Distinct Retinoid X Receptor-Retinoic Acid Receptor Heterodimers Are Differentially Involved in the Control of Expression of Retinoid Target Genes in F9 Embryonal Carcinoma Cells

HIDEKI CHIBA, JOHN CLIFFORD, DANIEL METZGER, AND PIERRE CHAMBON*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, Illkirch, France

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The F9 murine embryonal carcinoma cell line represents a well-established system for the study of retinoid signaling in vivo. We have investigated the functional specificity of different retinoid X receptor (RXR)-retinoic acid (RA) receptor (RAR) isotype pairs for the control of expression of endogenous RA-responsive genes, by using wild-type (WT), $RXR\alpha^{-/-}$, $RAR\alpha^{-/-}$, $RAR\gamma^{-/-}$, $RXR\alpha^{-/-}RAR\alpha^{-/-}$, and $RXR\alpha^{-/-}RAR\gamma^{-/-}$ F9 cells, as well as panRXR and RAR isotype (α , β , and γ)-selective retinoids. We show that in these cells the control of expression of different sets of RA-responsive genes is preferentially mediated by distinct RXR-RAR isotype combinations. Our data support the conclusion that RXR-RAR heterodimers are the functional units transducing the retinoid signal and indicate in addition that these heterodimers exert both specific and redundant functions on the expression of particular sets of RA-responsive genes. We also show that the presence of a given receptor isotype can hinder the activity of another isotype and therefore that functional redundancy between retinoid receptor isotypes can be artifactually generated by gene knockouts.

The F9 murine embryonal carcinoma (EC) cell line, a well-established model system for the analysis of retinoid signaling, differentiates upon retinoic acid (RA) treatment into three distinct extraembryonic (primitive, parietal, and visceral) endodermal cell types, depending on the culture conditions (for reviews, see references 16, 17, and 40). Retinoid-induced differentiation is accompanied by an apoptotic response and a decrease in the rate of proliferation (references 1, 9, and 38 and references therein), as well as by the induction of expression of a number of genes (references 2, 16, and 38 and references therein). F9 cells express all RA receptor isotypes ($RAR\alpha$, $RAR\beta$, and $RAR\gamma$), activated by either all-*trans* retinoic acid (tRA) or its 9-*cis* isomer (9c-RA), as well as all retinoid X receptor isotypes ($RXR\alpha$, $RXR\beta$, and $RXR\gamma$), activated by 9c-RA only (30, 44, 45). RXR-RAR heterodimers bind much more efficiently in vitro to RA response elements (RAREs) than do their respective homodimers (references 5, 6, 13–15, 18, 21, 24, 27, and 28 and references therein), and several lines of evidence support the idea that these heterodimers represent the functional units transducing the retinoid signal in vivo (references 6 and 19 and references therein). For instance, we have previously shown that a combination of a pan-RXR agonist and suboptimal concentrations of RAR isotype-selective agonists synergistically induce the expression of several endogenous RA-responsive genes in F9 and P19 EC cells (37, 43), which indeed strongly suggests that RXR-RAR heterodimers are the functional units responsible for transducing the retinoid signal which triggers the expression of RA-responsive genes in vivo.

We have recently engineered F9 cells lacking either $RXR\alpha$ and $RAR\alpha$ or $RXR\alpha$ and $RAR\gamma$ and shown that different RXR-RAR pairs exert both specific and redundant functions

in differentiation, proliferation, and apoptosis of these cells (8). To further dissect the physiological roles of the various RXRs and RARs at the molecular level, we have compared here the expression of a number of endogenous RA-responsive genes in $RXR\alpha^{-/-}RAR\alpha^{-/-}$ and $RXR\alpha^{-/-}RAR\gamma^{-/-}$ cells with their expression in wild-type (WT), $RXR\alpha^{-/-}$ (9), $RAR\alpha^{-/-}$ (4), and $RAR\gamma^{-/-}$ (3, 42) cells. We show that distinct RXR-RAR pairs are involved in the control of expression of different sets of RA-responsive genes. Taken together with results obtained by treatment of WT and mutant F9 cells with pan-RXR- and RAR isotype-selective retinoids, our data support the conclusion that RXR-RAR heterodimers are the functional units mediating the retinoid signal in vivo, and indicate that RXR-RAR heterodimers have both specific and redundant functions in retinoid control of gene expression. Interestingly, we also show that the presence of a given receptor isotype can hinder the activity of another isotype, and therefore that functional redundancy between retinoid receptor isotypes can be artifactually generated by gene knockouts.

