MOLECULAR AND CELLULAR BIOLOGY, Dec. 1995, p. 6481–6487 Vol. 15, No. 12
0270-7306/95/$04.00+0

Synergistic Activation 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

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Received 1 June 1995/Returned for modification 8 August 1995/Accepted 30 August 1995

Retinoic acid receptor (RAR)-retinoid X receptor (RXR) heterodimers bind to cognate response elements in vitro more efficiently than do RAR or RXR homodimers, and both RAR and RXR partners have been shown to activate various promoters in transiently transfected cells. We have now investigated whether ligand-dependent activation of both heterodimeric partners is involved in induced expression of endogenous RA-responsive genes and in P19 and F9 cell differentiation. On their own, low concentrations of retinoids selective for either RARα, RARβ, or RARγ did not induce or very inefficiently induced the expression of several RA target genes or triggered differentiation. An RXR-specific synthetic retinoid was similarly inefficient at any concentration. In contrast, at the same concentrations, various combinations of RAR (RARα, RARβ, or RARγ) and RXR selective retinoids resulted in synergistic induction of all retinoic acid (RA) target genes examined, as well as in cell differentiation. However, the magnitude of this synergistic activation varied depending on both the RAR-RXR combination and the promoter context of the responsive genes. Promiscuous activation of the three RARs, or concomitant activation of RARα and RARγ, at selective retinoid concentrations also resulted in induction of gene expression and cell differentiation. Taken together, our results are consistent with the conclusion that the RAR and RXR partners of RAR-RXR heterodimers can synergistically activate transcription of RA-responsive genes and can induce differentiation of P19 and F9 cells. Our results also indicate that there is a significant degree of functional redundancy between the three RAR types which, however, varies with the nature of the RA target genes.

Retinoids, the derivatives of vitamin A, are required for cell growth and differentiation. The differentiation of P19 and F9 embryonal carcinoma (EC) cells upon treatment with retinoic acid (RA) is generally concomitant with a reduction in cell proliferation and an altered expression of several differentiation-specific RA-responsive genes (reviewed in reference 19). The various biological effects of RA and synthetic retinoids are mediated by two families of ligand-dependent regulators, the RA receptors (RARs) and the retinoid X receptors (RXRs), which are members of the nuclear receptor superfamily (for reviews, see references 10, 17, 18, 23, 29, 33, and 38). RARs bind and are activated by all-*trans*-RA (t-RA) and 9-*cis*-RA (9C-RA) with K_d values in the range of 0.2 to 0.7 nM, whereas RXRs bind and are activated by 9C-RA only with K_d values of 1.4 to 2.4 nM (1, 2, 21, 31). The various members of the two retinoid receptor families efficiently bind as either RAR-RXR heterodimers or RXR homodimers to cognate response elements in vitro (9, 24, 30, 34, 41–43) and in transfected cells overexpressing RXRs and/or RARs (14, 15). RAR-RXR heterodimers efficiently bind to and activate transcription in the presence of t-RA or 9C-RA from directly repeated half-sites [PuG(G/T)TCA motif] with a spacing of 5 bp (DR5 elements), 2 bp (DR2 elements), or 1 bp (DR1 elements) (14, 15; reviewed in references 18, 23, 29, 33, and 36). In response to 9C-RA, RXR homodimers can activate transcription from directly repeated PuGGTCA motifs separated by 1 bp (DR1 elements) (15, 18, 29, 33, 36, 43). Furthermore, RXRs also heterodimerize with other members of the nuclear receptor superfamily, thus providing a means for cross talk between several signalling pathways (for reviews, see references 17, 23, 29, and 33).

Using the synthetic ligands Am80 (20), CD666 (4), BMS189,453 (11), and BMS188,649 (28; also known as SR11237), which, at appropriate concentrations, are RARα-, RARγ-, RARβ-, and RXR (α, β, and γ)-selective agonists respectively, we have investigated whether the ligand-dependent activation of both RAR and RXR is needed for inducing the expression of RA-responsive genes and triggering morphological differentiation of P19 and F9 embryonal carcinoma (EC) stem cells. We demonstrate that at selective concentrations (low concentrations), RAR synthetic retinoids can induce the expression of a few endogenous RA target genes on their own to a low level but do not promote P19 or F9 cell morphological differentiation. The RXR-selective ligand at any concentration is also inefficient in triggering target gene activation and differentiation. In contrast, activation of both RAR and RXR with various combinations of synthetic retinoids at selective concentrations results in strong synergistic inductions of expression of RA-responsive genes and also in morphological differentiation of P19 and F9 cells. The extent of this synergism is variable

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with different combinations of synthetic retinoids and appears to be dependent on the response element and promoter context of the target genes. However, at nonselective concentrations, the RAR-specific retinoids Am80 and CD666 can activate RA target genes and can also trigger the morphological differentiation of both P19 and F9 cells, showing that activation of the RAR partner can be sufficient for RAR-RXR heterodimers to induce transcriptional activation of target genes. Interestingly, the concomitant activation of RARα and RARγ at selective concentrations also results in a synergistic activation of target genes and morphological differentiation of both P19 and F9 cells.

