The EMBO Journal vol.15 no.16 pp.4142–4155, 1996

RXRα-null F9 embryonal carcinoma cells are resistant to the differentiation, anti-proliferative and apoptotic effects of retinoids

John Clifford, Hideki Chiba, Dorothy Sobieszczuk, Daniel Metzger and Pierre Chambon[1]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch-Cedex, France

[1]Corresponding author

The F9 murine embryonal carcinoma (EC) cell line, a well established model system for the study of retinoic acid (RA)-induced differentiation, differentiates into cells resembling three types of extra-embryonic endoderm (primitive, parietal and visceral), depending on the culture conditions and RA concentration used. A number of previously identified genes are differentially expressed during this process and serve as markers for the different endodermal cell types. Differentiation is also accompanied by a decreased rate of proliferation and an apoptotic response. Using homologous recombination, we have disrupted both alleles of the retinoid X receptor (RXR) α gene in F9 cells to investigate its role in mediating these responses. The loss of RXRα expression impaired the morphological differentiation of F9 EC cells into primitive and parietal endoderm, but has little effect on visceral endodermal differentiation. Concomitantly the inducibility of most primitive and parietal endoderm differentiation-specific genes was impaired, while several genes upregulated during visceral endodermal differentiation were induced normally. We also demonstrate that RXRα is required for both the anti-proliferative and apoptotic responses in RA-treated F9 cells. Additionally, we provide further evidence that retinoic acid receptor (RAR)–RXR heterodimers are the functional units transducing the effects of retinoids in F9 cells.

Keywords: apoptosis/cellular proliferation/RXRα/RXR–RAR heterodimers/synthetic retinoids

Introduction

Retinoids have been shown to modulate a wide variety of biological processes, including morphogenesis in the embryo, cell proliferation, differentiation, homeostasis and malignant transformation (for reviews see Lotan, 1980; Chambon, 1994; Gudas *et al.*, 1994). Retinoids exert their effects through two classes of ligand-dependent transactivators, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both members of the nuclear receptor superfamily (Leid *et al.*, 1992a; Chambon, 1994; Mangelsdorf and Evans, 1995; Kastner *et al.*, 1995; and references therein). RARs are activated by all-*trans* retinoic acid (tRA) and its 9-*cis* isomer (9C-RA), while RXRs are only activated by 9C-RA. The three members (types) of each class of retinoid receptors designated RARα, β and γ and RXRα, β and γ, are encoded by different genes. Each gene can generate multiple mRNA splice variants encoding for receptor isoforms with unique N-terminal A regions (Leid *et al.*, 1992a; Gronemeyer and Laudet, 1995; and references therein). The various RAR and RXR types and isoforms are highly conserved in evolution, and display distinct spatio-temporal expression patterns in developing organisms and in the adult (reviewed in Kastner *et al.*, 1995), which suggested that each receptor exerts some distinct physiological functions (Leid *et al.*, 1992a). RXRs can dimerize with RARs, thyroid hormone receptors, vitamin D receptors, peroxisome proliferator-activated receptors, and a number of 'orphan' receptors (Chambon, 1994; Giguère, 1994; Glass, 1994; Leblanc and Stunnenberg, 1995; Mangelsdorf and Evans, 1995; and references therein). Upon heterodimerization, RXRs have been shown to modulate the DNA-binding and transcriptional activating functions of these receptors *in vitro* and in transfected cells. Thus RXRs are likely to play a key role in the control of several signaling pathways.

The F9 murine embryonal carcinoma (EC) cell line provides an interesting model system for analysis of retinoid receptor functions at the cellular and molecular level. F9 cells resemble the pluripotent stem cells of the inner cell mass of the early embryo and differentiate into three distinct endodermal cell types upon treatment with tRA (reviewed by Hogan *et al.*, 1983). Treatment of cells in monolayer culture with 0.1–1 μM tRA induces primitive endodermal differentiation (Strickland and Mahdavi, 1978). Treatment with 0.1–1 μM tRA and 250 μM dibutyryl cAMP (bt_2cAMP) induces a parietal endodermal phenotype, which in the embryo is derived from primitive endoderm (Strickland *et al.*, 1980). When F9 cells are cultured in suspension as aggregates, treatment with 50 nM tRA induces a visceral endodermal (VE) phenotype in the outermost cell layer (Hogan *et al.*, 1981; Rogers *et al.*, 1990). The retinoid-induced differentiation of F9 cells, as well as a large number of other cell lines (Amos and Lotan, 1991), is accompanied by a dramatic decrease in the rate of proliferation, characterized by a diminished rate of DNA synthesis and an increase in the fraction of cells in the G_1 phase of the cell cycle (Linder *et al.*, 1981; Rosenstraus *et al.*, 1982; Atencia *et al.*, 1994). Moreover, it has been reported recently that tRA can trigger the apoptotic response, or programmed cell death, in F9 cells (Atencia *et al.*, 1994).

Inactivation of RARα and RARγ by homologous recombination (HR) in F9 cells has indicated a more prominant role for RARγ than for RARα in mediating tRA-induced differentiation and control of gene expression in those cells (Boylan *et al.*, 1993, 1995; Taneja *et al.*, 1995). However, tRA-induced growth arrest was not

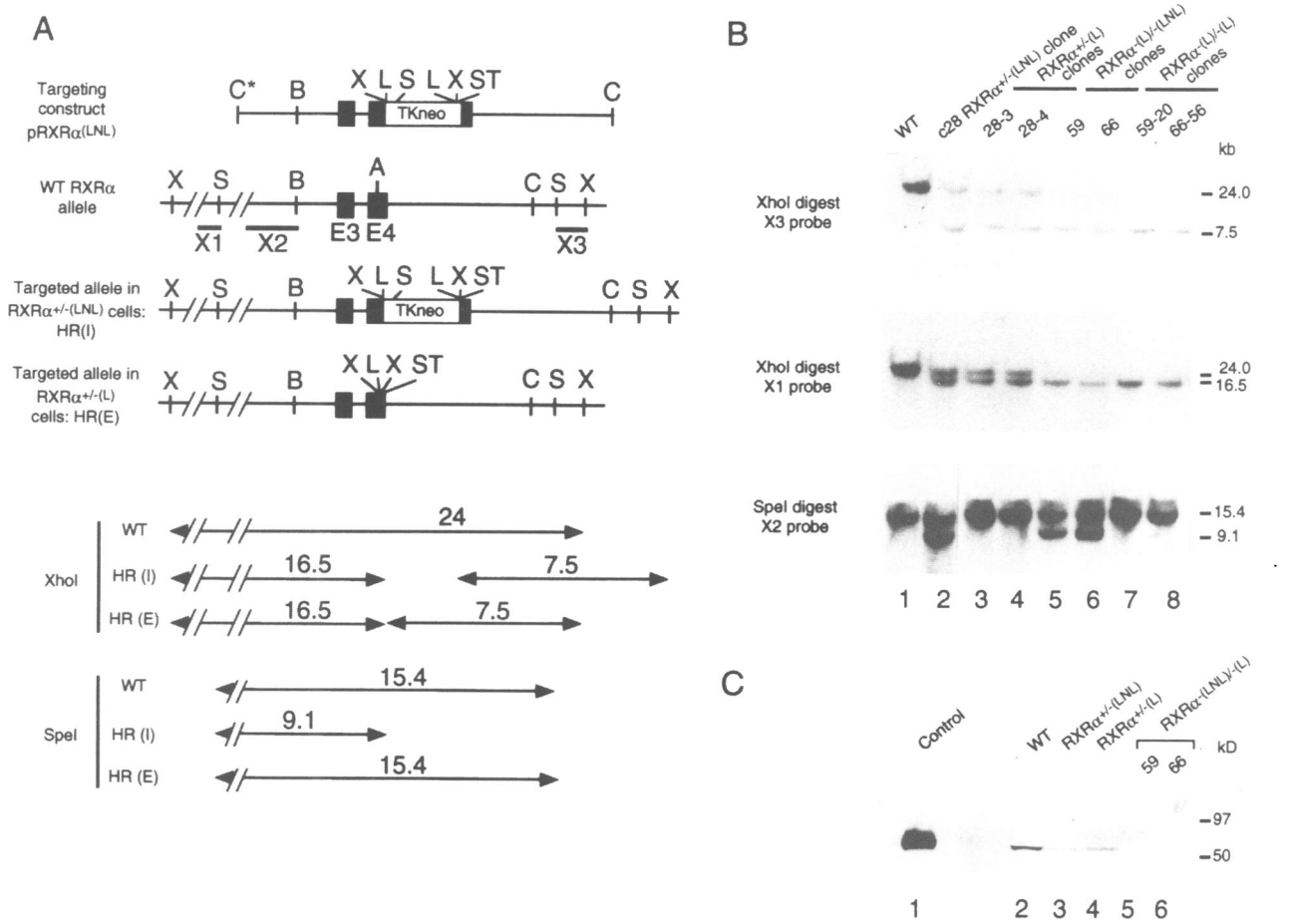

Fig. 1. Disruption of both alleles of the RXRα gene by HR. (**A**) Schematic diagram of the WT RXRα genomic locus, the pRXRα$^{(LNL)}$ targeting vector, the recombined locus after integration [HR(I)] and after Cre-mediated excision [HR(E)]. Dark boxes indicate exons. Restriction enzyme sites and the location of probes X1–X3 are indicated. X1 and X3 probes correspond to 0.6 kb *Sca*I–*Spe*I and 1.2 kb *Spe*I–*Xho*I fragments derived from the RXRα genomic clones λ6-pBSK and λ3-pBSK, respectively. X2 probe corresponds to a 2.1 kb *Sal*I–*Bam*HI fragment derived from pRXRα$^{(LNL)}$. The numbers in the lower part of diagram are in kilobases (kb). Abbreviations: A, *Acc*I; B, *Bam*HI; C, *Cla*I; L, *loxP* recombination site; S, *Spe*I; ST, three tandem translation stop codons inserted in frame after Cre excision; X, *Xho*I. * indicates that this restriction site is derived from vector sequences and is not present in the WT gene sequence. (**B**) Southern blot analysis indicating the disruption of the RXRα gene. The genotypes of different cell lines (for example 28) and their subclones (28-3,4 etc.) are indicated at the top of each lane, and correspond to all three panels. The WT and homologous recombined fragments resulting from *Xho*I digestion and probing with X3 and X1 are indicated in kb (upper and middle panels respectively). The WT, and recombined fragments which result from *Spe*I digestion and probing with X2 are indicated in the lower panel. (**C**) Western blot analysis of WT, RXRα$^{+/-(L)}$ and RXRα$^{-(L)/-(LNL)}$ cell lines. Lane 1 contains 2 μg of whole cell extract from Cos cells transfected with the mRXRαø expression construct, and lanes 2–6 contain 70 μg of the indicated nuclear extracts. Protein quantitation was determined by the Bradford assay. RXRα protein was detected using the rabbit polyclonal antibody RPRXα(A) (Rochette-Egly *et al.*, 1994), followed by chemiluminescence detection. Mol. wt is designated in kilodaltons.

affected by the loss of either RARγ or RARα (Boylan *et al.*, 1995). We have now inactivated the RXRα gene and shown that RXRα is required for mediating the tRA-induced differentiation of F9 cells into primitive and parietal endoderm, as well as for the tRA induction of a number of genes. Differentiation into VE, on the other hand, is delayed, but otherwise nearly unaffected by the loss of RXRα. Interestingly, we also demonstrate that both the anti-proliferative and apoptotic responses to tRA observed during primitive endodermal differentiation are impaired in cells lacking RXRα.

