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The Retinol Signaling Pathway in Mouse Pluripotent P19 Cells

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ABSTRACT

atRA (all-*trans*-retinoic acid), the active metabolite of retinol (vitamin A), is essential for embryogenesis and maintenance of cellular phenotype in adults. Chemicals that interfere with the metabolism of retinol to atRA, therefore, are a human health concern. During development of a screen for disruptors of this signaling pathway, we investigated whether the mouse pluripotent P19 cell metabolizes retinol to atRA and thus can be used in a cell-based screen for disruptors of the pathway. We found that retinol induced the identical pattern of homeobox gene expression as atRA and its precursor, retinal. Retinol was 160-fold less potent than atRA as an inducer, however. In spite of its lower potency, increased *Hoxa1* gene expression was detected 30 min after retinol exposure and increased 40-fold by 2 h. *Rdh10* and *Aldh1a2/Raldh2*, which together convert retinol to atRA in the embryo, were the predominant alcohol and aldehyde dehydrogenases expressed in P19 cells. The cell expressed high mRNA levels of retinol binding proteins, *Rbp1* and *Rbp4*, and the 13,14-dihydroretinol saturase, *Retsat*. It also expressed all *Rar* and *Rxr* isotypes, *Crabp1&2*, the three *Cyp26* genes, and both β-carotene-cleaving genes, *Bcmo1* and *Bco2*. The basal expression levels and retinol responsiveness of 25 pathway-related genes were quantitated by RT-qPCR. A test of the *Aldh1a2* inhibitor, citral, showed that the disruption of the pathway was easily detected and quantitated showing that the P19 cell provides an in vitro model system for identifying and exploring the mechanism of action of chemicals that interfere with this critical cellular pathway. J. Cell. Biochem. 112: 2865–2872, 2011.

KEY WORDS: STEM CELL; RETINOL; HOMEOBOX; SIGNALING; PLURIPOTENT

Retinol (vitamin A), an essential nutrient in vertebrates, must be obtained from dietary sources. It is stored predominantly in the liver as retinyl esters and is transported in the blood, as retinol, bound to retinol binding protein 4 (Rbp4). Retinol is taken up by cells through Stra6, which is a cell-surface receptor for Rbp4 and a transporter of retinol [Kawaguchi et al., 2007]. In the canonical retinol signaling pathway (RSP), retinol is oxidized to retinaldehyde by alcohol dehydrogenases and subsequently to all-*trans*-retinoic acid (atRA) by retinaldehyde dehydrogenases. atRA, the major active metabolite of vitamin A, is further oxidized by Cyp26 cytochrome p450 enzymes to polar metabolites for elimination or other biological functions. Additional natural metabolites of retinol and atRA have been identified, some of which are biologically active in some assays [Ross et al., 2000; Liu et al., 2009; Moise et al., 2009]. To maintain the cellular atRA concentration within defined limits, a balance appears to exist between its synthesis and metabolism. Abnormal cellular atRA levels induced in pregnant animals by a vitamin A deficient diet [Wilson et al., 1953] or high-administered doses of atRA [Collins and Mao, 1999] cause developmental defects in the offspring. Adult animals are also adversely affected by vitamin A deficiency [Wolbach and Howe, 1933] or excess atRA [Collins and Mao, 1999].

Retinol signaling plays an important role in the establishment and maintenance of cellular phenotype in embryonic and adult vertebrate tissues. atRA functions as the activating ligand for a family of ligand-activated transcription factors, the retinoic acid receptors (RARs), which form heterodimers with the retinoid X receptors (RXRs) to regulate gene transcription. Through its activation of the receptors, atRA regulates the expression of over 500 protein-coding genes [Balmer and Blomhoff, 2002] and a potentially larger number of non-coding regulatory RNAs [Cawley et al., 2004] to establish and maintain the various cellular phenotypes of an organism.

Much of the research interest in retinol signaling has focused on understanding its essential role in orchestrating embryonic development. It is clear from vitamin A deficiency (VAD) studies and studies in which components of the signaling pathway have been disrupted [Duester, 2008; Niederreither and Dolle, 2008] that it

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is essential for the normal development of all organ systems in the embryo and that components of the pathway must function together correctly for a successful developmental outcome.

This dependency on vitamin A is not limited to embryogenesis but continues through life. Studies on VAD in adult animals show the continued importance of this signaling pathway for the maintenance of cellular phenotype and function in adult cells and tissues. VAD in adult rats causes most epithelia to undergo reversible focal keratinizing squamous metaplasia [Wolbach and Howe, 1933]. Retinol is also necessary for the development and maintenance of lung alveoli throughout life; VAD in adult rats causes a condition resembling emphysema [Maden and Hind, 2004], which is reversible by vitamin A supplementation. Vitamin A signaling in adults is also necessary for the maintenance of innate and adaptive immunity [Stephensen, 2001] and recent studies have identified components of the vitamin A pathway as important regulators of adipogenesis and obesity [Ziouzenkova et al., 2007; Park et al., 2009; Moise et al., 2010]. A large body of evidence [Drager, 2006; Maden, 2007] also shows retinol signaling to be essential for normal brain function in adult animals. VAD in adult rodents, for example, adversely affects neurogenesis in the hippocampus leading to deficits in learning and memory, which can be reversed by atRA or vitamin A supplementation [Chiang et al., 1998; Misner et al., 2001; Cocco et al., 2002; Jacobs et al., 2006; Bonnet et al., 2008]. The age-related decline in cognitive function and onset of some neurodegenerative diseases such as Parkinson's and Alzheimer's have been hypothesized to be caused, in part, by disruption of the RSP [Maden, 2007].

