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Reexpression of retinoic acid receptor (RAR)γ or overexpression of RARα or RARβ in RARγ-null F9 cells reveals a partial functional redundancy between the three RAR types

(embryonal carcinoma cells/retinoic acid target genes/differentiation)

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ABSTRACT **Disruption of retinoic acid receptor (RAR)γ in F9 embryonal carcinoma cells leads to aberrent differentiation and reduced activation of expression of several all-*trans*-retinoic acid (RA)-induced genes. We have analyzed the expression of several additional RA-responsive genes in RARα- and RARγ-null F9 cells. The RA-induced activation of *Cdx1*, *Gap43*, *Stra4*, and *Stra6* was specifically impaired in RARγ-null cells, supporting the idea that each RAR may regulate distinct subsets of target genes. To further investigate the role of RARγ in F9 cell differentiation, "rescue" cell lines reexpressing RARγ2 or overexpressing either RARα1 or RARβ2 were established in RARγ-null cells. Reexpression of RARγ or overexpression of RARα restored both target-gene activation and the differentiation potential. In contrast, overexpression of RARβ only poorly restored differentiation, although it could replace RARγ for the activation of target genes. Functional redundancy between the various RARs is discussed.**

All-*trans*-retinoic acid (RA)-induced differentiation of F9 embryonal carcinoma (EC) cells *in vitro* is believed to be primarily mediated by retinoic acid receptors (RARs) and retinoic X receptors (RXRs), which belong to the nuclear receptor superfamily (1–4). The evolutionary conservation of these receptors suggests that each of them may regulate the expression of particular subsets of target genes in specific cell types, thereby mediating different biological activities (5, 6). During differentiation, RA induces a change in both cell morphology and expression of several genes (7). However, not all genes responding to RA during differentiation have been identified (8). To determine the possible role played by each RAR or RXR species in regulating cell growth and differentiation of F9 cells, we have disrupted RARγ (9) and RARα (10) genes in F9 EC cells, and identified subsets of target genes whose expression is altered specifically in either RARα$^{-/-}$ or RARγ$^{-/-}$ cells. Furthermore, we have shown that only RARγ-null cells lose their ability to differentiate upon RA treatment.

In the present study, we have identified several additional RA target genes, *Tcf2* (gene for hepatocyte nuclear factor 1β), *Cdx1*, *Gap43*, *Stra4*, and *Stra6* (8), whose induction is also altered in RARγ-null cells. We have then investigated whether RARγ plays a specific role in mediating the RA signal by reexpressing RARγ or by overexpressing either RARα or RARβ in RARγ$^{-/-}$ cells. We demonstrate that the RA inducibility of target genes and the morphological differentiation were restored in cells either reexpressing RARγ or overexpressing RARα. In contrast, RARβ overexpression was in general less efficient at rescuing the expression of RA target genes and did not restore morphological differentiation.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Differentiation. The plasmids used to establish the "rescue" lines were pD403A, pD404A, and pD405A (a gift from D. Lohnes; Institut de Génétique et de Biologie Moleculaire et Cellulaire, Illkirch-Cedex, France), which contain the RARα, -β2 and -γ2 cDNAs under the control of the *Pgk1* promoter (11). The plasmids were introduced into RARγ$^{-/-}$ cells by electroporation (9), along with pD503 (a gift from D. Lohnes), which confers resistance to 0.8 μg of puromycin per ml. Clones carrying the transgene at a high copy number were amplified. Wild-type (Wt), RARα$^{-/-}$, and RARγ$^{-/-}$ F9 cells were maintained as reported (9, 10). For differentiation studies, all cell lines were grown in the presence of 10^{-6} M RA for 96 h, with a change of medium after 48 h. Control cells were treated with ethanol (vehicle).

Electrophoretic Mobility-Shift Assays (EMSAs). Nuclear extracts were prepared from Wt, RARγ$^{-/-}$, α25, β30, and γ51 cells grown in the absence or presence of RA (9). EMSA was carried out as described (12) by using an oligonucleotide corresponding to the Hoxa1/RARβ retinoic acid response element (RARE) (13, 14) and mouse monoclonal antibodies (mAbs) directed against the F and A regions of RARα1 [Ab 9α(F) + Ab 15α(A1)], RARβ2 [Ab 8β(F) + Ab 6β(A2)], or RARγ2 [Ab 2γ(F) + Ab 10γ(A2)] (15–17).