MATERIALS AND METHODS

Cell culture and retinoid treatment. P19 EC cells were grown in monolayer in Dulbecco's modified Eagle's medium–F-12 medium containing 44 mM sodium bicarbonate and 7.5% fetal calf serum and maintained as described previously (37). F9 cells were grown in monolayer in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (7, 8). Retinoids (t-RA, 9C-RA, Am80, BMS189,453, CD666, and BMS188,649) were dissolved in ethanol and added to fresh medium containing fetal calf serum treated with 5% charcoal; control cells were treated with ethanol alone (final concentration, 0.1%). For differentiation studies, P19 and F9 cells (10^4 cells per 10-cm-diameter dish) were treated with retinoids for 96 h with a change of medium after 48 h.

RNA isolation. RNA was isolated by the guanidinium thiocyanate method followed by ultracentrifugation through a CsCl solution (12) and was dissolved in water.

Semiquantitative RT-PCR. Conditions for reverse transcription-PCR (RT-PCR) were as described previously (6). Primers (19- to 20-mers) with a G+C content of approximately 50% (whenever possible) were used, such that amplified products were 200 to 500 bp in length to ensure efficient PCR amplification. For every set of experiments, the quantity of RNA was normalized according to the signal obtained by Southern blot hybridization after RT-PCR of 36B4 mRNA (35), which encodes a mouse acidic ribosomal phosphoprotein and whose expression is not affected by retinoid treatment. After normalization, the same set of RNAs was used for RT-PCR amplification of RA-inducible transcripts. In general, 2 to 3 μg of total RNA was combined with 6 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 2 mM each deoxynucleoside triphosphate [pH 8.0]), 50 pmol of each primer, and water to a total volume of 60 μl. RNA was denatured at 94°C for 3 min. Primer was annealed to RNA by gradual (10-min) cooling to 50°C followed by addition of a mixture of 3.5 U of avian myeloblastosis virus reverse transcriptase (Pharmacia) and 2.5 U of *Taq* polymerase (Cetus) for 15 min at 50°C. Subsequent amplification was carried out for 15 to 25 cycles, depending on the abundance of the mRNA of a given gene. The program for one cycle was as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Aliquots of PCR products (20 μl) were taken from each tube at the end of the 72°C step of at least two cycles between the 15th and 25th cycles (depending on the RNA transcript analyzed, in order to ascertain that the amplification was in the linear range) and transferred to Hybond N^+ nylon membrane. Blots were prehybridized and hybridized at 42°C in solutions containing 50% formamide and cognate random-primed ^{32}P-labelled cDNA probes. The blots were washed at 50°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, and signals of PCR products were quantified with a bio-imaging analyzer (BAS 2000; Fuji Ltd.). The signal intensities of the various RNA samples from retinoid-treated cells were compared with that of the ethanol-treated control, which was given a value of 1.

RESULTS

Synergistic activation of RA-responsive genes by synthetic retinoids specific for RARs and RXRs in P19 cells. At low concentrations, the synthetic retinoids Am80 (reference 20 and our unpublished data) and CD666 (reference 4 and our unpublished data) selectively activate RARα and RARγ, respectively, whereas BMS189,453 (termed hereafter BMS453) (11) and BMS188,649 (termed hereafter BMS649; also known as SR11237) (28) specifically activate RARβ and all RXRs, respectively, regardless of their concentrations (references 11 and 28 and our unpublished results). In addition, BMS453 is a potent inhibitor of both RARα and RARγ (11). RT-PCR (6) was used to estimate the relative amount of RNA transcribed from the RA-inducible Stra1 and Stra2 (two novel RA-inducible genes [6]), CRABPII, RARβ, and Hoxa-1 (references 7, 8, 26, and 27 and references therein) in P19 cells treated with various doses of these synthetic retinoids (Fig. 1) (data not shown). The level of RNA induction was expressed relative to that in ethanol-treated control cells (Table 1). At nonselective concentrations, both Am80 and CD666 induced the expression of all five genes as efficiently as did t-RA (results not shown), whereas at low concentrations (1 nM Am80 and 1 or 10 nM CD666), the Stra1, Stra2, and CRABPII genes were not significantly induced and induction of the RARβ and Hoxa-1 genes was severely reduced. On the other hand, treatment of P19 cells with BMS453 (0.5 μM) or BMS649 (1 μM) resulted in very little or no induction in all cases (with the exception of the Stra1 and Stra2 genes in the case of BMS453), although these two ligands specifically and efficiently activated RARβ (11) and either RXRα, RXRβ, or RXRγ, respectively, when expressed in transfected HeLa cells (data not shown; note that all three RXR types are expressed in P19 cells [3]).