Given the essential role that retinol signaling plays during embryogenesis and throughout adult life, any interference with this essential pathway is a potential human health concern. While vitamin A deficiency or excess in humans might not be a significant medical problem in the developed world, the extent to which chemical exposures can interfere with the pathway in cells and tissues of otherwise healthy individuals to create localized deficiency or excess of retinol and its metabolites is largely unknown and unexplored. To understand the extent of the problem caused by unintentional exposure to chemicals that can interact with vertebrate cells to disrupt the RSP, a short-term, high-throughput in vitro assay would be useful for screening large chemical libraries. A cell that expresses essential components of the pathway and thus supports the conversion of retinol to transcriptionally active metabolites is obviously essential. The P19 mouse pluripotent embryonal carcinoma cell was shown to be induced to differentiate into neurons and glial cells when cultured as embryoid bodies in the presence of atRA [McBurney, 1993]. Nerve cell differentiation in this cell also was subsequently found to be inducible by retinol, however the level of differentiation was significantly less than that induced by atRA [Jones-Villeneuve et al., 1983] suggesting that the ability of the P19 cell to use retinol for signaling was impaired. In search for a cell line around which to develop an assay for chemicals that can disrupt the RSP, we have re-evaluated the effect of retinol on the P19 cell using changes in gene expression as an endpoint to determine if this line is sufficiently responsive to retinol to be used for the assay. Using induced changes in *Hox* gene expression as more sensitive endpoints, we show that retinol stimulates the rapid and easily quantitated upregulation of *Hox* gene expression, although it is approximately 160-fold less potent than atRA as an inducer. This demonstrates that the P19 cell has a functional pathway that can convert retinol to transcriptionally active metabolites. We also identify and quantitate the relative basal levels of the various alcohol and aldehyde dehydrogenase, *Cyp26*, and retinol/atRA binding-protein genes that are expressed in P19 cells and quantitate the retinol-induced changes in the expression of these genes. We further show that the genes for *Retsat* (all-*trans*-13,14-dihydroretinol saturase) and the mouse β-carotene-cleaving enzymes, *Bcmo1* and *Bco2*, are expressed and that *Rbp4*, which is thought to be expressed predominantly in liver and adipose tissue, also is expressed at significant levels in P19 cells. Finally, a dose–response analysis of the effect of citral, an inhibitor of *Aldh1a2*, shows that the inhibitory effects of this chemical can be easily detected and quantitated in the P19 cell.

MATERIALS AND METHODS

CELL CULTURE

P19 mouse pluripotent embryonal carcinoma (EC) cells [McBurney, 1993] were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured, without antibiotics, in MEM α medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (ATCC). The serum retinol concentration, assayed by the supplier, was 62 nM. All experiments were carried out on monolayer cultures. Retinol, retinaldehyde, retinoic acid, and citral were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in DMSO at 10 mM under red light illumination and stored in the vapor phase of liquid nitrogen.

RNA ISOLATION, CDNA SYNTHESIS, AND END-POINT PCR

RNA was isolated from P19 cells with TRI Reagent RT (Molecular Research Centre; Cincinnati, OH) and cDNA synthesized using SuperScript III (Invitrogen) reverse transcriptase following the manufacturer's protocol. PCR reactions were initiated at 95°C for 5 min followed by 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension was done at 72°C for 5 min. The RT-PCR-dot blot method for detecting qualitative changes in homeobox gene expression was described previously [Reese and Ramos-Valle, 2007].

DIRECT CDNA SYNTHESIS ON CELL LYSATES

For cDNA synthesis from cell lysates, cells were seeded at 4×10^4 cells/well in 96-well plates. Following over-night culture and subsequent exposure to test agents the next day, cell monolayers were washed in ice cold PBS, lysed in 50 μl cells-to-cDNA II lysis buffer (Ambion, Austin, TX), and the lysates processed according to the manufacturer's protocol to inactivate RNase activity. Lysates were treated with 3 U DNase I to remove genomic DNA. For cDNA syntheses, 4μl of lysate was used in a 20 μl RT reaction containing 5 μM oligo$(dT)_{18}$ and SuperScript III as above.

QUANTITATIVE REAL-TIME PCR (QPCR) ANALYSIS OF GENE EXPRESSION

PCR reactions were carried out in 96-well plates in a Roche LightCycler 480 Real-time PCR System II instrument using the SYBR

Green I protocol (Roche Diagnostics, Indianapolis, IN). Each 20 μl PCR reaction contained 1× Roche SYBR Green I Master Mix PCR reagent, 0.5 μM gene-specific primer pairs, and an RT reaction aliquot. PCR primer sequences for all genes assayed in this study and their amplification efficiencies are listed in Supplemental Table I. Relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method using *Gapdh* as the internal control. Melt-curve analysis was done on all primer pairs to eliminate those that produced nonspecific products.

RESULTS

RESPONSE OF P19 CELLS TO RETINOL, RETINALDEHYDE, AND RETINOIC ACID

Retinoic acid has been shown to upregulate the expression of several homeobox genes in P19 cells [Reese and Ramos-Valle, 2007]. To determine if this was also true for retinol and retinaldehyde, cells were cultured in the presence of retinol, retinaldehyde, or retinoic acid for 8 h and analyzed for changes in mRNA expression using an RT-PCR-dot blot method developed for the detection of qualitative changes in *Hox* and *non-Hox* homeobox gene expression [Reese and Ramos-Valle, 2007]. As shown in Figure 1, both retinol and retinaldehyde stimulated significant increases in the expression of *Hoxa* and *Hoxb* cluster genes indicating that the P19 cell can metabolize retinol to retinoic acid. Additional homeobox-containing genes, *Cdx1*, *Gbx2*, *Lhx3*, and *Hb9*, also showed apparent increased expression while *Gbx1* and *Lhx1* appeared to be downregulated. Although the levels of increased *Hoxa* and *Hoxb* gene expression stimulated by the three retinoids appeared to be comparable based on the intensity of the dot-blot signals, the amplification and gene detection end-points used in this method allow the detection of only gross changes in gene expression and are not quantitative. Further, because retinoic acid is the active metabolite of retinol and does not require further metabolism, it may be expected to initiate new gene expression earlier, thus generating higher levels of *Hox* expression than was observed on the dot-blots. To determine the relative potency of the three retinoids, the time course for the increase in mRNA synthesis of one of the *Hoxa*-cluster genes, *Hoxa1*, was analyzed by qPCR to quantitate relative changes in gene expression. Figure 2 shows that retinoic acid was clearly the most potent inducer and that retinol, which first must to be metabolized to retinaldehyde and then to retinoic acid, was the least effective and retinaldehyde was intermediate in potency. The data show that the P19 cell expresses all necessary genes for the canonical RSP.