RESULTS

Expression of RA-Responsive Target Genes in Wt, RARγ$^{-/-}$, and RARα$^{-/-}$ F9 cells. We have analyzed the expression of a number of RA-responsive genes by using reverse transcription–PCR (8) in Wt, RARγ$^{-/-}$, and RARα$^{-/-}$ F9 cells (Fig. 1). As reported (9, 10), there was a selective loss of induction of the *Hoxa1*, *Hoxa3* (data not shown), and *Gata4* genes in RARγ$^{-/-}$ cells, and the expression of the *Cdx1*, *Gap43*, *Stra4*, and *Stra6* genes was similarly affected in these cells. *Crabp2* and *Hoxb1* (data not shown) were the only genes whose inducibility was specifically lowered in RARα$^{-/-}$ cells (Fig. 1; see also ref. 10).

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Abbreviations: RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; Wt, wild type; EMSA, electrophoretic mobility shift assay; EC, embryonal carcinoma; mAb, monoclonal antibody; RARE, retinoic acid response element.

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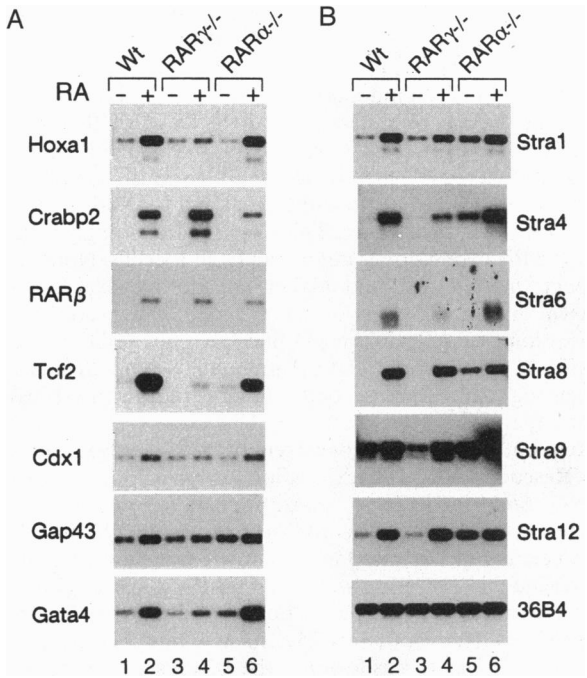

FIG. 1. Differential expression of various RA-responsive genes in Wt, RARγ$^{-/-}$, and RARα$^{-/-}$ F9 cells. RNA transcripts for each gene were analyzed by reverse transcription–PCR as described (8) in untreated cells (−) or after induction (+) with 10^{-6} M RA for 24 h, except for *Crabp2* expression, where the cells were treated for 48 h.

The inducibility of *Tcf2* was altered in both RARγ- and RARα-null lines, albeit more strongly in the RARγ$^{-/-}$ line. In contrast, no alteration of RARβ expression was seen in RARγ- or RARα-null cells (see also ref. 9). Among the identified RA-responsive genes (8), the induction of *Stra1*, *Stra8*, *Stra9*, and *Stra12* genes was not significantly altered in RARγ$^{-/-}$ or RARα$^{-/-}$ cells, but in contrast to RARβ, there was a significant increase in their expression in the absence of RA in the RARα$^{-/-}$ line. This result suggests that unliganded RARα could act as a negative regulator of these genes. Note that the expression of the *Stra4* gene was similarly derepressed in RARα$^{-/-}$ cells in the absence of RA.

