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Targeted Disruption of Retinoic Acid Receptor α (RARα) and RARγ Results in Receptor-Specific Alterations in Retinoic Acid-Mediated Differentiation and Retinoic Acid Metabolism

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F9 embryonic teratocarcinoma stem cells differentiate into an epithelial cell type called extraembryonic endoderm when treated with retinoic acid (RA), a derivative of retinol (vitamin A). This differentiation is presumably mediated through the actions of retinoid receptors, the RARs and RXRs. To delineate the functions of each of the different retinoid receptors in this model system, we have generated F9 cell lines in which both copies of either the RARα gene or the RARγ gene are disrupted by homologous recombination. The absence of RARα is associated with a reduction in the RA-induced expression of both the CRABP-II and Hoxb-1 (formerly 2.9) genes. The absence of RARγ is associated with a loss of the RA-inducible expression of the Hoxa-1 (formerly Hox-1.6), Hoxa-3 (formerly Hox-1.5), laminin B1, collagen IV(α1), GATA-4, and BMP-2 genes. Furthermore, the loss of RARγ is associated with a reduction in the metabolism of all-*trans*-RA to more polar derivatives, while the loss of RARα is associated with an increase in metabolism of RA relative to wild-type F9 cells. Thus, each of these RARs exhibits some specificity with respect to the regulation of differentiation-specific gene expression. These results provide an explanation for the expression of multiple RAR types within one cell type and suggest that each RAR has specific functions.

Retinoic acid (RA) is one of the most potent biologically active forms of vitamin A. RA exhibits a wide range of activities. RA influences the proliferation and differentiation of a variety of cell types including embryonic stem cells, teratocarcinoma stem cells, keratinocytes, bone progenitor cells, various types of carcinomas, and promyelocytic leukemia cells (3, 14, 21, 26, 31, 72). RA is currently being employed in the clinical treatment of many forms of cancer (30, 31, 71, 72). In addition, RA may play important roles with respect to axis formation and limb development during embryogenesis (29, 63–65).

Two types of RA-binding proteins exist in most cells: the small cytoplasmic high-affinity RA-binding proteins CRABP-I and CRABP-II (5, 24) and the nuclear receptors RARα, -β, and -γ and RXRα, -β, and -γ. Evidence suggests that one function of CRABP-I is the facilitation of the metabolism of all-*trans*-RA to more polar derivatives, thereby possibly regulating the intracellular concentration of RA available to the nuclear receptors (5–7, 20). The function of CRABP-II is still under investigation; this protein may also play a role in RA metabolism (13). The RARs and the RXRs are members of the steroid/thyroid/vitamin D superfamily and function as ligand-dependent transcription factors (9, 11, 14, 26, 35, 37, 41, 48, 62). The RARs bind all-*trans*-RA and its isomer 9-*cis*-RA with high affinity, while the highest-affinity ligand for the RXRs is 9-*cis*-RA (28, 43).

Because the sequences of RARs are highly conserved among different species and each of the RARs exhibits a spatially and temporally specific pattern of expression during embryonic development, each RAR may possess some unique functions (11, 17, 59). Such functions presumably involve the control of the expression of a subset of mRNAs regulated during the differentiation process. Current experimental evidence lends support for this idea. Within each RAR and RXR protein two transcriptional activator functions exist (41, 53). The first is located within the N-terminal A/B region, and the second is located in the C-terminal region. These domains possess the ability to transactivate different RA-responsive promoter elements. This activation presumably occurs through the formation of RXR-RAR or RAR-RAR protein heterodimers (27). Thus, the diversity of RA's effects can potentially be understood in terms of the various RXR-RAR and RAR-RAR combinations within the cell.

This appears to be true for RARγ; the targeted disruption of both copies of the RAR γ gene in F9 teratocarcinoma stem cells leads to a defect in the RA-associated activation of the Hoxa-1 (formerly Hox-1.6), laminin B1, and collagen type IV(α1) genes (8). However, not all differentiation-specific genes are altered in the RARγ −/− cell line, supporting the idea that each receptor is responsible for regulating different sets of gene targets. Some specificity with respect to RARγ function is also observed in mice. Disruption of the RARγ gene in mice produces specific developmental defects characterized by congenital malformations, growth deficiency, premature death, and male sterility but, interestingly, does not generate malformations which are similar to those in Hoxa-1-deficient mice (45, 46). The lack of any additional developmental changes in these RARγ-deficient mice may result from some receptor functional redundancy associated with the up-

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regulation of the expression of the two remaining RARs, RARα and RARβ, at critical times during embryogenesis.

RARα mRNAs are ubiquitously expressed in the developing mouse embryo as well as in most adult-mouse tissues (17, 42, 59, 76). While disruption of the α1 isoform of RARα resulted in normal development of the mice (44, 47), targeted disruption of the entire RARα gene resulted in early postnatal lethality and testis degeneration (47). Interestingly, a mutation in RARα is also associated with acute promyelocytic leukemia (2, 15, 36, 71). An acute promyelocytic leukemia-specific chromosomal translocation between chromosomes 15 and 17 forms two chimeric mRNAs: PML-RARα and RARα-PML. This suggests that the mutant RARα protein contributes to the development of acute promyelocytic leukemia by preventing normal promyelocytic cell differentiation. Collectively, these data support the idea that RARα regulates one or more critical events in the cellular differentiation pathway.