COMPARATIVE RESPONSIVENESS OF P19 CELLS TO RETINOL AND RETINOIC ACID

To characterize further the response of P19 cells to retinol, change in the expression of *Hoxa1* was assayed by RT-qPCR over an extended range of retinol concentrations along with retinoic acid over the same concentration range. The dose–response curves for the effect of retinol and retinoic acid on *Hoxa1* expression during a 6 h exposure period are shown in Figure 3. The retinoid concentration that stimulated a 50% increase (EC_{50}) in *Hoxa1* expression relative to untreated controls was calculated from the best-fit value of the

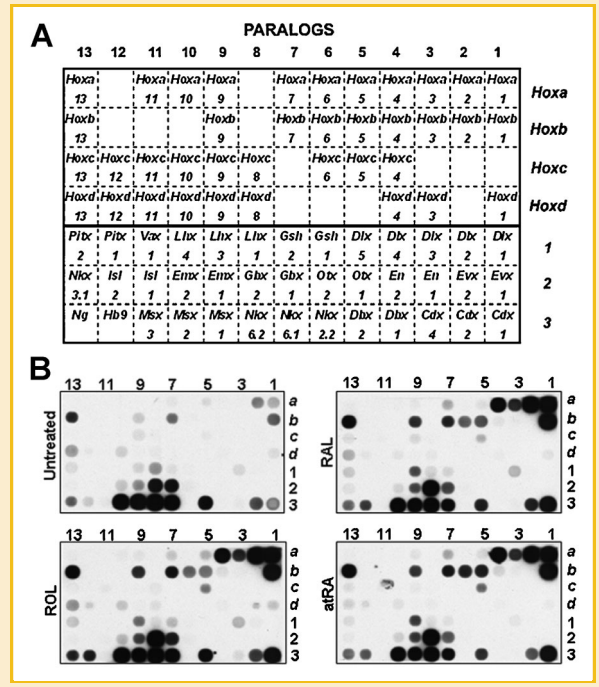

Fig. 1. Patterns of homeobox gene expression induced by retinol, retinal, and atRA. A: Configuration of homeobox-gene dot blot showing position of oligonucleotide sequences. The four *Hox* gene clusters are aligned in rows and marked *Hoxa–Hoxd* and gene numbers are indicated at the top; rows 1–3 indicate non-*Hox* homeobox sequences [Reese and Ramos-Valle, 2007]. B: Effects of each retinoid on homeobox gene expression. Cells were cultured with 1 μM of each retinoid for 8 h and processed for RT-PCR and hybridization as described in Reese and Ramos-Valle [2007]. Abbreviations: *Ng*, *Nanog*; ROL, retinol; RAL, retinaldehyde.

dose–response curve. The 95% confidence interval was 119–199 nM for the retinol EC_{50} and 0.63–1.39 nM for the retinoic acid EC_{50}, showing that retinoic acid was approximately 160-fold more potent than retinol as an inducer of *Hoxa1* expression in this cell culture system.

TIME COURSE FOR THE RETINOL INDUCTION OF HOXA-CLUSTER GENE EXPRESSION

The response of *Hoxa1* to retinol suggests that the expression of this gene, or another *Hoxa*-cluster gene, could be used as an end-point for a short-term, high-throughput assay for detecting chemicals that disrupt retinol signaling. It was of interest, therefore, to compare early retinol-induced changes in expression of the first four genes of the *Hoxa*-cluster to determine which gene would be the most suitable for a short-term assay. In a previous study on the human EC cell line, NT2/D1, the induction of *HOXA1* expression by 10 μM retinoic acid was first detected, using the RNase protection assay, 6 h after exposure to the retinoid but not after 3 h exposure [Simeone et al., 1991]. *HOXA1* was the first gene in the A-cluster to be upregulated by retinoic acid followed, after increasing periods of delay, by successively more 5' genes. The time course for the induction of the first four genes on the *Hoxa* cluster by 1 μM retinol in P19 cells using RT-qPCR to quantitate changes in expression is shown in Figure 4. Unlike the onset of expression in NT2/D1 cells,

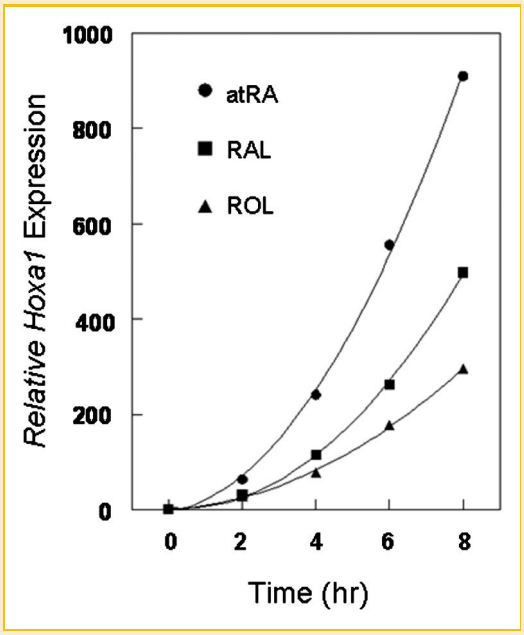

Fig. 2. Time course for *Hoxa1* induction by retinol, retinaldehyde, and atRA. Cells were cultured with 1 μM of each retinoid for the indicated times and changes in gene expression analyzed by RT-qPCR.