Results

Targeted disruption of the RXRα gene in F9 EC cells

The pRXRα$^{(LNL)}$ targeting construct was used to disrupt the RXRα gene by HR so as to introduce three tandem translation stop codons within the first zinc-binding motif encoded by exon 4 (see Materials and methods and Figure 1A). In order to target both RXRα gene alleles, the Cre/*loxP* system (Sauer and Henderson, 1990) was employed, which permitted the removal of the neomycin resistance gene from the first targeted allele, before targeting the second allele with the same construct. The occurrence of the HR event, and the Cre-mediated deletion, were checked by Southern blotting as exemplified in Figure 1. A 3′ probe (X3) located outside of the region of the targeting construct detected a single wild-type (WT) *Xho*I fragment of 24.0 kb and a 7.5 kb fragment in the case of HR (Figure 1A and B, upper panel, compare lane 1 with lanes 2–8). HR was confirmed by the use of a 5′-outside probe (X1) on the same Southern blot to detect the WT and mutant fragments of 24.0 and 16.5 kb, respectively (Figure 1A and B, middle panel).

Three out of 144 neomycin resistant clones were positive

for the desired recombination event. One heterozygote cell line [clone 28 RXRα$^{+/-(LNL)}$] was then transiently transfected with a Cre recombinase expression construct (pPGK-Cre), to excise the *loxP*-flanked PGK–TKneo cassette. Cells were grown in the presence of gancyclovir to select for the loss of the TK gene [+/–(LNL) signifies a single allele targeted prior to Cre excision, and +/–(L) signifies the removal of one *loxP* site and sequences between the *loxP* sites in this allele]. Approximately 47% of the gancyclovir-resistant clones had undergone the expected Cre-mediated recombination at the mutated allele as determined by Southern blotting (data not shown). The loss of a *Spe*I site internal to the *loxP* sites was verified using the X2 probe, which detects a *Spe*I fragment of 15.4 kb in the WT allele and in targeted alleles after Cre-mediated excision (WT and RXRα$^{+/-(L)}$ respectively), and a 9.1 kb fragment in targeted alleles before Cre excision [RXRα$^{+/-(LNL)}$ in Figure 1A and B, lower panel, compare lane 2 with lanes 3 and 4]. Note that at least one of the *Xho*I sites immediately flanking both of the *loxP* sites remains after the Cre-mediated excision (Figure 1A and B, upper and middle panel lanes 3–8 compared with lower panel, lanes 3–8). The absence of other *Spe*I fragments detectable by the X2 probe indicates that random integrations of the pRXRα$^{(LNL)}$ construct did not take place, which was confirmed with a probe derived from the neomycin resistance gene sequence (data not shown). The second allele of the RXRα gene was targeted as above in a subclone of the clone 28 RXRα$^{+/-(L)}$ cell line (28-3), using the pRXRα$^{(LNL)}$ targeting vector, followed by Cre excision, to yield RXRα$^{-(L)/-(LNL)}$ and RXRα$^{-(L)/-(L)}$ clones, respectively (Figure 1B, lanes 5–8).

The absence of RXRα protein expression in RXRα$^{-/-}$ cell lines was verified by Western blotting using the polyclonal antibody RPRXα(A) (Rochette-Egly *et al.*, 1994), directed against region A of the RXRα protein (Figure 1C). No 50 kDa RXRα protein could be detected for RXRα$^{-/-}$ clones 59 and 66 (compare lanes 5 and 6 with lanes 1–4, and data not shown). Note that the level of protein in heterozygote cells was intermediate between those of WT and RXRα$^{-/-}$ cells (Figure 1C, compare lanes 3 and 4 with lanes 2, 5 and 6).

RXRα-null F9 cells are impaired in their morphological differentiation into primitive and parietal endoderm

WT and RXRα$^{-/-}$ cells [RXRα$^{-(L)/-(L)}$, clone 59–19] were grown in monolayer culture and treated with either 1 μM tRA alone (Figure 2B and G) or in combination with 250 μM bt$_2$cAMP (Figure 2C and H), to induce differentiation into primitive or parietal endoderm, respectively (see Introduction). Interestingly, RXRα-null cells did not exhibit the changes in morphology which normally accompany tRA-induced differentiation (compare Figure 2A–C with F–H). After a 5-day treatment, a majority of RXRα$^{-/-}$ cells retained a clustered or compact appearance, similar to that of the untreated controls (Figure 2, compare G and H with F). All RXRα$^{-/-}$ clones analyzed (66, 81, 95, 190, 66–56) exhibited the same impaired differentiation response to tRA or tRA and bt$_2$cAMP, and similar results to those shown in Figure 2 were obtained using 9C-RA (1 μM) instead of tRA (data not shown).

Longer tRA treatment of RXRα$^{-/-}$ cells never resulted in a fully differentiated cell population. By 10 days of continuous treatment with tRA, clusters of compact, morphologically undifferentiated cells were interspersed with a minority of cells displaying a differentiated morpho-

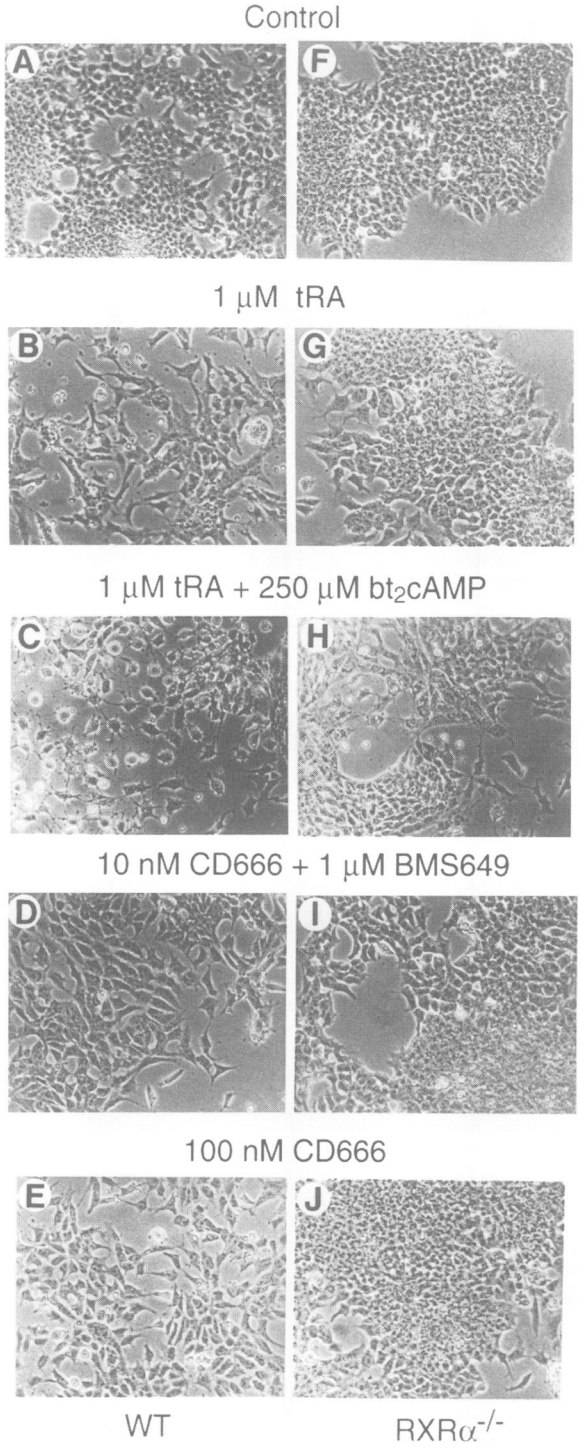

Fig. 2. RXRα$^{-/-}$ cells are resistant to tRA-induced primitive and parietal endodermal differentiation and RXRα is a required partner for retinoid-induced morphological differentiation of F9 cells. WT (A–E) and clone 59-19 RXRα$^{-(L)/-(L)}$ (F–J) cells were seeded in 10 cm culture dishes and treated with control vehicle (**A** and **F**), 1 μM tRA alone (**B** and **G**), 1 μM tRA and 250 μM bt$_2$cAMP (**C** and **H**), 10 nM CD666 and 1 μM BMS649 (**D** and **I**) or with 100 nM CD666 (**E** and **J**). The cells were photographed under a phase-contrast microscope at 125× magnification.

Table I. Effect of various retinoids on the morphological differentiation, proliferation and apoptotic response of F9 EC cells

Cell line	Treatment[a]	Differentiation[b]	% Prolif. inhibition[c]	Apoptosis[d]
F9 WT	1 μM tRA	++	88	+
	10 nM CD666	+	62	–
	1 μM BMS649	–	0	–
	10 nM CD666 + 1 μM BMS649	++	79	+/–
	100 nM CD666	++	76	+/–
RXRα$^{-/-}$	1 μM tRA	+/–	52	–
	10 nM CD666	+/–	16	–
	1 μM BMS649	–	0	–
	10 nM CD666 + 1 μM BMS649	+/–	29	–
	100 nM CD666	+/–	38	–
RARγ$^{-/-}$	1 μM tRA	+/–	82	+
	1 μM BMS649	–	0	–
	1 nM AM80	–	0	n.d.
	100 nM AM80	+/–	51	n.d.
	1 nM AM80 + 1 μM BMS649	+/–	75	n.d.
	100 nM AM80 + 1 μM BMS649	+/–	77	n.d.

[a]F9 WT, clone 95 RXRα$^{-(L)/-(LNL)}$ and RARγ$^{-/-}$ cells were treated for 5 days in monolayer culture to induce differentiation into primitive endoderm with the indicated retinoids.
[b]Differentiation was scored according to the proportion of cells assuming the differentiated morphology after 5 days of retinoid treatment. 70–90% of cells, ++; 15–25% of cells, +; not more than 10%, +/–; no visible effect, –.
[c]Percentage of proliferation inhibition was calculated as described in Materials and methods.
[d]The presence (+) or absence (–) of apoptosis was determined by both the appearance of a ladder of fragmented DNA, as in Figure 8A and B, and by the presence of apoptotic nuclei and subcellular fragments upon staining of fixed cells with Hoechst 33258, as in Figure 8C. +/– indicates that the induction of apoptosis was sharply reduced compared with WT. n.d., not determined.

logy (data not shown). The 'non-differentiated' morphology persisted for as long as the cells were tRA treated (15 days), and at a wide range of plating densities (data not shown). Thus, the loss of RXRα results in a drastic reduction in the capacity of the cell population to differentiate, rather than in a simple delay in the differentiation process.

Retinoid-induced morphological differentiation of F9 cells requires RXRα as a synergistic partner for RARs

Previous studies have shown that RAR–RXR heterodimers bind more efficiently to retinoic acid response elements (RAREs) than RAR or RXR homodimers, both *in vitro* and in transfected cells overexpressing RARs and/or RXRs (Yu *et al.*, 1991; Leid *et al.*, 1992b; Durand *et al.*, 1992, 1994 and references therein). Furthermore, recent studies using RAR type-selective and pan-RXR-specific synthetic retinoids have shown that RAR and RXR strongly synergize upon ligand binding to induce differentiation and gene transcription in F9 cells, thus suggesting that RAR–RXR heterodimers are the functional units (Roy *et al.*, 1995). We therefore determined whether RXRα is required for this synergism. As expected, WT F9 cells treated for 4 days with a combination of 10 nM CD666, a RARγ-selective retinoid, and 1 μM BMS649 (also called SR11237), a pan-RXR agonist (Lehmann *et al.*, 1992), morphologically differentiated (compare Figure 2A and B with D, and Table I), while each of these retinoids administered separately has little or no effect (Table I; and see Roy *et al.*, 1995). Under similar conditions almost no differentiation was observed in RXRα$^{-/-}$ cells, providing evidence that RARγ–RXRα heterodimers may mediate the effects of this combination of retinoids (Figure 2I and Table I). It has also been previously demonstrated that at the non-selective concentration of 100 nM, under conditions where all RARs but not RXRs are activated, CD666 can induce the morphological differentiation of F9 cells (Roy *et al.*, 1995; Figure 2E and Table I). Interestingly, RXRα$^{-/-}$ F9 cells were almost unaffected by treatment with 100 nM CD666, indicating that an unliganded RXRα partner is still required to induce differentiation at an elevated concentration of a RAR-specific ligand (Figure 2, compare E with J, and Table I).