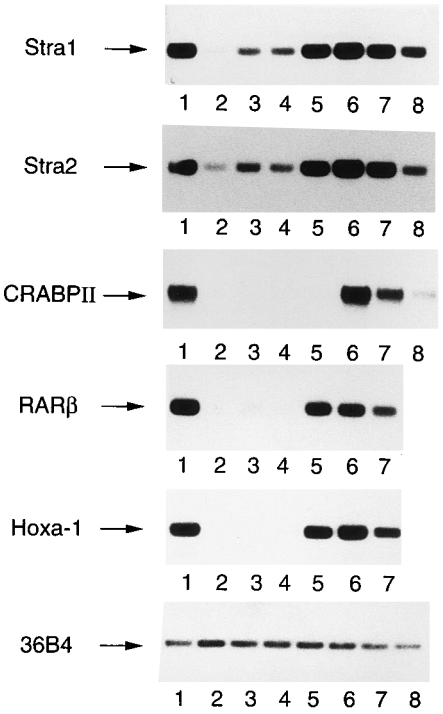

FIG. 1. RT-PCR determination of RNA transcripts of Stra1, Stra2, CRAB PII, RARβ, and Hoxa-1. P19 cells were treated for 24 h with either 100 nM Am80, ethanol vehicle, 0.5 μM BMS453, 1 nM Am80 plus 0.5 μM BMS453, 1 nM Am80 plus 10 nM CD666, 1 nM Am80 plus 1 μM BMS649, 10 nM CD666 plus 1 μM BMS649, or 0.5 μM BMS453 plus 1 μM BMS649 (lanes 1 to 8, respectively). RNA transcripts of the invariably expressed 36B4 gene (35) were used to normalize the RT-PCR assays.

In marked contrast to the above results, the combination of either Am80 (1 nM), CD666 (10 nM), or BMS453 (0.5 μM) with BMS649 (1 μM) resulted in synergistic inductions whose magnitudes (numbers in parentheses in Table 1) ranged from 2- to 48-fold, depending on the gene considered and the nature of the combination. In general, the synergism was highest with the Am80-BMS649 combination and lowest with the BMS453-BMS649 combination, with the exception of the RARβ gene, whose autoinduction in the presence of the RXR-specific ligand appeared to be particularly efficient. Note in this respect that the distinct profiles of synergistic activation of the RARβ and Hoxa-1 genes clearly illustrate the importance of the promoter context in the induction brought about by the three RAR types, since both genes possess the same RA response

TABLE 1. RNA induction by selective synthetic retinoids in P19 cells[a]

Retinoid	Concn	Fold induction relative to ethanol[b]				
		Stra1	Stra2	CRABPII	RARβ	Hoxa-1
Ethanol (vehicle)		1	1	1	1	1
t-RA	50 nM	12	5	2	7	35
9C-RA	50 nM	37	10	4	22	78
Am80	1 nM	2	1	1	7	7
	100 nM	22	10	40	40	101
CD666	1 nM	1	1	1	1	1
	10 nM	2	1	1	7	9
	1 μM	17	5	12	32	46
BMS453	0.5 μM	3	3	1	1	2
BMS649	1 μM	1	1	<2	<2	<2
Am80 + BMS453	1 nM + 0.5 μM	3	2	2	1	2
Am80 + CD666	1 nM + 10 nM	25 (6)	11 (11)	3 (3)	25 (2)	84 (5)
Am80 + BMS649	1 nM + 1 μM	50 (25) [100]	19 (19) [100]	48 (48) [100]	30 (4) [100]	119 (17) [100]
CD666 + BMS649	10 nM + 1 μM	33 (17) [66]	11 (11) [58]	13 (13) [27]	17 (2) [57]	51 (6) [43]
BMS453 + BMS649	0.5 μM + 1 μM	20 (7) [40]	8 (3) [42]	3 (3) [6]	18 (18) [60]	20 (10) [17]

[a] Relative levels of RNA transcripts for Stra1, Stra2, CRABPII, RARβ, and Hoxa-1 as estimated by semiquantitative RT-PCR. P19 cells were treated for 24 h with various retinoids as indicated.