In an attempt to understand the function of RARα within the context of the RA signalling mechanism, we have developed F9 stem cells that have disruptions in both of the RARα alleles. The characterization of these cell lines will allow the identification of RARα target genes and lead to a better understanding of the functions of each of the receptors. We also compare the ability of RARγ −/− and RARα −/− F9 stem cells to respond to signalling by all-*trans*-RA versus 9-*cis*-RA and examine the roles of these receptors in the metabolism of RA.

MATERIALS AND METHODS

Cell culture and generation of disrupted lines. The RARα disruption vector p826B2 is described in detail elsewhere (47). F9-Wt cells were cultured under standard conditions (6). Ten million cells in 500 μl of electroporation buffer plus 20 μg of linearized p826B2 plasmid were electroporated by using a Bio-Rad gene pulser set at 200 V and 960 μF (52). Cells were plated at a density of 10^6/150-cm^2 tissue culture plate for 36 h and selected in G418 (300 μg of active drug per ml) for 18 to 21 days. Individual colonies were harvested for Southern blot analysis. The second allele was targeted by growing the single-copy disruption line F9-Wt-α-85 in high levels of G418 (2 mg of active drug per ml) for 18 days (52). Individual colonies were harvested and analyzed as described above.

Differentiation of F9 stem cells. F9-Wt, F9-Wt-α-85, F9-Wt-α-85-2, and F9-Wt-α-85-6 stem cells were cultured and treated with either 1 μM all-*trans*-RA or 1 μM 9-*cis*-RA as described elsewhere (6, 61), and RNA was prepared at the indicated times (6). Southern and Northern (RNA) blot analyses were performed as described elsewhere (6).

Gel mobility assays. Whole-cell extracts from cultures of F9-Wt and RARα −/− cells were prepared as previously reported (57). Mobility shift assays were performed as described by Garner and Revzin (22) by using the Hoxa-1/RARE-β double-stranded oligodeoxynucleotide corresponding to the RA response element (RARE) of the Hoxa-1 and RARβ genes (16, 38) as described elsewhere (67). Five micrograms of each of the protein extracts was preincubated with 50,000 cpm of a ^{32}P-labeled oligonucleotide probe, and then 1 μl of ascites fluid monoclonal antibodies directed towards the F region of the RAR α1, β2, or γ1 protein (23, 56, 57) was added. The protein complexes were resolved on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA.

RT-PCR. Reverse transcriptase PCR (RT-PCT) was performed under standard conditions by using avian myeloblastosis virus RT and *Taq* polymerase with 2 μg of RNA and 50 pmol of each primer. The primers used for RARα were 5′ ATCGAGACCCAGAGCAGCAG 3′ (B domain) and 5′ CCTGGTGCGCTTT GCGAACC 3′ (D domain), and those for CRABP-II were 5′ ATGAATTCGG AGACAGCAAAGTATCTTTA 3′ and 5′ ATAAGCTTAAATCACACAGACT ACAAGG 3′. The amplifications were done for 20 and 30 cycles, aliquots were electrophoresed on a 1.5% agarose gel, and samples were transferred onto Hybond N membranes. The blots were hybridized to the corresponding random-primed RARα or CRABP-II cDNAs.

Plasmids. The following plasmids were used in the Northern analyses: ERA-1-993-E1.0 for Hoxa-1 (39, 40), B15.5 for laminin B1, pGEM-CRABP-II for CRABP-II (12), HBA for Hox-1.5 (Hoxa-3) (from Frank Ruddle), I5-7 for collagen IV(α1), pBSK5 for oct 3/4 (from Y. Bergman), BMP-2-68 for BMP-2, BMP-4-70 for BMP-4 (from J. Wozney), Hox 2.9 (435) for Hoxb-1 (from J. Grippo), p2-5 for REX-1, and MT_2-GATA-4 for GATA-4 (from S. Orkin).

Extraction of retinoids and high-performance liquid chromatography (HPLC) chromatography. F9-Wt, RARα (−/−), and RARγ (−/−) cells were plated in dishes (10 by 15 mm) at a density of 5×10^5 per plate. Twenty-four hours later, the cells were incubated in the presence of 50 nM [11,12-^{3}H]all-*trans*-RA (Du Pont-New England Nuclear) (50 Ci/mmol) for the indicated times. At the appropriate times, the media were collected and the cells were rinsed with phosphate-buffered saline (PBS) and then scraped into 500 μl of PBS. Retinoids were immediately extracted from both cells and media according to the method of McClean et al. (50). Briefly, 350 μl of acetonitrile-butanol (50:50, vol/vol) was added to 500 μl of cells or media, the mixtures were vortexed for 30 s, 300 μl of a saturated potassium phosphate solution was added, and the mixtures were vortexed again. After centrifugation (10,000 × *g* for 10 min), the organic layer was removed, dried in a Speed-Vac, and stored for no more than 3 days at −70°C.

Each sample was dissolved in 50 μl of ethanol for analysis by HPLC. The HPLC system included two Beckman 110B pumps and an analytical 5-μm reversed-phase C_{18} column (Vydac, Hesperia, Calif.). A linear gradient from 67% acetonitrile (ammonium acetate, 15 mM, pH 6.5) to 100% acetonitrile in 25 min was employed at a flow rate of 1.5 ml/min. A radiochromatography detector (Packard A-500) was used to count labeled retinoids.