Establishment of RARα1, RARβ2, and RARγ2 Rescue Lines. To investigate whether the complete or partial loss of inducibility of *Hoxa1*, *Hoxa3*, *Tcf2*, *Cdx1*, *Gap43*, *Gata4*, *Stra4*, and *Stra6* genes in RARγ$^{-/-}$ cells reflected a "gene-dosage" effect or a selective control by RARγ, stable "rescue" lines were established from RARγ$^{-/-}$ cells by using vectors encoding RARγ2, RARα1, or RARβ2 cDNA (2, 5) under the control of the *Pgk1* promoter (11). The levels of RAR transcripts in each rescue line were compared with the expression of RARα, RARβ, or RARγ in Wt cells by an RNAse protection assay (Fig. 2*A*). The rescue cell lines α25 and α53 (Fig. 2*A*, lanes 2 and 3, respectively) constitutively expressed high levels of RARα1 transcript when compared with Wt cells (lane 1). Since F9 cells contain very low levels of RARβ2 transcript in the absence of RA (Fig. 2*A*, lane 4), the constitutive RARβ2 expression in the rescue lines (lanes 7–9) was compared with that of Wt cells treated with RA (lane 5). Line β30 exhibited a constitutive level of RARβ2 transcript similar to that of RA-induced Wt cells, while lines β17 and β36 expressed RARβ2 at a much lower level. Two lines, γ29 and γ51, showed low and high levels of RARγ2 transcripts, respectively. The expression level in the γ51 line was nearly identical to that of Wt cells. Lines α25, β30, and γ51 were retained for further study.

Western blotting with polyclonal antibodies (15–17) was used to compare the levels of each receptor in the rescue lines

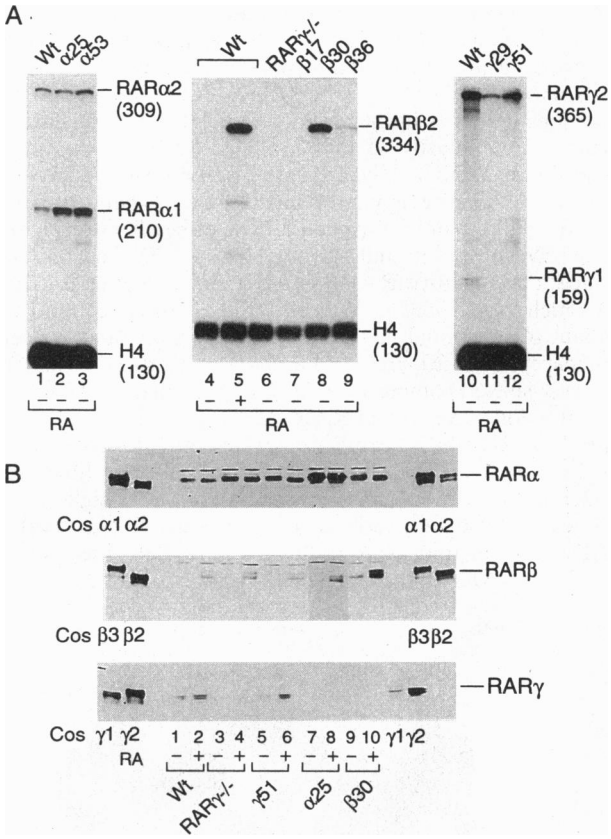

FIG. 2. (*A*) Expression of RARα, RARβ, and RARγ transcripts in the rescue lines, as estimated by RNAse protection assay. Conditions of hybridization and templates for antisense riboprobes for RARα, RARβ, RARγ, and histone H4 have been reported (18). Lines α25 and α53 (lanes 2 and 3) showed a high level of RARα1 expression compared with Wt (lane 1). Constitutive RARβ2 expression in the β rescue lines (lanes 7–9) was compared with RARβ2 expression in Wt cells after treating the cells with 10^{-6} M RA for 24 h (lane 5). RARγ2 expression in the γ29 and γ51 lines (lanes 11 and 12) was compared with Wt cells (lane 10). The identities and lengths (in nt) of the protected fragments are indicated. The amount of RNA used in each assay was normalized by using histone H4 transcripts. (*B*) Western blot analysis of nuclear proteins isolated from Wt, RARγ$^{-/-}$, α25, β30, and γ51 cell lines. Nuclear extracts from each cell line were prepared (15, 16) and RARs were detected with rabbit polyclonal antibodies specific to RARα [(RPα(F)], RARβ [RPβ(F)], and RARγ [RPγ(F)] (15–17). Cells were grown in the absence or presence of 10^{-6} M RA for 24 h. A high level of RARα was seen in the α25 line by using RARα-specific antisera. Line β30 showed constitutive expression of RARβ (lane 9) which was markedly induced upon RA treatment (lane 10). The RARγ expression detected in line γ51 was equivalent to that in Wt cells. (Note that the low signal in lane 5 is not representative, since it was similar to that seen in lane 6 in other assays.) The upper band in each panel corresponds to a nonspecific immunoreaction. Control lanes in respective panels show transfected COS-1 cells expressing RARα1 and RARα2, RARβ3 and RARβ2, or RARγ1 and RARγ2.