MATERIALS AND METHODS

Cell culture. F9 cells were cultured as previously described (3, 9). The retinoids (tRA, BMS753, BMS453, BMS961, and BMS649) were dissolved in ethanol.

Western blotting. Whole-cell extracts from F9 cells and transfected COS-1 cells were isolated, and Western blot analysis was performed with various polyclonal and monoclonal antibodies as previously described (3, 12, 34–36) (see Fig. 1).

RT-PCR. RNA preparation and reverse transcription-PCR (RT-PCR) were performed as previously described (2, 9, 37, 42). The PCR primers for Hoxa-1, CRABP II, $RAR\beta$ 2, Cdx1, and 36B4 were described previously (9). The primers for Stra4, Stra6, and Stra8 were gifts from P. Bouillet. The other primers were as follows: Hoxb-1, 5'-TACCTGAGCCGTGCCCGAGG-3' (nucleotides [nt] 1300 to 1320) and 5'-GTCTTCGAGAGAAGTGGTC-3' (nt 1562 to 1581) (31); CRBP I, 5'-CCTGTGGACTTCAACGGGTA-3' (nt 14 to 33) and 5'-GTGTACTTTCTTAAACACTT-3' (nt 396 to 415) (39); and HNF1 β , 5'-GAAGATGCGCCGAACCGGT-3' (nt 697 to 716) and 5'-ATGGTGACTGATTGTCGAAG-3' (nt 1088 to 1107) (10). To make sure that amplifications were in the linear range, aliquots of PCR products were taken from each tube at three cycles between the cycles 15 and 25 depending on the genes analyzed (2, 9, 37, 42). End-labelled oligonucleotide probes for Hoxa-1 and 36B4 and random-primed cDNA probes for Hoxa-1, CRABP II, $RAR\beta$ 2, and Cdx1 were generated as previously described (9). The following cDNAs were random primed for probing

* Corresponding author. Mailing address: Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch Cedex, France. Phone: (33) 388 65 32 13, (33) 388 65 32 15, or (33) 388 65 32 10. Fax: (33) 388 65 32 03. E-mail: IGBMC@IGBMC.U-STRASBG.FR.

FIG. 1. Western blot analysis indicating that undisrupted retinoid receptors are similarly expressed in WT and RXR-RAR mutant F9 cells. Lanes 1 to 14 contain 70 μ g of whole-cell extracts from WT and mutant F9 cells treated for 24 h with control vehicle (lanes 1, 3, 5, 7, 9, 11, and 13) or 1 μ M tRA (lanes 2, 4, 6, 8, 10, 12, and 14), as indicated, and lanes 15 to 17 contain 2 μ g of whole cell extracts from COS cells transfected with either mRXR α , mRXR β , or mRXR γ expression vectors (25). RAR α , RAR β , RAR γ , RXR α , and all three RXR proteins were detected with rabbit polyclonal antibodies RP α (F) (12), RP β (F) (35) and RP γ (F) (34) and mouse monoclonal antibodies 4RX3A2 (36) and 1RX6G12 (36), respectively, followed by chemiluminescence detection. NS, non-specific interaction.

Southern blots: CRBPI (39), HNF1 β , Stra4, Stra6, and Stra8 (2). Transcript levels were quantified with a BAS 2000 bioimaging analyzer (Fuji Ltd.) and were normalized to the corresponding 36B4 mRNA levels.

RNAse protection assay. An RNAse protection assay was performed with antisense riboprobes for RAR β 2 and histone H4 as previously described (26). Transcript levels were quantified with a GS-700 imaging densitometer (Bio-Rad) and were normalized to the corresponding H4 mRNA levels.

RESULTS

The purpose of the present study is to investigate the functional specificity of different RXR-RAR pairs for mediating the induction of expression of a number of RA-responsive genes by using RXR-RAR mutant F9 cells as well as pan-RXR- and RAR isotype-selective retinoids. Since the loss of a given receptor isotype(s) might affect the expression of other isotypes, we first checked the protein levels of RXRs and RARs in WT and mutant F9 cells upon 24 h of tRA treatment by Western blot analysis with available antibodies. In each mutant F9 cell line, the undisrupted retinoid receptors were expressed at levels similar to those found in WT cells with the exception of RAR β in RXR α ^{-/-}-RAR γ ^{-/-} cells (Fig. 1 and data not shown), indicating that the observed differences in the RA response of the various mutants cannot be ascribed to changes in the expression levels of the remaining receptors.