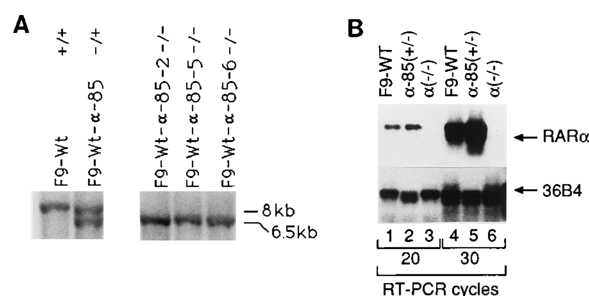


FIG. 1. Generation of RARα −/− cell lines. (A) Southern blot analysis demonstrating the successful disruption of the RARα gene. The homologous recombined fragment and WT genomic fragment are indicated. (B) RARα mRNA expression in the F9-Wt, F9 α-85 +/−, and F9 α −/− lines. The levels of RARα transcripts were monitored by RT-PCR in the F9-Wt, F9 α-85 +/−, and F9 α −/− lines by using the primers 5′ ATCGAGACCCAGAGCAGCAG 3′ and 5′ CCTGGTGCGCTTTGCGAACC 3′. The amounts of RNA were normalized with internal control primers to the ribosomal phosphoprotein mRNA 36B4. Results for both 20 cycles and 30 cycles of PCR are shown. The RT-PCR assay was performed on both the RAR α-85-2 −/− and α-85-6 −/− lines; results for only the α-85-2 −/− line are shown here.

RESULTS

Generation of targeted disruptions in the RARα gene. A replacement type disruption vector, described previously (47), was employed to introduce the neomycin resistance gene into exon 8 of the RARα allele. Insertion of the neomycin resistance gene within this exon will disrupt all encoded RARα isoforms. F9-Wt cells were electroporated with 20 μg of linearized p826B2 and selected in G418 for 21 days. Resistant colonies were isolated and screened by Southern blotting for the expected change in the size of the wild-type (WT) *Xba*I genomic fragment. Figure 1A shows the presence of the homologous recombinant 6.5-kb *Xba*I fragment in the F9-Wt-α-85 −/+ line in addition to the WT 8.0-kb fragment. To target the second RARα allele, F9-Wt-α-85 −/+ cells were selected in high concentrations of G418 for 21 days (8, 52), resulting in six surviving colonies. All six cell lines showed a recombination event which resulted in the loss of the second WT 8.0-kb genomic fragment (Fig. 1A). Two RARα −/− lines were chosen for further study.

In order to show that the RARα −/− lines lacked detectable RARα mRNA, RT-PCR was performed, as this technique is more sensitive than Northern analysis (Fig. 1B). Whereas bands at the appropriate position for RARα were detected in the F9-Wt and RARα-85 +/− lines, no band was observed in the RARα −/− line (Fig. 1B). Thus, the RARα −/− lines do not express the RARα transcripts which are expressed in F9-Wt cells.

As it is difficult to detect the low levels of RARα protein

even in WT F9 cells by Western (immunoblot) analysis, this method could not be employed to assay the RARα −/− cells for the loss of RARα protein. A gel mobility assay was employed instead, using monoclonal antibodies specific for each RAR to test for the absence of RARα protein. The RARβ/Hoxa-1 RARE was used for the gel shift assays, and protein extracts were prepared from the F9-Wt and the RARα −/− lines. Preliminary results showed that there was no supershift with the RARα monoclonal antibody in the RARα −/− lines (data not shown).

Analysis of the growth of the RARα −/− lines. Changes in the RA responsiveness of the RARα −/− cell lines were first investigated by measuring cell growth in the presence and absence of all-*trans*-RA. A characteristic of RA-treated F9-Wt cells is a marked slowing of cellular growth as differentiation progresses. F9-Wt and RARα −/− cells were grown in the presence of both high (10^{-6} M RA) and low (10^{-8} M RA) concentrations of the drug for 6 days. There were no significant (greater than 5%) differences between the rates of growth of the RARα −/− lines and the F9-Wt cells after RA addition (data not shown). As the RARγ line was also growth inhibited by RA with kinetics comparable to those of RA-treated WT cells (8), it is possible that RARβ regulates cell growth. When we obtain an RARβ-disrupted F9 line, this idea can be tested. Alternatively, there may be receptor redundancy with respect to the regulation of cell growth.

Characterization of the differentiation of the F9 RARα −/− lines. The RARα −/− lines were investigated in detail to assess their ability to differentiate in response to RA, as measured by the activation of RA-responsive, differentiation-specific genes. When the RARα −/− lines were tested by Northern blot analysis for changes in the expression of differentiation-related mRNAs, only a few differences from WT F9 cells were observed (Fig. 2A). Of the differentiation-specific marker genes tested, only Hoxb-1 and CRABP-II mRNA expression was altered in the RARα −/− lines (Fig. 2B and C). In the RARα −/− lines, the level of the induction of the CRABP-II gene was threefold lower and the Hoxb-1 mRNA level was two- to threefold lower than the levels in F9-Wt cells treated for 48 h with RA (Fig. 2B and C).

We had previously shown by Northern analyses that the Hoxb-1 (formerly Hox 2.9) and CRABP-II genes were induced to a greater extent and that their mRNA expression was elevated for a longer time after RA expression in the RARγ −/− line than in the F9-Wt line (8). We next compared the levels of CRABP-II transcripts in the F9-Wt, RARγ −/−, and RARα −/− lines by quantitative RT-PCR (Fig. 2D). At 48 h after RA addition, the RARγ −/− line exhibits a CRABP-II transcript level which is three- to fourfold higher than that in F9-Wt cells; the CRABP-II level in the two RARα −/− lines is fourfold lower than that in F9-Wt cells, in agreement with the Northern analyses.