with those of Wt and RARγ$^{-/-}$ cells, with or without RA treatment (Fig. 2*B*). A high level of RARα protein was detected only in the α25 line, both in the absence and in the presence of RA (Fig. 2*B*, lanes 7 and 8). Constitutive expression of RARβ (Fig. 2*B*, lane 9) was detected in the β30 line, at a level equivalent to that of RARβ in Wt cells treated with RA (compare lanes 9 and 2 of Fig. 2*B*), which agrees with the RNAse protection results (Fig. 2*A*). Note that the RA-induced expression of RARβ in the β30 line (Fig. 2*B*, lane 10) was much higher than RARβ expression in either Wt cells or α25 and γ51 rescue lines. The RARγ protein was detected in the γ51 line at a level comparable with that of Wt cells.

A further comparison and quantitation of functional receptor levels in α25, β30, and γ51 rescue and Wt cells were performed using EMSA and mAbs specific for either RARα, RARβ, or RARγ. A specific RARα–RARE complex was seen in all cell lines in the absence of RA treatment (Fig. 3*A*, arrow). The intensity of this complex, however, was much higher in the α25 line (Fig. 3*A*, lane 14), indicating a high level of RARα. This level appeared much higher (when compared with Wt cells) than that expected from either RNAse protection or Western blot analysis (Fig. 2 *A* and *B*). The β30 line yielded a constitutive RARβ–RARE complex (Fig. 3*A*, lane 11) which was absent in Wt cells (Fig. 3*A*, lane 3), and the amount of functional RARγ was similar in γ51 and Wt cells (compare lanes 20 and 4, Fig. 3*A*). Quantitation of the antibody-shifted complexes indicated that relative to the level of expression of functional RARγ in the γ51 cell line—i.e., Wt level—the constitutive expression of functional RARβ2 in the β30 line was 50% lower, whereas the expression of functional RARα in the α25 line was approximately 5-fold higher.

Since the β30 line showed a significantly higher level of RARβ protein upon RA treatment (Fig. 2*B*), the level of functional RARβ in the rescue lines was also estimated in the presence of RA (Fig. 3*B*). In agreement with the results shown in Fig. 2, the constitutive level of RARβ in the β30 line (Fig. 3*B*, lane 14) was roughly equivalent to that seen in Wt and other lines upon RA induction (Fig. 3*B*, lanes 8, 12, 20, and 24). However, in the presence of RA, the level of the RARβ-specific complex in the β30 rescue line was much higher than the 2-fold increase which would result from the simple additivity of the β30 line constitutive level and RA-inducible level in Wt or other rescue lines. This observation strongly suggests that RARβ expression is autocatalytic in F9 cells. Note in this respect that an ≈10-fold higher constitutive expression of RARα in the α25 line or an ≈2-fold higher constitutive expression of RARγ in the γ51 line (see above) did not result in such a high level of RARβ induction (Figs. 2*B* and 3*B*), suggesting that RARβ is a better inducer of its own expression than either RARα or RARγ.