Induction of Hoxa-1, CRBPI, HNF1 β , and Stra4 is preferentially mediated by RXR α -RAR γ pairs. After 24 h of tRA treatment (1 μ M), the induction of Hoxa-1, CRBPI, HNF1 β , and Stra4 transcripts was reduced in RXR α ^{-/-} (3-, 2-, 5-, and 2-fold decreases, respectively) and RAR γ ^{-/-} (3-, 2-, 13-, and 2-fold decreases, respectively; see also references 3 and 42) cells but not in RAR α ^{-/-} cells (Fig. 2). In RXR α ^{-/-}-RAR α ^{-/-} cells, the induction of Hoxa-1 and HNF1 β was similarly decreased and CRBPI and Stra4 induction was slightly further reduced when compared to RXR α ^{-/-} cells. In contrast, very little, if any, induction of the four transcripts was observed in RXR α ^{-/-}-RAR γ ^{-/-} cells. Thus, these four genes

appear to be preferentially activated by RXR α -RAR γ pairs, and combinations of RXR(β + γ) with RAR(α + β) are inefficient at least at the levels present in F9 cells. However, our data also show that when either RXR α or RAR γ is lacking, it can be partially replaced by the remaining RXRs or by the remaining RARs, respectively, thus indicating that there is some functional redundancy between RXR isotypes and between RAR isotypes (Table 1) (43). In any event, taken together with the results of our previous studies with mutant F9 EC cells and receptor-specific synthetic retinoids (9, 37, 43), the present demonstration of the preferential involvement of the RXR α -RAR γ pairs in the induction of Hoxa-1, CRBPI, HNF1 β , and Stra4 expression strongly supports the conclusion that RXR-RAR heterodimers are actually the functional units which transduce the retinoid signal upon ligand binding to both partners.

Induction of Cdx1 and Stra8 is preferentially mediated by RXR(β + γ)-RAR γ pairs. The induction of Cdx1 and Stra8 after 24 h of tRA treatment was similarly decreased in RAR γ ^{-/-} (four- and twofold-lower levels, respectively) and RXR α ^{-/-}-RAR γ ^{-/-} (four- and twofold-lower levels, respectively) cells but not in RAR α ^{-/-}, RXR α ^{-/-}, and RXR α ^{-/-}-RAR α ^{-/-} cells (Fig. 2). The induction was also reduced in RAR γ ^{-/-} and RXR α ^{-/-}-RAR γ ^{-/-} cells after 12 h of tRA treatment (data not shown).

To further dissect the functions of RARs and RXRs in the induction of Cdx1 and Stra8, WT cells were treated for 24 h with tRA or retinoids selective for RAR α (BMS188,753 [BMS753] [43]), RAR β (BMS189,453 [BMS453] [7]), RAR γ (BMS188,961 [BMS961] [43]), and all three RXRs (BMS188,649 [BMS649], also known as SR11237 [23, 37]). BMS961 (RAR γ) induced the expression of Cdx1 and Stra8 genes much more efficiently than did BMS753 (RAR α) (Fig. 3; compare lanes 15 to 17 with lanes 3 to 5). A combination of BMS961 (RAR γ) and BMS649 (panRXR) synergistically induced the expression of both genes in a dose-dependent manner (compare lanes 18 to 20 with lanes 2 and 15 to 17), and the combination of 100 nM BMS961 and 1 μ M BMS649 was as effective as 100 nM tRA treatment (compare lane 20 with lane 21). BMS753 (RAR α) and BMS649 (pan-RXR) synergized much less efficiently than did the BMS961-BMS649 (RAR γ -pan-RXR) combination (compare lanes 6 to 8 with lanes 2 to 5 and 18 to 20). In contrast, no induction was detectable upon treatment with BMS453 (RAR β) alone or in combination with BMS649 (pan-RXR) (lanes 9 to 14). Thus, RXR-RAR γ pairs appear to play a major role in mediating the induction of the expression of Cdx1 and Stra8 genes, and RXR α is dispensable for the induction of these genes. These results, together with the observation that the inductions of Cdx1 and Stra8 are at least similar or even more efficient in RXR α ^{-/-} and RXR α ^{-/-}-RAR α ^{-/-} cells compared with those in WT cells (Fig. 2, compare lanes 4 and 6 with lane 2), suggest that the combination RXR(β + γ)-RAR γ is preferentially involved in the induction of expression of the Cdx1 and Stra8 genes and also that RXR α may partially prevent efficient synergisms between RXR(β + γ) and RAR γ (Table 1). Note, however, that inactivations of the RXR β and RXR γ genes in combination with the RAR γ knockout are required to rule out the possibility that the RXR α -RAR γ pair also mediates the induction of Cdx1 and Stra8 genes but with a lower efficiency.

