BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 349–354 (1996)
ARTICLE NO. 1804

Retinoic Acid Receptors β and γ Distinguish Retinoid Signals for Growth Inhibition and Neuritogenesis in Human Neuroblastoma Cells

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Received October 17, 1996

Retinoids induce marked growth inhibition and neuritic differentiation in human neuroblastoma cells. Expression patterns of nuclear retinoic acid receptors (RAR) in embryonic and adult tissues suggests that RAR subtypes α, β and γ have tissue-specific functions. We have transfected a human neuroblastoma tumor cell line with a vector expressing either human RAR α, β or γ cDNAs. In the absence of exogenous retinoid, RARβ transfectants demonstrated marked growth inhibition without morphologic evidence of differentiation, whereas transfectant clones overexpressing RARs α and γ had no significant reduction in cell growth rates. Although RARγ transfectants were sensitive to the growth inhibitory effects of exogenous retinoids, these cells demonstrated resistance to the neuritogenic retinoid effects. Only RARβ transfectants exhibited increased sensitivity to retinoids added *in vitro.* These results suggest that distinct neuritogenic and growth inhibitory signalling pathways exist in neuroblastoma cells and that RARβ expression may be necessary for the retinoid growth inhibitory pathway.

Retinoids are vital for the growth and differentiation of a variety of normal adult and embryonic tissues (1), and have potent antiproliferative effects on many malignant cell types (2). Retinoids mediate their widespread effects on cells by regulating the transcription of target genes through a complex system of ligand-inducible nuclear transcription factors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (3). *In vitro* evidence indicates that retinoid signalling involves heterodimerisation of a particular RAR subtype (α, β and γ) with an RXR subtype (α, β and γ) in the presence of retinoid ligand. This complex binds directly to *cis*-acting, retinoic acid-responsive DNA elements in the promoter/enhancer regions of tissue-specific target genes. Some, but not all, of the embryologic abnormalities seen in retinoid-deficient animals have been seen in mice with germline homozygous deletion of each RAR gene (4-6). While RARα is ubiquitously expressed in adult tissues, RARβ and RARγ expression is much more restricted (1,7,8), suggesting that one mechanism for converting a general, to a tissue-specific, retinoid differentiation signal may be through expression of different RARs. Accumulated evidence indicates that, in adult tissues, RARα expression is important for myeloid differentiation (9), while RARβ plays a role in epidermal differentiation (10). In both myeloid and epidermal tissues malignant cellular transformation can occur as a consequence of abnormal RAR expression and function.

Human neuroblastoma cells undergo growth inhibition and neuritic differentiation when treated with retinoids *in vitro* (11). We and others have shown that neuroblastoma primary tumor tissue and cell lines express RARα, and to a lesser degree RARγ (12-15). RARβ is expressed at a very low level in most neuroblastoma cell lines, and, is markedly induced by retinoid treatment of cells *in vitro* (12). In the present study we sought to directly compare

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0006-291X/96 $18.00

the effects of individual overexpressed RAR subtypes on neuroblastoma cells by analysing the growth and differentiation properties of neuroblastoma transfectants overexpressing each RAR subtype.

MATERIALS AND METHODS

Cell culture and transfection. The human neuroblastoma tumor cell line used in these experiments was the N-myc-amplified, BE(2)-C cell line kindly provided by Dr J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). BE(2)-C cells were cultured at 37°C in 5% CO_2 as an adherent monolayer in Dulbecco modified Eagle medium (DMEM) supplemented with L-glutamine and 10% fetal calf serum. Transfections were performed as described (14).

Plasmids. The full-length human cDNAs for RARβ (kindly provided by Prof P. Chambon, INSERM, Strasbourg, France), RARα and RARγ (both kindly provided by Dr R. Evans, Salk Institute, La Jolla, CA, USA) were cloned into the *Pvu*II multi-cloning site of the pMEP4 episomal Epstein-Barr virus-based expression vector (kindly provided by Dr M. Tykocinski, Case Western University, Cleveland, Ohio, USA). The pMEP4 plasmid contains the Hygromycin B resistance gene and utilises the human metallothionein IIA promoter to direct cloned gene expression (16).

Chemicals. All-*trans* retinoic acid (aRA) and 13-*cis* retinoic acid (13RA) were purchased from Sigma (St. Louis, MO, USA). 9-*cis* retinoic acid (9RA) was kindly donated by Hoffman-LaRoche (Australia). The three RAR subtype-specific synthetic retinoids, CD336 (RARα), CD2314 (RARβ) and CD666 (RARγ) were supplied by CIRD-GALDERMA Company (Sophia-Antipolis, France).