Disruption of RARα does not affect the expression of many of the differentiation-specific genes (Fig. 2A), supporting the idea that each receptor regulates different sets of mRNAs. Moreover, the RA-associated induction of some genes, such as RARβ, REX-1 (32), J6 (68, 69), and J31 (70) (also called SPARC [51]), is not altered in either the RARα −/− or RARγ −/− lines (8; also, this study). These genes may be regulated via RARβ.

Effects of all-*trans*-RA versus 9-*cis*-RA on gene expression and examination of additional differentiation markers in the RARα −/− and RARγ −/− lines. Several research groups have recently demonstrated the ability of the RARs to form heterodimers with the RXRs leading to gene activation (18, 27, 49). In an attempt to understand the functions of RXRs in the F9 differentiation system, the F9-Wt, RARα −/−, and RARγ −/− cell lines were treated with either all-*trans*-RA (1 μM) or 9-*cis*-RA (1 μM). Northern blot analysis was used to assess changes in gene expression (Fig. 3).

In this experiment, some additional differentiation-specific gene markers were examined, including GATA-4 (4), oct 3/4 (54, 60), BMP-2 (58, 75), BMP-4 (58, 75), and Hoxa-3 (formerly Hox 1.5) (19). As was shown previously (see above references), the GATA-4, BMP-2, and Hoxa-3 genes are induced in response to RA in F9-Wt cells. GATA-4 and Hoxa-3 are transcription factors with zinc finger and homeobox DNA-binding motifs, respectively, and BMP-2 is a growth factor in the transforming growth factor β superfamily. In contrast, the BMP-4 and oct 3/4 genes have previously been shown to exhibit a decrease in expression in response to RA. BMP-4 is a member of the transforming growth factor β superfamily, and oct 3/4 is a member of the octamer family of transcription factors (60).

The Hoxa-3, GATA-4, and BMP-2 genes are dramatically induced in response to all-*trans*-RA in F9-Wt cells (Fig. 3). In the RARα −/− line, these three genes are also induced in response to all-*trans*-RA. However, in the RARγ −/− line, the Hoxa-3, GATA-4, and BMP-2 genes are only minimally induced in response to all-*trans*-RA (Fig. 3A to C). The induction of these three genes in the RARγ −/− line is less than 10% of that in the F9-Wt in response to all-*trans*-RA. Thus, these genes are similar to Hoxa-1 and laminin B1 (Fig. 3D and E) in their lack of RA inducibility in the RARγ −/− line. Only a slight decrease in oct 3/4 expression, about two- to threefold, in all three cell types is seen (Fig. 3A). A three- to fourfold decrease in BMP-4 is also observed in all three cell lines at 24 h after addition of all-*trans*-RA or 9-*cis*-RA (Fig. 3A). Thus, we have identified several additional genes whose activation in response to RA is dramatically reduced in the absence of RARγ expression: Hoxa-3, GATA-4, and BMP-2.

When the responses of specific genes to all-*trans*-RA versus 9-*cis*-RA are examined, several conclusions can be drawn. First, although the levels of induction of both the Hoxa-1 and the Hoxa-3 genes are similar 24 h after all-*trans*-RA versus 9-*cis*-RA addition in F9-Wt cells, after 96 h of drug treatment the expression of these two Hox genes is still elevated in the cells treated with 9-*cis*-RA whereas the expression has declined dramatically in cells treated with all-*trans*-RA. A similar result is not observed for the GATA-4, BMP-2, or laminin B1 genes, however. Thus, it appears that all-*trans*-RA is less effective than 9-*cis*-RA in sustaining the expression of the Hox genes at late times such as 96 h. The RARα −/− line behaves in a fashion similar to the F9-Wt line with respect to the activation of the Hox genes and the GATA-4 gene in response to all-*trans*-RA versus 9-*cis*-RA.

Metabolism of RA in the RARα −/− and RARγ lines. Some target cells have the ability to metabolize all-*trans*-RA to more polar compounds (7, 25, 55, 74). There is indirect evidence that at least some of the enzymes involved in this process are RA inducible (1, 25, 74) as the half-life of [^{3}H]RA is shorter in cells which have been pretreated with RA than in cells which have not previously been exposed to RA. The RARα −/− and the RARγ −/− lines were employed to examine the roles of each of the RARs in the metabolism of all-*trans*-RA. Cells were incubated in the presence of [^{3}H]-all-*trans*-RA for specific times, after which both the intracellular and the extracellular levels of [^{3}H]-all-*trans*-RA and its more polar metabolites were quantitated by reverse-phase HPLC (Fig. 4). Compared with F9-Wt control cells, the RARγ −/− cells metabolize all-*trans*-RA at a slower rate, producing lower levels of polar metabolites. In comparison, the RARα −/− cells metabolize