Induction of CRABP II is preferentially mediated by both RXR α -RAR α and RXR α -RAR γ pairs. The induction of CRABP II after 48 h of treatment was decreased (~twofold) in RAR α ^{-/-} cells when compared with WT cells but not in RAR γ ^{-/-} cells (Fig. 2) (4, 42). The induction was also reduced (ca. twofold) in RXR α ^{-/-} cells (9) and was nearly abolished in

FIG. 2. Regulation of RA-responsive genes is differentially affected in RXRα^{-/-}, RARα^{-/-}, RARγ^{-/-}, RXRα^{-/-}-RARα^{-/-}, and RXRα^{-/-}-RARγ^{-/-} F9 cells. (A) Total RNA from WT and mutant cells treated with control vehicle or 1 μM tRA for 24 h, except for CRABPII expression (48 h of treatment), was subjected to RT-PCR analysis for the indicated genes. (B) RT-PCR analysis of RA-responsive genes was performed as in panel A for at least three independent experiments (the number [n] of experiments is indicated). The mRNA levels were expressed relative to the amount present in tRA-treated WT cells, which was taken as 100. The open and solid bars correspond to transcript levels in vehicle- and tRA-treated cells, respectively.

either RXRα^{-/-}-RARα^{-/-} or RXRα^{-/-}-RARγ^{-/-} cells. Thus, the induction of CRABPII expression appears to be preferentially mediated by RXRα-RARα and RXRα-RARγ pairs, in contrast to the preferential induction of Hoxa-1, CRBPI, HNF1β, and Stra4 expression by RXRα-RARγ heterodimers and of Cdx1 and Stra8 by RXR(β+γ)-RARγ heterodimers (see above). However, it is also clear that RARα

and RARγ can substitute for each other when RXRα is expressed and that RXR(β+γ) can partially substitute for RXRα when RARα and RARγ are expressed (Table 1). This complex situation could with two preferential RXRα-RARα and RXRα-RARγ pairs reflect the presence of two synergizing RAREs (DR1 and DR2 elements) in the mouse CRABPII gene (11).

TABLE 1. Specific and functionally redundant RXR-RAR isotype pairs involved in the control of expression of RA-responsive genes in F9 cells

RA-responsive genes	Efficient RXR-RAR pairs	Other less efficient or inefficient RXR/RAR pairs ^d
Hoxa-1, CRBPI, HNF1 β , Stra4	RXR α -RAR γ	RXR(β + γ)-RARs (A) RXRs-RAR(α + β) (A) RXR(β + γ)-RAR(α + β) (B)
Cdx1, Stra8	RXR(β + γ)-RAR γ	RXRs-RAR α (A) RXRs-RAR β (B) RXR(β + γ)-RAR(α + β) (A)
CRABPII	RXR α -RAR α RXR α -RAR γ	RXR(β + γ)-RAR(α + β) (B) RXR(β + γ)-RAR(β + γ) (A)
RAR β 2	RXR α -RAR γ RXR(β + γ)-RAR γ RXRs-RAR α ^a RXRs-RAR β ^b	RXR(β + γ)-RAR(α + β) (A)
Hoxb-1 ^c	RXR α -RAR α RXR α -RAR γ	
Stra6 ^c	RXR α -RAR γ	RXR(β + γ)-RARs (A) RXRs-RAR(α + β) (A) RXR(β + γ)-RAR(α + β) (B)

^aPartially hindered by the presence of RAR γ (43).^bBlocked by the presence of RAR γ (43).^cMaximal induction is prevented by the presence of RAR α .^dA, less efficient; B, inefficient.

