Retinoic Acid-induced Expression of Apolipoprotein D and Concomitant Growth Arrest in Human Breast Cancer Cells Are Mediated through a Retinoic Acid Receptor RARα-dependent Signaling Pathway*

(Received for publication, November 27, 1995, and in revised form, August 28, 1996)

Yolanda S. López-Boado, Michael Klaus‡, Marcia I. Dawson§, and Carlos López-Otín¶

From the Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33006-Oviedo, Spain, ‡Pharma Division, Preclinical Research, F. Hoffmann-La Roche, 4002 Basel, Switzerland, and §Life Sciences Division, SRI International, Menlo Park, California 94025

Apolipoprotein D (apoD) is a human plasma protein, belonging to the lipocalin superfamily, that is produced by a specific subtype of highly differentiated breast carcinomas and that is strongly up-regulated by retinoic acid (RA) in breast cancer cells. In this work, we have examined the molecular mechanisms mediating the induction of apoD gene expression by retinoids in T-47D human breast cancer cells. Northern blot analysis revealed that Ro40-6055, a synthetic retinoid that selectively binds and activates the retinoic acid receptor RARα, induced the accumulation of apoD mRNA in breast cancer cells in a time- and dose-dependent manner. The time course analysis demonstrated that apoD mRNA was induced 14-fold over control cells after 48 h of incubation with 10^{-8} M Ro40-6055. As little as 10^{-11} M of this retinoid induced apoD mRNA 5-fold over the control, whereas incubation with 10^{-7} M Ro40-6055 induced maximally 15-fold over control cells. RARα-selective antagonists counteracted the inductive effects of all-trans-RA, 9-cis-RA, and Ro40-6055 on the expression of apoD, when present at the same concentration as the retinoid agonists. By contrast, RARβ, RARγ, and RXR-selective retinoids did not affect apoD gene expression. The retinoid agonist Ro40-6055 had an antiproliferative effect on T-47D cells, with maximal growth inhibition of approximately 60% obtained after 7 days of incubation with 10^{-7} M. This antiproliferative effect could be counteracted by a 100-fold excess of the antagonist Ro41-5253. Treatment of the cells with retinoids that do not bind the nuclear retinoic acid receptors did not affect apoD expression, despite the fact that they did have a strong antiproliferative effect on T-47D cells. On the basis of these results, a role for RARα on apoD gene expression induction by retinoids in breast cancer cells is proposed.

Apolipoprotein D (apoD) is a glycoprotein of about 30 kDa, polymorphic in charge, that was originally isolated from human plasma by McConathy and Alaupovic (1). According to its structural characteristics, apoD is a member of the lipocalin family of proteins, which have a common function of transporting small hydrophobic molecules in different body fluids (2). The specific ligand associated with apoD remains unclear, and a variety of substances including cholesterol, cholesteryl esters, heme-related compounds, progesterone, and arachidonic acid have been described as putative ligands for this protein (2–5). Similarly, the functional role of apoD is also unclear (6, 7), although it is involved in a number of apparently unrelated biological and pathological processes. Thus, in the plasma, apoD mainly associates with high density lipoprotein particles and forms complexes with other apolipoproteins (8) and with lecithin-cholesterol acyltransferase (9), which has led to the suggestion that apoD may be involved in the early steps of the cholesterol transport from peripheral tissue to the liver (10). The production of apoD also has been correlated with some growth and regeneration processes. In particular, levels of apoD increase several-fold during the regeneration of peripheral nervous tissue in the rat (11, 12). Finally, our finding that apoD accumulates to extremely high concentrations in cyst fluid from women with gross cystic disease of the breast has led to the hypothesis that this protein may be involved in steroid hormone binding and transport in human mammary tissue (13–15).