Loss of RXRα results in impaired RA induction of several genes during primitive endodermal differentiation

The expression of a number of tRA-induced genes was compared over a time course of tRA (1 μM) treatment for WT and RXRα$^{-/-}$ cells. Reverse transcription-polymerase chain reactions (RT–PCR) were performed on total RNA to semi-quantitatively estimate the expression of the genes indicated in Figure 3 (see Materials and methods). The induction of most genes tested was affected by the loss of RXRα expression. The induction of both collagen type IVα1 and laminin B1, two markers of endodermal differentiation (Strickland and Mahdavi, 1978; Wang and Gudas, 1983), was ~6-fold and ~8-fold lower, respectively, in RXRα$^{-/-}$ cells than in WT cells after 96 h of tRA treatment (Figure 3A). Similarly, the induction of another tRA-responsive gene, TGF-β2 (Glick *et al.*, 1989), was ~7-fold lower in RXRα$^{-/-}$ cells than in WT cells (Figure 3A). The induction of several other genes which contain RAREs in their promoter region was also diminished in RXRα$^{-/-}$ cells. The Hoxa-1 and Hoxb-1 homeogenes are known to be rapidly induced by tRA in F9 cells (LaRosa and Gudas, 1988a; Langston and Gudas, 1992; Marshall *et al.*, 1994; Boylan *et al.*, 1995). The tRA induction of Hoxa-1 was much lower in RXRα$^{-/-}$ than in WT cells (Figure 3A). In contrast to WT cells, the induction of Hoxb-1 and CRABPII (Giguère *et al.*, 1990) was very low by 24 h of tRA treatment in RXRα$^{-/-}$ cells, and by 48 h both genes were ~2-fold less efficiently induced than

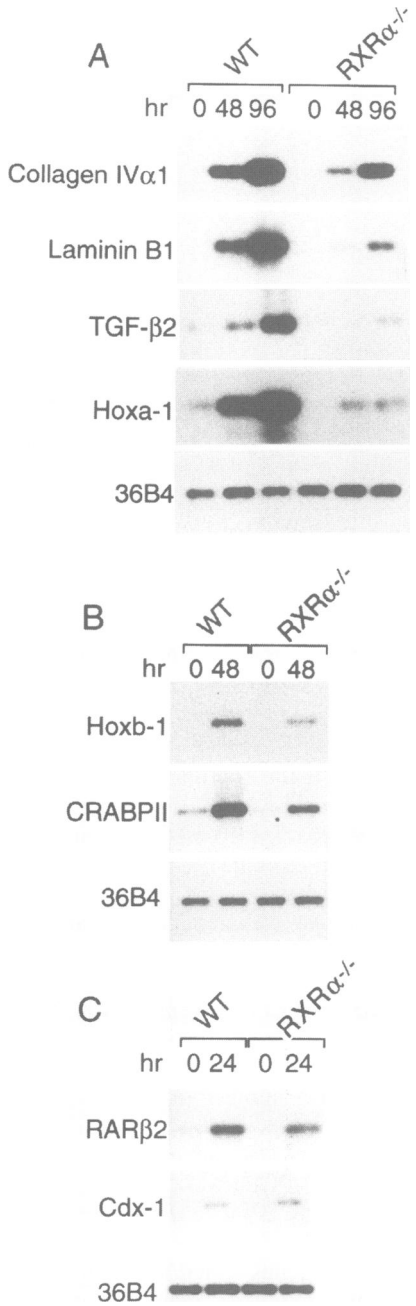

Fig. 3. RXRα$^{-/-}$ cells grown in monolayer culture are impaired in the tRA-induction of several genes. (**A**) Total RNA from WT and clone 59-19 RXRα$^{-(L)/-(L)}$ cells treated with 1 μM tRA for the indicated times was subjected to semi-quantitative RT–PCR analysis for the genes indicated. Of the total PCR 15% was electrophoresed and blotted onto nylon membranes as previously described (Bouillet *et al.*, 1995), followed by hybridization with the indicated probes. (**B** and **C**) RT–PCR analysis of early tRA-inducible genes was performed as in (A) for the indicated times of tRA treatment, followed by hybridization with the indicated probes. Similar results were observed for each gene for at least three RXRα$^{-/-}$ cell lines, in at least three separate experiments. The level of 36B4 expression is indicated for each RNA sample as a control for quantitation.

WT cells (Figure 3B and data not shown). The induction of RARβ2, which contains the same RARE as Hoxa-1 in its 5′ promoter region (deThé *et al.*, 1990; Langston and Gudas, 1992), was less dramatically decreased (Figure 3C). In contrast, Cdx-1 expression, known to be upregulated by tRA in P19 and F9 cells (Bouillet *et al.*, 1995; Taneja *et al.*, 1995; Meyer and Gruss, 1993), was apparently not affected in RXRα$^{-/-}$ cells (Figure 3C). It appears therefore that RXRα is required during F9 cell primitive endodermal differentiation for the induction of most tRA-responsive genes, and that this requirement effects differentially the various genes tested.

Lack of RXRα has little effect on VE differentiation

In contrast to primitive and parietal endodermal differentiation, RXRα$^{-/-}$ cells appear to differentiate normally into VE. Their morphology, when grown as aggregates for 10 days in the presence of 50 nM tRA to induce VE differentiation, was indistinguishable from that of WT cells (Figure 4A), although there was a 1–2 day delay in their morphological differentiation (data not shown). In order to better characterize the VE differentiation of the null mutants, the expression of three differentiation markers, collagen IVα1, laminin B1 and α-fetoprotein (AFP) was analyzed by RT–PCR (Figure 5), and the expression of one marker, laminin B1, was also determined by immunohistochemical staining of sectioned aggregates (Figure 4B). Induction of collagen IVα1 mRNA was slightly reduced in RXRα$^{-/-}$ cells (~1.5-fold and ~1.2-fold lower levels than in WT cells after 6 and 10 days of culture in the presence of tRA, respectively (Figure 5). AFP and laminin B1 mRNA levels were also slightly lowered in RXRα$^{-/-}$ cells compared with the WT cells (AFP mRNA level was ~1.7-fold lower after 10 days of tRA treatment, and laminin B1 mRNA levels were ~1.4-fold and ~1.2-fold lower after 6 and 10 days of tRA treatment, respectively; Figure 5). In agreement with the RT–PCR results, both WT and RXRα$^{-/-}$ aggregates display very similar patterns of laminin B1 expression, with the highest fluorescence intensity in the area underlying the outer layer of VE cells (Figure 4B, arrows), as previously reported for F9 WT cells (Hogan *et al.*, 1981; Rogers *et al.*, 1990). In conclusion, the levels of collagen IVα1, laminin B1 and AFP mRNA are only slightly reduced in tRA-treated RXRα$^{-/-}$ cells grown as aggregates, and RXRα$^{-/-}$ F9 cells do appear to differentiate normally into VE, in contrast to their drastically impaired differentiation into primitive and parietal endodermal cell types.

RXRα-null cells are less sensitive to the anti-proliferative effect of RA and exhibit a higher basal rate of proliferation than WT F9 cells

WT population doubling time (DT) was 15 h, whereas that of RXRα$^{-/-}$ lines ranged from 12 to 13 h (Table II). Interestingly, in the absence of retinoid treatment, the basal rate of proliferation of every RXRα$^{-/-}$ cell line tested was higher than that of the parental WT F9 cell line (Figure 6A–C, and data not shown).

Since tRA treatment is known to decrease the rate of proliferation of F9 cells (see Introduction), we compared the anti-proliferative effect of tRA on RXRα$^{-/-}$ and WT F9 cells. The inhibition of proliferation relative to vehicle treated-controls (% in parentheses, Figure 6A) was markedly lower for RXRα$^{-/-}$ cells after 5 days of tRA treatment (32–48%, depending on the clone) than for WT cells (87%). Similar results were observed for eight different RXRα$^{-/-}$ clones (data not shown). Figure 6B illustrates the inhibition of proliferation for cells which

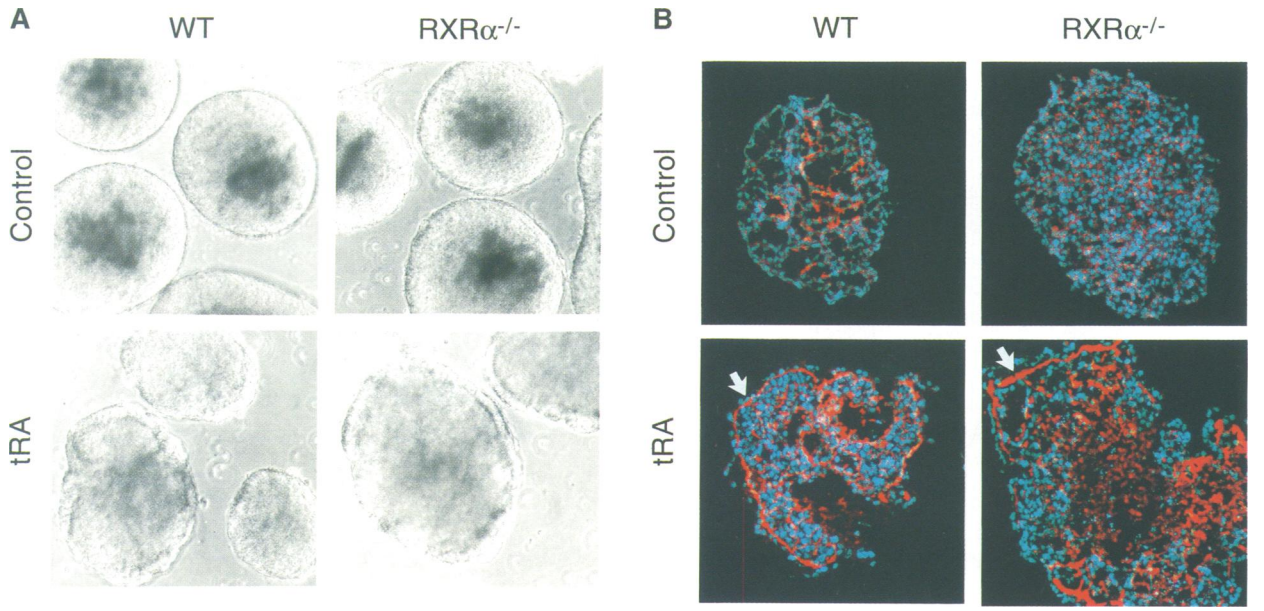

Fig. 4. RXRα$^{-/-}$ VE aggregrates are morphologically similar to WT as revealed by phase contrast microscopy and immunohistochemical staining of laminin B1. (**A**) WT and clone 66 RXRα$^{-(L)/-(LNL)}$ cells were grown in the absence or presence of 50 nM tRA for 10 days, with a change of media every 2 days. The aggregates were photographed under a phase-contrast microscope at 125× magnification. (**B**) WT and clone 66 RXRα$^{-(L)/-(LNL)}$ cells treated as in (A) were sectioned, immunostained using specific anti-laminin antibodies (red), and the nuclei stained with Hoescht dye (blue). White arrows indicate the location of the basement membrane that forms between the outer layer of VE cells and the non-differentiated inner mass of cells.

were replated at an equal density after 5 days of continuous tRA treatment, and counted after an additional 5 days of tRA treatment. The proliferation of RXRα$^{-/-}$ cells was markedly reduced after such a 10 day tRA treatment, but in contrast, that of the WT cells was fully inhibited (Figure 6B).