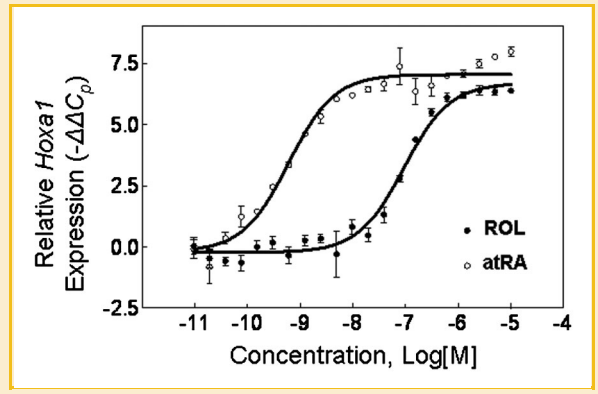

Fig. 3. Retinol and atRA dose–response curves for the upregulation of *Hoxa1* expression. Cells were cultured for 6 h with the indicated concentration of retinol or atRA. RT-qPCR analysis was done on lysates of cells cultured in 96-well plates as described in Materials and Methods section. Values are mean ± S.E., n = 3.

increases in *Hoxa1*, *Hoxa2*, and *Hoxa3* expression were all detected by 30 min exposure to retinol; only the onset of *Hoxa4* expression was delayed. Each gene displayed a unique pattern of expression during the 2 h culture period suggesting that different transcription regulatory mechanisms might be involved.

EXPRESSION OF RSP GENES IN P19 CELLS

End-point PCR was carried out on cDNA synthesized from purified total RNA to assay for the expression of genes that may have a role in the RSP. Genes surveyed included retinoid binding and receptor genes, alcohol dehydrogenases, retinaldehyde dehydrogenases, and Cyp26 cytochrome P450 genes. Because of their involvement with retinol synthesis or metabolism, three additional genes, *Bcmo1* (β,β-carotene 15,15'-monooxygenase) and *Bco2* (β,β-carotene 9',10'oxygenase), which cleave β-carotene symmetrically and asymmetrically, respectively, and *Retsat* (all-*trans*-13,14-dihydroretinol saturase) were assayed for expression. Figure 5 shows the gene transcripts that were amplified in P19 cells using end-point RT-PCR. Genes sharing the same or similar/analogous functions were grouped together in Figure 5. Different criteria were used for the placement of *Stra6* and *Retsat*. *Stra6* was grouped with the retinol binding proteins, *Rbp4* and *Rbp1*, because it presumably binds retinol for transport/translocation into the cell; *Retsat* was included with the retinol/alcohol dehydrogenases because it also metabolizes retinol. The following genes, previously shown to be expressed in P19 cells, were detected: Membrane retinol receptor/translocator, *Stra6* [Bouillet et al., 1997; Kawaguchi et al., 2007]; cellular retinol and retinoic acid binding proteins, *Rbp1*/*Crbp* and *CrabpI* [Wei et al., 1989]; *Rarα,β,γ* and *Rxrα,β* [Gudas et al., 1994]; retinaldehyde dehydrogenases, *Aldh1a1*, *Aldh1a2*, and *Aldh1a3* [Xi and Yang, 2008]; and *Cyp26s* [Sonneveld et al., 1999; Xi and Yang, 2008]. We also detected the expression of additional pathway genes for retinol (*Rbp4*) and cellular retinoic acid (*Crabp2*) binding proteins, retinol dehydrogenases (*Rdh1*and *Rdh10*), alcohol dehydrogenases (*Adh1*, *Adh4*, *Adh7/3*), *Retsat*, *Rxrγ*, and genes that catalyze the oxidative cleavage of β-carotene (*Bcmo1*, *Bco2*).

EFFECT OF RETINOL ON RSP GENE EXPRESSION

A number of pathway-related genes are known to be regulated by atRA in P19 or other cell types [Wei et al., 1989; Bouillet et al., 1997; Sonneveld et al., 1999; Xi and Yang, 2008]. To quantitate the change

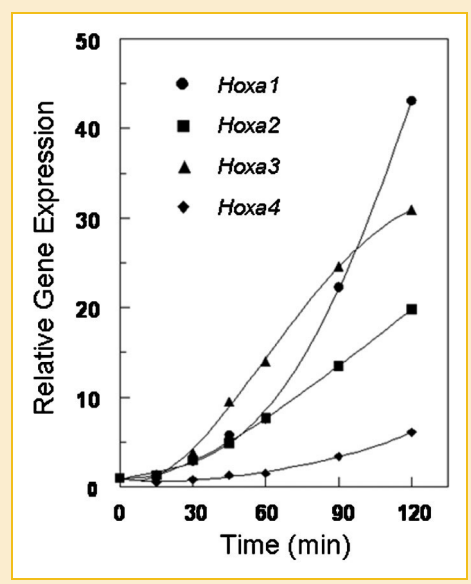

Fig. 4. Time course for the upregulation of *Hoxa*-cluster genes by retinol. Cells were cultured with 1 μM retinol for the indicated times and changes in the expression of the first four genes of the *Hoxa* cluster were analyzed by RT-qPCR on purified RNA.