[b] The level of RNA induction was expressed relative to the amount of RNA transcripts present in ethanol-treated cells, which was taken as 1. Numbers in brackets correspond to levels of induction relative to those achieved with 1 nM Am80 plus 1 μM BMS649 taken as 100%. Numbers in parentheses correspond to the extent of synergism over that achieved with 1 nM Am80 or 10 nM CD666 alone.

element (13, 26). In any event, these synergistic inductions are consistent with the conclusion that transactivation is brought about by heterodimers and also that the ligand-dependent activation functions of the RAR and RXR can strongly cooperate to stimulate the transcription of the different RA-responsive genes examined here. This cooperativity, which was maximum at 0.1 to 1 μM BMS649, could already be observed in the nanomolar range (1 to 10 nM) (Fig. 2). Interestingly, the profiles of synergistic activation as a function of increasing BMS649 concentrations in the presence of 1 nM Am80 were different for the three RA-responsive genes in Fig. 2, with *Stra4* (a novel RA-responsive gene [6]) being more responsive than Stra2 and with RARβ being the least responsive.

When compared with the Am80-BMS649 combination, no further increases in the levels of induction were observed when Am80 and CD666 were concomitantly added at selective concentrations to P19 cells together with the RXR-specific retinoid BMS649 (data not shown). In contrast, with the exception of the CRABPII gene, a surprising synergism was observed when the cells were treated with a combination of Am80 and CD666 at low concentrations (Table 1; Fig. 1). The level of induction was greater than the expected sum of the stimulation brought about by Am80 and CD666 on their own, albeit in general lower than that obtained with the Am80-BMS649 or CD666-BMS649 combinations. Note that no synergism could be observed between Am80 and BMS453 (Table 1) or between CD666 and BMS453 (results not shown), as expected from the known inhibitory effect of BMS453 on RARα and RARγ activity (11).

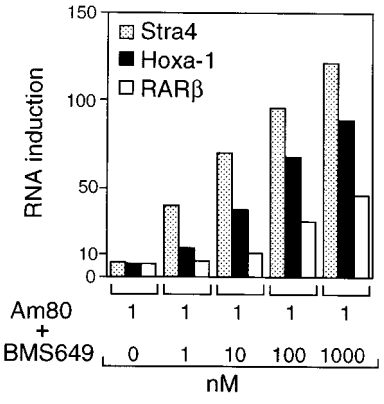

FIG. 2. Dose-response results showing the synergistic effect of 1 nM Am80 and increasing concentrations of BMS649 (0, 1, 10, 100, and 1,000 nM, as indicated) on the induction of Stra4, Hoxa-1, and RARβ RNA transcripts. P19 cells were treated with retinoids for 24 h as indicated. RNA was extracted, and RT-PCR was used to estimate the relative amount of each gene transcript, taking the level present in ethanol vehicle-treated cells as 1.

Synergistic effects of RAR- and RXR-selective retinoids on P19 and F9 EC cell differentiation. P19 and F9 cells were also treated with the same combinations of RAR- and RXR-selective ligands, to investigate whether RARs and RXRs could similarly synergize to promote the occurrence of a complex process, namely, the RA-induced morphological differentiation of EC cells in vitro (reference 19 and references therein). P19 cells retained their undifferentiated morphology when treated with either 1 nM Am80, 10 nM CD666, 0.5 μM BMS453, or 1 μM BMS649, whereas the use of nonselective concentrations of Am80 (100 nM) or CD666 (1 μM) resulted after 96 h in a morphological differentiation comparable to that achieved by 1 μM t-RA (Fig. 3) (data not shown). In contrast, P19 cells treated with either the RARα-RXR (1 nM Am80–1 μM BMS649) or the RARγ-RXR (10 nM CD666–1 μM BMS649) agonist pair did show morphological differentiation (Fig. 3). However, no differentiation was observed when the cells were treated with the RARβ-RXR agonist pair (0.5 μM BMS453–1 μM BMS649 [data not shown]). The combination of the RARα (1 nM Am80) and RARγ (10 nM CD666) agonists, which on their own had no effects on P19 cell morphology, also resulted in a clear morphological differentiation (Fig. 3), whereas the 1 nM Am80–0.5 μM BMS453 and 10 nM CD666–0.5 μM BMS453 combinations were inactive, as expected (data not shown).

Essentially the same pattern of morphological differentiation, including the lack of morphological differentiation upon treatment with the RARβ-RXR agonist pair (0.5 μM BMS 453–1 μM BMS649), was observed when F9 cells were simi-