As seen above for the induction of morphological differentiation, 10 nM CD666 and 1 μM BMS649 (pan-RXR-specific) synergized to inhibit the proliferation of WT F9 cells, and this effect clearly requires the presence of RXRα (Table I, and data not shown). WT F9 cells were also growth-inhibited by 100 nM CD666 (although to a slightly lower extent than with 1 μM tRA), while RXRα$^{-/-}$ cell growth was markedly less inhibited (Figure 6C and Table I). Thus, as in the case of the differentiation response, the anti-proliferative response also requires RXRα, and may therefore involve RAR–RXRα heterodimers. It should also be noted that treatment with the pan-RXR agonist BMS649 alone had no effect on the proliferation rate of either WT or RXRα$^{-/-}$ cells (Table I), indicating that liganding of RXRs is not sufficient for eliciting the anti-proliferative response.

In order to better estimate the effect of tRA on cellular proliferation, the rate of [^{3}H]thymidine incorporation was compared for WT and a representative RXRα$^{-/-}$ cell line. A marked reduction in [^{3}H]thymidine incorporation was observed for the WT, but not RXRα$^{-/-}$ cells after 5 days of tRA treatment (Figure 6D). The amount of incorporated [^{3}H]thymidine was calculated on a per cell basis, in order to control for differences in cell number both between control and tRA-treated cultures, and between WT and RXRα$^{-/-}$ cultures. Clearly, at least part of the anti-proliferative response to tRA in WT cells is related to a decrease in the rate of cell division, which is much less affected in the case of RXRα$^{-/-}$ mutants.

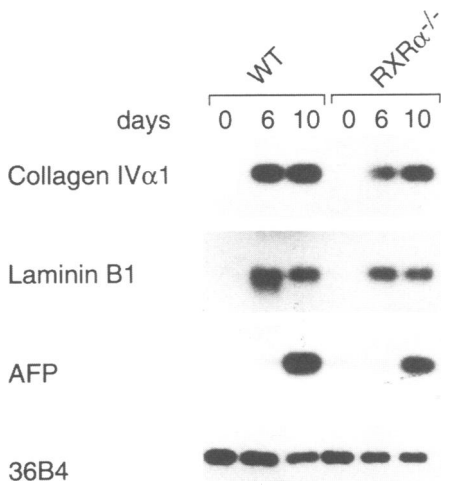

Fig. 5. The loss of RXRα has a minimal effect on the induction of VE markers of differentiation by tRA. WT and clone 66 RXRα$^{-(L)/-(LNL)}$ cells were grown in suspension culture as aggregates for the indicated times in the presence of 50 nM tRA before collection of total RNA, followed by RT–PCR analysis.

Table II. Comparison of cell number and DT of F9 WT and RXRα$^{-/-}$ cells after a 5-day period of growth in monolayer culture

Cell line	Cell number (×10^{-6}) ± SEM	DT (h)
WT	0.64 ± 0.03	15
RXRα$^{-(L)/-(LNL)}$ c81	2.30 ± 0.30	12
RXRα$^{-(L)/-(LNL)}$ c95	1.80 ± 0.05	12.5
RXRα$^{-(L)/-(L)}$ c59-11	1.40 ± 0.03	13

The population DT for the untreated cell cultures shown in Figure 6A was calculated according to Kuchler (1977).

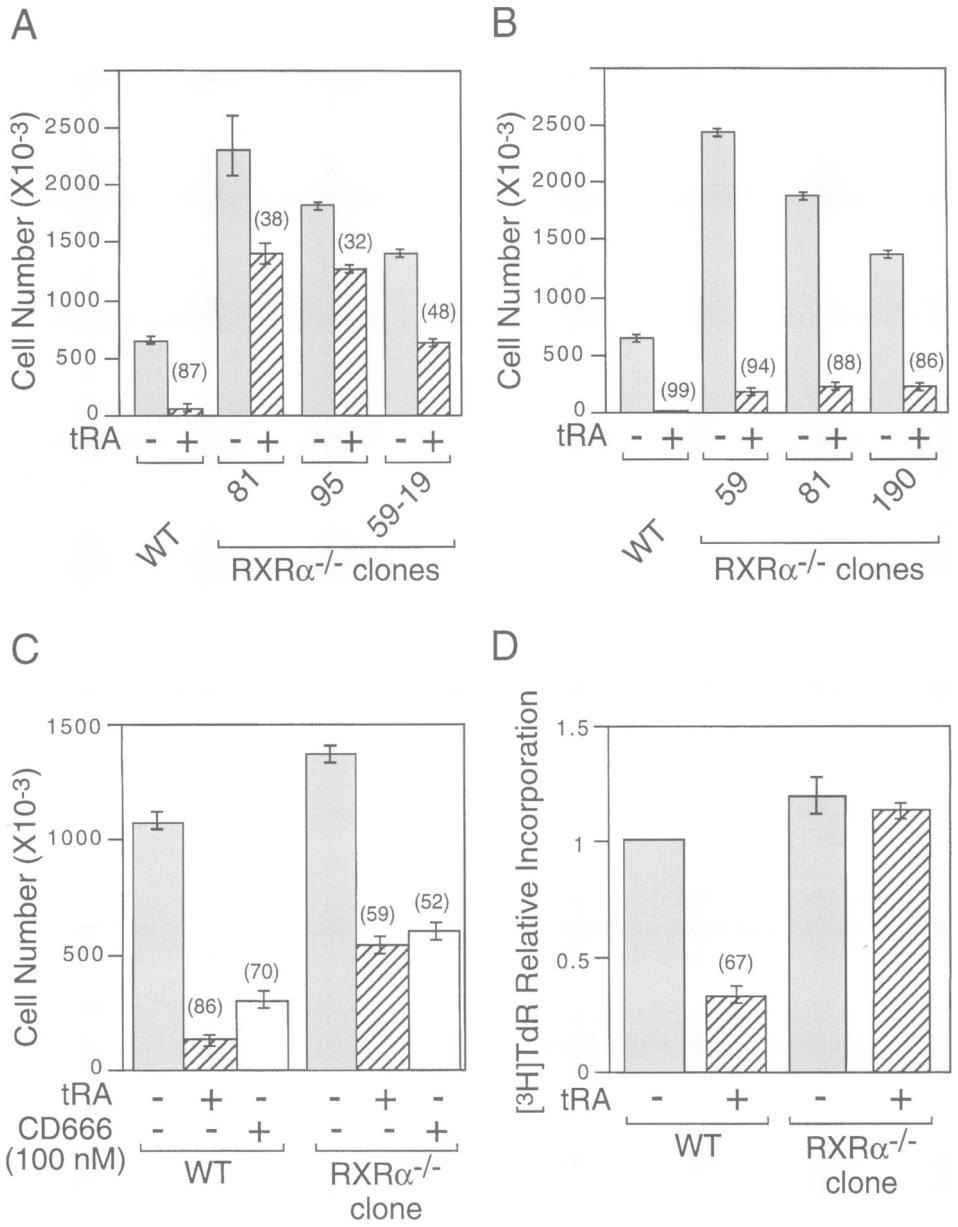

Fig. 6. RXRα$^{-/-}$ cells are impaired in the anti-proliferative response to tRA and CD666, and have a higher basal proliferation rate than WT cells. (**A**) The number of cells after 5 days of culture in the presence (striped bars) or absence (dark bars) of tRA are indicated for WT and clone 81 RXRα$^{-(L)/-(LNL)}$ (81), clone 95 RXRα$^{-(L)/-(LNL)}$ (95) and clone 59–19 RXRα$^{-(L)/-(L)}$ (59-19). The bars represent the mean ± SEM for triplicate cultures within the same experiment. The numbers in parentheses indicate the percentage of growth inhibition for the tRA-treated cultures relative to the untreated controls. (**B**) F9 WT and the indicated RXRα$^{-/-}$ cell lines were cultured as in (A) for 5 days followed by replating in triplicate, at identical densities (2.5×10^3 cells/well) into 3 cm culture wells, and the tRA treatment continued for an additional 5 days before counting. (**C**) F9 WT and clone 59–19 RXRα$^{-(L)/-(L)}$ cells were treated and counted as in (A), in the absence of retinoids (dark bars), or in the presence of 1 μM tRA (striped bars) or 100 nM CD666 (open bars). (**D**) F9 WT and clone 59–19 RXRα$^{-(L)/-(L)}$ cells were cultured in the presence or absence of 1 μM tRA for 5 days, followed by [^{3}H]thymidine labeling. The error bars represent the mean ± SEM for three different experiments, setting the amount of [^{3}H]thymidine ([^{3}H]TdR) incorporated per 1000 cells equal to 1 for F9 WT control cells.

Since the proliferation rate of eukaryotic cells is controlled at specific points in the cell cycle, and previous studies have shown that tRA treatment of F9 cells results in the lengthening of the G_1 portion of the cell cycle (Linder *et al.*, 1981; Rosenstraus *et al.*, 1982), the cell cycle profile of untreated and tRA-treated RXRα$^{-/-}$ cells was compared with that of WT cells by cell cycle flow cytometry. tRA treatment for 4 and 6 days resulted in an accumulation of cells in the G_1 phase for both WT and RXRα$^{-/-}$ cells, but the shift to the G_1 phase was more pronounced for WT cells (Figure 7, compare A, B and C with D, E and F). The proportion of cells remaining in the proliferative phases of the cell cycle after tRA treatment ($S+G_2+M$) was higher for the RXRα$^{-/-}$ line, 61 and 52% at 4 and 6 days tRA treatment, respectively, compared with 38 and 34% for WT cells.

RXRα is required for the induction of apoptosis by tRA in F9 cells

Since at least part of the differences in cell number observed between tRA-treated RXRα$^{-/-}$ and WT cells could also be due to variation in the rate of cell death,

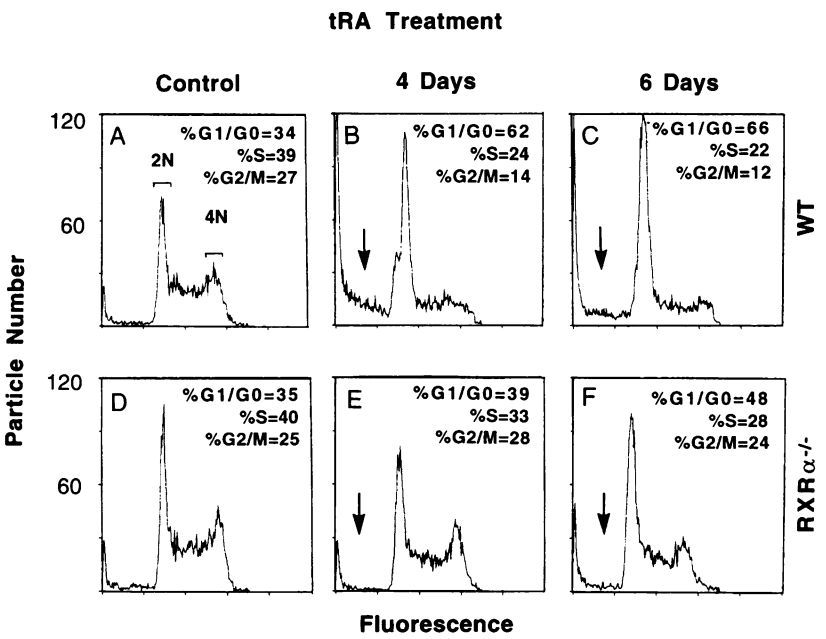

Fig. 7. FACS analysis reveals that the tRA-induced accumulation in G_1 is reduced for RXRα$^{-/-}$ cells compared with WT. RXRα$^{-/-}$ cells also have a diminished accumulation of apoptotic particles in the response to tRA treatment. Subconfluent cultures of WT (**A–C**), and clone 81 RXRα$^{-(L)/-(LNL)}$ cells (**D–F**), were grown for 4 and 6 days in the presence of 1 μM tRA, and analyzed by FACS. The *x*-axis indicates the integrated fluorescence intensity and the *y*-axis particle number. Approximately 20 000 particles are represented in each histogram. The values indicated for the percentage of cells in G_1/G_0, S and G_2/M are accurate within ±10–15%. Arrows in B, C, E and F indicate the position of sub-2N size DNA-containing particles or 'apoptotic bodies'.

we compared the tRA-induced apoptotic response of WT and RXRα$^{-/-}$ cells. Strikingly, the appearance of the characteristic apoptotic 'ladder' of fragmented DNA was observed for WT, but not RXRα$^{-/-}$ cells, upon a 5-day treatment with tRA (Figure 8A). Cells were plated at different densities to achieve low (~50% confluent cells; L) and high (confluent cells; H) cell densities by the end of the experiment, for both untreated and tRA-treated cultures, in order to control for effects of cell density on apoptosis [see Materials and methods for details; overconfluent F9 WT and RXRα$^{-/-}$ cells will apoptose due to nutrient and/or mitogen depletion (data not shown)]. Note the presence of faint bands of fragmented DNA in all lanes, especially the high density lanes, indicating a background level of apoptosis even in the absence of tRA (Figure 8A).