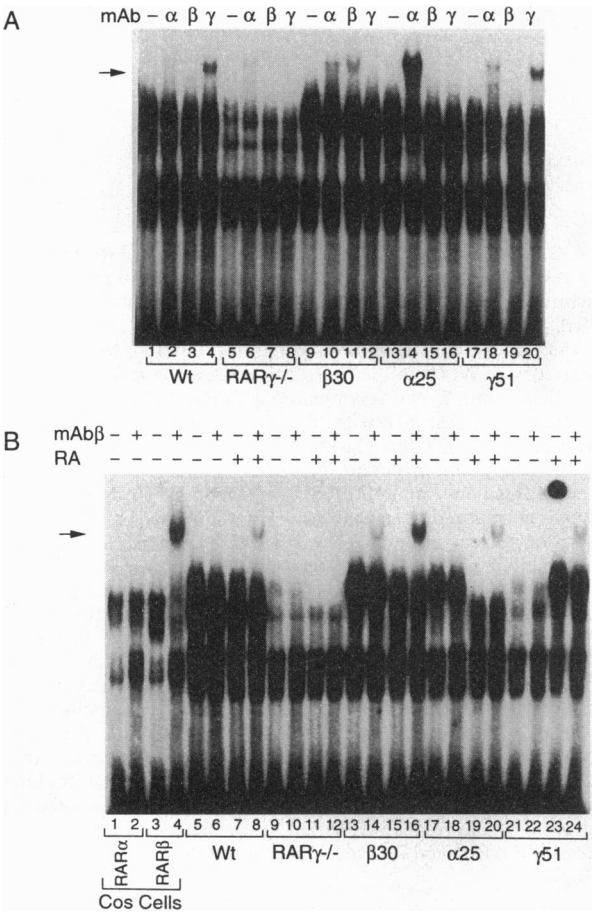

FIG. 3. (*A*) Comparative levels of RARE-binding activity of the RARs in rescue lines versus Wt and RARγ$^{-/-}$ cells. The arrow indicates the supershifted complex formed in the presence of mAb to RARα, RARβ, or RARγ. (*B*) Expression of RARβ is autocatalytic. The levels of RARβ RARE-binding activity after induction of cells with RA was determined by EMSA in presence of RARβ mAb. The constitutive expression in the β30 line was equivalent to the RA-induced expression in all the other lines. [Note that the expression of RARβ in the RARγ$^{-/-}$ cells (lane 12) was aberrently low and was similar to that seen in Wt (lane 8) in other assays.] The RA-induced expression of RARβ in the β30 line (lane 16) was much higher than in the other lines. Control lanes (1–4) correspond to transfected COS-1 cells expressing RARα and RARβ.

Restoration of the Responsiveness of RA Target Genes in the Rescue Lines. The expression of *Hoxa1*, *Hoxa3*, *Cdx1*, *Gap43*, *Stra4*, and *Gata4*, whose induction was abolished in RARγ$^{-/-}$ cells, as well as the expression of *Tcf2* and *Stra6*, which exhibited a reduced inducibility (Fig. 1) was analyzed in the rescue lines. By using an RNAse protection assay (Fig. 4*A*), *Hoxa1* transcripts appeared to be induced at Wt levels in the presence of RA, both in α25 and γ51 lines. However, the induction of *Hoxa1* was lower in the β30 rescue line (Fig. 4*A*, lane 6). Similarly, the RA responsiveness of *Hoxa3* was restored to Wt levels (Fig. 4*A*, lane 2) in both α25 (lane 4) and γ51 (lane 8) rescue lines, but only to a lower level in the β30 line (lane 6). The induction of *Cdx1*, *Gap43*, *Tcf2*, *Stra4*, *Stra6*, and *Gata4* transcripts was restored in all three rescue lines, as estimated by reverse transcription–PCR analysis (compare Fig. 4*B* with Fig. 1). However, as was the case for *Hoxa1* and *Hoxa3*, the induction of the *Gap43*, *Tcf2*, and *Stra6* genes was less efficient in the β30 line. In contrast, the *Cdx1*, *Stra4*, and *Gata4* genes were induced to Wt levels in all three rescue lines.