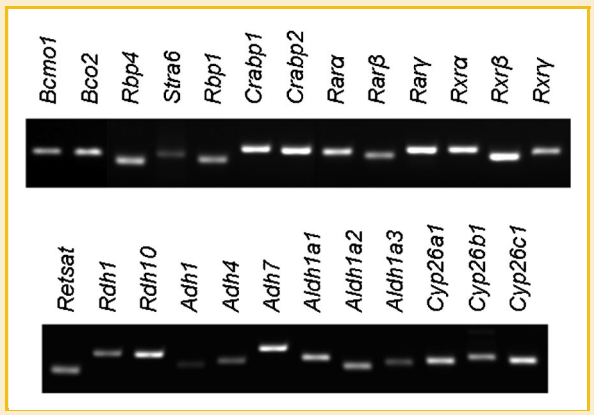

Fig. 5. Expression of candidate RSP genes in P19 cells. RT-PCR was done on P19-cell RNA using primer pairs for candidate RSP genes (see Supplemental Table I) and fractionated on agarose gels. Control PCR reactions on reverse transcriptase-minus RT samples produced no PCR products showing that the templates for the PCR products shown here were not from contaminating genomic DNA (data not shown).

in expression induced by retinol for the genes identified in Figure 5, cells were cultured in the absence or presence of 1 μM retinol for 24 h and changes in gene expression analyzed by RT-qPCR. Significant changes in gene expression were detected in at least one member of each group except the β-carotene-cleaving and retinol/alcohol-metabolizing groups, which were unresponsive to retinol (Table I, retinol-induced change in expression).

RELATIVE BASAL LEVELS OF RSP GENE TRANSCRIPTS

To provide some insight into the relative steady state expression levels of genes within each grouping in Figure 5, relative transcript abundance was calculated from the difference in crossing points (C_p) between each gene within a group and the least abundant gene transcript (highest C_p value) in that group. The genes in each group are ranked in Table I (relative transcript abundance) relative to the least abundant transcript in the group, which is assigned a value of one; the data are expressed as $2^{\Delta Cp}$. Although multiple genes were expressed for each step or function in the pathway, one gene was usually expressed at several hundred to a 900-fold higher level than the other genes in its group, indicating a major role for the gene in the pathway.

DETECTION OF CHEMICAL-INDUCED INHIBITION OF THE RSP

The goal of this study was to determine if the P19 cell has a functional RSP that could be used for detecting chemicals whose mode of action is the disruption of this important signaling pathway. Having established that the P19 cell has a functional pathway, we tested the ability of the cell to detect the inhibitory action of a positive test chemical, citral. This chemical is a known inhibitor of the Aldh1a2/Raldh2 enzyme [Gagnon et al., 2002] and, therefore, it also inhibits the synthesis of atRA from retinol. Citral was tested over an extended range of concentrations to determine the sensitivity of the P19 cell to detect a disruptor of the RSP. The MTT assay for cellular toxicity was also done over the full range of concentrations and showed no evidence of toxicity (data not shown). The data in Figure 6 show the dose–response curve for the citral inhibition of *Hoxa1* induction by retinol. The IC_{50} for the inhibition of the RSP in the P19 cell was 4.2 μM.

TABLE I. RSP Gene Inducibility by Retinol and Relative Abundance of Basal-Level Transcripts

Gene	Retinol-induced change in expression[a]	Relative transcript abundance[b]
Bco2	1.13 ± 0.16	917
Bcmo1	1.03 ± 0.14	1
Rbp1	4.14 ± 0.31**	940
Rbp4	0.81 ± 0.04*	49
Stra6	14.92 ± 2.17**	1
Rdh10	1.24 ± 0.15	471
Retsat	1.08 ± 0.06	321
Rdh1	1.37 ± 0.32	7
Adh4	0.63 ± 0.11	2
Adh7	1.60 ± 0.59	1
Adh1	1.50 ± 0.66	1
Aldh1a2	4.74 ± 0.08**	288
Aldh1a1	0.96 ± 0.12	27
Aldh1a3	2.51 ± 0.19**	1
Crabp2	10.07 ± 0.79**	2
Crabp1	7.60 ± 0.09**	1
Rarα	2.00 ± 0.18**	635
Rarγ	1.63 ± 0.10**	393
Rarβ	91.04 ± 7.47**	1
Rxr γ	0.95 ± 0.08	108
Rxrβ	1.50 ± 0.45	4
Rxrα	2.52 ± 0.61*	1
Cyp26a1	22.00 ± 2.13**	67
Cyp26c1	14.25 ± 0.17**	5
Cyp26b1	50.22 ± 1.23**	1

[a]Cells were cultured without or with 1 μM retinol 24 h and isolated RNA subjected to RT-qPCR. The statistical significance of retinol-induced change in gene expression was determined relative to expression in uninduced cells by Student's *t*-test. Values are mean ± S.D., n = 3.
[b]Genes in each group are ranked relative to the least abundant transcript, i.e., with the highest crossing point (Cp) in the group, which is assigned a value of one; the data are expressed as $2^{\Delta Cp}$.
*$P < 0.05$.
**$P < 0.01$.

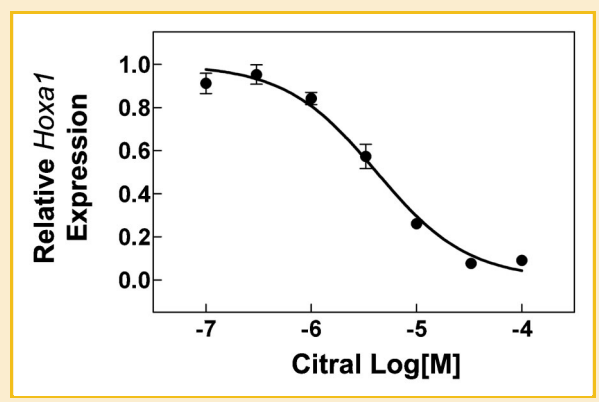

Fig. 6. Effect of citral on the induction of *Hoxa1* expression by retinol. Citral was added to cultures grown in 96-well plates at the indicated final concentrations. One hour later, retinol was added at a final concentration of 0.33 μM and incubation was continued for 6 h. RT-qPCR analysis was done on cell lysates as described in Materials and Methods section. Values are mean ± S.E., n = 3.