Apoptosis is also known to be associated with the appearance of membrane-bound subcellular fragments containing DNA in the culture media. Such 'apoptotic bodies' can be visualized, and their relative amount determined by flow cytometry. The arrows in Figure 7 point to the sub-2N particles appearing in culture supernatants of tRA-treated cells. These 'apoptotic bodies' accounted for 37 and 26% of the total number of particles for WT cells after 4 and 6 days of tRA treatment respectively, whereas they amounted to only 7 and 13% of all particles for RXRα$^{-/-}$ cells after 4 and 6 days of tRA treatment (Figure 7B and C compared with E and F).

We also compared the tRA-induced apoptosis in RXRα$^{-/-}$ and RARγ$^{-/-}$ cells. As reported, tRA-treated RARγ$^{-/-}$ F9 cells are resistant to differentiation and are also impaired for the induction of several tRA-responsive genes (Boylan *et al.*, 1993, 1995; and Table I). However, unlike RXRα$^{-/-}$ cells, RARγ$^{-/-}$ cells are almost as sensitive as WT to the anti-proliferative effect of tRA (Boylan *et al.*, 1995). Interestingly, tRA treatment of the RARγ$^{-/-}$ cells resulted in the appearance of an apoptotic DNA ladder, similar to that observed for the WT cells (Figure 8B, compare lanes 2 and 6). Thus, RXRα, but not RARγ, is indispensible for the tRA-induced apoptotic response of F9 cells. However, the induction of apoptosis clearly requires the presence of a RAR, since the RXR-specific BMS649 alone did not induce apoptosis in WT F9 cells (Table I).

Further evidence of the differential requirement for RXRα and RARγ for tRA-induced apoptosis is presented in Figure 8C. WT, RXRα$^{-/-}$ and RARγ$^{-/-}$ F9 cells grown under the same conditions as in Figures 7 and 8A and B, were stained with the DNA-binding fluorochrome Hoechst 33258. Panel A is a schematic diagram representing the key morphological alterations associated with the apoptotic process. These include the condensation of chromatin along the inner surface of the nuclear membrane, the fragmentation of the cell to produce membrane-bound apoptotic bodies, some of which contain DNA, and finally endocytosis by neighboring cells (Kerr *et al.*, 1995). Panel B is a high magnification of tRA-treated F9 cells after Hoechst staining, revealing the characteristic, highly condensed DNA lining the inner surface of the nuclear membrane. The WT and RARγ$^{-/-}$ cells exhibited this morphology to a far greater extent than RXRα$^{-/-}$ cells (Figure 8C, compare panels D and H with F, arrows). The appearance of the DNA-containing particles, which are likely to be remnants of apoptosing cells, is also visible. Apoptotic particles and condensing chromatin were rarely observed in the tRA-untreated controls for all cell lines.

RARs are functionally redundant for mediating anti-proliferative effects of retinoids

RARγ$^{-/-}$ cells were treated with a combination of 1 nM AM80 (a RARα-selective retinoid) and 1 μM of the

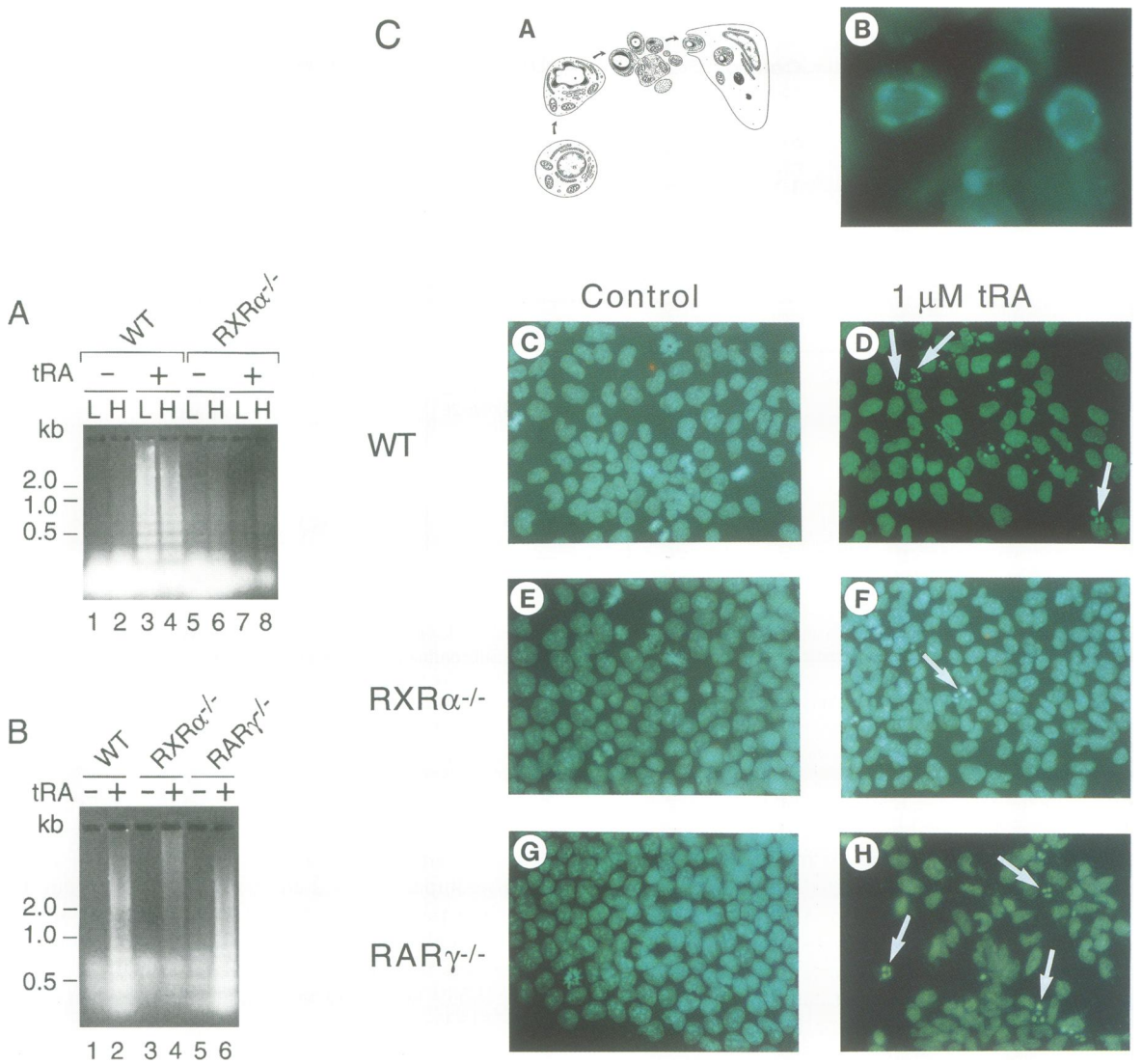

Fig. 8. RXRα$^{-/-}$ cells, but not RARγ$^{-/-}$ cells, are defective in the apoptotic response to tRA. (**A**) WT F9 and clone 81 RXRα$^{-(L)/-(LNL)}$ cells were plated to allow growth to confluence (high density, H) and to 50% confluence (low density, L) after 5 days of culture, either in the absence or presence of 1 μM tRA. Low molecular weight DNA (5 μg/lane) was electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide. Molecular weight is indicated in kilobases (kb). (**B**) WT F9 cells, clone 81 RXRα$^{-(L)/-(LNL)}$ cells, and an RARγ$^{-/-}$ cell line were plated as in (A) to yield subconfluent cultures after 5 days in the presence or absence of tRA. DNA (5 μg/lane) was purified and electrophoresed as in (A). (**C**) WT F9 cell, clone 81 RXRα$^{-(L)/-(LNL)}$ cells, and an RARγ$^{-/-}$ cell line were grown on gelatinized plastic coverslips for 5 days in the absence (panels C, E and G) or presence of 1 μM tRA (panels D, F and H) followed by fixation and staining with Hoechst dye, and photography on a fluorescence microscope at a 120× magnification. Panel A is a schematic diagram illustrating the major morphological alterations accompanying apoptosis (adapted with permission from Figure 1 of Kerr *et al.*, 1995). Arrows indicate condensed chromatin in the nuclei of apoptosing cells or in apoptotic bodies. Panel B, a 600× magnification of tRA-treated F9 cells, highlighting the morphology of nuclei containing condensed chromatin.

pan-RXR agonist BMS649, and assessed for their anti-proliferative response (Figure 6A). Interestingly, both tRA and the 1 nM AM80/1 μM BMS649 combination inhibited proliferation in RARγ$^{-/-}$ cells to nearly the same extent as seen for tRA in WT F9 cells, even though the differentiation response remained drastically impaired (Table I). A higher dose of AM80 (100 nM), which can activate the remaining RAR types (RARα and RARβ) in these cells, in combination with BMS649, did not result in greater inhibition of proliferation, indicating that RARα, in combination with RXRs, can mediate the anti-proliferative effect of retinoids. Thus, RARs are functionally redundant for mediating this anti-proliferative effect, since it was also not affected by disruption of the RARα gene (Boylan *et al.*, 1995).

Discussion

A key role for RARγ–RXRα heterodimers in primitive and parietal endodermal differentiation of F9 cells

F9 EC cells which do not express RXRα have been generated by gene targeting in order to investigate the function of RXRα at the cellular level. RXRα$^{-/-}$ cells are drastically impaired in their differentiation response to high doses of tRA both in the absence and presence of bt_2cAMP, when grown in monolayer culture to induce primitive and parietal endodermal cell types, respectively. In contrast, when treated with low doses of tRA, the differentiation of RXRα$^{-/-}$ cells in aggregate culture into VE, although delayed by 1–2 days, appeared otherwise

normal. This finding may reflect a distinct lineage specificity of function for RXRα, such that the control of genes implicated in primitive/parietal endodermal differentiation might require specifically RXRα, whose absence could not be fully compensated by functionally redundant RXRβ and/or RXRγ. On the other hand, mouse embryos null for RXRα, as well as for RXRβ or RXRγ expression, are defective only in a limited range of tissues, which suggests a high degree of functional redundancy between RXR types (Kastner *et al.*, 1994, 1996; Sucov *et al.*, 1994). In fact, the culture conditions employed for VE differentiation may represent a closer approximation to conditions found in the early embryo. The outer layer of cells in the aggregates, which are destined to become VE, are in contact with underlying cells and a lower concentration of tRA in the medium. This is in contrast to cells growing in monolayer culture, which are in contact with a gelatinized plastic surface and a sustained high concentration of tRA in the medium, two conditions not found in the early embryo. It is likely that the acquisition of the normal 3-dimensional stucture and gene expression pattern for the VE cells is dependent not only on transduction of the retinoid signal, but also on cell–cell contacts and on contact with the underlying basement membrane, which eventually forms directly beneath the outer layer of cells (Hogan *et al.*, 1981; Rogers *et al.*, 1990). Thus, as in RXRα-null embryos, other regulatory mechanisms (possibly involving RXRβ and γ) may compensate for the absence of RXRα during VE differentiation. The final possibility remains that in the case of VE differentiation, RXRs are not involved and that RARs are the only retinoid receptors mediating the tRA signal. It will be necessary to generate double and triple knockouts of RXRs in order to address this possibility.