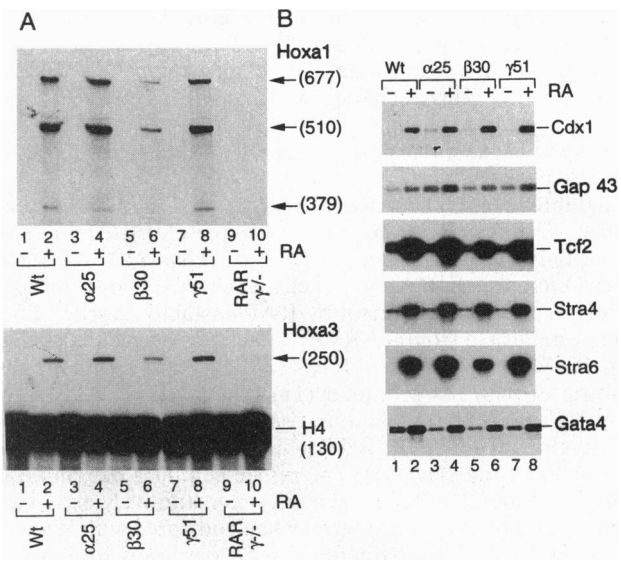

FIG. 4. (*A*) Detection of *Hoxa1* and *Hoxa3* transcripts in Wt, α25, β30, γ51, and RARγ$^{-/-}$ cells by RNAse protection assay. The template for *Hoxa1* riboprobe (gift from T. Lufkin; Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch Cedex, France) contains a 1.7-kb genomic fragment corresponding to nucleotides 44–1231 of the cDNA (19), which generates four protected fragments of 677 nt, 510 nt, 379 nt, and 95 nt. The *Hoxa3* template was a gift from S. J. Gaunt (20). Cells were grown in the absence or presence of 10^{-6} M RA for 24 h as indicated. (*B*) Level of RNA transcripts of *Cdx1*, *Gap43*, *Tcf2*, *Stra4*, *Stra6*, and *Gata4*, as determined by reverse transcription–PCR (8). RNA was isolated from untreated Wt, α25, β30, and γ51 cells before or after treatment with 10^{-6} M RA for 24 h.

Differentiation of α25, β30, and γ51 Rescue Lines. Since the expression of any of the three RAR types in the rescue lines restored the inducibility of RA target genes, the capacity of rescue cells to differentiate in the presence of 10^{-6} M RA for 96 h was compared with the differentiation of Wt and RARγ$^{-/-}$ cells (Fig. 5). Wt cells (Fig. 5*A*) morphologically differentiated following treatment with RA (Fig. 5*B*), whereas, as reported (9), the RARγ$^{-/-}$ cells mostly retained their undifferentiated characteristics (compare Fig. 5 *C* and *D*). In contrast, morphological differentiation could be observed in the three rescue cell lines (Fig. 5 *E–J*). However, the β30 line (compare Fig. 5 *E* and *F*) poorly differentiated, retaining to a large extent an undifferentiated stem cell morphology. The γ51 (Fig. 5 *G* and *H*) and α25 (Fig. 5 *I* and *J*) lines apparently differentiated to a similar extent as Wt cells.

The extent of differentiation of the rescue lines was also estimated from the expression of the transcripts of two differentiation markers, collagen type IV (α1) and laminin B1. Northern blot analysis (Fig. 6) showed a clear increase in collagen type IV (α1) RNA in the α25 and γ51 lines after 48 h of RA treatment, although to a lower level than in differentiated Wt cells. In contrast, the induction of collagen type IV (α1) transcripts was much lower in the β30 line, in agreement with the poor morphological differentiation of this rescue cell line. Similarly, the levels of expression of laminin B1 transcript were lower in the rescue lines, with the lowest level of induction observed in the β30 rescue line.