The relationship of apoD to pathological processes involving the human mammary gland has been extended further by the finding of a subtype of breast carcinomas that are able to produce and secrete this glycoprotein (16–19). Because intratumor apoD values are significantly higher in well differentiated carcinomas than in those moderately or poorly differentiated (19), we have recently examined the possibility that substances with potent differentiating effects, like retinoic acid and other vitamin A derivatives, could be involved in apoD expression by breast cancer cells. In fact, we have found that all-trans-, 9-cis-, and 13-cis-retinoic acid strongly induce the expression of apoD in estrogen receptor-positive human breast cancer cells. Furthermore, this increased expression of apoD was accompanied by an inhibition of cell proliferation and a progression through a more differentiated phenotype in these cells, which has led us to suggest that the mechanisms controlling RA-induced growth arrest, cell differentiation, and apoD synthesis may be directly coordinated in human breast cancer cells (20). The mechanisms responsible for this marked induction of apoD mRNA by retinoids are presently unknown. However, it seems likely that their elucidation could contribute to clarify the role of these compounds in the biochemical path-
always leading to apoD-increased expression in specific conditions, such as growth arrest and cell differentiation of breast cancer cells, as well as in peripheral nerve regeneration processes (11, 12, 21, 22).

Here we examine the possibility that induction of apoD by retinoid acid analogs in breast cancer cells is a direct event mediated by the nuclear retinoic acid receptors. By using a series of receptor-selective agonist and antagonist retinoids, we present evidence for the involvement of a RARα-dependent signaling pathway in the stimulation of apoD gene expression by retinoids in breast cancer cells. We also show that activation of this pathway leads to a significant inhibition of breast cancer cell proliferation. Finally, on the basis of the lack of apoD induction by retinoids which do not bind to nuclear retinoic acid receptors but are still able to inhibit cell proliferation, we propose that apoD may be a biochemical marker of RAR-mediated specific pathways of growth arrest in breast cancer cells.

EXPERIMENTAL PROCEDURES

Materials—All media and supplements for cell culture were obtained from Life Technologies, Inc. except for fetal calf serum, which was from Boehringer Mannheim (Mannheim, Germany). All-trans-retinoic acid (t-RA) and sulforhodamine B were from Sigma. 9-cis-Retinoic acid (9-cis-RA), Ro40-6055, Ro40-6973, Ro41-5253, Ro46-5471, Ro48-2249, Ro48-5757, and Ro40-8757 were from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). RXR-retinoids SR11235 and SR11236 and the anti-API retinoid SR11238 were from SRI International (Menlo Park, CA). Plasmid pBL-CAT201-RARE (RAR-tk-CAT) was kindly provided by Dr. A. Muñoz (Instituto de Investigaciones Biomédicas, Madrid, Spain). Plasmid CRBPII-tk-CAT (RXRE-tk-CAT) was a gift from Dr. X. Zeng (La Jolla Cancer Research Foundation, La Jolla, CA). Expression plasmid containing RXRa was kindly donated by Dr. P. Chambon (Institute of Genetics and Molecular Cellular Biology, Illkirch, France). [γ-32P]dCTP (3000 Ci/mmol), the random priming labeling kit and the nylon membranes for RNA blots were from Amerham International (Buckinghamshire, United Kingdom).

Cell Culture and Cell Growth Estimation—Human breast cancer cells T-47D were obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 μg/ml gentamicin. Cells were subcultured weekly by incubation at 37 °C for 2 min with 0.0125% trypsin in 0.02% EDTA, followed by addition of complete medium, washing, and reuspension in fresh medium. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2. In experiments to test the effect of retinoids on cell number, T-47D cells were plated in 24-well plates and allowed to adhere to substrate for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Afterward, the serum concentration was reduced to 2%, and retinoids were added at different concentrations. Cells were incubated for 7 days in the presence of retinoids with medium changes every 2 days. At the end of the incubation period, the total number of cells was estimated by the fluorometric protein assay essentially as described by Skehan et al. (23).

Cell Transfection—For transient transfection studies, T-47D cells were plated at 30% confluence in 6-well plates 20 h before the experiment. The cells were transfected with 1 μg of RARE-tk-CAT plasmid DNA (24) or 1 μg of CRBPII-tk-CAT plasmid DNA (25) and 1 μg of a RXRa expression plasmid (26) and 4 μl of LipofectAMINE (Life Technologies, Inc.) in 1 ml of Opti-MEM medium (Life Technologies, Inc.) per well. In addition, 1 μg of a β-galactosidase expression plasmid DNA (BSV-βgal) per well was co-transfected to monitor transfection efficiency. After a 5-h incubation at 37 °C and 5% CO2, 1 ml of fresh medium containing 10% fetal calf serum was added to the dish, and the incubation was allowed to proceed for another 20 h. Retinoids were then added to the dishes at different concentrations, and, after 48 h, the cells were collected with a cell scraper in ice-cold phosphate-buffered saline and washed twice in cold phosphate-buffered saline. Cell extracts were prepared by 3 cycles of quick freezing and thawing in a buffer containing 15 mM Tris-HCl pH 8.0, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride, followed by centrifugation for 15 min at 12,000 × g. CAT activity in cell extracts was determined essentially as described in Sambrook et al. (27). Quantification of the signals in the thin layer chromatography plates was carried out with a Packard InstantImager electronic autoradiography system (Packard Instrument, Meriden, CT).