Assays of cell growth and neurite formation. Neurite extension was measured as previously reported (13). Cell growth rates were measured, either by counting viable, trypan blue-negative cells, or by using the Alamar Blue reagent (Accu Med International Inc., Iowa, USA) at defined time points following plating. In the Alamar Blue assay, cells were initially plated at a density of 750 cells per well in 96-well culture dishes. On the day of cell counting 20μl of the Alamar Blue reagent was added to each well and then incubated with the cells for 5 hours in 5% CO_2 at 37°C. Comparative values for cell growth in each well were determined by a Titertek Multiskan MCC/340 MKII (ICN Pharmaceuticals Inc., CA, USA) plate reader which measured light absorbance in each well at 570 nm. The mean and standard error of mean (SEM) values of each experiment were determined after at least three replicates. All cell growth and neurite assays were performed in the continuous presence of 10μM $ZnSO_4$ in the culture medium. This concentration of $ZnSO_4$ had no effect on cell growth of control cells and was used to induce cloned gene expression from the human metalliothionein IIA promoter.

Analysis of gene expression by reverse transcriptase/polymerase chain reaction. RARα, RARβ and RARγ mRNA expression levels were assayed using a competitive, reverse transcriptase/polymerase chain reaction (RT/PCR) technique previously described (14,17,18) which involved determining a ratio between the level of expression of each of the RAR genes and that of the control β2-microglobulin gene in DNAse-treated total RNA samples. The PCR primer sequences for β2-microglobulin, RARβ and RARγ have been described (14,15). The gene-specific RARα forward PCR primer was 5′GCGGGCACCTCAATGGGTAC 3′, and the reverse PCR primer 3′ TATCGTGTGGTAGGG-GTCGG 5′. This primer pair generated a 120 bp PCR product extending from bp 264-384 of the published RARα sequence (19).

RESULTS AND DISCUSSION

Transfectant clones of the BE(2)-C neuroblastoma cell line overexpressing either RARα, RARβ, or RARγ were isolated, perpetuated in mass culture in the presence of Hygromycin B, and then examined for the presence of the vector. Southern analysis confirmed the presence of the Hygromycin B resistance gene in all transfectant clones (data not shown). RT/PCR analysis performed 24 hours after the addition of 10μM $ZnSO_4$ to the culture medium, demonstrated that each clone overexpressed the relevant transfected RAR when compared to a control clone transfected with the empty vector [MEP] (Fig. 1).

To determine which RAR was most effective in mediating the retinoid growth inhibitory signal in neuroblastoma cells we compared cell growth rates of each pair of RAR transfectant clones with the control MEP clone (Fig. 2). Overexpression of RARβ caused marked growth inhibition in RARβ clone 8. RARβ clone 3 had a smaller increase in RARβ expression than clone 8 (Fig. 1), and, a concomittantly lower level of growth inhibition, indicating a relationship between the level of RARβ expression and the level of growth inhibition. The morphology of the RARβ transfectants was similar to the control MEP clone. The mean proportion of cells exhibiting neurites in the absence of additional retinoid was 5% for both RARβ clones and 7% for control MEP cells. Thus, RARβ overexpression inhibited cell growth in the absence