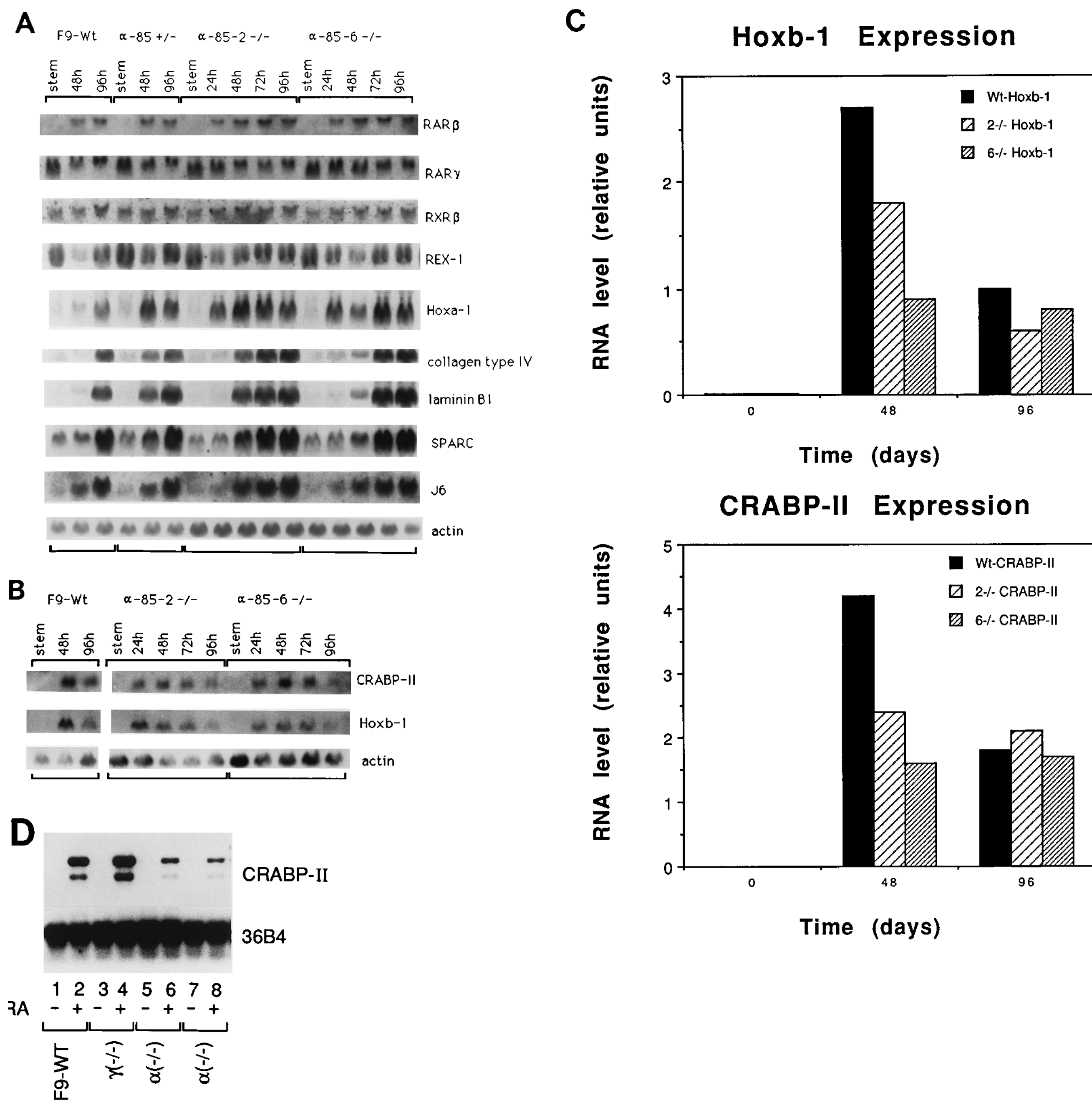


FIG. 2. Effects of all-*trans*-RA on the expression of differentiation-specific genes in RARα −/− cells. F9-Wt cells, the RARα heterozygote line α-85 +/−, and the two homozygous negative RARα lines α-85-2 −/− and α-85-6 −/− were treated with 1 μM all-*trans*-RA, and RNA was harvested at the indicated times and analyzed on Northern blots. Twenty micrograms of RNA was loaded in each lane. (A) Expression of RARβ, RARγ, RXRβ, REX-1, Hoxa-1, J31, J6, collagen type IV(α1), and laminin B1 mRNAs. Actin mRNA levels do not change after RA treatment (39); differences in actin mRNA levels in various lanes reflect RNA loading differences from lane to lane. Exposure times of the autoradiograms varied from 8 h for actin to 4 days for Hoxa-1. (B) Northern analysis of Hoxb-1 and CRABP-II gene expression at different times after RA addition. (C) Northern blot data from panel B quantitated by densitometry and expressed relative to actin mRNA levels. Quantitation of only the stem, 48-h, and 96-h samples is shown so that the RARα −/− lines can be compared with the samples of the F9 WT line in panel B. (D) CRABP-II expression in F9-Wt, F9γ −/−, and two F9α −/− lines by RT-PCR. Cells of the F9-Wt, F9γ −/−, and two F9 α −/− lines were grown in the absence (−) or presence (+) of 1 μM all-*trans* RA for 48 h. The expression of CRABP-II was analyzed with the primers 5′ ATGAATTCGGAGACAGCAAAGTATCTTTA 3′ and 5′ ATAAGCTT AAATCACACAGACTACAAGG 3′; 20 amplification cycles were carried out. The amount of RNA in each reaction was normalized to the ribosomal phosphoprotein mRNA 36B4, which is not altered upon treatment with RA. This experiment was performed three times with similar results.

all-*trans*-RA at a faster rate, producing higher levels of polar RA derivatives. By 7 h after RA addition, 50% more polar derivatives are produced in the RARα −/− line than in the F9-Wt cells, whereas only half as many polar derivatives of RA are produced in the F9 RARγ −/− line as in F9-Wt cells (Fig. 4B). Thus, the loss of the RARα gene enhances RA metabolism, whereas the loss of the RARγ gene reduces the rate of RA metabolism.