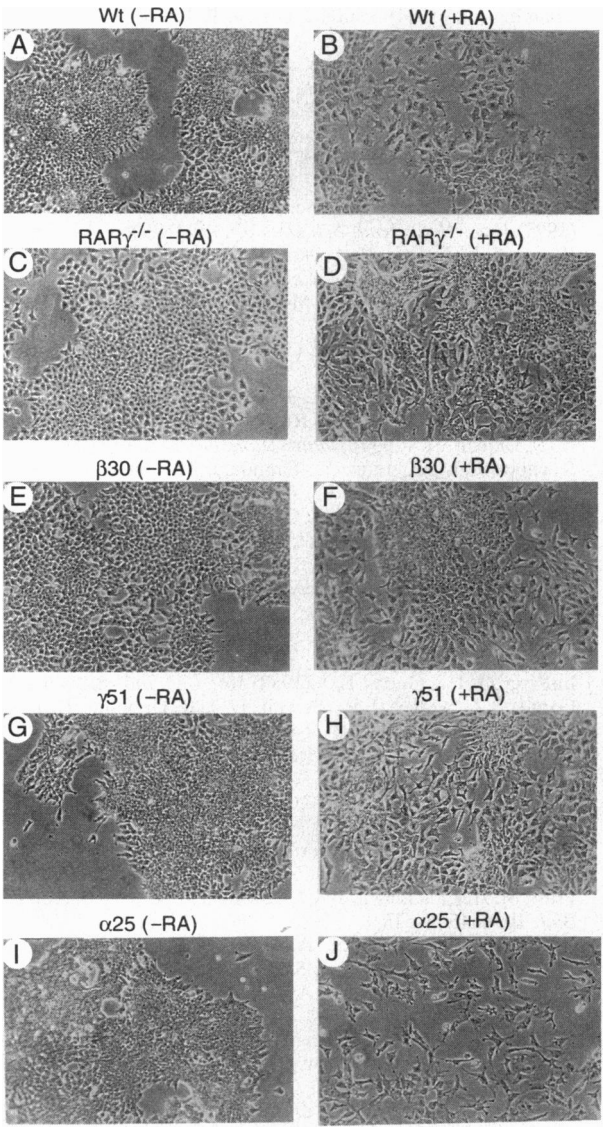

FIG. 5. (*A–J*) RA-induced morphological differentiation of Wt (*B*), RARγ$^{-/-}$ (*D*), γ51 (*H*), β30 (*F*), and α25 (*J*) cell lines after treatment with 10^{-6} M RA for 96 h. The corresponding untreated control cells are shown in *A*, *C*, *G*, *E*, and *I*, respectively.

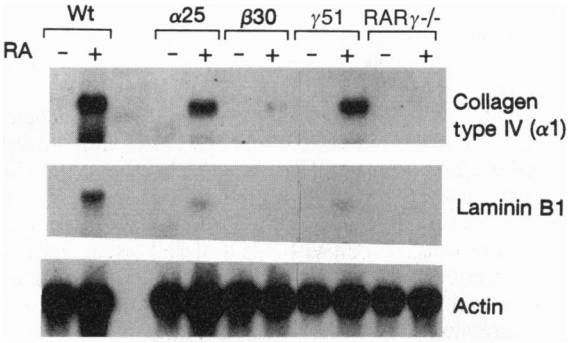

FIG. 6. Northern blot analysis of the relative levels of the transcripts of two differentiation-specific markers, collagen type IV (α1) and laminin B1 from Wt, α25, β30, γ51, and RARγ$^{-/-}$ cells treated with 10^{-6} M RA for 48 h. The conditions for Northern blotting and the laminin B1 and collagen type IV (α1) probes have been described (9, 10). The actin probe was used as an internal control to standardize the amount of RNA in each lane.

DISCUSSION

Differential Control of RA-Target Genes by RARα and RARγ. The loss of RARγ results in a lowered RA inducibility of a number of genes, such as *Hoxa1*, *Hoxa3*, *Tcf2*, *Cdx1*, *Gap43*, *Gata4*, *Stra4*, and *Stra6*, which indicates that these genes may be direct or indirect RARγ targets. In contrast, the RA-induced expression of *Crabp2* and *Hoxb1* (ref. 10, data not shown), which is specifically lowered in RARα$^{-/-}$ cells, may be mediated by RARα. It is noteworthy that the expression of a third set of genes, *Stra1*, *Stra8*, *Stra9*, and *Stra12* (Fig. 1*B*), was not grossly affected by the loss of either RARγ or RARα, suggesting that for these genes either RARα and RARγ are functionally equivalent or RA induction is mediated by RARβ. Alternatively or concomitantly, the expression of these genes may be negatively regulated by unliganded RARα since there was a marked increase in their expression in the RARα$^{-/-}$ cells in the absence of RA. RARβ expression remained unaltered in both RARα- and RARγ-null lines, suggesting that either RARα and RARγ are functionally redundant in their capacity to mediate RARβ induction or expression of RARβ is autocatalytic. This latter possibility is strongly supported by the observation that the constitutive expression of RARβ in the β30 line results in a much higher RA-induced level of RARβ than that brought about by the constitutive expression of either RARα or RARγ in the α25 and γ51 cell lines (see Figs. 2*B* and 3*B*). All of these results support and extend our previous conclusion (9, 10) that RARα and RARγ mediate the induction of different subsets of RA target genes in F9 EC cells, even though RARγ is by far the most abundant RAR in these cells.