DISCUSSION

In this study we show that retinol induces the same pattern of homeobox gene expression as its proximal metabolite, retinal, and major active metabolite, atRA, in P19 cells demonstrating that this cell has a functional RSP. The cell expresses multiple retinol/alcohol dehydrogenase and retinaldehyde dehydrogenase genes thought to be necessary for the conversion of retinol to retinal and atRA. The basal expression of the microsomal retinol dehydrogenase gene, *Rdh10*, is several hundred-fold higher than the expression of *Rdh1* and the three cytosolic alcohol dehydrogenase genes (*Adh4*, *Adh7/3*, *Adh1*) suggesting that Rdh10 is the enzyme primarily responsible the conversion of retinol to retinal in the P19 cell. The expression of this gene was recently shown to be essential for normal development and is thought to be the enzyme that is predominantly responsible for the conversion of retinol to retinal in the embryo [Cammas et al., 2007; Sandell et al., 2007]. *Aldh1a2/Raldh2*, the enzyme which catalyzes the oxidation of retinal to atRA in the embryo [Niederreither and Dolle, 2008], is also the predominant retinaldehyde dehydrogenase transcript detected in P19 cells. The P19 cell, therefore, expresses high levels of the two genes thought to catalyze the conversion of retinol to atRA in the embryo. The possibility cannot be excluded, however, that additional alcohol and aldehyde dehydrogenases expressed in P19 cells such as *Rdh1*, *Adh7/3*, and *Aldh1a1* may also contribute to the synthesis of atRA.

The observation that both β-carotene-cleaving genes were expressed in the pluripotent P19 cell and that the basal level of *Bco2* gene transcripts was almost a 1,000-fold higher than that of Bcmo1 is of interest. Bcmo1 catalyzes the initial step in the synthesis of retinol by symmetrically cleaving β-carotene and other provitamin A dietary carotenoids in the mucosa of the small intestine to retinal, which is converted to retinol and stored in the liver. Not all absorbed dietary carotenoids are metabolized in the intestine, however. Some enter the blood associated with chylomicrons and both low- and high-density lipoproteins and are carried in the circulation to the tissues [Bendich and Olson, 1989; Lindqvist and Andersson, 2004]. Mammalian tissues, including human tissues, express both β-carotene-cleaving enzymes [Lindqvist and Andersson, 2004; von Lintig and Vogt, 2004; Lindqvist et al., 2005] suggesting that retinol synthesis can also occur locally at the tissue and organ level. Although both enzymes are expressed in many of the same tissues, significant differences exist. Bco2 is expressed at lower levels than Bcmo1 and in some tissues its expression is restricted to a small subset of cells [Lindqvist et al., 2005]. Also, Bco2 is expressed in some adult tissues that do not express Bcmo1 and do not appear to be sensitive to VAD. In addition to cleaving β-carotene asymmetrically, Bco2 can also carry out the oxidative cleavage of lycopene [Kiefer et al., 2001] indicating a broader substrate specificity than Bcmo1. These distinct properties of Bco2 have led to the suggestion that the enzyme may have other functions in addition to catalyzing the synthesis of retinol [Lindqvist et al., 2005]. The significance of *Bcmo1* and *Bco2* expression in the pluripotent P19 cell, especially the unusually high level of *Bco2* expression relative to *Bcmo1*, is unknown. Further studies are needed to define the function of Bco2 in this cell and whether its high level of expression is connected in any way to the pluripotent phenotype of the P19 cell, which can be induced to differentiate into neurons, glia, cardiac and skeletal muscle, adipocytes, endoderm, or endothelial cells by various agents and culture conditions [McBurney, 1993; Jho and Malbon, 1997; Choi et al., 2008; Bouchard and Paquin, 2009].

Retinol was found to be significantly less potent than its metabolites, retinal, and atRA, as an inducer of *Hoxa1* expression in P19 cells; compared to atRA, retinol was approximately 160-fold less effective as an inducer. Differences in cellular uptake, availability/sequestration, storage, and metabolism between atRA and retinol probably account for the difference in potency. A difference in uptake between retinol and atRA would appear not to be a factor in this in vitro study, however, since both free retinol and atRA rapidly traverse cell membranes [Noy and Xu, 1990; Noy, 1992] and thus probably have comparable access to the intracellular compartment. After cellular uptake, however, the two molecules are handled differently. atRA interacts with two high affinity cellular retinoic acid binding proteins, Crabp1 and Crabp2. Crabp1 has been proposed to facilitate the metabolism of atRA by members of the Cyp26 family of P450 enzymes to polar retinoic acid derivatives while atRA-bound to Crabp2, which is expressed at a twofold higher level than Crabp1 in P19 cells, is delivered to the RARs without further metabolism [Noy, 2000]. Retinol, on the other hand, binds with high affinity to the cellular retinol binding protein, Rbp1/Crbp1. This binding protein, which is abundantly transcribed in P19 cells, has been proposed to act as a retinol chaperone. By sequestering retinol, Rbp1/Crbp1 is thought to protect cells from the membranolytic effects of free retinol and to "channel" retinol to enzymes that catalyze its metabolism and storage [Noy, 2000]. Other data, however, indicate that free retinol, and not Rbp1-bound retinol, may be the preferred molecular form for metabolism [Gallego et al., 2006]. Significant amounts of retinol are probably not stored in P19 cells in view of preliminary data (unpublished) showing a very low level expression of *Lrat* (lecithin:retinol acetyltransferase), which catalyzes the esterification of retinol for storage. The bulk of retinol taken up by P19 cells, therefore, is probably metabolized. In addition to retinal and atRA, retinol has been reported to be metabolized to a number of transcriptionally active and inactive compounds in various cell types [Buck et al., 1991; Derguini et al., 1995; Achkar et al., 1996; Ross et al., 2000; Liu et al., 2009; Moise et al., 2009] and, although the profile of retinol metabolites in P19 cells has not been determined, both active and inactive metabolites would be expected to be produced in this cell as well. The generation of high levels of inactive retinol metabolites might be a contributing factor for the low potency of retinol, relative to atRA, in the present study. Retinol saturase (Retsat), which is expressed at a level comparable to that of Rdh10 in P19 cells, has been shown to catalyze the conversion of retinol to (*R*)-all-*trans*-13,14-dihydroxyretinol, (*R*)-DROL, which is then metabolized to the dihydroretinoic acid derivative, (*R*)-DRA [Moise et al., 2009]. In cell-based assays, (*R*)-DRA was shown to be significantly less potent than atRA as an activator of RAR-regulated genes apparently because the dihydrometabolite is not translocated to the nucleus efficiently. It has been suggested that the synthesis of (*R*)-DRA may be a mechanism for diverting the metabolism of retinol to the synthesis of less active ligands thus reducing the expression of some