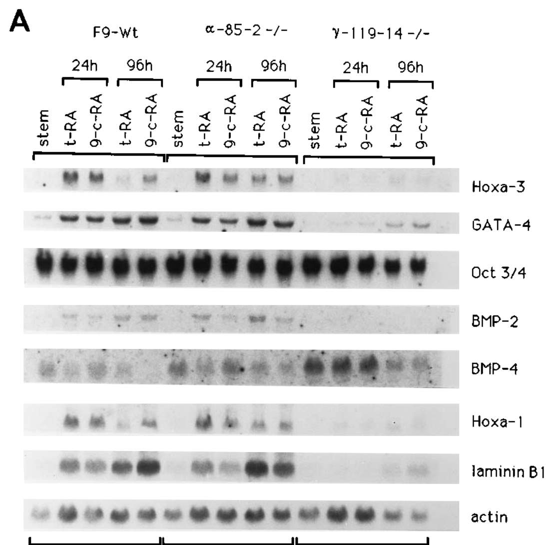
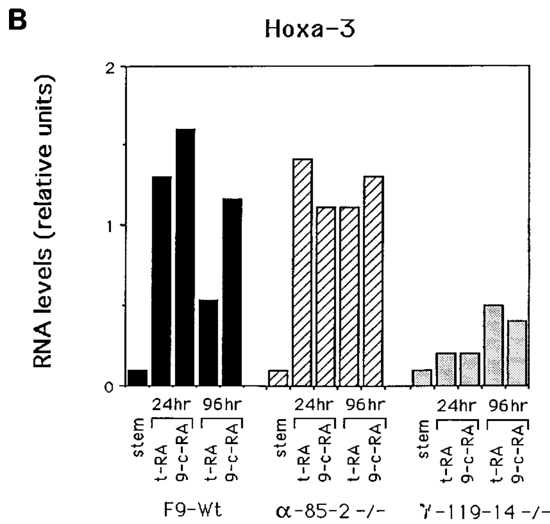
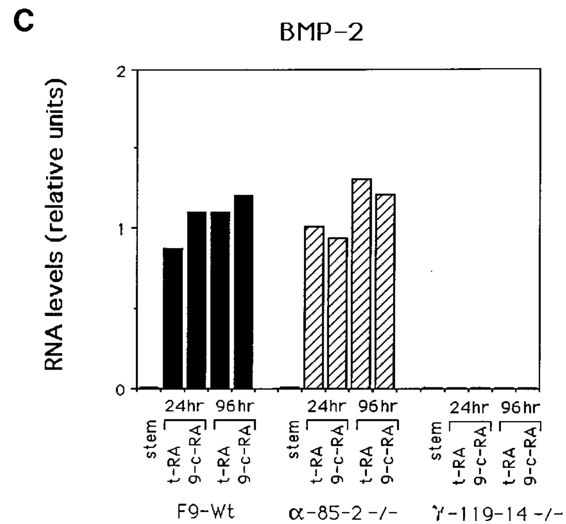
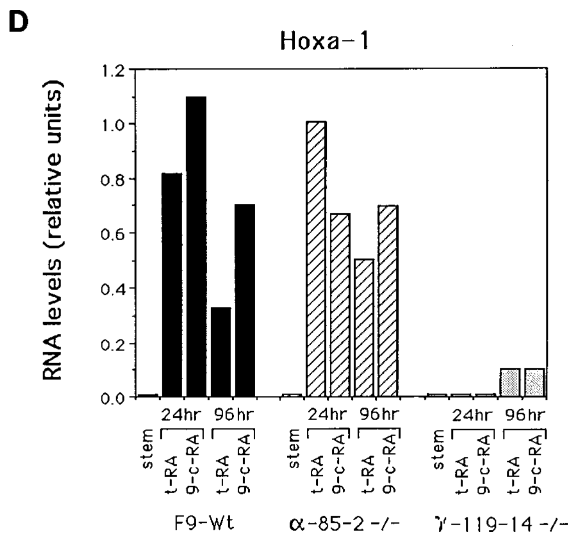
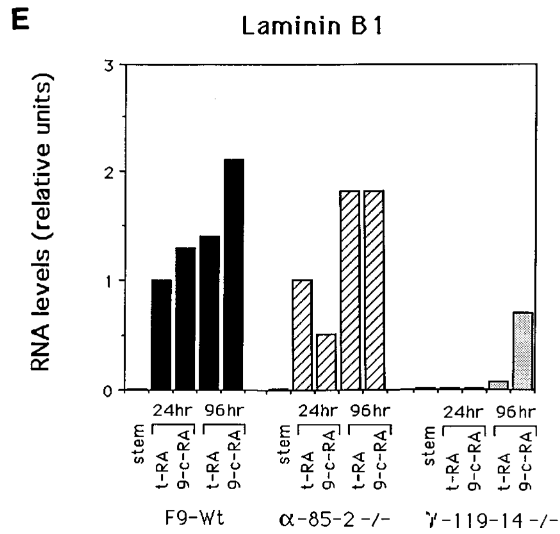


FIG. 3. Comparison of the effects of all-*trans*-RA and 9-*cis*-RA during the differentiation of RARα −/− and RARγ −/− cells. F9-Wt, RARα −/−, and RARγ −/− cells were treated with either 1 μM all-*trans*-RA or 1 μM 9-*cis*-RA for 24 or 96 h. (A) Gene expression was monitored by Northern blot analysis as described previously. Actin mRNA is presented to normalize for RNA loading differences from lane to lane, as actin mRNA levels do not change in response to RA. (B to E) The data from panel A were quantitated by densitometry and expressed relative to actin mRNA levels. This experiment was performed twice.