Functional Redundancy Between RARα, RARβ, and RARγ. Since RARγ is the predominant RAR in F9 cells, the decreased expression of *Hoxa1*, *Hoxa3*, *Gap43*, *Cdx1*, *Stra4*, and *Stra6* in RARγ$^{-/-}$ cells may correspond to a "quantitative" requirement for any of the three RARs rather than a selective "qualitative" requirement for RARγ. Although the low level of RARα present in the RARγ$^{-/-}$ cells is clearly insufficient to substitute for RARγ (this study and ref. 9), the overexpression of RARα in the α25 rescue line (to levels approximately 5-fold higher than Wt RARγ) efficiently restores to Wt levels the RA responsiveness of all genes examined. Similarly, the overexpression of RARβ to constitutive

levels, which are approximately 50% lower than that of RARγ in Wt cells, and to RA-induced levels, which are much higher, can restore to Wt levels the expression of the genes which are not induced in the RARγ$^{-/-}$ cells, with the exception of *Hoxa1*, *Hoxa3*, *Tcf2*, and *Stra6*, which are less efficiently induced. Thus, although RARα and RARβ cannot substitute for RARγ when expressed at Wt levels, they can clearly mediate the RA-induction of all the RA-target genes whose expression is abrogated in RARγ$^{-/-}$ cells when expressed at much higher levels, as seen with α25 and β30 rescue lines. Such functional redundancy may account for the paradoxical observations that a RARγ-null mutation results in a loss of *Hoxa1* expression in F9 cells, but cannot generate a *Hoxa1*-null phenotype in the mouse (21–23). Some degree of functional redundancy between RARγ and the other two RARs is also in agreement with the observation that, while no induction of *Hoxa1* could be seen in RARγ$^{-/-}$ F9 cells treated with RA for 24 h, a low level of induction was detectable after 48 h (9).

Several cases of functional redundancy between the three RARs have been previously reported. RA-induced expression of *Hoxa1*, which is blocked in the P19 cell line derivative RAC65 by a dominant-negative RARα allele (24), was restored by transient transfection of any of the three RAR genes (25). Similarly, the RA sensitivity of a mutant subclone of HL-60 defective in its ability to differentiate into granulocytes was restored by overexpression of RARα, RARβ, or RARγ, even though RARα is the predominant receptor in this line (26, 27). Interestingly, the extent of differentiation was found to be dependent on the level of the overexpressed receptor (26, 27). It should be stressed that in these two latter studies, as well as in the present study, the effects of the three RAR types were not compared by using cells expressing the same amount of one of the three RARs. Thus, although there is no doubt that overexpressed RARα or RARβ can substitute for RARγ, the true efficiency of this functional redundancy remains to be investigated. That the RA-induced expression of different genes may be specifically mediated, at least in part, by the different RAR types is indeed indicated, not only by the observation that the expression of the *Hoxb1* and *Crabp2* gene is not decreased in the RARγ$^{-/-}$ F9 cells (10), but also by the present differential response of *Hoxa1*, *Hoxa3*, *Tcf2*, and *Stra6* in the β30 rescue line when compared with Wt and the other rescue cell lines.

Although the activation of all "early" target genes examined here is restored to Wt levels in both α25 and γ51 cell lines, the differentiation of these cells, as monitored by the expression of differentiation-specific "late" responsive genes, is impaired when compared with Wt cells. Furthermore, it is striking that the β30 rescue line differentiates morphologically and biochemically much less efficiently than the α25 and the γ51 rescue lines. This is in contrast with the ability of RARβ to efficiently restore the induction of a number of RA-responsive genes, and suggests that some RA-dependent events either cannot be mediated by RARβ or would require much higher levels of RARβ than those achieved in the β30 rescue line. Clearly, although the various receptors appear to be redundant to some extent for mediating the induction of some RA target genes, their functional redundancy does not appear to extend to the more complex situation of RA-induced cell differentiation, which involves a cascade of RA-induced events.

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