RAR β 2 induction is mediated by multiple RXR-RAR pairs, but the presence of RAR γ hinders the activity of RAR α and RAR β in WT cells. There was no decrease in the induction level of RAR β 2 in RAR α ^{-/-} and RAR γ ^{-/-} F9 cell lines after 24 h of tRA treatment (Fig. 2) (3, 4, 43). The RAR β 2 induction was decreased in RXR α ^{-/-} (9) and RXR α ^{-/-}-RAR α ^{-/-} cells, and was further reduced in RXR α ^{-/-}-RAR γ ^{-/-} cells (two- to threefold-lower levels than in WT cells), as determined by RT-PCR and an RNase protection assay (Fig. 2, 4, and 5A). These results indicate the involvement of the RXR α -RAR γ pairs in RAR β 2 induction, as well as potential func-

FIG. 3. Expression of Cdx1 and Stra8 genes is efficiently induced in WT cells by the combination of the pan-RXR-specific agonist BMS649 and RAR γ -specific agonist BMS961. Total RNA from WT cells treated for 24 h with vehicle (ethanol) or the indicated retinoids was subjected to RT-PCR analysis of Cdx1 and Stra8 genes.

FIG. 4. Expression of RAR β 2 gene is inducible by tRA in RXR α ^{-/-}, RXR α ^{-/-}-RAR α ^{-/-}, and RXR α ^{-/-}-RAR γ ^{-/-} F9 cells. (A) A 10- μ g portion of total RNA from WT and mutant cells treated for 24 h with control vehicle or 1 μ M tRA was subjected to a RNase protection assay for RAR β 2 and histone H4 gene. The lengths of the protected fragments are indicated in nucleotides (nts). (B) The levels of RAR β 2 transcripts were expressed relative to the amount present in tRA-treated WT cells, which was taken as 100. The open and solid bars correspond to transcript levels in vehicle- and tRA-treated cells, respectively.

tional redundancy among RXR-RAR combinations for the induction of the expression of this gene (Table 1).

To further investigate this redundancy, RXR α ^{-/-}-RAR α ^{-/-} and RXR α ^{-/-}-RAR γ ^{-/-} cells were treated for 12 h with tRA or receptor-selective retinoids. In RXR α ^{-/-}-RAR α ^{-/-} cells, 10 nM BMS961 (RAR γ) alone induced RAR β 2 expression, and the addition of 1 μ M BMS649 (pan-RXR), which is inactive on its own, resulted in a twofold further increase in the induction (Fig. 5B, lanes 1, 3, 6, and 9). No induction was seen in RXR α ^{-/-}-RAR α ^{-/-} cells upon treatment with either 10 nM BMS753 (RAR α) alone (as expected) or 500 nM BMS453 (RAR β) alone or together with 1 μ M BMS649 (pan-RXR) (lanes 4, 5, 7, and 8). In RXR α ^{-/-}-RAR γ ^{-/-} cells, RAR β 2 expression was synergistically induced by 10 nM BMS753 (RAR α) and 1 μ M BMS649 (pan-RXR) treatment, each of which is inefficient on its own (Fig. 5C, lanes 1, 3, 4, and 7). Thus, the synergistic effects of the pan-RXR agonist BMS649 with either the RAR γ -specific BMS961 in RXR α ^{-/-}-RAR α ^{-/-} cells or the RAR α -specific BMS753 in RXR α ^{-/-}-RAR γ ^{-/-} cells support the conclusion that RAR β 2 induction can be transduced by RXR(β + γ)-RAR α or RXR(β + γ)-RAR γ heterodimers. This conclusion is in agree-

FIG. 5. Expression of the RARβ2 gene is inducible by retinoids in RXRα^{-/-}-RARα^{-/-} and RXRα^{-/-}-RARγ^{-/-} F9 cells. (A) Total RNA from WT and RXRα^{-/-}-RARγ^{-/-} cells treated with 100 nM tRA for the indicated times was subjected to RT-PCR analysis for RARβ2 expression. (B and C) RXRα^{-/-}-RARα^{-/-} (B) and RXRα^{-/-}-RARγ^{-/-} (C) cells were treated for 12 h with vehicle (ethanol) or the indicated retinoids, and the expression of RARβ2 was analyzed by RT-PCR. Similar results were obtained for three independent experiments.