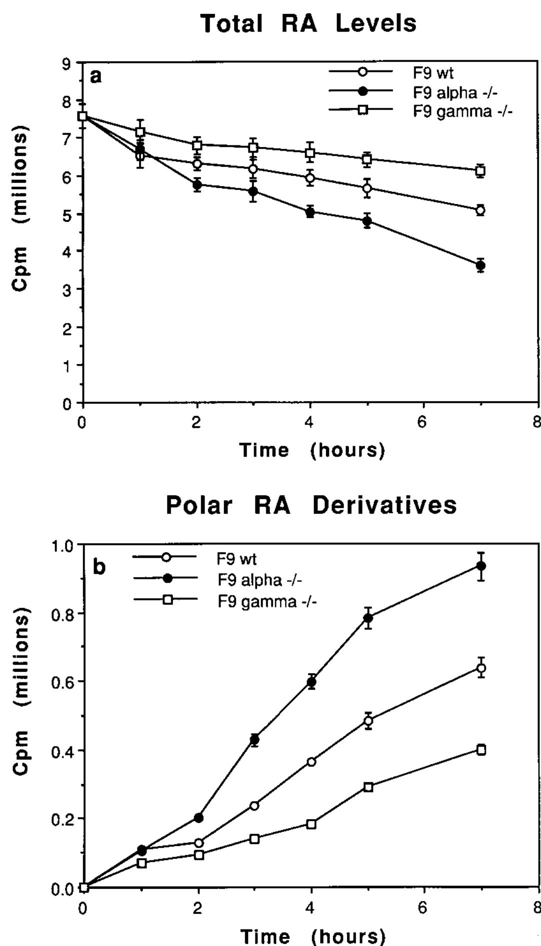


FIG. 4. Metabolism of all-*trans*-RA in F9-Wt, RARα −/−, and RARγ −/− cell lines. Cells were incubated in the presence of [^{3}H]RA for the indicated times, and labeled retinoids were extracted and analyzed by HPLC. The levels of total radiolabeled-RA (a) and total radiolabeled polar RA derivatives (b) are shown. [^{3}H]RA was separated from its radiolabeled metabolites by HPLC, and the [^{3}H]RA peak was quantitated at each time point both in samples of the cell extracts and in samples of the culture medium. The total [^{3}H]RA remaining at various times is plotted in panel a. In panel b the levels of the radiolabeled polar RA derivatives, separated from [^{3}H]RA and quantitated by HPLC, are shown at various times after [^{3}H]RA addition. This experiment was performed twice with similar results.

DISCUSSION

We are interested in describing in detail the molecular events which occur after addition of RA to embryonic teratocarcinoma stem cells, which leads to their eventual growth arrest and differentiation into endoderm, an epithelial-like cell layer of the late mouse blastocyst. RARs and RXRs mediate most, if not all, of RA's actions. As F9 teratocarcinoma cells express all three RARs and RXRs (RARα, -β, and -γ and RXRα, -β, and -γ), it is crucial to understand the specific roles of each of these receptors in the regulation of the differentiation process and the associated growth arrest. Thus, we are in the process of carrying out a series of experiments in order to disrupt both copies of each of the three RAR genes in F9 cells. We previously reported the initial characterization of two independently isolated RARγ −/− F9 cell lines (8). In this report, we have further characterized one of the RARγ −/− cell lines, and in addition, we have characterized two RARα −/− lines with respect to their growth and differentiation properties. We have found very specific differences between these two types of receptor-deficient lines in the regulation of gene expression after RA addition.

The loss of RARα or RARγ does not influence the RA-associated growth arrest. First, with respect to the growth arrest which occurs 2 to 3 days after RA addition to WT F9 cells, neither the absence of RARα nor the absence of RARγ results in a loss of this growth arrest associated with the differentiation of the cells. RA induces the growth arrest of a variety of cell types, and the ability of RA to arrest cell growth may be important in its action as a cancer chemopreventive and therapeutic agent. Thus, it is intriguing that the growth arrest associated with the differentiation process is not altered by the loss of either RARα or RARγ. Several possibilities remain: the growth arrest of F9 cells after RA addition may be mediated by RARβ, the arrest may be receptor mediated but may not depend on a specific receptor type, or the growth arrest may be an RA action that is not receptor mediated. These possibilities are currently being tested.

