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13. ABSTRACT *(Maximum 200 words)*

We have performed ßRARE gel shift analyses and partial RARß promoter sequence analysis for 4 and 5 breast cancer cell lines, respectively. We have introduced a functional RARß gene into four breast cancer cell lines by retroviral vector mediated transduction. We have analyzed 24 differential mRNA transcripts from the cell line, MCF7 cultured in the presence of retinoic acid. We have initiated studies to determine RARß expression in primary tumors.

Tumor cells exhibit a dramatically more complex binding to the promoter response element, RARE for all tumor lines tested to date (n=3) compared to normal mammary epithelial cells. We have found 2 mutations in the promoter of the cell line, MDA-MB-231. We show that reconstitution of an expressed RARß gene results in dramatic growth suppression of breast cell lines: MCF7 and MDA-MB-231, with induction of apoptosis in the MCF7 cells when they are cultured with retinoic acid. **Our findings continue to support the hypothesis that the RARß gene product is a candidate tumor suppressor gene for breast cancer.**

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Karen Swisshelm Sept. 18, 1995

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U.S. Army Medical Research & Materiel Command
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Presentation of Annual Report
September 21, 1995

Expression and Regulation of the Retinoic Acid Receptor Beta Gene in Human Mammary Epithelial Cells

P.I.: Karen Swisshelm

Introduction:

Retinoic acid receptors (RARs): The current model for retinoic acid or retinoid action in the prevention or "cure" of certain cancers is their ability to induce and maintain cellular differentiation. Differentiation is thought to be specifically programmed through a family of nuclear transcription factors, the retinoic acid receptors (RARs) and the previously named "orphan" receptors, the RXRs. There are three RARs and three RXRs (α, β, and γ). Each receptor is a distinct, single gene, mapped to different chromosomes. Multiple isoforms for each RAR have been identified. The isoforms differ in their N-terminal A region and are created by differential usage of tandem promoters and/or alternative splicing. The factors that determine and regulate isoform expression are unknown. Evolutionary data suggests that each RAR may have a distinct function that has been conserved throughout evolution because RARs show a high nucleotide sequence homology within a single subtype across different species and less so among themselves in the same species. RARs and RXRs and their subtypes differ in their affinity for different retinoid ligands. RARs bind both *all trans* retinoic acid(RA) and 9 cis RA while RXRs only bind 9 cis RA[1].

Retinoids and breast cancer: Breast cancer is the most frequent tumor in women, and there is intense effort to understand it's etiology in order to advance rational preventive as well as therapeutic measures. Important clues as to the importance of retinoids come from epidemiology. Dietary intake of carotenoids (plant source of vitamin A) is inversely associated with the risk of breast cancer. These observations have been made by many investigators (rev in:[2]) and most recently within the large nurses' study in Boston [3]. From a large body of animal carcinogenesis studies, retinoids are found to reduce mammary tumor burden in mice and rats [rev. in:4].

Retinoic acid receptors and breast cancer: The first reports addressing RAR expression in breast carcinoma demonstrated that: **i)** most carcinoma cell lines express RARα and **ii)** breast cancer cell lines that were estrogen receptor negative are apparently resistant to growth regulation by retinoic acid [5, 6]. It was noted within those reports that RARβ expression was diminished or absent in most of the tumor lines. We have shown that RARβ expression is present in normal human mammary cells in culture (from cultures established from 5 different individuals), in contrast to the tumor cells; and, quite dramatically, that RARβ expression increases as normal mammary cells "senesce" in culture, an *in vitro* aging phenomenon whereby cells fail to replicate in the presence of growth factors. We speculate that one program RARβ regulates is growth suppression, as observed in normal aging or senescence. It was futher demonstrated that suppression of RARβ was at the trancriptional level [7].

To date only one report has investigated the expression of the retinoic acid receptors in primary breast carcinoma, and that study determined that tumor samples with RARα expression were likely to also be estrogen receptor positive [8]. Estrogen receptor expression is very important in the biology of breast carcinomas. ER positive (ER^+)

carcinoma is more prevalent in post menopausal women while ER negative (ER^-) more so in younger women. Patients with ER^- tumors have a worse prognosis. It has been hypothesized that mechanisms regulating transcription of the ER gene influence the phenotype of breast carcinomas [9]. Thus the treatment of each tumor depends on its ER status as well as other histologic criteria.