The recent generation of RXRα and RXRα–RAR double mutant mice has provided the first evidence supporting the proposal that RXR–RAR heterodimers are the functional units which actually transduce the retinoid signal *in vivo* (Kastner *et al.*, 1994, 1995). That such signaling involves RAR–RXR heterodimers was also strongly suggested by our previous finding that the pan-RXR-specific retinoid BMS649 and limiting concentrations of the RARγ-selective retinoid CD666 very efficiently synergize to induce F9 cell differentiation (Roy *et al.*, 1995). The present results obtained with RXRα$^{-/-}$ F9 cells provide further genetic evidence for a physiological role of RXRs in the transduction of the retinoid signal *in vivo*. Indeed, the removal of RXRα abolishes the synergistic effect of BMS649 and limiting concentrations of CD666 on differentiation, providing evidence that those retinoids probably act through RAR–RXR heterodimers. Moreover, we have found that the differentiation which can be induced in WT F9 cells by non-selective high concentrations of CD666 in the absence of RXR ligand, is also abolished by the removal of RXRα. This provides compelling evidence supporting our previous assumption that RXRα is still required as a heterodimeric partner even when all RARs are activated (Roy *et al.*, 1995), although its ligand-dependent transcriptional activation function becomes dispensable. Combined with our previous finding that RARγ$^{-/-}$ cells are more severely impaired in their differentiation response than the RARα$^{-/-}$ cells (Boylan *et al.*, 1995), the present results strongly support the conclusion that RARγ–RXRα heterodimers correspond to the functional units which play a major role in transducing the tRA signal triggering F9 cell differentiation.

The impaired tRA-induced morphological differentiation of RXRα$^{-/-}$ cells into primitive and parietal endoderm-like cells is closely paralleled by a marked decrease in the induction of several tRA-responsive genes. However, the inducibility of RARβ2 is less affected and the induction of Cdx-1 is not affected at all by the loss of RXRα. In these two cases, RXRβ and/or RXRγ, which are present in the F9 cells (Wan *et al.*, 1994) may functionally substitute for RXRα. Interestingly, while the sequence and halfsite spacing of the RARE in the RARβ2 promoter is identical to that of Hoxa-1, its position relative to the start site of transcription is different (deThé *et al.*, 1990; Langston and Gudas, 1992). The Hoxa-1 RARE lies downstream of the coding exons, while the RARβ2 RARE is 6 bp upstream of the TATA box, suggesting that a greater amount of RAR–RXR heterodimer may be required for activation of transcription initiation at a distance. Knock-outs of the RXRβ and RXRγ genes in F9 cells are required to investigate this possibility further.

The marked reduction in TGF-β2 transcript induction in RXRα$^{-/-}$ cells is noteworthy, since no RARE has been identified in this gene (Noma *et al.*, 1991). Further studies will show whether the lack of induction of TGF-β2 in RXRα$^{-/-}$ cells corresponds to a secondary event. Further experiments are also necessary to determine whether the impaired anti-proliferative response to tRA in RXRα$^{-/-}$ cells is related to a decreased level of TGF-β2 (see below).

Mediation of anti-proliferative and apoptotic effects of RA in F9 cells by RARs and RXRs

There has long been an interest in understanding the mechanism by which retinoids control the proliferation of a variety of cells. Much of this interest derives from observations that retinoids can decrease the growth rate of numerous transformed cell lines (Amos and Lotan, 1991), can suppress experimental carcinogenesis in animals, as well as exhibit varying degrees of efficacy in chemoprevention and therapy of certain cancers (for reviews see Smith *et al.*, 1992; Hong and Itri, 1994; Love and Gudas, 1994). The anti-tumoral effect of retinoids in precancerous and cancer cells is thought to be related to the induction of differentiation and to the concomitant reduction in proliferation rate. However, retinoids can also induce apoptotic responses in cultured cell lines (Martin *et al.*, 1990; Atencia *et al.*, 1994; Nagy *et al.*, 1995), raising the possibility that at least part of the anti-carcinogenic effect of retinoids could be related to the induction of apoptosis. Besides playing an important role in many biological processes, including tissue remodeling and morphogenesis (for reviews see Fesus *et al.*, 1991; Raff *et al.*, 1993), apoptosis may indeed provide an additional mechanism by which many cancer treatments kill tumor cells (Fisher, 1994).

Although F9 cells have served primarily as a model system for retinoid-induced differentiation, the anti-proliferative effect of RA on these cells is well documented (Linder *et al.*, 1981; Rosenstraus *et al.*, 1982; Sleigh, 1992). In fact, one initial aim for generating RAR and RXR-null cells has been to gain an understanding of the growth inhibitory effect of retinoids, and possibly

determine how this effect may relate to differentiation. Somewhat unexpectedly, it was found that the knockout of RARα or RARγ does not affect RA-induced growth arrest, even though in the case of RARγ$^{-/-}$ cells, differentiation by RA is markedly impaired (Boylan *et al.*, 1993, 1995). In contrast, we have shown here that the loss of RXRα results in a substantially reduced anti-proliferative response, as well as an impaired apoptotic response, which most probably also contributes to the difference in cell number between tRA-treated WT and RXRα$^{-/-}$ cells. As is the case for differentiation, RXR–RAR heterodimers appear to mediate the retinoid effects on both inhibition of cell proliferation and apoptosis, since neither of these two effects could be induced by the RXR-specific retinoid BMS649 alone. Furthermore, the combination of a sub-optimal concentration of an RARα-selective retinoid (AM80) and the pan-RXR-specific retinoid (BMS649) strongly synergizes to inhibit proliferation (but not to induce differentiation) in the RARγ$^{-/-}$ cells, clearly indicating an involvement of RARα in the inhibition of proliferation. Thus, although the requirement of individual RARs and RXRs for mediating differentiation, inhibition of proliferation and apoptosis may be different, RAR–RXR heterodimers appear to be involved in all three cases. In this respect, it is interesting to note that, as previously observed for cell differentiation (see Roy *et al.*, 1995), inhibition of F9 cell proliferation can be achieved by a saturating concentration of a RAR-specific ligand, in the absence of a RXR ligand, whereas activation of both RARs and RXRs is required to obtain efficient apoptosis (Table I).

Since RXRs can serve as heterodimerization partners for several other members of the nuclear receptor superfamily (Leblanc and Stunnenberg, 1995), some aspects of the anti-proliferative and apoptotic responses of F9 cells to retinoids may nevertheless involve other RXR partners. Possible candidates for such a partnership include the orphan receptors, Nurr1 and NGFI-B (Nur77), which can heterodimerize with RXRs and confer RXR-specific ligand-inducibility to RARE reporter constructs (Forman *et al.*, 1995; Leblanc and Stunnenberg, 1995; Perlmann and Jansson, 1995). Interestingly, the Nurr1 and NGFI-B genes are rapidly upregulated by mitogens, suggesting a role for these proteins in regulating cellular proliferation (Hazel *et al.*, 1988; Law *et al.*, 1992). The phenotype of RXRα$^{-/-}$ cells, wherein both proliferation characteristics and retinoid-induced differentiation are altered, could therefore also reflect a defect at the convergence point of the mitogen and retinoid signaling pathways. In any event, regardless of whether RAR-independent pathways controlling proliferation and apoptosis exist, it is clear from the present study that, at least in F9 cells, RXRα plays an important role in the control of these processes, in addition to its role in mediating the differentiation response. Finally, we cannot unequivocally rule out that, in the process of targeting and selection for neomycin resistant clones, some other genetic alteration has occurred, which is unrelated to the disruption of the RXRα gene. However, the finding that all independently isolated RXRα$^{-/-}$ clones exhibit an elevated proliferation rate, and that the heterozygote clone from which they were derived exhibits a rate of proliferation intermediate between those of F9 WT and RXRα$^{-/-}$ cells (data not shown), makes this possibility unlikely.

As was the case for the RARγ$^{-/-}$ F9 cells (Boylan *et al.*, 1993), it is difficult to reconcile the phenotype of RXRα$^{-/-}$ cells with that of RXRα$^{-/-}$ mice, which do not exhibit any obvious defect in cell proliferation (Kastner *et al.*, 1995). The maintainance of normal cell proliferation, differentiation and death in the embryo are tightly regulated by a variety of extracellular signals not present in the cell culture environment. This may explain the absence of a widespread proliferative defect in RXRα$^{-/-}$ mice, where cells are subject to far more complex proliferation controls.

Materials and methods

Cell culture

F9 cells were cultured as previously described (Boylan *et al.*, 1993). F9 cells were induced to differentiate into primitive and parietal endoderm by treatment with 1 μM tRA alone, or in combination with 250 μM N^6,$O^{2'}$-bt$_2$cAMP (Sigma), respectively (Strickland *et al.*, 1980). Cells were refed with fresh media containing drugs every 2 days. Induction of VE differentiation was essentially performed as previously described (Rogers *et al.*, 1990). Single-cell suspensions were seeded at a density of 1×10^5 cells/ml, treated with 50 nM tRA, and fed every 2 days with fresh medium and tRA. The aggregate suspensions were split into two cultures after 4 days growth and maintained at this density throughout the experiments. The retinoids (tRA, AM80, BMS649 and CD666) were dissolved in ethanol to a concentration of 1 mM and diluted to the appropriate final concentrations in culture media at time zero of retinoid treatment.

Targeting of the RXRα gene locus

The targeting vector, pRXRα$^{(LNL)}$, was constructed as follows: a phage containing an 11.8 kb genomic fragment containing RXRα exons 2, 3 and 4, was isolated from a genomic library established in λEMBL3 from the P19 EC cell line. After partial filling in, a full length *Sal*I insert derived from this phage was subcloned into the partially filled *Bam*HI site of a pBluescript II SK+ (Stratagene) derivative in which the *Xho*I site was destroyed. A unique *Xho*I site, followed by three in frame stop codons, was introduced at the *Acc*I site located at nucleotide 637 (Mangelsdorf *et al.*, 1990) in exon 4 of RXRα by inserting the oligonucleotides 5′-ATTATTATTACTCGAGTGATGATG-3′ and 5′-ATCATCATCACTCGAGTAATAATA-3′, which also destroys the *Acc*I site. An *Xho*I fragment containing a fusion of the herpes simplex virus thymidine kinase (TK) and bacterial neomycin phosphotransferase gene (neo) driven by the phosphoglycerate kinase promoter, and flanked by *loxP* sites (Metzger *et al.*, 1995), was inserted into this *Xho*I site.