RESULTS

In previous work, we showed that retinoids up-regulate the expression of the apoD gene at the transcriptional level in human breast cancer cells. We also showed that the induction of apoD mRNA expression was independent of the de novo synthesis of proteins (20). These observations strongly suggest that the induction of apoD by RA in breast cancer cells may be a direct event, possibly mediated by nuclear retinoic acid receptors. To determine whether the receptors expressed by T-47D cells are functionally intact and to establish the type of response element that can be transactivated by these receptors, we initially studied the activity of different retinoids in T-47D cells transfected with DNA constructs containing the CAT reporter gene placed under the control of either a RARE or a RXRE (24, 25). The transactivation of the reporter gene was determined after treatment of the transfected cells with a number of agonistic and antagonistic retinoids (Fig. 1). A 10^{-8} M concentration of all-trans-RA, 9-cis-RA, and the RARα-selective retinoid Ro40-6055 caused a 15-, 19-, and a 12-fold increase in the transactivation of RARE-tk-CAT, respectively, as shown in Fig. 1A (lower panel). In addition, treatment of the transfected cells with the RARα-selective antagonist Ro41-5253, extensively abolished the transactivation by the retinoids of the reporter gene placed under the control of the RARE. Nevertheless, it is interesting to note that concentrations of Ro40-5253 at least 100-fold higher than the concentrations of Ro40-6055 or all-trans-RA were necessary to completely block the transactivation (Fig. 1A and data not shown). We also studied the activity of the different retinoids in T-47D cells transfected with the construct containing the CAT gene under the control of the RXRE element of the cellular retinol-binding protein type II (CRBPII-tk-CAT). As shown in Fig. 1B, upon cotransfection with an expression plasmid containing RXRa, treatment of the transfected cells with 10^{-8} M of the RXRα-selective retinoid LG100064 produced a significant increment of CAT activity (about 17-fold). By contrast, treatment of the transfected cells with all-trans-RA, the RARα-selective retinoid Ro40-6055, the RARα-selective retinoid (Ro48-2249), and the RARγ-selective retinoid (Ro44-4753) did not produce any significant increase in the CAT activity placed under the control of the RXRE (Fig. 1B). In similar experiments in RXRa-transfected cells, the RARα-selective retinoid Ro40-6055 was able to consistently induce CAT expression controlled by the RARE by 12-fold, while the RARβ-selective and RARγ-selective retinoids induced an increase in CAT activity of only 5- and 3-fold, respectively (Fig. 1C). Taken together, these results suggest the existence in T-47D cells of functional retinoid signaling pathways, possibly mediated by different homo- or heterodimeric combinations of the receptors expressed by these
breast cancer cells. Thus, the transactivation of the RARE by all-trans-RA, 9-cis-RA, and the RARα-selective retinoid Ro40-6055 is very likely mediated by RARα, since it can be specifically competed by the RARα antagonist Ro41-5253, although contribution of RARβ- and γ-selective pathways cannot be definitively ruled out. By contrast, the RXRE seems to be exclusively transactivated by the RXR-specific retinoid LG100064, possibly through RXRα heterodimers (29, 30).

