a 3–5-fold increase in the production of [^{3}H]RA from [^{3}H]retinaldehyde was observed in the pSG5-AHD-M1 COS cell transfectants (Fig. 5B, compare panel A with C). A much greater increase (roughly 100-fold) in the production of [^{3}H]RA from [^{3}H]retinaldehyde was seen in the pSG5-AHD-2 COS cell transfectants (Fig. 5B, panel A versus B), even though the amounts of transfected messages were similar in the two transfectants (Fig. 5A). Thus, AHD-2, expressed in cultured cells, is more active than AHD-M1 in the conversion of retinaldehyde to RA. Moreover, this is the first demonstration that two different members of the aldehyde dehydrogenase gene family, when cloned and expressed, are capable of converting retinaldehyde to RA in intact cells.

Metabolism of [^{3}H]retinaldehyde in undifferentiated F9 stem cells transfected with expression vectors for AHD-M1 or AHD-2. Experiments similar to those described above for COS cells were performed using F9 cells. In the F9 wild-type stem cells that were mock transfected, a small amount of RA was generated from retinaldehyde (Fig. 6, compare panel A with B and C). However, when the F9 cells were transfected with either the AHD-2 or the AHD-M1 expression construct, 15-fold and 3-fold higher levels of [^{3}H]RA, respectively, were observed, compared with the mock-transfected F9 stem cells (Fig. 6). We also observed some expression of the AHD-2 and AHD-M1 mRNAs in the transfected F9 cells, but the mRNA levels were lower than those shown in Fig. 5A for the transfected COS cells (data not shown). As was the case in COS cells, the AHD-2 enzyme was more active than the AHD-M1 enzyme in converting [^{3}H]retinaldehyde to [^{3}H]RA. These results show that these cloned cDNAs can function in F9 stem cells. These results also suggest that the low level of oxidation of retinaldehyde to RA in F9 stem cells (Fig. 6A) may be related to the expression of AHD-M1. However, the possibility that other aldehyde dehydrogenases participate in this conversion of retinaldehyde to RA cannot be ruled out at this time.

Discussion

We have reported the isolation of a cDNA clone encoding a murine mitochondrial aldehyde dehydrogenase, by differential expression and conserved oligonucleotide screening of a cDNA library constructed from RACT-treated F9 mRNA. This cloned cDNA contains an ORF that encodes a protein of 517 amino acid residues with a molecular mass of 57 kDa. Northern blot analysis demonstrated that the AHD-M1 mRNA is expressed constitutively in several mouse cell lines, including F9, P19, and AB1, and that there is a 3–5-fold induction of AHD-M1 mRNA expression in RA-treated F9 cells. Expression of this cDNA clone in cultured COS cells resulted in a 3–5-fold increase in the oxidation of [^{3}H]retinaldehyde to RA, as assayed by HPLC.

In comparing the deduced amino acid sequence of AHD-M1 with those of human and rat mitochondrial aldehyde dehydrogenase (known as ALDH-2) (50, 51), only 31 and 15 residues, respectively, of the 517 amino acids were found to be different (94% and 97% amino acid identity). In contrast, the gene encoding the murine enzyme AHD-2 shares only 64% sequence identity with its mitochondrial counterpart. Other mitochondrial enzymes also share less identity with their cytosolic counterparts than they do with the corresponding mitochondrial enzymes from different species. The function of the mitochondrial enzyme, compared with the cytosolic enzyme, is not known. Retinol, retinaldehyde, and RA are all lipophilic molecules, and it is possible that metabolism of retinaldehyde could take place in mitochondria.