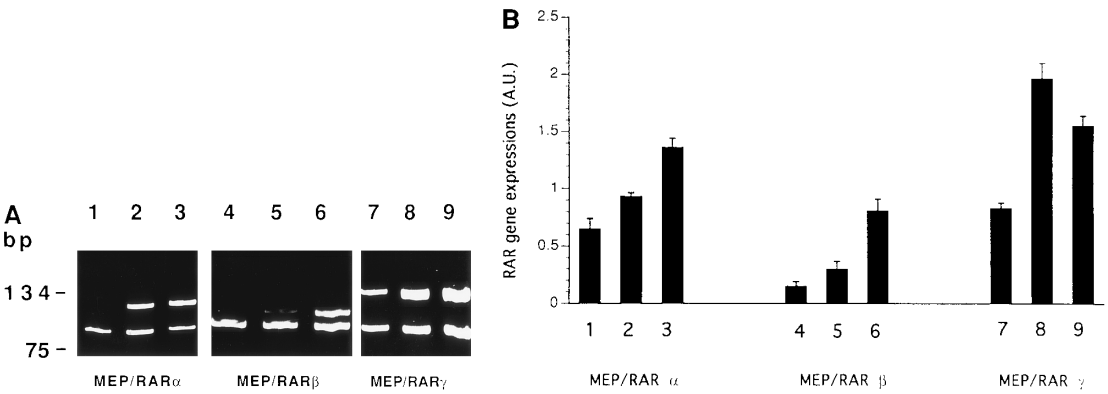

FIG. 1. Overexpression of transfected RARs detected by reverse transcriptase/polymerase chain reaction (RT/PCR) on total RNA from neuroblastoma cell transfectants. (A) Representative RT/PCR assays for expression of RARα (lanes 1-3), RARβ (lanes 4-6) and RARγ (lanes 7-9) in control MEP cells (lanes 1, 4 and 7), MEP/RARα clones 1 (lane 2) and 2 (lane 3), MEP/RARβ clones 3 (lane 5) and 8 (lane 6), and MEP/RARγ clones 2 (lane 8) and 5 (lane 9). cDNA samples obtained from exponentially growing RAR transfectants, 24 hours after the addition of 10μM $ZnSO_4$, were subjected to independent competitive RT/PCR analyses as previously described using RAR subtype-specific PCR primers together with β2-microglobulin control primers (14,17,18). An aliquot of the PCR product was then electrophoretically size-fractionated on a polyacrylamide gel as shown here. Densitometry was performed on the photographic negatives and a ratio determined for the RAR and β2-microglobulin bands. (B) Each PCR experiment was performed at least three times and a mean and standard error of mean determined for each RAR:β2-microglobulin ratio.

of excess retinoid, without inducing neuritic differentiation. The two RARα and RARγ transfectant clones exhibited cell growth rates and morphology which were similar to control MEP cells. We next evaluated whether increased expression of RAR subtypes enhanced the sensitivity of the cell to retinoids by testing RAR transfectants for growth inhibition and neuritogenesis at the end of one week of continuous exposure to low concentrations (0.1μM) of the three naturally occuring retinoids aRA, 9RA and 13RA (Fig. 3A and 3C). Only RARβ transfectants demonstrated enhanced retinoid sensitivity when compared to control MEP cells for growth inhibition and neuritogenesis. The RARγ transfectant clone was resistant to the neuritogenic effects of all three naturally occuring retinoids (Fig. 3C).

We hypothesised that synthetic, RAR subtype-specific retinoids may have improved efficacy when used in neuroblastoma cells transfected with the corresponding RAR subtype. The synthetic retinoids CD336 (RARα), CD2314 (RARβ) and, CD666 (RARγ) have previously been shown to have RAR subtype-specific *in vitro* DNA binding activity using COS-7 cell nuclear extracts, and, RAR subtype-specific transactivation properties in HeLa cells (20,21). We measured cell growth and neuritogenesis in RAR transfectants after one week of continuous treatment with these three synthetic retinoids (0.1μM) (Fig. 3B and 3D). The RARα- and RARγ-specific retinoids were more effective growth inhibitors than the RARβ-specific retinoid in control MEP cells. Treatment of each RAR transfectant with its corresponding synthetic RAR-specific retinoid did not significantly increase the level of growth inhibition compared with the effects of aRA, 9RA and 13RA. However, CD336 (RARα) and CD666 (RARγ) were more effective at inducing neuritogenesis than aRA, 13RA and 9RA in control MEP cells.

The present study provides further support for the notion of separate retinoid signalling pathways for neuritogenesis and growth inhibition in neuroblastoma cells (14,22). In our experiments, overexpressed RARγ inhibited retinoid-induced neuritogenesis, but not retinoid-induced growth inhibition, while overexpressed RARβ induced growth inhibition without neuritogenesis, in the absence of added retinoid. Our results, combined with the observed