To investigate the mechanism of activation of the apoD gene by retinoic acid, we first examined by Northern blot analysis the effect of a number of different agonistic and antagonistic retinoids on the expression of the apoD gene in breast cancer cells. As shown in Fig. 2A, incubation of T-47D cells with 10^{-8} M concentrations of the RARα-selective agonists Ro40-6055 and Ro40-6055 induced an expression of the apoD gene comparable to that observed with all-trans-RA (1.1 and 0.7-fold, respectively). 9-cis-RA was about a 2-fold more potent inducer of apoD mRNA than the remaining retinoids. In addition, the RARα-selective antagonists Ro41-5253 and Ro46-5471 prevented all-trans-RA-induced accumulation of apoD mRNA, as well as the increase in the levels of apoD mRNA induced by 9-cis-RA and the RARα-selective agonists (Fig. 2A and data not shown). Fig. 2A also shows that treatment of the cells with the retinoid antagonists Ro41-5253 and Ro46-5471 alone did not have any effect on the expression of the apoD gene. After these results, we examined the possibility that retinoids selective for other RAR and RXR pathways could be also effective in apoD induction in breast cancer cells. To do that, T-47D cells were treated with 10^{-8} M concentrations of the RARβ-selective (Ro48-2249) or the RARγ-selective (Ro44-4753) retinoids, and the apoD mRNA levels were examined by Northern blot. However, these retinoids did not have any marked effect on apoD gene expression (Fig. 2B). Similarly, the RXR-selective agonists SR11235, SR11246, and LG100064 were unable to induce apoD expression in T-47D cells (Fig. 2B).

To further characterize the effect of the RARα-selective retinoids on apoD gene expression, T-47D cells were incubated for 48 h in the presence of different concentrations of the RARα-selective agonist Ro40-6055, and total cellular RNAs were purified and analyzed by Northern blot. As shown in Fig. 3A, treatment of the T-47D cells with the selective retinoid resulted in an accumulation of apoD mRNA at concentrations ranging from 10^{-7} to 10^{-11} M. Incubation of the cells with 10^{-7} M Ro40-6055 resulted in a maximal accumulation of approximately 15-fold over the control cells, whereas concentrations as low as 10^{-11} M still produced an accumulation of apoD mRNA of about 5-fold over the control. Comparable results were obtained upon treatment of the T-47D cells with the other RARα-selective agonist, Ro40-6973 (data not shown). The time course of the induced accumulation of apoD mRNA was also studied in the presence of 10^{-9} M Ro40-6055. As shown in Fig. 3B, there was a consistent increase with time in the steady-state apoD mRNA levels in treated T-47D cells, the maximal effect (about 12-fold over the control) being observed after 48 h and remaining constant for at least 72 h (data not shown). The extent of apoD mRNA accumulation induced by the RARα-selective retinoid was thus comparable to that previously observed for all-trans-RA (20). We also examined whether the antagonistic effect of the RARα-selective retinoid Ro41-5253 on the RA-induced accumulation of apoD mRNA was dose-dependent. To this end, T-47D cells were treated for 48 h with 10^{-7} M all-trans-RA and different concentrations of Ro41-5253. As shown in Fig. 4, equimolar concentrations of the RARα-selective antagonist blocked almost completely the induction of apoD mRNA accumulation by all-trans-RA, whereas treatment of the breast cancer cells with concentrations of the antagonist 10- to 100-fold lower than the concentration of RA resulted in lesser antagonism (about 2- and 4-fold lower, respectively).

We also investigated whether the effect of the RARα-selective agonists Ro40-6055 and Ro40-6973 on apoD mRNA induction was correlated with inhibition of cell proliferation. T-47D cells were incubated for 7 days with retinoids, and the total number of cells was determined at the end of the incubation period. As shown in Fig. 5A, treatment of the T-47D cells in exponential growth with Ro40-6055 resulted in a marked decrease in cell number that was concentration-dependent. Max-
antiproliferative effect of the RAR
selective retinoid antagonist Ro41-5253, which did not have
significant (p < 0.01). It is interesting that the RARβ-selective agonist (Ro48-2249) also showed antiproliferative activity, although the effect is less remarkable than the one produced by the RARα-selective retinoid Ro40-6055 (Fig. 5C). By contrast, the RARγ-selective agonist Ro44-4753 did not have a significant effect on cell growth at any concentrations tested (Fig. 5D).