Electroporation of F9 cells with pRXRα$^{(LNL)}$, selection of neomycin resistant clones and analysis were done as described (Metzger *et al.*, 1995). RXRα$^{+/-(LNL)}$ cells (5×10^6) were electroporated with 5 μg of the Cre enzyme expression vector pPGK-Cre, and replated at a density of 5×10^3 cells per 10 cm plate, followed 24 h later by treatment with 2 μM gancyclovir for 8 days. Correct excision events yielding RXRα$^{+/-(L)}$ cells were determined by Southern blotting of DNA from individual gancyclovir-resistant clones. A subclone of the clone 28 RXRα$^{+/-(L)}$ cell line (c28-3) was re-electroporated as above with pRXRα$^{(LNL)}$, followed by Cre-mediated excision, to yield RXRα$^{-(L)/-(L)}$ cell lines (also called RXRα$^{-/-}$) lacking any integrated antibiotic resistance genes.

Western blotting

Western blot analysis was performed as previously described (Kastner *et al.*, 1994) using the rabbit polyclonal antibody RPRXα(A), directed against the N-terminal A/B domain of RXRα (Rochette-Egly *et al.*, 1994). Chemiluminescence detection was performed according to the manufacturer's instructions (Amersham).

RT–PCR

RNA preparation, RT–PCR and Southern blotting was performed as described previously (Bouillet *et al.*, 1995; Roy *et al.*, 1995). The PCR primers were as follows: collagen IVα1, 5′-ATGAATTCTCAGCGT-CTGGCTTCTGCTG-3′ (nt 155–174) and 5′-ATGGATCCGTTGCATC-CTGGGATACCTG-3′ (nt 500–519) (Killen *et al.*, 1988); laminin

B1, 5′-ATGAATTCTCACTGCAGACATGGTGAAG-3′ (nt 4946–4965) and 5′-ATGGATCCCTCACTTATGTCCTTAAGGA-3′ (nt 5489–5508) (Sasaki *et al.*, 1987); TGF-β2, 5′-ATGAATTCGAAATGTGCAGGATAATTGC-3′ (nt 2149–2168) and 5′-ATTCTAGATTACAAGACTTGACAATCAT-3′ (nt 2433–2451) (Kelly *et al.*, 1990); Hoxa-1, 5′-CTACTTACCAGACTTCTGGA-3′ (nt 301–320) and 5′-CAAAGGTCTGCGCTGGAGAA-3′ (nt 665-684) (LaRosa and Gudas, 1988b); Hoxb-1, 5′-ATGAATTCTCAGAACCCAGCACTCTCAC-3′ (nt 1130–1149) and 5′-ATTCTAGAAGTCTTCGAGAGAACTGGTC-3′ (nt 1562–1581) (Murphy and Hill, 1991); RARβ2, 5′-GCCGAATGGCAGCACCG-3′ (nt 589–605) and 5′-GCTCTCTGTGCATTCCTGCT-3′ (nt 953–972) (Zelent *et al.*, 1991); CRABPII, 5′-GGAGACAGCAAAGTATCTTTA-3′ (nt 29–49) and 5′-GGAACATCAGACACACTAAA-3′ (nt 806–825) (Giguère *et al.*, 1990); Cdx-1, 5′-ACTGCCTACCTAGGACAAGT-3′ (nt 860–879) and 5′-GTGAGGCTGGAAGAGGAGAC-3′ (nt 1211–1220) (Duprey *et al.*, 1988); AFP, 5′-ATGAATTCACATCAGTGTCTGCTGGCAC-3′ (nt 308–327) and 5′-ATTCTAGAGCGAGTTTCCTTGGCAACAC-3′ (nt 750–769) (Gorin *et al.*, 1981); 36B4, 5′-CAGCTCTGGAGAAACTGCTG-3′ (nt 290–309) and 5′-GTGTACTCAGTCTCCACAGA-3′ (nt 826-845) (Krowczynska *et al.*, 1989). The following oligonucleotides, internal to their respective PCR primers, were end-labeled for probing the Southern blots: Collagen IVα1, 5′-GGTTGCAAGGTGTCATTGGATTTCC-3′ (nt 305–330); Laminin B1, 5′-TCAGCAGATGCCAGGAGGAAAGCTG-3′ (nt 5311–5335); TGF-β2, 5′-CTCAACACACCAAAGTCCTCAGCCT-3′ (nt 2290–2314); Hoxb-1, 5′-CCCAGTTCCATCACCTCTTGAATTG-3′ (nt 1508–1532); AFP, 5′-GCTGACAACAAGGAGGAGTG-3′ (nt 529–538); and 36B4 5′-ATGTGAAGTCACTGTGCCAG-3′ (nt 426–445). RARβ2, Hoxa-1, CRABPII and Cdx-1 RT–PCR were probed with random-primed mRARβø total plasmid (Zelent *et al.*, 1989), the *Eco*RI–*Hin*dIII fragment from pERA-1-993 (LaRosa and Gudas, 1988b), pGEM-CRABPII (Boylan *et al.*, 1995) and a 267 bp fragment from the mouse Cdx-1 cDNA (Duprey *et al.*, 1988) as templates, respectively. All fold inductions were calculated after normalizing to the corresponding 36B4 mRNA levels.

Immunohistochemistry

Aggregates of F9 WT and RXRα$^{-/-}$ cells, cultured in the presence or absence of 50 nM tRA for 11 days, were washed in PBS and quick-frozen in OCT compound on dry ice before sectioning into 5 μm sections and immunostaining with a rabbit polyclonal antibody against all forms of laminin (Sigma) and CY3 indocarbocyanine-conjugated donkey anti-rabbit IgG as second antibody (Jackson Immunoresearch Labs, Inc). The sections were also stained with Hoechst 33258 dye to reveal the location of nuclei, and photographed on a fluorescence microscope at a magnification of 150×.

Analysis of cellular growth

Cell counting experiments were performed for untreated and tRA-treated cells as follows: cells were plated at identical densities (2.5×10^3 cells/well) in triplicate 3 cm culture wells, fed with fresh media on days 2 and 4, and on day 5, trypsinized, resuspended to appropriate dilutions and counted on a Coultronics particle counter (Coultronics France, SA). The percentage growth inhibition by tRA was calculated using the equation: (1-R/C)×100, where R and C represent the number of cells in tRA-treated and control cultures, respectively. The DT was calculated according to Kuchler (1977). The cell cycle profile of WT and null mutant clones was determined by cell cycle flow cytometry based on cellular DNA content, using an Epics Profile II cell sorter (Coulter Electronics, Inc.), equipped with a 15 W argon laser at a wavelength of 488 nm, and an emission wavelength of 575 nm. Subconfluent cultures of untreated or tRA-treated cells were trypsinized, resuspended and combined with their supernatents, and fixed in 70% ethanol at 4°C. Following two washes in PBS, the cells and subcellular particles were incubated in 1 mg/ml RNase A (59 Kunitz U/mg, Sigma Chemical Co.) for 30 min at 37°C, centrifuged and resuspended in PBS at a concentration of ~10^6 cells per ml. Ethidium bromide was added to a final concentration of 50 μg/ml immediately prior to sample analysis. The percentage of cells in the different phases of the cell cycle was determined from the raw data using the Epics (R) Elite Flow Cytometry software. [^{3}H]thymidine incorporation assays were performed essentially as described (Heath, 1987), with the following modifications. F9 cells were plated in six replicate 1 cm wells and after 5 days of growth in the presence or absence of 1 μM tRA, three of six wells were treated with 2 μCi/well [^{3}H]methylthymidine (20.0 Ci/mmol, Dupont NEN) 10 h prior to the end of the experiment. The TCA-precipitable material from the labeled wells was measured by scintillation counting and the cell number determined as above for the corresponding unlabeled replicate wells.

Analysis of apoptosis

For the DNA fragmentation assay, F9 cells were plated either in the presence or absence of 1 μM tRA for 6 days, scraped in PBS, combined with the culture supernatants and spun at 250 *g* for 5 min. Low molecular weight DNA, extraction and analysis were performed as described (Nagy *et al.*, 1995). For Hoechst staining of nuclei, cells were grown on gelatinized, 1 cm diameter plastic disks (Nunc), either in the presence or absence of 1 μM tRA for 5 days. After fixation in 4% paraformaldehyde, cells were stained with 5 μg/ml Hoechst 33258 dye for 1 min, washed three times with PBS and photographed on a fluorescence microscope.

Acknowledgements

We are grateful to P.Reczek (Bristol Meyers Squibb, Pharmaceutical Research Institute, Buffalo, NY) for the gift of BMS649; to B.Shroot (CIRD Galderma, Valbonne, France) for CD666; to K.Shudo (University of Tokyo, Tokyo, Japan) for AM80; to P.Bouillet, J.Brocard, M.P.Gaub, P.Kastner, B.Mascrez, C.Rochette-Egly, B.Roy and R.Taneja for various oligonucleotides and plasmids and RPRXα(A) antibody; to C.Ebel and C.Waltzinger for help with FACS analysis; and to the P.Gruss laboratory for the Cdx-1 cDNA. We thank M.P.Gaub, C.Rochette-Egly and H.Gronemeyer for helpful discussions and critical reading of the manuscript. Thanks to F.Rijli, S.Kato, C.Mendelsohn, C.Lampron, J.Mountford and all other members of the retinoid group for helpful discussions. We thank the cell culture group for providing cells, F.Ruffenach for oligonucleotide synthesis, the secretarial staff for typing and C.Werlé, S.Metz, B.Boulay and J.M.Lafontaine for preparing the figures. Special thanks to P.Leroy for genomic cloning and help with the initial constructions. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Human Frontier Science Program and Bristol Myers Squibb. H.C. was supported by a fellowship from the Centre National de la Recherche Scientifique, J.C. by a fellowship from the Association pour la Recherche sur le Cancer and the US National Institutes of Health (IF32GM15857).

References

Amos,B. and Lotan,R. (1991) Retinoid-sensitive cells and cell lines. *Methods Enzymol.*, **190**, 217–225.

Atencia,R., Garcia-Sanz,M., Unda,F. and Arechaga,J. (1994) Apoptosis during retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *Exp. Cell Res.*, **214**, 663–667.

Bouillet,P., Oulad-Abdelghani,M., Vicaire,S., Garnier,J.-M., Schuhbaur,B., Dollé,P. and Chambon,P. (1995) Efficient cloning of cDNAs of retinoic acid-responsive genes in P19 embryonal carcinoma cells and characterization of a novel mouse gene, Stra1 (Mouse LERK-2/Eplg2). *Dev. Biol.*, **170**, 420–433.

Boylan,J., Lohnes,D., Taneja,R., Chambon,P. and Gudas,L.J. (1993) Loss of RARγ function by gene disruption results in aberrant *Hoxa-1* expression and differentiation upon retinoic acid treatment. *Proc. Natl Acad. Sci. USA*, **90**, 9601–9605.

Boylan,J., Lufkin,T., Achkar,C.C., Taneja,R., Chambon,P. and Gudas,L.J. (1995) Targeted disruption of retinoic acid receptor α (RARα) and RARγ results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Mol. Cell. Biol.*, **15**, 843–851.

Chambon,P. (1994) The retinoid signaling pathway: molecular and genetic analyses. *Semin. Cell Biol.*, **5**, 115–125.

deThé,H., Vivanco-Ruiz,M., Tiollais,P., Stunnenberg,H. and Dejean,A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature*, **343**, 177–180.

Duprey,P., Chowdhury,K., Dressler,G.R., Balling,R., Simon,D., Guenet,J.-L. and Gruss,P. (1988) A mouse gene homologous to the drosophila gene caudal is expressed in epithelial cells from the embryonic intestine. *Genes Dev.*, **2**, 1647–1654.

Durand,B., Saunders,M., Leroy,P., Leid,M. and Chambon,P. (1992) All-*trans* and 9-*cis* retinoic acid induction of CRABPII transcription is

mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell*, **71**, 73–85.