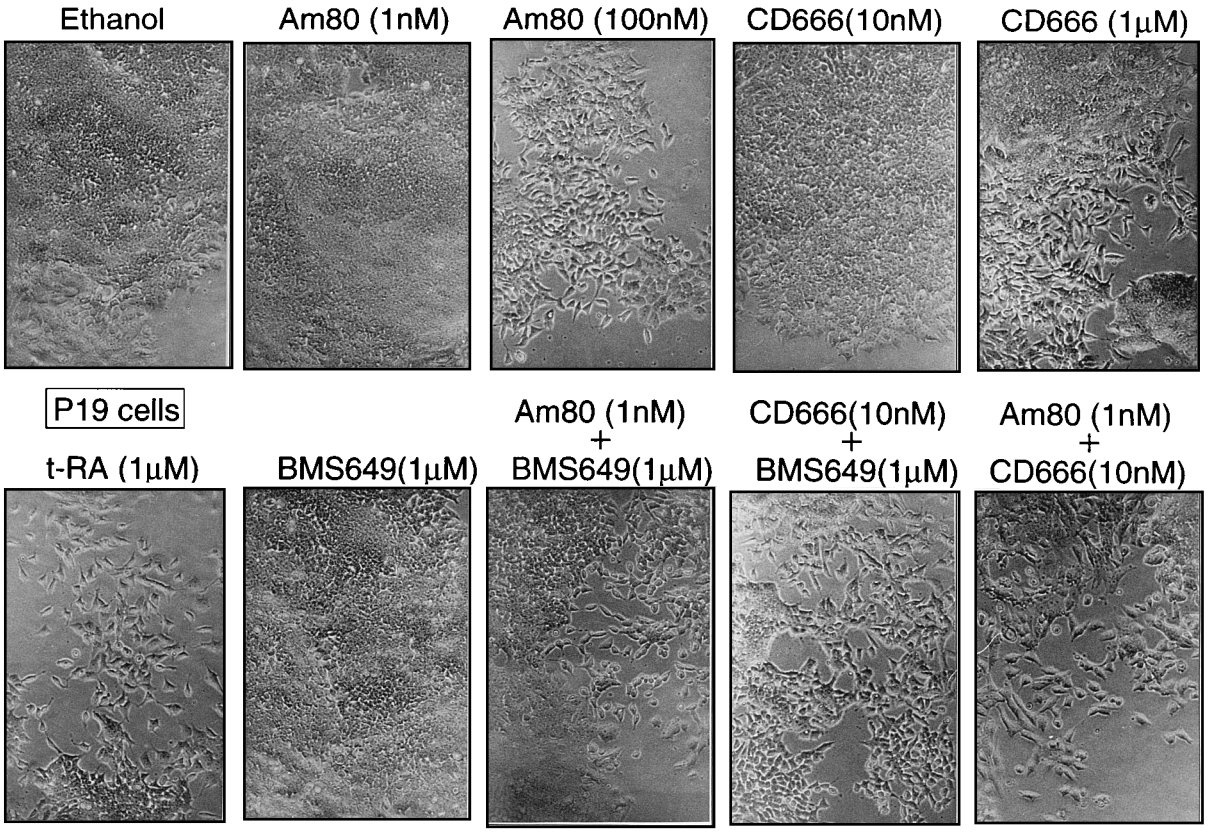

FIG. 3. Morphological differentiation of P19 cells exposed for 96 h to ethanol vehicle, Am80 (1 nM), Am80 (100 nM), CD666 (10 nM), CD666 (1 μM), t-RA (1 μM), BMS649 (1 μM), Am80 (1 nM)-BMS649 (1 μM), CD666 (10 nM)-BMS649 (1 μM), and Am80 (1 nM)-CD666 (10 nM), as indicated. Differentiation in the presence of either 100 nM Am80 or 1 μM CD666 was comparable to that observed with 1 μM t-RA. Am80 (1 nM) and CD666 (10 nM) synergized with 1 μM BMS649 to induce differentiation, although BMS649 (1 μM) alone had no effect. Similarly, Am80 (1 nM) and CD666 (10 nM) acted synergistically, although Am80 (1 nM) and CD666 (10 nM) alone did not result in any visible differentiation.

larly treated (Table 2; Fig. 4B) (data not shown). However, the differentiation brought about by the various combinations of synthetic retinoids at selective concentrations appeared to be less efficient than that resulting from t-RA, 9C-RA, or nonselective concentrations of Am80 or CD666 (Table 2). These morphological data were supported by the determination of the level of expression of two differentiation-specific markers (7, 8), collagen type IVα1 and laminin B1, after 24 and 48 h of retinoid treatment (Fig. 4B; Table 2). The expression of these two genes was clearly induced upon treatment of the cells with

TABLE 2. Synergistic effect of selective synthetic retinoids on F9 cell differentiation

Retinoid(s) (concn[s])	Relative level of RNA at 24 and 48 h[a]		Morphological differentiation (96 h)[b]
	Collagen IVα1	Laminin B1	
Ethanol (vehicle)	1, 1	1, 1	None
t-RA (1 μM)	5, 7.1	2.5, 22	++
9C-RA (1 μM)	2.8, 4.8	2.3, 9.2	++
Am80 (1 nM)	1, 1	1, 1	None
Am80 (100 nM)	4, 2.5	10, 9.4	++
CD666 (10 nM)	1, 1	1, 1	+/−
CD666 (1 μM)	5, 6.2	6.5, 17	++
BMS649 (1 μM)	1, 1	1, 1	None
BMS453 (0.5 μM)	1, 1	1, 1	None
Am80 (1 nM) and BMS649 (1 μM)	1.0, 2.5	1.3, 4.8	+
CD666 (10 nM) and BMS649 (1 μM)	1.7, 3.9	2.0, 5.6	+
BMS453 (0.5 μM) and BMS649 (1 μM)	1, 1	1, 1	None
Am80 (1 nM) and CD666 (10 nM)	4, 5	2.5, 10	+