Finally, to obtain further support for the involvement of RARs on the induction of the apoD gene by RA, T-47D cells were incubated with two retinoids (Ro40-8757 and SR11238) which display potent antiproliferative activity in breast cancer cells, but are inactive in transactivation assays (Fig. 6). The first of these compounds is an arrotinoid with strong antiproliferative activity in a number of human cancer cell lines in vitro (31), but does not bind to the nuclear retinoic acid receptors (32). Similarly, the anti AP1-selective retinoid SR11238 is unable to transactivate the RARs despite effectiveness in triggering growth arrest of breast cancer cells (33). As shown in Fig. 6A, treatment of T-47D cells with 10^{-7} M Ro40-8757 and SR11238 did not have any effect on the expression of the apoD gene. Cell growth analysis, however, confirmed their potent antiproliferative effect on T-47D cells (Fig. 6B). Furthermore, the retinoids Ro40-8757 and SR11238 did not exhibit any transacting activity of the RARE or the RXRE reporter genes (data not shown). These results provide additional evidence for the occurrence of separable pathways of retinoid-induced growth arrest in breast cancer cells, with apoD mRNA levels being up-regulated only in those cases in which RAR-mediated signaling pathways are involved.

**DISCUSSION**

We have previously reported that RA induces the expression of apoD in human breast cancer cells, concomitantly with an increase in the status of cell differentiation and a decrease in the cell growth rate (20). Although the molecular mechanisms responsible for this positive effect on apoD expression have not yet been elucidated, it is likely that retinoic acid receptors may be involved. In this work, we have examined the effect of agonistic and antagonistic receptor-selective retinoids, to shed light on the receptor(s) involved in these actions. The results presented herein indicate that the induction of apoD expression by retinoids in the T-47D estrogen receptor-positive human breast cancer cell line is mediated by a retinoid signaling pathway in which the RARα receptor is involved. Thus, we have found that the RARα-selective retinoids Ro40-6055 and
Ro40-6973 (32) are very strong inducers of apoD expression. Furthermore, the induction of apoD by all-trans-RA, 9-cis-RA, and the RARγ-selective retinoids was completely abolished by the RARα-selective antagonists Ro41-5253 and Ro46-5471. The precise mechanism by which these retinoids exert their antagonistic effect is not completely understood; however, Ro41-5253 competes with RA specifically for binding to RARα (34), leading to the suggestion that the antagonist binds to this receptor but it is unable to induce transactivation (34, 35). In any case, regardless of the specific mechanism through which the antagonists act on nuclear receptors, the finding that they counteract the effects of all retinoid analogs with the ability to induce apoD gene expression, provides additional evidence of the involvement of RARα in the transduction of the retinoid signal.

The finding that RARs is important in mediating RA responses in breast cancer cells agrees very well with previous results indicating that this receptor may be involved in relevant retinoid-mediated pathways in different mammary carcinoma cell lines (36, 37). In addition, the RXR-selective retinoids are unable to elicit significant growth inhibitory events (38), suggesting that these pathways do not mediate the growth-related retinoid effects on these cells. Furthermore, the RARβ receptor is not expressed at significant levels in breast carcinoma specimens as well as in different breast cancer cell lines including T47-D, at least in basal conditions (39–42). Finally, retinoids do not induce growth arrest in estrogen receptor-negative cell lines which display a high level expression of RARγ (39), thus making unlikely a potential effect of RARγ in retinoid-mediated growth arrest in breast cancer cells, although putative defects in other transcription factors interacting with RARγ cannot be excluded. These data, together with the results presented in this work with agonists and antagonists selective for diverse RAR, are therefore consistent with the proposal that RARα plays a major role in the induction of apoD by retinoids in breast cancer cells. However, it is remarkable that the presence of functional RARα in breast cancer cells is not sufficient to make these cells responsive to retinoids in terms of apoD expression, although they become sensitive to growth-inhibitory effects. In this regard it should be mentioned that the absence of a significant level of RARα in estrogen receptor-negative cancer cells seems to be the cause of the nonresponsiveness of these cells to RA-mediated growth arrest (43, 44). In fact, transfection of this receptor subtype in estrogen receptor-negative cancer cells confers a significant degree of responsiveness to retinoids (36, 45), probably through the up-regulation of the RARβ gene (46). However, and somewhat unexpectedly, analysis of the ability of these transfectants to overproduce apoD upon RA treatment revealed that the expression of this gene remained unaffected. Thus, it is tempting to speculate that additional factors are required to restore a full RA-responsive phenotype in these cells. In this regard, of special interest is the very recent finding of a series of activator or corepressor protein factors that seem to be essential to modulate the response to retinoids (47–49), as has been shown for the estrogen receptor (50, 51). Further studies and molecular characterization of these factors will be required to address the possibility that their presence or absence in the different breast cancer cell lines could explain the variability in response to retinoids.