ment with our previous results showing a synergistic activation of RARβ2 expression by the same combinations of pan-RXR with either RARα- or RARγ-specific ligands (43). Interestingly, 500 nM BMS453 (RARβ) alone induced RARβ2 expression in RXRα^{-/-}-RARγ^{-/-} cells, and the addition of 1 μM BMS649 (panRXR) resulted in a twofold increase of this induction (lanes 5 and 8). It appears, therefore, that RARβ2 expression not only can be induced by a variety of RXR-RARα or RARγ pairs but also is auto-inducible (Table 1). However, this autoinducibility occurs only in RARγ^{-/-} (43) or RXRα^{-/-}

cells, indicating that the presence of RARγ blocks the activity of RARβ and that functional redundancy can be artifactually generated by gene knockouts, as previously noted (43). As expected, no activation was observed in RXRα^{-/-}-RARγ^{-/-} cells upon treatment with BMS961 (RARγ) alone or together with BMS649 (pan-RXR) (lanes 6 and 9).

Hoxb-1 induction is preferentially mediated by both RXRα-RARα and RXRα-RARγ pairs, but the presence of RARα prevents maximal induction. The expression of Hoxb-1 was induced to the same extent in RARγ^{-/-} and WT cells after 24 h of 1 μM tRA treatment (Fig. 2) (3). On the other hand, the induction was drastically reduced in RXRα^{-/-} and RXRα^{-/-}-RARα^{-/-} cells (17- and 19-fold decreases, respectively) and was not detectable in RXRα^{-/-}-RARγ^{-/-} cells, indicating that the presence of RXRα is important for the induction of Hoxb-1. Interestingly, after 48 h of 1 μM tRA treatment, Hoxb-1 expression in RXRα^{-/-} cells reached a level which was only twofold lower than in WT cells (9), suggesting that Hoxb-1 expression might be autoregulated in F9 cells, as appears to be the case in the mouse embryo hindbrain (33). In contrast to our previous report showing that the induction of Hoxb-1 transcript after 48 h of tRA treatment was slightly decreased in RARα^{-/-} cells when compared with WT cells (4), there was an enhanced induction of Hoxb-1 in RARα^{-/-} cells treated with tRA for 24 h (Fig. 2, compare lanes 9 and 10 with lanes 1 and 2). Similar results were obtained by either RT-PCR with three different sets of primers or Northern blotting (data not shown). Hoxb-1 transcripts were clearly induced in RARα^{-/-} cells but not in WT cells after 3 and 6 h of 100 nM tRA treatment, and their levels were nine- and threefold higher in RARα^{-/-} cells than in WT cells after 12 and 24 h of treatment, respectively (Fig. 6A).

To further investigate the roles of RARs and RXRs in the regulation of Hoxb-1 expression, WT and RARα^{-/-} F9 cells were treated for 12 h with tRA or receptor-selective retinoids. Note that the pan-RXR agonist, BMS649, which is inactive on its own for the induction of a number of RA-responsive genes (see above and references 37 and 43), weakly induced Hoxb-1 expression in RARα^{-/-} cells (Fig. 6C, compare lane 14 with lane 12). Whether this poor induction is mediated by RXR homodimers or RXR-RAR heterodimers, in which RAR would be activated by traces of tRA that are known to be present in the serum used in the culture medium, is unknown. The expression of Hoxb-1 was synergistically upregulated in WT cells and much more strongly in RARα^{-/-} cells by the combination of 1 μM BMS649 (pan-RXR) and 10 nM BMS961 (RARγ) treatment (compare lanes 9 and 20 with lanes 1, 3, 6, 12, 14, and 17). A similar synergistic induction was seen in WT cells with 1 μM BMS649 (pan-RXR) and 10 nM BMS753 (RARα) treatment (compare lane 7 with lanes 3 and 4). The combination of BMS649, BMS753, and BMS961 retinoids induced Hoxb-1 expression in WT cells at least as efficiently as does 100 nM tRA treatment (compare lane 11 with lane 2). On the other hand, addition of 500 nM BMS453 (RARβ) had no effect on the induction of Hoxb-1 in WT and RARα^{-/-} cells (compare lanes 5, 8, 16, and 19 with lanes 1, 3, 12, and 14, respectively).