[a] The levels of collagen IVα1 and laminin B1 RNA transcripts estimated by RT-PCR were expressed relative to those found in ethanol-treated nondifferentiated cells, which were taken as 1. F9 cells were treated with retinoids as indicated for 24 or 48 h.

[b] The morphological differentiation of the cells was monitored for 96 h after retinoid treatment. ++, +, and ± indicate that 70 to 80, 50 to 70, and ~10% of the cell population appeared differentiated at 96 h, respectively.

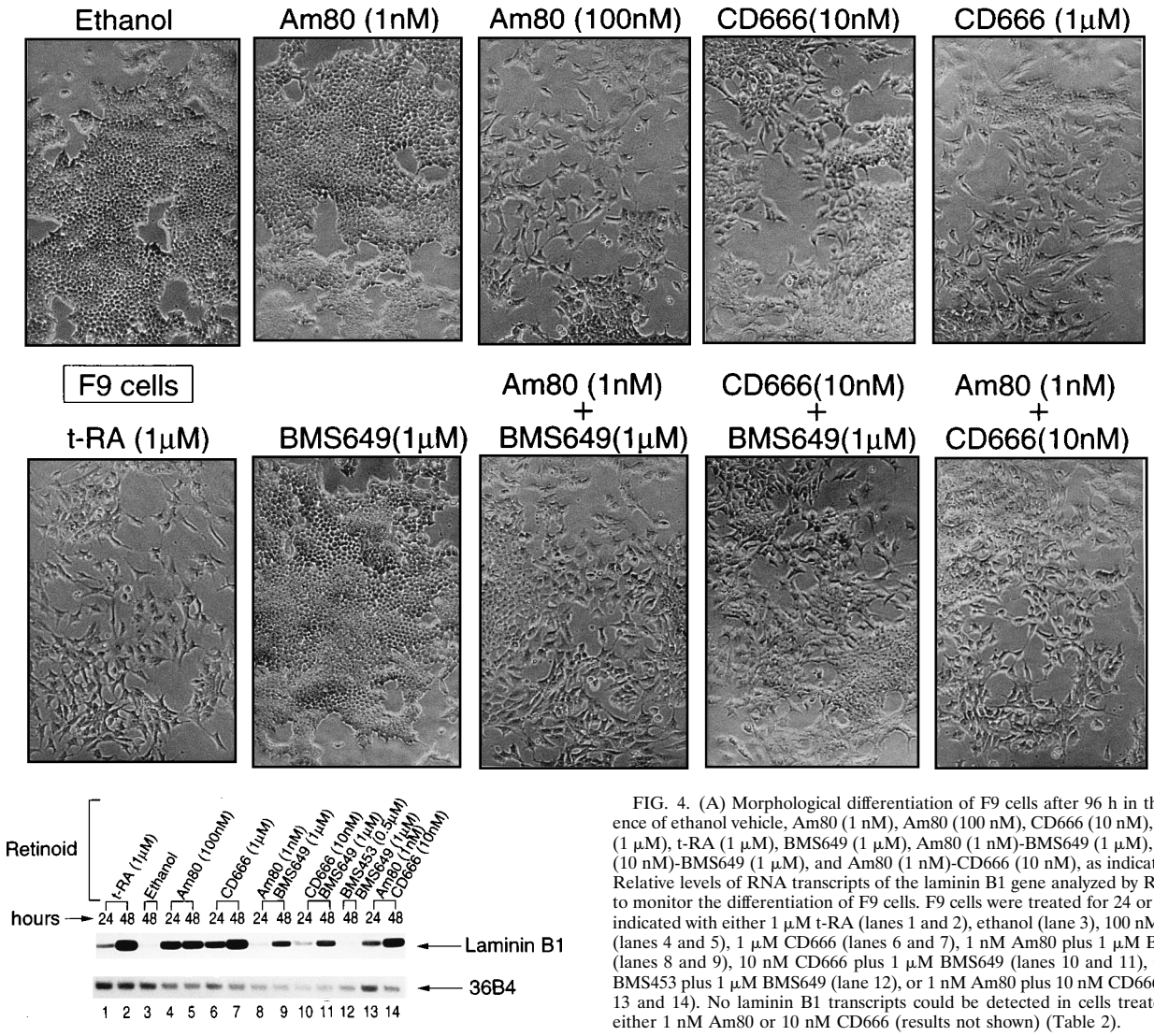

FIG. 4. (A) Morphological differentiation of F9 cells after 96 h in the presence of ethanol vehicle, Am80 (1 nM), Am80 (100 nM), CD666 (10 nM), CD666 (1 μM), t-RA (1 μM), BMS649 (1 μM), Am80 (1 nM)-BMS649 (1 μM), CD666 (10 nM)-BMS649 (1 μM), and Am80 (1 nM)-CD666 (10 nM), as indicated. (B) Relative levels of RNA transcripts of the laminin B1 gene analyzed by RT-PCR to monitor the differentiation of F9 cells. F9 cells were treated for 24 or 48 h as indicated with either 1 μM t-RA (lanes 1 and 2), ethanol (lane 3), 100 nM Am80 (lanes 4 and 5), 1 μM CD666 (lanes 6 and 7), 1 nM Am80 plus 1 μM BMS649 (lanes 8 and 9), 10 nM CD666 plus 1 μM BMS649 (lanes 10 and 11), 0.5 μM BMS453 plus 1 μM BMS649 (lane 12), or 1 nM Am80 plus 10 nM CD666 (lanes 13 and 14). No laminin B1 transcripts could be detected in cells treated with either 1 nM Am80 or 10 nM CD666 (results not shown) (Table 2).

the different combinations of synthetic retinoids used at selective concentrations (with the expected exception of the BMS453-BMS649 combination); however, in agreement with the morphological differentiation data, the induction of collagen IVα1 and laminin B1 transcripts was somewhat lower in the cells treated with the Am80-BMS649 and CD666-BMS649 combinations.

DISCUSSION

We have shown here that treatment of P19 cells with a selective concentration of Am80 (1 nM) or CD666 (1 or 10 nM) which results in a suboptimal activation of RARα or RARγ, respectively, leads either to no induction (Stra1, Stra2, and CRABPII) or a weak induction (RARβ, Hoxa-1) of RA-responsive genes (Table 1). At optimal concentration, the RARβ-specific retinoid BMS453 (0.5 μM) weakly induced Stra1 and Stra2 expression, while none of the genes examined here could be induced