Durand,B., Saunders,M., Gaudon,C., Roy,B., Losson,R. and Chambon,P. (1994) Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activation domain and influence of the nature of the response element on AF-2 activity. *EMBO J.*, **13**, 5370–5382.

Fesus,L., Davies,P.J.A. and Piacentini,M. (1991) Apoptosis: molecular mechanisms in programmed cell death. *Eur. J. Cell Biol.*, **56**, 170–177.

Fisher,D.E. (1994) Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539–542.

Forman,B.M., Umesono,K., Chen,J. and Evans,R.M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, **81**, 541–550.

Giguère,V. (1994) Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocrine Rev.*, **15**, 61–79.

Giguère,V., Lyn,S., Yip,P., Siu,C.-H. and Amin,S. (1990) Molecular cloning of cDNA encoding a second cellular retinoic acid-binding protein. *Proc. Natl Acad. Sci. USA*, **87**, 6233–6237.

Glass,C.K. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrine Rev.*, **15**, 391–407.

Glick,A.B., Flanders,K.C., Danielpour,D., Yuspa,S.H. and Sporn,M.B. (1989) Retinoic acid induces transforming growth factor-β2 in cultured keratinocytes and mouse epidermis. *Cell Regul.*, **1**, 87–97.

Gorin,M.B., Cooper,D.L., Eiferman,F., van de Rijn,P. and Tilghman,S.M. (1981) The evolution of α-fetoprotein and albumin I. A comparison of the primary amino acid sequences of mammalian α-fetoprotein and albumin. *J. Biol. Chem.*, **256**, 1954–1959.

Gronemeyer,H. and Laudet,V. (1995) Nuclear receptors. *Protein Profile*, **2**, 1173–1308.

Gudas,L.J., Sporn,M.B. and Roberts,A.B. (1994) Cellular biology and biochemistry of the retinoids. In Sporn,M.B. and Roberts,A.B. (eds), *The Retinoids: Biology, Chemistry, and Medicine*. Raven Press, New York, pp. 443–520.

Hazel,T.G., Nathans,D. and Lau,L. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl Acad. Sci. USA*, **85**, 8444–8448.

Heath,J.K. (1987) Experimental analysis of teratocarcinoma cell multiplication and purification of embryonal carcinoma-derived growth factor. In Robertson,E.J. (ed.), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRL Press, Oxford, UK, pp. 183–206.

Hogan,B.L., Taylor,A. and Adamson,E. (1981) Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature*, **291**, 235–237.

Hogan,B.L.M., Barlow,D. and Tilly,R. (1983) F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surveys*, **2**, 115–140.

Hong,W.K. and Itri,L.M. (1994) Retinoids and human cancer. In Sporn,M.B. and Roberts,A.B. (eds), *The Retinoids: Biology, Chemistry, and Medicine*. Raven Press, New York, pp. 597–630.

Kastner,P., Grondona,J.M., Mark,M., Gansmuller,A., LeMeur,M., Decimo,D., Vonesch,J., Dollé,P. and Chambon,P. (1994) Genetic analysis of RXRα developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell*, **78**, 987–1003.

Kastner,P., Mark,M. and Chambon,P. (1995) Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell*, **83**, 859–869.

Kastner,P. *et al.* (1996) Abnormal spermatogenesis in RXRβ mutant mice. *Genes Dev.*, **10**, 80–92.

Kelly,D., Campbell,W.J., Tiesman,J. and Rizzino,A. (1990) Regulation and expression of transforming growth factor type-β during early mammalian development. *Cytotechnology*, **4**, 227–242.

Kerr,J.F.R., Gobé,G.C., Winterford,C.M. and Harmon,B.V. (1995) Anatomical methods in cell death. *Methods Cell Biol.*, **46**, 1–27.

Killen,P.D., Burbelo,P., Sakurai,Y. and Yamada,Y. (1988) Structure of the amino-terminal portion of the murine α1(IV) collagen chain and the corresponding region of the gene. *J. Biol. Chem.*, **263**, 8706–8709.

Krowczynska,A.M., Coutts,M., Makrides,S. and Brawerman,G. (1989) The mouse homologue of the human acidic ribosomal phosphoprotein PO: a highly conserved polypeptide that is under translational control. *Nucleic Acids Res.*, **17**, 6408.

Kuchler,R. (1977) Development of animal cell populations *in vitro*. In Kuchler,R. (ed.), *Biochemical Methods in Cell Culture and Virology*. Dowden, Hutchinson and Ross Inc., Stroudsburg, PA, pp. 90–113.

Langston,A.W. and Gudas,L.J. (1992) Identification of a retinoic acid responsive enhancer 3′ of the murine homeobox gene Hox-1.6. *Mech. Dev.*, **38**, 217–228.

LaRosa,G.L. and Gudas,L.J. (1988a) An early effect of retinoic acid: Cloning of an mRNA (Era-1) exhibiting rapid and protein synthesis-independent induction during teratocarcinoma stem cell differentiation. *Proc. Natl Acad. Sci. USA*, **85**, 329–333.

LaRosa,G.L. and Gudas,L.J. (1988b) Early retinoic acid-induced F9 teratocarcinoma stem cell gene ERA-1: alternate splicing creates transcripts for a homeobox-containing protein and one lacking the homeobox. *Mol. Cell. Biol.*, **8**, 3906–3917.

Law,S.W., Conneely,O.M., DeMayo,F.J. and O'Malley,B.W. (1992) Identification of a new brain-specific transcription factor, NURR1. *Mol. Endocrinol.*, **6**, 2129–2135.

Leblanc,B.P. and Stunnenberg,H.G. (1995) 9-cis retinoic acid signaling: changing partners causes some excitement. *Genes Dev.*, **9**, 1811–1816.

Lehmann,J.M., Jong,L., Fanjul,A., Cameron,J.F., Lu,X.P., Haefner,P., Dawson,M.I. and Pfahl,M. (1992) Retinoids selective for retinoid X receptor response pathways. *Science*, **258**, 1944–1946.

Leid,M., Kastner,P. and Chambon,P. (1992a) Multiplicity generates diversity in the retinoic acid signaling pathways. *Trends Biochem. Sci.*, **17**, 427–433.

Leid,M. *et al.* (1992b) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell*, **68**, 377–395.

Linder,S., Krondahl,U., Sennerstam,R. and Ringertz,N.R. (1981) Retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *Exp. Cell Res.*, **132**, 453–460.

Lotan,R. (1980) Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta*, **605**, 33–91.

Love,J.M. and Gudas,L.J. (1994) Vitamin A, differentiation and cancer. *Curr. Biol.*, **6**, 825–831.

Mangelsdorf,D.J. and Evans,R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841–850.

Mangelsdorf,D.J., Ong,E.S., Dyck,J.A. and Evans,R.M. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature*, **345**, 224–229.

Marshall,H., Studer,M., Popperl,H., Aparicio,S., Kuroiwa,A., Brenner,S. and Krumlauf,R. (1994) A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature*, **370**, 567–571.

Martin,S.J., Bradley,J.G. and Cotter,T.G. (1990) HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin. Exp. Immunol.*, **79**, 448–453.

Metzger,D., Clifford,J., Chiba,H. and Chambon,P. (1995) Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre-recombinase. *Proc. Natl Acad. Sci. USA*, **92**, 6991–6995.

Meyer,B.I. and Gruss,P. (1993) Mouse Cdx1 expression during gastrulation. *Development*, **117**, 191–203.

Murphy,P. and Hill,R.E. (1991) Expression of the mouse labial-like homeobox-containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain. *Development*, **111**, 61–74.

Nagy,L., Thomazy,V.A., Shipley,G.L., Fesus,L., Lamph,W., Heyman, R.A., Chandraratna,R.A.S. and Davies,P.J.A. (1995) Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. *Mol. Cell Biol.*, **15**, 3540–3551.

Noma,T., Glick,A.B., Geiser,A.G., O'Reilly,M.A., Miller,J., Roberts,A.B. and Sporn,M.B. (1991) Molecular cloning and structure of the human transforming growth factor-β2 gene promoter. *Growth Factors*, **4**, 247–255.

Perlmann,T. and Jansson,L. (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes Dev.*, **9**, 769–782.

Raff,M.C., Barres,B.A., Burne,J.F., Coles,H.S., Ishizaki,Y. and Jacobson,M.D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science*, **262**, 695–700.

Rochette-Egly,C., Lutz,Y., Pfister,V., Heyberger,S., Scheuer,I., Chambon,P. and Gaub,M.-P. (1994) Detection of retinoid X receptors using specific monoclonal and polyclonal antibodies. *Biochem. Biophys. Res. Commun.*, **204**, 525–536.

Rogers,M.B., Watkins,S.C. and Gudas,L.J. (1990) Gene expression in visceral endoderm: A comparison of mutant and wild-type F9 embryonal carcinoma cell differentiation. *J. Cell Biol.*, **110**, 1767–1777.

Rosenstraus,M.J., Sundell,C.L. and Liskay,R.M. (1982) Cell-cycle characteristics of undifferentiated and differentiating embryonal carcinoma cells. *Dev. Biol.*, **89**, 516–520.

Roy,B., Taneja,R. and Chambon,P. (1995) Synergistic activation of expression of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor α (RARα)-, RARβ-, or RARγ-selective ligand in combination with a retinoid X receptor-specific ligand. *Mol. Cell. Biol.*, **15**, 6481–6487.

Sasaki,M., Kato,S., Kohno,K., Martin,G.R. and Yamada,Y. (1987) Sequence of the cDNA encoding the laminin B1 chain reveals a multidomain protein containing cysteine-rich repeats. *Proc. Natl Acad. Sci. USA*, **84**, 935–939.

Sauer,B. and Henderson,N. (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.*, **2**, 441–449.

Sleigh,M.J. (1992) Differentiation and proliferation in mouse embryonal carcinoma cells. *BioEssays*, **14**, 769–775.

Smith,M.A., Parkinson,D.R., Cheson,B.D. and Friedman,M.A. (1992) Retinoids in cancer therapy. *J. Clin. Oncol.*, **10**, 839–864.

Strickland,S. and Mahdavi,V. (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell*, **15**, 393–403.

Strickland,S., Smith,K.K. and Marotti,K.R. (1980) Hormonal induction of differentiation in teratocarcinoma stem cells: Generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell*, **21**, 347–355.

Sucov,H.M., Dyson,E., Gumeringer,C.L., Price,J., Chien,K.R. and Evans,R.M. (1994) RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev.*, **8**, 1007–1018.

Taneja,R., Bouillet,P., Boylan,J.F., Gaub,M.-P., Roy,B., Gudas,L.J. and Chambon,P. (1995) 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. *Proc. Natl Acad. Sci. USA*, **92**, 7854–7858.

Wan,Y.-J., Wang,L. and Wu,T.-C. (1994) The expression of retinoid X receptor genes is regulated by all-trans and 9-cis-retinoic acid in F9 teratocarcinoma cells. *Exp. Cell Res.*, **210**, 56–61.

Wang,S.-Y. and Gudas,L.J. (1983) Isolation of cDNA clones specific for collagen IV and laminin from mouse teratocarcinoma cells. *Proc. Natl Acad. Sci. USA*, **80**, 5880–5884.

Yu,V.C. *et al.* (1991) RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell*, **67**, 1251–1266.

Zelent,A., Krust,A., Petkovich,M., Kastner,P. and Chambon,P. (1989) Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. *Nature*, **339**, 714–717.

Zelent,A., Mendelsohn,C., Kastner,P., Krust,A., Garnier,J.-M., Ruffenach,F., Leroy,P. and Chambon,P. (1991) Differentially expressed isoforms of the mouse retinoic acid receptor β are generated by usage of two promoters and alternative splicing. *EMBO J.*, **10**, 71–81.

Received on March 5, 1996