---

Fig. 4. Dose dependence of the effect of the RARα-selective antagonist Ro41-5253 on the induction of apolipoprotein D mRNA levels by all-trans-RA. T-47D cells were cultured for 48 h with 10^{-9} M all-trans-RA and different concentrations of Ro41-5253. Total RNA from the cells was isolated and analyzed as described in the legend to Fig. 2. Filters were hybridized consecutively with the apoD and the actin probes.

---

Fig. 5. Effect of different retinoids on T-47D cell proliferation. 24 h after plating, T-47D cells were incubated for 7 days with different retinoids. At the end of the incubation period, cell numbers were determined with a fluorimetric protein assay. A, T-47D cells were incubated with different concentrations of the RARα-specific agonist Ro40-6055. B, T-47D cells were incubated in the presence of the RARα-specific agonist Ro40-6973. C, T-47D cells were treated with different concentrations of Ro48-2249. The effect of 10^{-7} M Ro40-6055 is shown for comparison. D, T-47D cells were incubated with different concentrations of the RARβ-specific agonist Ro44-4753. The effect of 10^{-7} M Ro40-6055 is shown for comparison. E, T-47D cells were treated with different concentrations of the RARα-specific antagonist Ro41-5253. F, T-47D cells were treated with different combinations of Ro40-6055 and Ro41-5253. The data are expressed as the means of quadruplicate wells in three independent experiments. Error bars indicate standard deviations. AU, arbitrary units.
in these cells. The availability of genes like apoD that are specifically induced through one of these routes, could be of great interest to better define the molecular events associated with each of them. Further studies will be required to clarify the potential value of this member of the lipocalin family of proteins as a biochemical marker of RAR-mediated growth arrest and cell differentiation in breast cancer cells.

Acknowledgments—We thank Dr. S. Gascon for support, Dr. U. Reichert (Galderna, Sophia Antipolis, France) for the generous gift of retinoid analogs, and Sondoles Alvarez for expert technical assistance. We also thank Drs. P. Chambon, A. Muñoz, and X. Zhang for kindly providing some of the plasmids used in this work.

including the ability to produce large amounts of apoD.

The observation that apoD expression in breast cancer cells is controlled by a RARα-dependent signaling pathway supports the hypothesis that each of the multiple subtypes and isoforms of retinoid receptors performs a specific function (52). Despite the fact that RAR-targeted disruption studies in mice have suggested a significant degree of functional redundancy between the diverse members of this protein family, the high degree of amino acid sequence conservation of a given receptor subtype or isoform among different species, as well as their specific spatiotemporal patterns of expression during embryogenesis and in adult tissues, suggest that the various receptor subtypes play unique roles and may indeed modulate the activity of distinct genes in the diverse cell types (53, 54). Other genes whose expression seems to be specifically mediated by RARα include alkaline phosphatase in F9 teratocarcinoma cells (55), tissue-type plasminogen activator in endothelial cells (56), and tissue transglutaminase in a tracheobronchial epithelial cell line (57).

Finally, in this work we have also tried to provide further insights into the relationship between apoD induction, growth arrest, and cell differentiation mediated by retinoids in breast cancer cells. The finding that retinoid analogs like Ro40-8757 with the ability to inhibit breast cancer cell proliferation in a RAR-independent manner (31, 32) as well as anti-AP1 dissociating retinoids such as SR11238 (33), do not induce apoD expression provides additional support for the idea that there are different retinoid signaling pathways, namely retinoid acid receptor-dependent and independent, mediating growth arrest