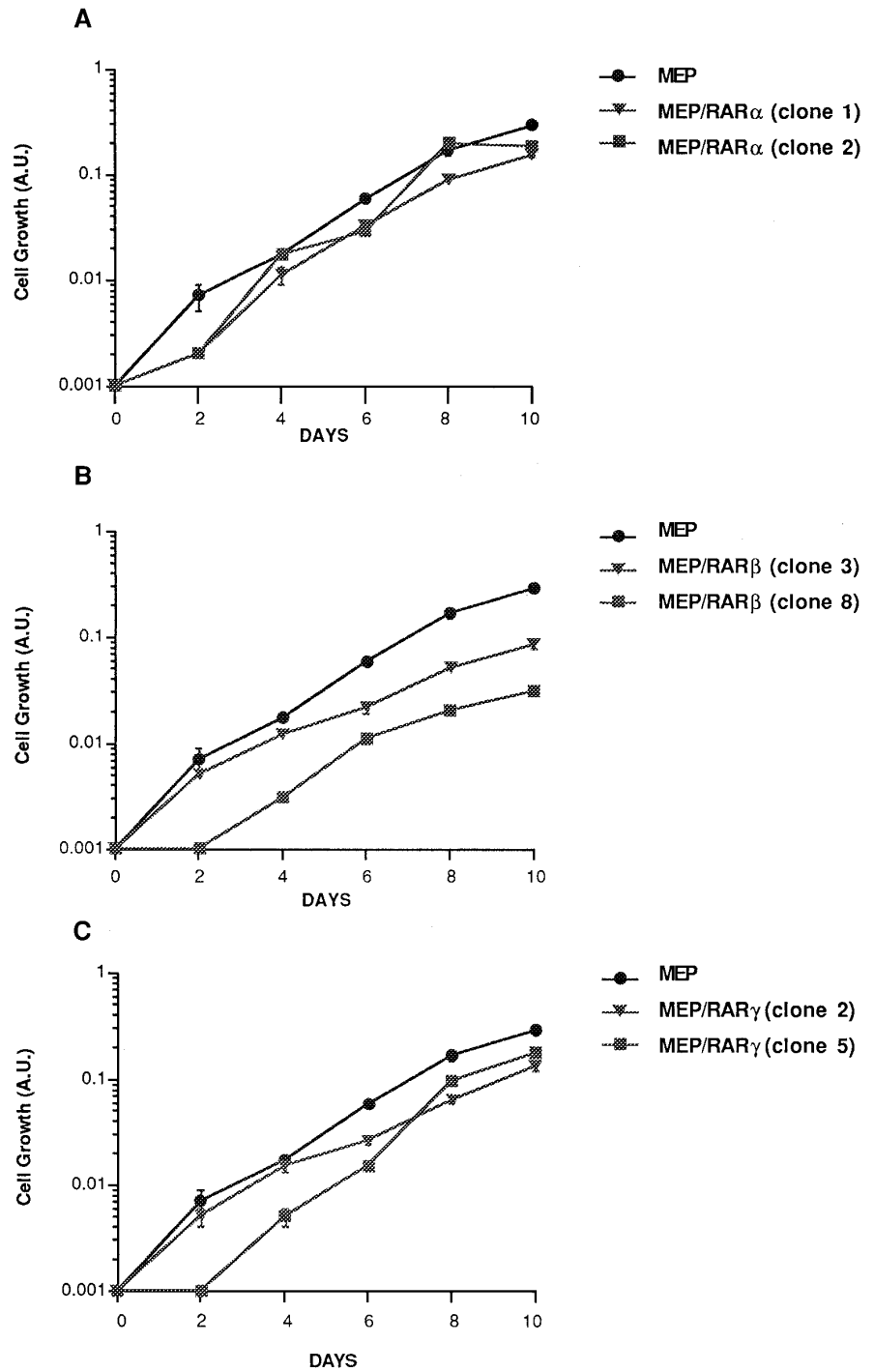

FIG. 2. Growth curves for RAR transfectants clones. Cell growth was measured at defined time points using the Alamar Blue reagent as described in materials and methods. Day 0 represents the day of plating. All data points represent the mean and standard error of mean of at least three replicate experiments.