by the RXR-specific ligand BMS649 (1 μM). In marked contrast, the expression of all genes was efficiently induced by the same concentrations of either one of the selective RAR ligands in combination with the RXR-specific ligand. However, the magnitude of the activation (relative activation is indicated by bracketed numbers in Table 1) and the extent of the synergism (numbers in parentheses in Table 1) varied greatly depending on both the nature of the activated receptors (RARα-RXR, RARβ-RXR, or RARγ-RXR) and the context of the promoter of the responsive gene. In all cases, activation was highest with the Am80-BMS649 (RARα-RXR) combination (Table 1), probably reflecting the quantitative predominance of RARα in P19 cells (22), while it was, in general, the lowest with the BMS453-BMS649 (RARβ-RXR) combinations, most notably in the case of the CRABPII gene. The promoter context dependence of the extent of synergistic activation is best illustrated by the comparison of the RARβ and Hoxa-1 genes (Table 1; see also Fig. 2), which have the same DR5 RA response element (13, 26). In the case of RARβ, the highest degree of synergism is achieved with the RARβ-RXR combination, which can be correlated with our previous observation showing that RARβ transcription is autocatalytic (39).

However, taken together, our results very often show a clear functional redundancy between the various RAR-RXR combinations for activation of expression of the RA-responsive

genes examined here, thus corroborating our previous report, which shows that the induction of RA-responsive genes can be rescued (at least in part) in RARγ null F9 cells by overexpression of either RARα or RARβ (39). In this respect, it is particularly noteworthy that the induction of Hoxa-1, which is markedly reduced in RARγ null F9 cells (7), is readily induced here by the Am80-BMS649 (RARα-RXR) combination. Nevertheless, these functional redundancies vary considerably depending on the gene considered (note, for instance, that CRABPII expression is very poorly induced by the BMS453-BMS649 combination), in keeping with our previous conclusion that the responsiveness of specific subsets of RA target genes may be differentially mediated in F9 cells by the different RAR types (7, 8, 39).