**REFERENCES**

1. McConathy, W. J., and Alasopovic, P. (1973) *FEBS Lett.* **77**, 178–182
2. Drayna, D., Fielding, C., McLean, J., Baer, B., Castro, G., Chen, E., Comstock, L., Henzel, W., Kurh, W., Rhein, L., Wio, K., and Lown, R. (1986) *J. Biol. Chem.* **261**, 1635–1639
3. Peitsch, M. C., and Boguski, M. S. (1990) *New Biol.* 2, 197–206
4. Dilley, W. G., Haagensen, D. E., Cox, C. E., and Wells, S. A. (1990) *Breast Cancer Res. Treat.* 16, 253–260
5. Morais-Cabral, J. H., Atkins, G. I., Sánchez, L. M., López-Boado, Y. S., López-Otín, C., and Sawyer, L. (1995) *FEBS Lett.* **366**, 53–56
6. Wex, P. K., Provoost, P., Tremblay, N. M., Camato, R. N., Milne, R. W., Muller, H. W. (1990) *EMBO J.* 9, 2479–2484
7. Boles, J. K., Notterpek, L. M., and Anderson, L. J. (1990) *J. Biol. Chem.* **265**, 17805–17815
8. Balbin, M., Freije, J., M., Fueyes, A., Sánchez, L. M., and López-Otín, C. (1990) *Biochem. J.* **271**, 803–807
9. Sánchez, L. M., Diez-Itza, I., Vizoso, F., and López-Otín, C. (1992) *Clin. Chim. Acta* **305**, 695–698
10. Lopez-Boado, Y. S., Tcheknavorian, A., and López-Otín, C. (1994) *Biochem. J.* **299**, 95–100
11. Silva, J. S., Cox, C. S., Wells, S. A., Faull, D., Dilley, W. G., McCarty, K. S., Jr., Eppig, J. T., Glaubitz, L. C., and McCarty, K. S., Jr. (1989) *Science* **245**, 443–449
12. Lea, O. A., Kvinnsland, S., and Thorsen, T. (1987) *Cancer Res.* **47**, 6189–6192
13. Mazzoljan, G., and Haagensen, D. E. (1990) *Annu. N. Y. Acad. Sci.* **586**, 188–197
14. Diez-Itza, I., Vizoso, F., Merino, A. M., Sánchez, L. M., Tcheknavorian, A., Sánchez, J., Reiert, C., and López-Otín, C. (1994) *Am. J. Pathol.* **144**, 310–320
15. López-Boado, Y. S., Tcheknavorian, A., and López-Otín, C. (1994) *J. Biol. Chem.* **269**, 26871–26878
16. Simard, J., Dauvois, S., Haagensen, D. E., Lévesque, C., Merand, Y., and Labrie, F. (1990) *Endocrinology* **126**, 3923–3931
17. Simard, J., de Launoy, Y., Haagensen, D. E., and Labrie, F. (1992) *Endocrinology* **130**, 1115–1121
18. Sheehan, P., Stremm, R., Studier, D. M., Mahon, J., Vistica, D., Warren, J. T., Bakesh, H., Kenney, S., and Boyd, M. R. (1990) *J. Natl. Cancer Inst.* **82**, 1107–1112
19. Husmann, M., Lehnahn, J., Hoffmann, T., Trzukerman, M., and Pfahl, M. (1991) *Mol. Cell. Biol.* 11, 4097–4103
20. Chang, Z., Lehnahn, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992) *Nature* **358**, 587–590
21. Lehnahn, J., Long, J., Fanjul, A., Cameron, J. F., Ping-Lu, X., Haefner, P., and Pfahl, M. I. (1992) *Science* **258**, 1944–1946
22. Eliason, J. F., Kaufmann, F., Tanaka, T., and Tsukaguchi, T. (1993) *Br. J. Cancer* **67**, 1293–1298
23. Crettaz, M., Baran, A., Siegenschuh, G., and Hunziker, W. (1990) *Biochem. J.* **267**, 391–397
24. Fanjul, A., Dawson, M. I., Hobbs, P. D., Long, J., Cameron, J. F., Harlev, E., Graupner, G., Lu, X. P., and Pfahl, M. (1994) *Nature* **372**, 107–111
25. Apfél, C., Bauer, F., Crettaz, M., Fournel, L., Kamber, M., Kaufmann, F., LeMotte, P., Pinson, W., and Klaus, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7129–7133
26. Keidel, S., LeMotte, P., and Apfél, C. (1994) *Mol. Cell. Biol.* **14**, 287–298
27. Sheik, M. S., Shao, Z., Li, X., Dawson, M., Setten, A. M., Wu, S., Conley, B. A., Garcia, M., Rochefort, H., and Fontana, J. A. (1994) *J. Biol. Chem.* **269**, 21440–21447