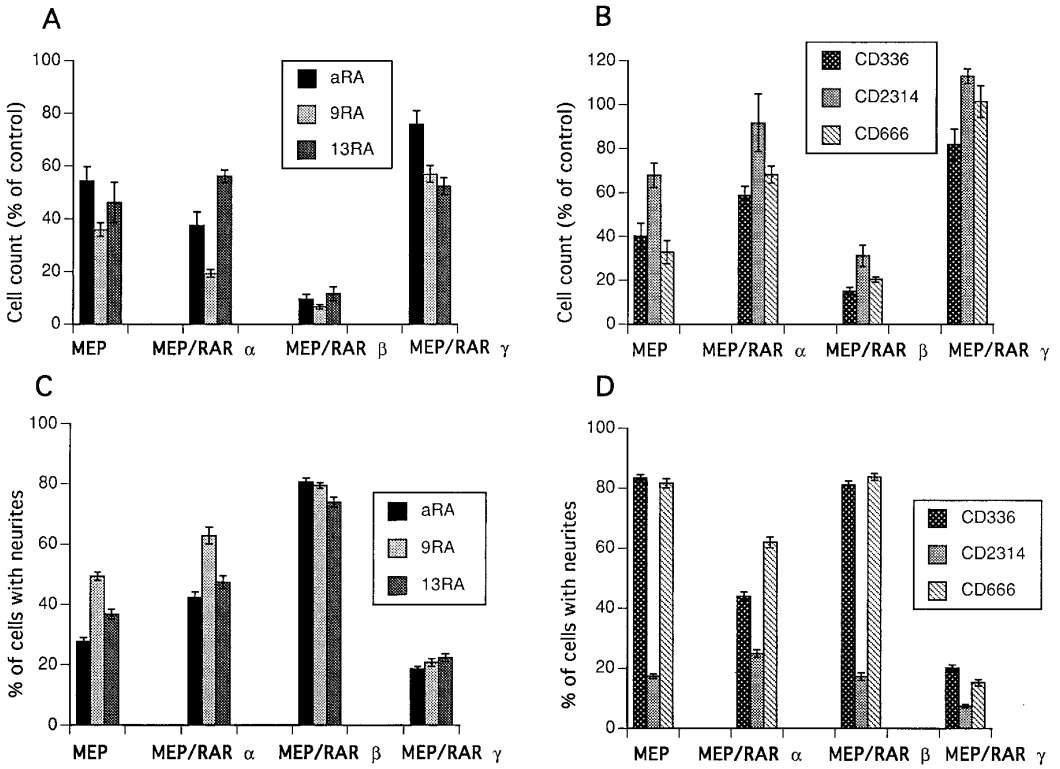

FIG. 3. Comparative cell growth and neurite formation of RAR transfectants following 7 days of retinoid treatment. Cell growth was determined by counting viable cells after 7 days of (A) natural or (B) synthetic retinoid treatment and the values were expressed as a percentage of untreated control MEP cell numbers after 7 days of growth. Cells were scored as positive for neurite formation, following (C) natural or (D) synthetic retinoid treatment, if the neuritic process was longer than the cell body and the value for each experiment was expressed as the number of cells positive for neurite formation as a percentage of the total number of cells counted. All data points represent the mean and standard error of mean of at least three replicate experiments.

induction of endogenous RARβ expression in neuroblastoma cells treated with aRA, suggest that RARβ expression may be necessary for conveying, or amplifying, the retinoid-induced growth inhibitory signal.

Endogenous RARβ gene expression is markedly induced as an early response in many cell types treated with retinoids, pointing to a tissue non-specific effect of RARβ in retinoid signalling. In breast cancer cells RARβ overexpression restored retinoid-induced growth inhibition (23,24), but did not alter cell growth in the absence of additional retinoid. In neuroblastoma cells RARβ overexpression, in the absence of excess retinoid, caused growth inhibition in neuroblastoma cells and enhanced retinoid sensitivity. Moreover, only low levels of RARβ expression were required to affect cell growth, suggesting that neuroblastoma cells may be much more sensitive to the effects of RARβ overexpression than breast cancer cells.

In the presence of the corresponding overexpressed RAR subtype, RAR subtype-selective retinoid ligands did not substantially enhance retinoid effectiveness in neuroblastoma cells. Since the original assays for ligand-receptor interactions were not performed in neuroblastoma cells our result may simply reflect inconsistencies between *in vitro* biochemical assays and *in vivo* cell biologic measures of retinoid efficacy. The accumulated evidence supports the model of RAR-RXR heterodimers as the functional transcriptional regulator of retinoid action (3).

Indeed, another explanation for the lack of efficacy of the receptor-selective ligands may be differing RXR expression levels between different cell lines. Our data does not support the contention by some workers (25) that the biochemical and cellular effects of retinoid receptor-selective ligands directly reflect signals mediated by corresponding RAR or RXR subtypes. Taken together our data indicates that the nuclear RARs are important determinants of the cellular response to retinoids in malignant tissues and provides further support for a link between RARβ-activated retinoid signalling pathways and retinoid-induced growth inhibition.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health & Medical Research Council (GMM, MDN, MH), New South Wales State Cancer Council (GMM, BWS, MDN, MH), Sydney Children's Hospital Foundation (GMM), Leo & Jenny Leukaemia and Cancer Research Foundation (GMM), and the Childrens Cancer Foundation Australia (GMM, BWS, MDN, MH).