Thus, the above data indicate that the retinoid-induced Hoxb-1 expression is preferentially mediated by RXRα-RARα and RXRα-RARγ heterodimers (Table 1). However, the observation that Hoxb-1 induction by BMS961 (RARγ) alone or in combination with BMS649 (panRXR) was much stronger in the absence of RARα than in the presence of liganded or unliganded RARα (Fig. 6C, compare lanes 17 and 20 to 22 with lanes 6 and 9 to 11, respectively) indicates that the presence of RARα also decreases the maximal level of Hoxb-1

FIG. 6. Expression of Hoxb-1 and Stra6 genes is markedly induced in RAR $\alpha^{-/-}$ F9 cells. (A) Total RNA from WT, RAR $\alpha^{-/-}$, and RAR $\gamma^{-/-}$ cells, treated with 100 nM tRA for the indicated times, was subjected to RT-PCR analysis for expression of the Hoxb-1 gene. Experiments were performed three times, and the levels of the Hoxb-1 transcript were expressed relative to the amount present in WT cells upon 24 h of treatment, which was taken as 100. (B) RT-PCR analysis of Stra6 expression for WT and RAR $\alpha^{-/-}$ cells was performed as in panel A. (C) Total RNA from WT and RAR $\alpha^{-/-}$ cells treated for 12 h with vehicle (ethanol) or the indicated retinoids was subjected to RT-PCR analysis of the Hoxb-1 and Stra6 genes. Similar results were obtained for three independent experiments.

induction, irrespective of the presence of an RAR α agonist (Table 1). Two RAREs have been identified in the 3'- and 5'-flanking regions of the Hoxb-1 gene and correspond, respectively, to positive and negative regulatory elements for segment-restricted expression of Hoxb-1 in the hindbrain (29, 41). Thus, the negative effect of RAR α on maximal Hoxb-1 induction may reflect a competition of RXR α -RAR α heterodimers with transcriptionally more efficient RXR α -RAR γ het-

erodimers for binding to the 3' RARE; alternatively or in addition, RAR α might exert its negative effect by binding to the "negative" 5' RARE, irrespective of the presence of the ligand.

Stra6 induction is preferentially mediated by RXR α -RAR γ pairs, and the presence of RAR α prevents maximal induction. The induction of Stra6 was decreased in RAR $\gamma^{-/-}$ (42), RXR $\alpha^{-/-}$, and RXR $\alpha^{-/-}$ -RAR $\alpha^{-/-}$ cells after 24 h of tRA treatment (2-, 4-, and 4-fold decreases, respectively [Fig. 2]), whereas it was abrogated in RXR $\alpha^{-/-}$ -RAR $\gamma^{-/-}$ cells. Interestingly, the induction of Stra6, like that of Hoxb-1, was markedly increased in RAR $\alpha^{-/-}$ cells (Fig. 2, compare lanes 9 and 10 with lanes 1 and 2). Stra6 transcripts were detected in RAR $\alpha^{-/-}$ cells but not in WT cells after 3 and 6 h of tRA treatment, and their levels were eight- and threefold higher for RAR $\alpha^{-/-}$ cells than for WT cells after 12 and 24 h of treatment, respectively (Fig. 6B).

Stra6 induction in WT and RAR $\alpha^{-/-}$ cells was also analyzed with receptor-selective retinoids. BMS649 (panRXR) was ineffective for the induction of Stra6 in WT and RAR $\alpha^{-/-}$ cells (Fig. 6C, compare lanes 3 and 14 with lanes 1 and 12, respectively). The expression of Stra6 was induced in WT and RAR $\alpha^{-/-}$ cells by treatment with 10 nM BMS961 (RAR γ) alone or in combination with 1 μ M BMS649 (pan-RXR) (lanes 6, 9, 17, and 20). The combination of 1 μ M BMS649 (pan-RXR) with either 10 nM BMS753 (RAR α) or 500 nM BMS453 (RAR β) had no or very little effect on the induction of Stra6 in WT and RAR $\alpha^{-/-}$ cells (compare lanes 5, 8, 16, and 19 with lanes 1, 3, 12, and 14). Thus, it appears that Stra6, like Hoxa-1, CRBPI, HNF1 β , and Stra4, is preferentially activated by RXR α -RAR γ heterodimers but also that in the absence of either RXR α or RAR γ , RXR(β + γ)-RARs heterodimers or RXR-RAR(α + β) heterodimers, respectively, are to some extent functionally redundant (Table 1). The induction of Stra6 by BMS961 (RAR γ) alone or in combination with BMS649 (pan-RXR) was much stronger in the absence of RAR α than in the presence of liganded or unliganded RAR α (compare lanes 7, 8, 18, and 19 with lanes 1, 3 to 5, 12, and 14 to 16). Thus, RAR α prevents maximal Stra6 induction, irrespective of the presence of its ligand, as is the case for Hoxb-1 (Table 1). Characterization of the *cis* elements controlling the activity of the Stra6 promoter is required to elucidate the molecular mechanism responsible for this negative effect of RAR α on Stra6 expression.