The conversion of retinaldehyde to RA has been reported to be catalyzed by three types of enzymes, i.e., aldehyde dehydrogenases, aldehyde oxidase, and xanthine oxidase. Based on their primary structures and physical and immunological properties, the aldehyde dehydrogenases have been divided into three classes. The major class 2 mitochondrial enzyme is important in ethanol metabolism, the class 3 aldehyde dehydrogenases are associated with tumorigenesis (53), and the class 1 enzymes such as AHD-2 oxidize several pharmacologically important aldehydes (54). Lee *et al.* (37) identified and partially purified 13 different aldehyde dehydrogenases in mouse tissues. One of these, AHD-5, has characteristics similar to those of the AHD-M1 that we have cloned. However, because this AHD-5 activity has not yet been purified and the gene encoding this protein has not been cloned, we cannot be certain about its relationship to AHD-M1. Lee *et al.* (37) demonstrated that only two enzymes, AHD-2 and AHD-7, that were present in the soluble fraction of mouse liver could use retinaldehyde as a substrate. AHD-2 was the most important enzyme in mouse liver because it accounted for almost 95% of the total conversion of retinaldehyde to RA in this tissue. Although AHD-2 was shown to convert retinaldehyde to RA in protein extracts, it is uncertain how important this enzyme is *in vivo*, where retinaldehyde concentrations are lower than those used in the assays of liver extracts (37). However, in two L1210 cell lines that differed in their expression of AHD-2, the conversion of retinaldehyde to RA was shown to correlate with the expression of AHD-2 (55). This results suggests that AHD-2 is involved in the conversion of retinaldehyde to RA in intact cultured L1210 cells.

In the studies reported here, when the cDNA clone coding for AHD-2 was inserted into the expression vector pSG5 and transfected into cultured COS-1 cells, a 100-fold increase in conversion of [^{3}H]retinaldehyde to [^{3}H]RA could be detected by HPLC analysis. This is the first demonstration that the cloned AHD-2 enzyme can oxidize retinaldehyde to RA *in vivo* in intact cells. However, the expression of AHD-2 is quite tissue specific. The protein has been found in a number of mouse tissues, e.g., liver, lung, and testes, but it apparently is not expressed or is expressed at only very low levels in mouse kidney, stomach, ovary, heart, and brain (42). Our finding that there is no expression of AHD-2 mRNA in F9, P19, or AB1 stem cells or in cells induced to differentiate by RA indicates that this enzyme is not involved in the conversion of retinaldehyde to RA in these cell lines. AHD-M1 is expressed constitutively in F9 cells and can be induced 3–5-fold in response to RA. We have shown that the conversion of retinaldehyde to RA can be detected in F9 stem cells (Fig. 6) and increases 3–5-fold after cells are induced to differentiate by RA (data not shown). Thus, there is a correlation between the increased expression of this enzyme (AHD-M1) and the conversion of retinaldehyde to RA upon the RA-associated differentiation of the cells. In addition, the AHD-M1 cDNA clone, expressed in COS cells, is able to catalyze the oxidation of retinaldehyde to RA, suggesting that this enzyme may be involved in the metabolism of retinaldehyde to RA in F9 cells. However, we cannot exclude the possibility that another unidentified enzyme is the relevant enzyme for the oxidation of retinaldehyde in F9 cells,