RAR-controlled genes [Moise et al., 2009]. This and similar mechanisms for diverting retinol metabolism from the synthesis of atRA and other transactivating retinoids could have a role in protecting pluripotent stem cells from spontaneously differentiating in the presence retinol. Overall, the difference in potency between retinol and atRA in the induction of *Hoxa1* measured in this study is likely to be determined by a number of cell-type specific factors including the time that retinol resides in the Rbp1-retinol compartment, the efficiency of retinol metabolism to atRA and other transactivating metabolites, and the extent of metabolism to less active ligands.

Given its diversity of expressed RSP-associated genes, its rapid transcriptional response to retinol, and its ability to detect and quantitate the inhibitory action of citral on the retinol-induced upregulation of *Hoxa1*, the P19 cell appears to provide a sensitive in vitro model system for detecting and exploring the mechanism of action of chemicals that interfere with this critical cellular pathway.

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REFERENCES

Achkar CC, Derguini F, Blumberg B, Langston A, Levin AA, Speck J, Evans RM, Bolado J Jr, Nakanishi K, Buck J, Gudas LJ. 1996. 4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors. Proc Natl Acad Sci USA 93:4879–4884.

Balmer JE, Blomhoff R. 2002. Gene expression regulation by retinoic acid. J Lipid Res 43:1773–1808.

Bendich A, Olson JA. 1989. Biological actions of carotenoids. FASEB J 3:1927–1932.

Bonnet E, Touyarot K, Alfos S, Pallet V, Higueret P, Abrous DN. 2008. Retinoic acid restores adult hippocampal neurogenesis and reverses spatial memory deficit in vitamin A deprived rats. PLoS One 3:e3487.

Bouchard F, Paquin J. 2009. Skeletal and cardiac myogenesis accompany adipogenesis in P19 embryonal stem cells. Stem Cells Dev 18:1023–1032.

Bouillet P, Sapin V, Chazaud C, Messaddeq N, Decimo D, Dolle P, Chambon P. 1997. Developmental expression pattern of Stra6, a retinoic acid-responsive gene encoding a new type of membrane protein. Mech Dev 63:173–186.

Buck J, Derguini F, Levi E, Nakanishi K, Hammerling U. 1991. Intracellular signaling by 14-hydroxy-4,14-retro-retinol. Science 254:1654–1656.

Cammas L, Romand R, Fraulob V, Mura C, Dolle P. 2007. Expression of the murine retinol dehydrogenase 10 (Rdh10) gene correlates with many sites of retinoid signalling during embryogenesis and organ differentiation. Dev Dyn 236:2899–2908.

Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, Kampa D, Piccolboni A, Sementchenko V, Cheng J, Williams AJ, Wheeler R, Wong B, Drenkow J, Yamanaka M, Patel S, Brubaker S, Tammana H, Helt G, Struhl K, Gingeras TR. 2004. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116:499–509.

Chiang MY, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF, Evans RM. 1998. An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. Neuron 21:1353–1361.

Choi SC, Choi JH, Shim WJ, Lim DS. 2008. P19 embryonal carcinoma cells: A new model for the study of endothelial cell differentiation. Biotechnol Lett 30:1169–1175.

Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L, Fadda F. 2002. Vitamin A deficiency produces spatial learning and memory impairment in rats. Neuroscience 115:475–482.

Collins MD, Mao GE. 1999. Teratology of retinoids. Annu Rev Pharmacol Toxicol 39:399–430.

Derguini F, Nakanishi K, Hammerling U, Chua R, Eppinger T, Levi E, Buck J. 1995. 13,14-Dihydroxy-retinol, a new bioactive retinol metabolite. J Biol Chem 270:18875–18880.

Drager UC. 2006. Retinoic acid signaling in the functioning brain. Sci STKE 2006:pe10.

Duester G. 2008. Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921–931.

Gagnon I, Duester G, Bhat PV. 2002. Kinetic analysis of mouse retinal dehydrogenase type-2 (RALDH2) for retinal substrates. Biochim Biophys Acta 1596:156–162.

Gallego O, Belyaeva OV, Porte S, Ruiz FX, Stetsenko AV, Shabrova EV, Kostereva NV, Farres J, Pares X, Kedishvili NY. 2006. Comparative functional analysis of human medium-chain dehydrogenases, short-chain dehydrogenases/reductases and aldo-keto reductases with retinoids. Biochem J 399:101–109.