DISCUSSION

In vitro studies have suggested that RXRs and RARs differentially activate various RAREs (22, 32). However, these studies were performed with cultured cells cotransfected with vectors overexpressing receptors and recombinant chimeric reporter genes, which is far from physiological conditions in terms of both receptor concentration and chromatin environment. Genetic studies in the mouse have indicated that the various RXR-RAR heterodimers, which can be generated by combinations of the multiple RAR and RXR isoforms (and their isoforms), could be differentially implicated in the highly pleiotropic effects triggered by RA in vivo. However, the complexity of the mouse prevents unequivocal conclusions concerning the role of different heterodimers in the events controlled by retinoids, notably because cell-autonomous and nonautonomous effects cannot be easily distinguished (for a discussion, see references 5, 19, and 20). We therefore chose the F9 EC cells, which represent a well-established retinoid-responsive cell-autonomous system, to investigate the possible specificity of RARs and RXRs in the control of expression of

a variety of endogenous RA target genes. To this end, we previously used F9 cells mutated for either RAR γ (3), RAR α (4), or RXR α (9), as well as RAR- or RXR-selective retinoids (37, 43). These studies suggested that RXR-RAR heterodimers are the functional units transducing the retinoid signal. To further strengthen this notion, we have used here compound RXR-RAR mutant F9 cells, together with combinations of a synthetic pan-RXR agonist with synthetic retinoid agonists specific for either RAR α , RAR β , or RAR γ . Our results (summarized in Table 1) lead to several important conclusions.

First, our study indicates that RXR-RAR heterodimers are indeed the functional units transducing the retinoid signals. This conclusion is supported by data generated with RXR-RAR mutants and comparing them to single RAR or RXR mutants, as well as by data which result from the use of the synthetic retinoids and extend our previous observations (37, 43). In all cases, the pan-RXR ligand, which is inactive on its own, together with suboptimal concentrations of an appropriate RAR isotype-selective retinoid, can synergistically induce the expression of certain RA-responsive genes.

The second important conclusion is that the various RXR-RAR heterodimers which can be formed in F9 EC cells exhibit some functional specificity, as the control of expression of different sets of RA-responsive genes appears to be preferentially mediated by distinct RXR-RAR isotype combinations (Table 1). The observation that the identity of the less efficient functionally redundant RXR-RAR isotype pairs also varies with the set of responsive genes considered further strengthens the conclusion that different RXR-RAR heterodimers have different patterns of target gene specificity. The molecular basis for these distinct responses of subsets of RA target genes to different combinations of RXR-RAR isotype heterodimers is unknown but could be related to differential affinities of the various heterodimers for the RAREs of these genes. Alternatively or concomitantly, the various RXR-RAR heterodimers could differ in their synergism with different transactivators bound to the promoter region of these genes.

The final conclusion is that the presence of a given receptor isotype can hinder (Hoxb-1 and Stra6 [Table 1]) or even completely block the activity of another isotype for inducing the expression of certain target genes (RAR β 2 [Table 1]). The latter observation, namely, that the presence of RAR γ hinders the potential redundant function of RAR α and fully blocks that of RAR β , clearly shows that functional redundancy between RAR isotypes can be artifactually generated by gene knockouts. Interestingly, this artifactual redundancy which allows RAR β to replace RAR γ for RAR β 2 induction in RAR γ ^{-/-} cells but not in WT F9 cells was not observed for the induction of Hoxa-1 expression (43), even though the Hoxa-1 RARE is identical to that of RAR β 2. This provides an additional example of functional specificity of the retinoid receptors present in a given cell.

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