Taken together, our results are consistent with our previous proposal that both RAR and RXR partners of RAR-RXR heterodimers act synergistically to activate transcription of RA target genes (14, 15), although we cannot exclude that the present synergisms reflect more complex mechanisms involving a cooperation between events independently induced by the RAR- and RXR-specific ligands. However, it is particularly striking that high concentrations of Am80 (100 nM) or CD666 (1 μM), which promiscuously activate all three RAR types, very efficiently induce the expression of all the genes examined here. Xiao et al. (40) have similarly shown that the binding of the RAR-specific ligand CD367 is sufficient on its own to allow activation of transcription by RAR-RXR heterodimers bound to the RARβ2 RA response element in cultured keratinocytes. It is plausible that the synergistic effect of the RXR partner becomes dispensable when all RAR partners of RAR-RXR heterodimers are liganded in a cell (and therefore transcriptionally active) whereas this synergism would be indispensable at lower concentrations of an RAR-selective ligand, which probably better reflects physiological RA concentrations. In this respect, it is noteworthy that the fraction of RXR which must be liganded to achieve an efficient synergistic activation varies greatly depending on the context of the induced promoter (Fig. 2). The synergistic effect of low concentrations of the RARα- and RARγ-selective ligands Am80 and CD666, which is at first sight surprising, most probably indicates that there is a threshold level of ligand-activated RARs (RARα and RARγ) which must be reached before the RXR partner becomes dispensable for efficient transcriptional activation. It is also striking that irrespective of its concentration, the RXR-specific ligand BMS649 has no effect on the expression of any of the RA-responsive genes studied here. This result is in keeping with our previous study showing that the RXR partner of RAR-RXR heterodimers bound to a response element consisting of directly repeated PuAGTTCA motifs separated by 5 bp (DR5T) could not activate transcription unless its RAR partner was itself liganded (14). Our results are also in agreement with those of Kurokawa et al. (25) and Forman et al. (16), who reported that RXR agonists alone are unable to activate RAR-RXR heterodimers. However, no or very little synergism between RAR and RXR heterodimeric partners was seen in these transient-transfection studies. In this respect, it should be stressed that it is difficult to compare our results with those obtained by transfection studies in which nonnatural episomal templates are exposed to high, nonphysiological concentrations of receptors.

The present study also shows that the same combinations of RAR- and RXR-selective ligands, which induce the expression of RA target genes, can trigger to various extents the morphological differentiation of P19 and F9 cells (Table 2). Again, the activation of RXR, which cannot induce differentiation on its own, is required to induce it at selective low concentrations of either the RARα or RARγ agonists. In contrast, activation of RXRs becomes dispensable for nonselective concentrations of either one of these agonists, as well as for a combination of selective low concentrations of RARα and RARγ agonists (Table 2). This latter observation also supports the conclusion that RARα and RARγ are to some extent functionally redundant. It is, however, noteworthy that activation of RARβ and RXRs (BMS453-BMS649 combination) cannot trigger the differentiation of P19 or F9 cells (Table 2) (data not shown), which is in agreement with the inability of RARβ overexpression to "rescue" the differentiation of RARγ null F9 cells (39). Taken together, our present observations are in keeping with those of Lotan et al. (32), who, using ME180 cervical carcinoma cells, have recently reported synergistic antiproliferative effects of a combination of RAR (TTAB)- and RXR (SR11217)-specific retinoids. Interestingly, these authors have also shown that the same combination of specific RAR and RXR retinoids results in a synergistic activation of a reporter gene containing a RARβ2 RA response element, which also supports the involvement of RAR-RXR heterodimers in this activation.

It is noteworthy that none of the various combinations of RAR-RXR-selective agonists could trigger the full morphological differentiation brought about by t-RA (Table 2), even though some of these combinations were as efficient as t-RA for inducing the expression of particular RA-responsive genes (Table 1) (data not shown). The present therapeutic uses of RA are largely limited by its adverse pleiotropic effects (5). Our present study clearly indicates that low doses of selective RAR agonists, acting synergistically with a RXR agonist, are more restricted than t-RA in their effects on both RA target gene expression and extent of cell differentiation. Specific combinations of synthetic receptor-selective retinoids may therefore extend the therapeutic use of retinoids by avoiding the numerous side effects of RA, whose administration indiscriminately activates all RARs and RXRs.

ACKNOWLEDGMENTS

B.R. and R.T. contributed equally to this study.

We thank J. L. Plassat and N. Chartoire for technical assistance. We are grateful to P. Reczek, Bristol-Myers-Squibb Pharmaceutical Research Institute, Buffalo, N.Y., for the generous gifts of BMS453 and BMS649; to B. Shroot, CIRD Galderma, Valbonne, France, for CD666; and to K. Shudo, University of Tokyo, Tokyo, Japan, for Am80. We also thank P. Bouillet for gifts of various plasmids and probes, H. Gronemeyer for discussions, and S. Ward for critical reading of the manuscript. We are grateful to the cell culture facility for providing cells, to F. Ruffenach for oligonucleotide synthesis, to R. Bucher and B. Boulay for illustrations, and to the secretarial staff for help with preparation of the manuscript.

This work was supported by funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Collège de France, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur la Cancer (ARC), the Fondation pour la Recherche Médicale (FRM), and the Human Frontier Science Program. R.B. was supported by a fellowship from the INSERM, and R.T. was supported by fellowships from the CNRS and FRM.

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