Gudas LJ, Sporn MB, Boberts AB. 1994. Cellular biology and biochemistry of the retinoids. The retioids: Biology, chemistry, and medicine, 2nd edition. Sporn MB, Roberts AB, and Goodman DS, editors. New York: Raven Press Ltd. pp. 443–520.

Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH, Evans RM. 2006. Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proc Natl Acad Sci USA 103:3902–3907.

Jho EH, Malbon CC. 1997. Galpha12 and Galpha13 mediate differentiation of P19 mouse embryonal carcinoma cells in response to retinoic acid. J Biol Chem 272:24461–24467.

Jones-Villeneuve EM, Rudnicki MA, Harris JF, McBurney MW. 1983. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol Cell Biol 3:2271–2279.

Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, Sun H. 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315:820–825.

Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO, Breithaupt DE, von Lintig J. 2001. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. J Biol Chem 276:14110–14116.

Lindqvist A, Andersson S. 2004. Cell type-specific expression of beta-carotene 15,15'-mono-oxygenase in human tissues. J Histochem Cytochem 52:491–499.

Lindqvist A, He YG, Andersson S. 2005. Cell type-specific expression of beta-carotene 9',10'-monooxygenase in human tissues. J Histochem Cytochem 53:1403–1412.

Liu L, Derguini F, Gudas LJ. 2009. Metabolism and regulation of gene expression by 4-oxoretinol versus all-trans retinoic acid in normal human mammary epithelial cells. J Cell Physiol 220:771–779.

Maden M. 2007. Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8:755–765.

Maden M, Hind M. 2004. Retinoic acid in alveolar development, maintenance and regeneration. Philos Trans R Soc Lond B Biol Sci 359:799–808.

McBurney MW. 1993. P19 embryonal carcinoma cells. Int J Dev Biol 37:135–140.

Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF, Evans RM. 2001. Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. Proc Natl Acad Sci USA 98:11714–11719.

Moise AR, Alvarez S, Dominguez M, Alvarez R, Golczak M, Lobo GP, von Lintig J, de Lera AR, Palczewski K. 2009. Activation of retinoic acid receptors by dihydroretinoids. Mol Pharmacol 76:1228–1237.

Moise AR, Lobo GP, Erokwu B, Wilson DL, Peck D, Alvarez S, Dominguez M, Alvarez R, Flask CA, de Lera AR, von Lintig J, Palczewski K. 2010. Increased adiposity in the retinol saturase-knockout mouse. FASEB J 24:1261–1270.

Niederreither K, Dolle P. 2008. Retinoic acid in development: Towards an integrated view. Nat Rev Genet 9:541–553.

Noy N. 1992. The ionization behavior of retinoic acid in lipid bilayers and in membranes. Biochim Biophys Acta 1106:159–164.

Noy N. 2000. Retinoid-binding proteins: Mediators of retinoid action. Biochem J 348(Pt 3): 481–495.

Noy N, Xu ZJ. 1990. Interactions of retinol with binding proteins: Implications for the mechanism of uptake by cells. Biochemistry 29:3878–3883.

Park PJ, Kong SW, Tebaldi T, Lai WR, Kasif S, Kohane IS. 2009. Integration of heterogeneous expression data sets extends the role of the retinol pathway in diabetes and insulin resistance. Bioinformatics 25:3121–3127.

Reese DH, Ramos-Valle M. 2007. A high-throughput method for monitoring changes in homeobox gene expression. Biochem Biophys Res Commun 357:882–888.

Ross SA, McCaffery PJ, Drager UC, De Luca LM. 2000. Retinoids in embryonal development. Physiol Rev 80:1021–1054.

Sandell LL, Sanderson BW, Moiseyev G, Johnson T, Mushegian A, Young K, Rey JP, Ma JX, Staehling-Hampton K, Trainor PA. 2007. RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development. Genes Dev 21:1113–1124.

Simeone A, Acampora D, Nigro V, Faiella A, D'Esposito M, Stornaiuolo A, Mavilio F, Boncinelli E. 1991. Differential regulation by retinoic acid of the homeobox genes of the four HOX loci in human embryonal carcinoma cells. Mech Dev 33:215–227.

Sonneveld E, van den Brink CE, Tertoolen LG, van der Burg B, van der Saag PT. 1999. Retinoic acid hydroxylase (CYP26) is a key enzyme in neuronal differentiation of embryonal carcinoma cells. Dev Biol 213:390–404.

Stephensen CB. 2001. Vitamin A, infection, and immune function. Annu Rev Nutr 21:167–192.

von Lintig J, Vogt K. 2004. Vitamin A formation in animals: Molecular identification and functional characterization of carotene cleaving enzymes. J Nutr 134:251S–256S.

Wei LN, Blaner WS, Goodman DS, Nguyen-Huu MC. 1989. Regulation of the cellular retinoid-binding proteins and their messenger ribonucleic acids during P19 embryonal carcinoma cell differentiation induced by retinoic acid. Mol Endocrinol 3:454–463.

Wilson JG, Roth CB, Warkany J. 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. Am J Anat 92:189–217.

Wolbach SB, Howe PR. 1933. Epithelial repair in recovery from vitamin A deficiency: An experimental study. J Exp Med 57:511–526.

Xi J, Yang Z. 2008. Expression of RALDHs (ALDH1As) and CYP26s in human tissues and during the neural differentiation of P19 embryonal carcinoma stem cell. Gene Expr Patterns 8:438–442.

Ziouzenkova O, Orasanu G, Sharlach M, Akiyama TE, Berger JP, Viereck J, Hamilton JA, Tang G, Dolnikowski GG, Vogel S, Duester G, Plutzky J. 2007. Retinaldehyde represses adipogenesis and diet-induced obesity. Nat Med 13:695–702.