![Fig. 6. Effect of the retinoids Ro40-8757 and SR11238 on cell proliferation and apolipoprotein D mRNA levels in T-47D cells. A, T-47D cells were incubated for 48 h with 10⁻⁷ M Ro40-8757, SR11238, and all-trans-RRA (t-RA), and total RNAs were analyzed as described in the legend to Fig. 2. Filters were hybridized with an apoD cDNA probe and a β-actin probe to verify equal loading of the samples. B, 24 h after plating, T-47D cells were incubated for 7 days in the presence of different concentrations of Ro40-8757, SR11238, and all-trans-RA. Retinoids were added to the growth medium in dimethyl sulfoxide solutions so that the final concentration of dimethyl sulfoxide was 0.1% in both control and treated cultures, and media were changed every 2 days. At the end of the incubation period, cell number was determined as described in the legend to Fig. 5. The data are expressed as the mean of quadruplicate wells in three independent experiments. Error bars indicate standard deviations. C, control; AU, arbitrary units.](image)
37. Dawson, M. I., Zhao, W., Pine, P., Jong, L., Hobbs, P. D., Rudd, C. K., Quick, T. C., Niles, R. M., Zhang, X., Lombardo, A., Ely, K. R., Shroot, B., and Fontana, J. A. (1995) Cancer Res. 55, 446-4451
38. Lotan, R., Dawson, M. I., Zou, C. C., Jong, L., Lotan, D., and Zou, C. F. (1995) Cancer Res. 55, 232–236
39. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. A., and Sutherland, R. L. (1992) Cancer Res. 52, 2236–2242
40. Roman, S. D., Ormandy, C. J., Manning, D. L., Blamey, R. W., Nicholson, R. I., Sutherland, R. L., and Clarke, C. (1993) Cancer Res. 53, 5940–5945
41. Shao, Z. M., Sheikh, M. S., Chen, J., Hussain, A., and Fontana, J. (1994) Exp. Cell Res. 219, 555–561
42. Sheikh, M. S., Shao, Z., Chen, J., Hussein, A., and Fontana, J. A. (1992) J. Cell. Biochem. 53, 394–404
43. Van der Burg, B., Van der Leede, B. M., Kwakkenbos-Iserbrucker, L., Salverda, S., de Laut, S. W., and Van der Saag, P. T. (1993) Mol. Cell. Endocrinol. 91, 149–157
44. Van der Leede, B. M., Folkers, G. E., Van den Brink, C. E., Van der Saag, P. T., and Van der Burg, B. (1995) Mol. Cell. Endocrinol. 109, 77–86
45. Liu, Y., Lee, M., Wang, H., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. (1996) Mol. Cell. Biol. 16, 1138–1149
46. Horlein, A. J., Naar, A. M., Heinzel, T., Torchio, J., Glass, B., Kurokawa, R., Ryan, A. M., Kamel, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Nature 377, 397–404
47. Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995) Nature 377, 451–454
48. Chen, J. D., and Evans, R. M. (1995) Nature 377, 455–457
49. Halachmi, S., Marden, E., Martin, G., Mackay, H., Abbondanza, C., and Brown, M. (1994) Science 264, 1455–1458
50. Cavailles, V., Dauvois, S., Danielian, P. S., and Parker, M. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10009–10013
51. Boylan, J. F., Luften, T., Achkar, C. C., Taneja, R., Chambon, P., and Gudas, L. J. (1995) Mol. Cell. Biol. 15, 843–851
52. Lohnes, D., Mark, M., Mendelsohn, C., Dollé, P., Dierich, A., Gory, P., Ganssmuller, A., and Chambon, P. (1994) Development 120, 2723–2748
53. Mendelsohn, C., Lohnes, D., Decimo, D., Luften, T., LeMeur, M., Chambon, P., and Mark, M. (1994) Development 120, 2749–2771
54. Gianni, M., Zanotta, S., Teras, M., Garattini, S., and Garattini, E. (1993) Biochem. Biophys. Res. Commun. 196, 252–259
55. Kooistra, T., Lansink, M., Arts, J., Sitter, T., and Toet, K. (1995) Eur. J. Biochem. 232, 425–432
56. Zhang, L., Mills, K. J., Dawson, M. I., Collins, S. J., and Jetten, A. M. (1995) J. Biol. Chem. 270, 6022–6029