The loss of RARγ is associated with more dramatic alterations in gene expression than the loss of RARα. With respect to the activation of RA-responsive, differentiation-specific genes, the effects of the loss of RARγ are much more substantial than those associated with the loss of RARα. In the RARγ −/− lines, two genes of the Hoxa cluster, Hoxa-1 and Hoxa-3, are not activated or are only minimally activated in response to RA. In addition, laminin B1, laminin B2 (73), and collagen type IV(α1), three genes which encode extracellular matrix proteins, are not activated in response to RA (8). The GATA-4 gene is not activated in response to RA in the RARγ −/− lines, and BMP-2 is not regulated normally in these lines (Fig. 3). In contrast, the loss of RARα expression is associated with reduced expression of only two genes, Hoxb-1 and CRABP-II, of those analyzed to date (Fig. 2). One explanation for this result is that the RARα protein is involved in the activation of both the Hoxb-1 and the CRABP-II genes in response to RA. In the RARγ −/− lines these two genes, Hoxb-1 and CRABP-II, are expressed for an unusually long time and at a higher level than in F9-Wt cells treated with RA (8) (Fig. 2D). One explanation for this result is that more receptor dimers containing RARα may exist in the RARγ −/− line, resulting in the enhanced, prolonged expression of these two genes in the RARγ −/− line. Studies by Durand et al. (18) have shown that there are two RAREs in the promoter of the CRABP-II gene and that the direct repeats in each of these RAREs are separated by 2 bp (DR2) and 1 bp (DR1). Thus, these RAREs are of the class that binds RAR-RXR heterodimers. 9-*cis*-RA was also shown to increase CRABP-II mRNA levels more efficiently than all-*trans*-RA (18). Our data suggest that RARα is one of the receptor types which interacts with one of the RXRs to activate CRABP-II gene expression in response to RA.

The genes that are not activated normally in response to RA in the RARγ −/− lines do not fall into any single category or type. Some of these genes are transcription factors (Hoxa-1, Hoxa-3, GATA-4), while some of the genes could be classified as more structural genes [laminin B1 and collagen type IV(α1)]. Another gene encodes a growth factor in the transforming growth factor β superfamily (BMP-2). The Hoxa-1 gene is expressed at early times after RA addition, while some of the genes [laminin B1, collagen type IV(α1), and BMP-2] are maximally expressed only after 2 or 3 days of RA treatment. We cannot state at the present time that RARγ is directly involved in the regulation of all of the aforementioned genes. Information concerning the actual DNA sequences involved in RA activation is available for only two of the genes affected by the loss of RARγ. The Hoxa-1 gene is activated by

RA through a 3′ enhancer that contains an RARE (38), and the laminin B1 gene possesses an RARE in its promoter (66, 67). Little is known about the mechanisms by which RA regulates collagen type IV(α1), GATA-4, and BMP-2. When more information is available about the specific sequences involved in the RA activation of these genes, sequence similarities which explain why these genes are all affected by the absence of RARγ may be found.

Some RA-responsive genes are not affected by the loss of RARα or RARγ. A number of RA-responsive genes, including J6, J31 (SPARC), REX-1, and RARβ, are not significantly affected by the absence of either RARα or RARγ. Again, there is no commonality among these genes. RARβ is an early, direct RA target (16, 34), while J6 and J31 are genes that are not activated by RA until approximately 2 days after RA addition (70). The REX-1 gene encodes a transcription factor which exhibits reduced expression after RA addition to F9-Wt, RARα −/− or RARγ −/− lines (32, 33). Our data suggest either that the RARβ gene is autoactivated by RARβ protein in F9 cells in response to RA or that RARα and RARγ are functionally equivalent with respect to RARβ induction. Since the RA responsiveness of the J6 (68) and J31 genes is under investigation but is currently not fully understood, it is difficult to explain why these genes are not affected by the lack of either RARα or RARγ.

RARγ influences the rate of metabolism of RA to more polar derivatives. That RA is metabolized to more oxidized derivatives in F9 cells was previously shown (25, 74). These studies also demonstrated that pretreatment with RA could decrease the half-life of a subsequent dose of RA. Evidence from Williams and Napoli and other laboratories indicates that at least some of the enzymes involved in the oxidation of RA are members of the P450 gene family, since a known P450 inhibitor, ketoconazole, can lengthen the half-life of RA in F9 cells (5, 74). The identities of these P450 enzymes that metabolize RA are not yet known. We have shown that the RARγ −/− line exhibits a lower rate of production of the polar metabolites than the F9 WT line after RA addition, whereas increased metabolism of RA is observed in the RARα −/− line (Fig. 4). Thus, both RARα and RARγ can influence RA metabolism.

Relationship of the embryonic teratocarcinoma model differentiation system to the results with transgenic animals. Our data in this F9 model differentiation system suggest that different Hox clusters are regulated by different RAR types, with the Hoxa cluster regulated through interactions with RARγ and the Hoxb cluster regulated via interactions with RARα. However, if this were true, we might expect to observe a more severe phenotype in the mice which have both copies of either RARα or RARγ disrupted (45, 47). As major morphological abnormalities have not been observed in these mice, it is possible that the cell culture model differentiation system does not reflect in all respects what occurs in vivo during mouse development with respect to the actions of RA. The relative levels of RARα and RARγ in F9 cells may be different from the levels in cells which respond to RA during embryogenesis. This may lead to functional redundancies among the different RARs at critical periods of development. This possibility is supported by recent observations which indicate that high levels of expression of RARα can compensate for the loss of RARγ in cultured F9 cells and, also, that the malformations in RARα and RARγ double-mutant fetuses are much more severe than those in animals mutated for either one of these two RARs (10).

In conclusion, we have demonstrated that specific alterations in gene expression are associated with the absence of either RARα or RARγ protein in F9 cells. Thus, under appropriate conditions, each of the RARs may perform some distinct biological functions. This possibility is interesting because it indicates that what we define as cell differentiation may be a complex response of a variety of different genes to all three RA receptor types and also suggests that it should be possible to interfere with only one portion of the differentiation pathway through the use of receptor-specific retinoid analogs.

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