REFERENCES

1. Gudas, L. J., Sporn, M. B., and Roberts, A. B. (1994) *in* The Retinoids: Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.), pp. 443–520, Raven Press, New York.
2. Smith, M. A., Parkinson, D. R., Cheson, B. D., and Friedman, M. A. (1992) *J. Clin. Oncol.* **10,** 839–864.
3. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) *in* The Retinoids: Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.), pp. 319–349, Raven Press, New York.
4. Chambon, P. (1993) *Gene* **135,** 223–228.
5. Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M., and Chambon, P. (1993) *Cell* **73,** 643–658.
6. Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J.-L., Dolle, P., and Chambon, P. (1994) *Cell* **78,** 987–1003.
7. Dolle, P., Ruberte, E., Leroy, P., Morris-Kay, G., and Chambon, P. (1990) *Development* **110,** 1133–1151.
8. Ruberte, E., Dolle, P., Chambon, P., and Morris-Kay, G. (1991) *Development* **111,** 45–60.
9. Grignani, F., Ferrucci, P. F., Testa, U., Giampaolo, T., Fagioli, M., Alcalay, M., Mencarelli, A., Grignani, F., Peschle, C., Nicoletti, I., and Pelicci, P. G. (1993) *Cell* **74,** 423–431.
10. Lotan, R., Xu, X.-C., Lippman, S. M., Ro, J. Y., Lee, J. S., Lee, J. J., and Hong, W. K. (1995) *N. Engl. J. Med.* **332,** 1405–1410.
11. Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. (1983) *Exp. Cell Res.* **148,** 21–30.
12. Clagett-Dame, M., Verhalen, T. J., Biedler, J. L., and Repa, J. J. (1993) *Arch. Biochem. Biophys.* **300,** 684–693.
13. Marshall, G. M., Cheung, B., Stacey, K. P., Norris, M. D., and Haber, M. (1994) *Anticancer Res.* **13,** 437–442.
14. Marshall, G. M., Cheung, B., Stacey, K. P., Camacho, M. L., Simpson, A. M., Kwan, E., Smith, S., Haber, M., and Norris, M. D. (1995) *Oncogene* **11,** 3729–3735.
15. Haber, M., Madafiglio, J., Cheung, B., Marshall, G. M., and Norris, M. D. (1994) *Prog. Clin. Biol. Res.* **385,** 245–252.
16. Groger, R. K., Morrow, D. M., and Tykocinski, M. L. (1989) *Gene* **81,** 285–294.
17. Bordow, S. B., Haber, M., Madafiglio, J., Cheung, B., Marshall, G. M., Norris, M. D. (1994) *Cancer Res.* **54,** 5036–5040.
18. Norris, M. D., Bordow, S. B., Marshall, G. M., Haber, P. S., Cohn, S. L., Haber, M. (1996) *N. Engl. J. Med.* **334,** 231–238.
19. Giguere, V., Ong, E. S., Segui, P., and Evans, R. M. (1987) *Nature* **330,** 624–629.
20. Schadendorf, D., Worm, M., Jurgovsky, K., Dippel, E., Michel, S., Charpentier, B., Bernardon, J.-M., Reichert, U., and Czarnetzki, B. M. (1994) *Int. J. Oncol.* **5,** 1325–1331.
21. Bernard, B. A., Bernardon, J.-M., Delescluse, C., Martin, B., Lenoir, M. C., Maignan, J., Carpentier, B., Pilgrim, W. R., Reichert, U., and Shroot, B. (1992) *Biochem. Biophys. Res. Commun.* **186,** 977–983.
22. Bader, S. A., Fasching, C., Brodeur, G. M., and Stanbridge, E. J. (1991) *Cell Growth & Diff.* **2,** 245–255.
23. Liu, Y., Lee, M. O., Wang, H. G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. K. (1996) *Mol. Cell. Biol.* **16,** 1138–1149.
24. Seewaldt, V. L., Johnson, B. S., Parker, M. B., Collins, S. J., and Swisshelm, K. (1995) *Cell Growth Diff.* **6,** 1077–1088.
25. Melino, G., Draoui, M., Bernardini, S., Bellincampi, L., Reichert, U., and Cohen, P. (1996) *Cell Growth Diff.* **7,** 787–796.