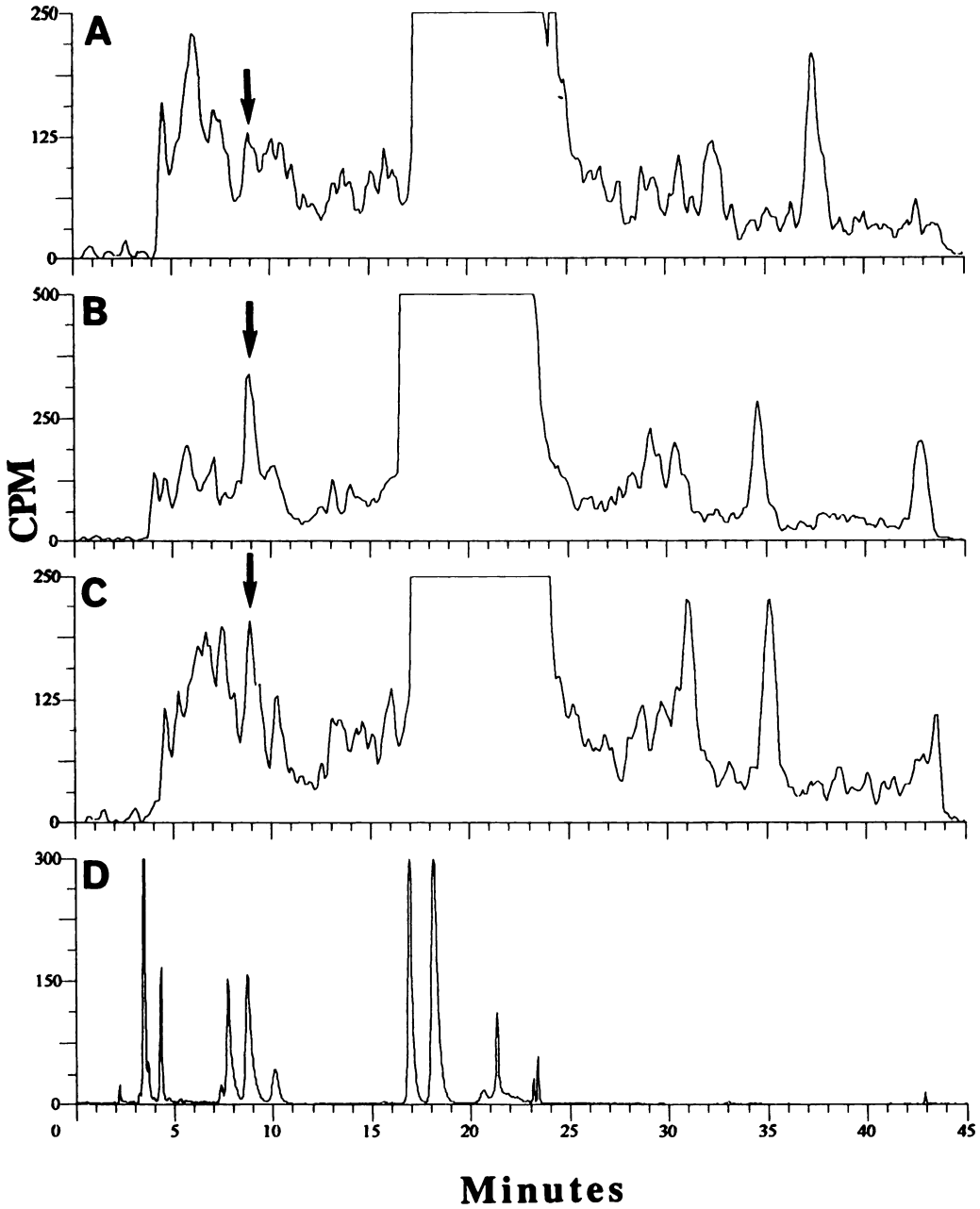

Fig. 6. HPLC analysis of the production of [^{3}H]RA from [^{3}H]retinaldehyde in transfected F9 stem cells. Twenty-four hours after F9 stem cells were either mock transfected or transfected with the pSG5-AHD-M1 or pSG5-AHD-2 expression vector, the cells were grown in the presence of 50 nM [^{3}H]retinaldehyde for various times. Cells were then harvested, retinoids were extracted, and the retinoids were separated by HPLC. Several time points were measured, but results from only the 3-hr time point are shown because results were similar at all time points. A, Mock-transfected F9 stem cells 3 hr after [^{3}H]retinaldehyde addition. *Arrow*, all-*trans*-RA. B, F9 stem cells transfected with pSG5-AHD-2 as described and then treated with [^{3}H]retinaldehyde for 3 hr. *Arrow*, all-*trans*-RA. Note the difference in the scale. C, F9 stem cells transfected with pSG5-AHD-M1 as described and then treated with [^{3}H]retinaldehyde for 3 hr. *Arrow*, all-*trans*-RA. D, Nonradioactive standards, as follows (from *left* to *right*): phenol red (a control for culture medium extraction of retinoids), 4-oxo-RA, 13-*cis*-RA, all-*trans*-RA, 13-*cis*-retinol, all-*trans*-retinol, and all-*trans*-retinaldehyde. (The minor peak eluting at 10 min is not one of the standards.)

especially because enzymes other than AHD-2 can carry out this reaction in the ventral retina (56). Proof that a particular enzyme is involved in RA synthesis will require that both gene copies of this enzyme are disrupted by homologous recombination in a cell line and that the ability to synthesize RA is lost or reduced as a result.

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Send reprint requests to: Lorraine J. Gudas, Department of Pharmacology, Cornell University Medical College, 1300 York Ave., E-409, New York, NY 10021.