Recently many studies have indicated interaction between retinoic acid receptors, estrogen receptors, estrogen and retinoids. Also retinoids and retinoic acid receptors have been shown to have antagonistic effect on estrogen responsive genes, e.g. transforming growth factor α (TGFα) and pS2, which, in turn, has profound effect on cell proliferation. RA suppresses the estradiol induced increase in expression of these genes [10] . The exact mechanism of how RA suppresses the estradiol induced increase in expression of these genes is not known. But it is likely that RA may inhibit the estradiol-induced growth of breast carcinoma cells through suppression of the estrogen responsive genes. It should also be noted that ER^- breast cancer cell lines have been shown to be unresponsive to RA-induced growth inhibition [6, 11, 12]. Most breast tumor lines studied for the expression pattern of retinoic acid receptors and ER status show a difference in RARα levels, which is higher in ER^+ and lower in ER^- cells [6, 8]. In addition RARα expression in ER^+ tumor cells can be induced by estradiol and abrogated by antiestrogens [8]. Previously it has been found that the human RARα 1 promoter contains a ERE-like sequence [13]. Up regulation of RARα could also be a possible mechanism by which estrogens sensitize ER^+ cells for growth inhibition by RA. This indicates that study of RARα status in patients is important for potential therapeutic use of retinoids [14].

As mentioned earlier RXRs can play a central role in hormone receptor action and thus regulate a number of genes. Recently inhibitory effect of RXRß on estrogen response elements (ERE) has been reported, thus effecting expression of many estrogen responsive genes [15]. RXRß induced repression of estrogen responsive genes was also shown to be through a ligand independent pathway and requiring involvement of unidentified factors , indicating that there are elements other than retinoids whose effects are mediated through RXRs. It is thus paramount, in light of the ongoing and increasing use of retinoids in breast cancer therapeutics, to understand whether or not a patient's normal or tumor tissue exhibits expression of specific RARs/RXRs, and what receptors are critical for maintaining growth control or differentiation.

Overview: This progress report addresses four areas of work accomplished over the past year:

1) We have began studies on the promoter of the RARß gene (**Task 1**): sequencing of tumor lines and analysis of gel shift patterns in a number of the breast tumor lines.

2) We have made major inroads towards **Task 2** by the introduction of a functional RARβ gene into breast tumor cell lines (**See Body/Conclusions/ and Appendix).**

3) We have examined expression of genes expressed in cells cultured in the presence of retinoic acid by the means of differential display (**Task 3**).

4) We have critically evaluated all of the state of the art methodology to assess levels of RARß gene expression in primary tumors, and have begun work on *in situ* hybridization and reverse transcriptase- polymerase chain reaction (RT-PCR), using breast tumor cell lines which do or do not express RARß mRNA (**Task 4**).

Body:

1) **Analysis of RARß promoter (Task 1)**

a) ***Gel shift analysis***: We have derived nuclear and well as cytoplasmic extracts from normal human mammary epithelial cells (HMECs) [AG11132 and AG11134, Coriell Cell repository] at early and late passage as well as nuclear extracts from the following breast tumor cell lines: Hs578T and MDA-MB231. Double stranded target RARß retinoic acid receptor response element (ßRARE), both mutant and wild-type, have been synthesized, end-labeled with 32P-gamma-dATP, and annealed. The following 5' to 3' upper strand sequences were used:

WT	GGGTAGGGTTCACCGAAAGTTCACTCG
Mutant:	GGGTAGGCTTACCCGAAAGTTCACTCG
*= non-identity	* **

Gel shift assays have revealed the following results:

i) nuclear extracts from normal HMECs exhibit a single nuclear protein complex or band when analyzed on a native polyacrylamide gel exposed to Xray film with the wild type ßRARE and approximatey 10 fold reduced binding to the mutant ßRARE.

ii) nuclear extracts from the tumor lines, Hs578T and MDA-MB-231 (both ER^-) exhibit reduced and a higher molecular weight binding to the wild type probe. Hs578T is one of the few (4/16) breast cancer cell lines with elevated expression of RARß--although we do not know at this point if this line encodes a mutant [and therefore dominant negative] receptor.

iii) nuclear extracts from the tumor line, MDA-MB-231, exhibit high molecular weight complexes with the mutant ßRARE at about 10 fold higher level than for the wild-type response element.

b) ***Sequence analysis of the RARβ promoter in breast cancer cell lines:***

The following primer sets have been designed (Primer program; Whitehead Institute) and synthesized to amplify: a 278 bp (I); 260 bp (II) and 1212 bp (III) fragments, respectively, of the promoter from genomic DNA derived from breast tumor cell lines:

Primer set I	forward:	5'- AGGAGCAGCGTCCCGGC-3'
	reverse	5'-CCTACTACTTCTGTCAC-3'
		inclusive for nucleotides -275 to +17 relative to the start of transcription:
Primer set II	forward:	5'- GTGGCCTGTGTGTTTGGGAC-3'
	reverse	5'-CTCGCAGTGTAGAAATCCAGG-3'
		inclusive for nucleotides -720 to -461 relative to the start of transcription
Primer set III	forward:	5'- GTGGCCTGTGTGTTTGGGAC-3'
	reverse	5'-CTCGCAGTGTAGAAATCCCAGG-3'
		inclusive for nucleotides -720 to + 535

Genomic DNA has been isolated from the following cell strains/lines: AG-11132, AG-11134, BT474, BT483, BT549, MCF7, MDA-MB-134, MDA-MB-231, MDA-MB-361, MDA-MB-435, T47D, and ZR 75-1.

We have determined the optimal polymerase chain reaction (PCR) conditions for each of the primer pairs above and amplified promoter DNA from 5 tumor lines. All experiments include a positive [D1 plasmid containing 5 kb of the promoter [16]] as well as negative control amplification.

Sequence analysis (ABI, automatic seqeunce analysis) using primer set I accompanied by computer sequence comparison (Genetics Computer Group, University of Wisconsin) has been accomplished for MDA-MB-231, from which we have identifed 2 mutations in comparision to the published sequence. They occur at nucleotides -16 and -17 and are highlighted in the sequence figure below. At this time, we do not know the functional signficance of these base pair changes, nor how they may be related to the overall RARß promoter function.

Figure 1. Nucleotide sequence of Promoter region of the human beta retinoic acid receptor beta. Location of primers for amplification of promoter sequence are indicated.

```
-743                                                    AGATCTGAAATCTCATTTTCTGTG
          primer set II, III forward
-719  TGGCTGTGTGTTTGGGACAGGGGTAACCAATTCCTGACTACTCTATATGCTGCATAGAA

-660 CCTGGAGAGGATTTTTCAAAGTAAATGAATCTCGAAAGCTGGATTGCAGAGCAAACGAG

-601 TGCAGTACAATTCAGCCAGGGGCTTGCAAGAGGAGAAAGAGAAAAAGACTCTGGAATGGA

-541 AAGTTTCCCAACCCAAGCCTTTCCCAAGGGGTAGCCATTCTCTGTTCTACAGTTTAGGG
           primer set II reverse
-482 CTTGCATGTGCTTTTTCTGGAGTCCAAAAATACATAAGTTATAACCAATTTAACAGACA

-423 GAAAGGCGCACAGAGGAATTTAAAGTGTGGGCTGGGGCGAGGCGGTGGGCGGGAGGC

-366 GAGCGGGCGCAGGCGGAACACCGTTTTCCAAGCTAAGCCCGCCGCAAATAAAAAGGCGTA
                                                  primer set I forward
-306 AAGGGAGAGAAGTTGGTGCTCAACGTGAGCCAGGAGCAGCGTCCCGGCTCCTCCCCTGC

-247 TCATTTTAAAAGCACTTCTTGTATTGTTTTTAAGGTGAGAAATAGGAAAGAAAACG

-191 CCGGCTTGTGCGCTCGCTGCCTGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCTGGGA
                                                  CRE              AP1
-132 GTTTTTAAGCTCTGTGAGAATCCTGGGAGTTGGTGATGTCAGACTAGTTGGGTCATTTG
                                RARE                          TATA Box
-73 AAGGTTAGCAGCCCGGGTAGGGTTCACCGAAAGTTCACTCGCATATATTAGGCAATT
                          primer set I reverse
-16 CAATCTTTCATTCTGTGTGACAGAAGTAGTAGGAAGTGAGCTGTTCAGAGGCAGGAGGG
                    ↳→
+46 TCTATTCTTTGCCAAAGGGGGGACCAGAATTCCCCCATGCGAGCTGTTTGAGGACTGGG

+105 ATGCCGAGAACGCGAGCGATCCGAGCAGGGTTTGTCTGGGCACCGTCGGGGTAGGATCC

            Primer set III reverse
....+515 CCTGGATTTCTACTGCGAGT+535
```

c) Luciferase reporter gene constructs for studying the specific negative regulatory elements utilized by breast tumor cells. We have been fortunate to obtain a series of luciferase reporter constructs generated in the laboratory of Dr. Monica Peacocke (Department of Dermatology, Columbia University) which span between -1.5kb to +155 bp.

2) **Introduction of functional RARß gene into breast tumor cells results in growth arrest and apoptosis (Task 2):** In order assess the functional significance of loss of expression of the RARß gene we introduced the full length human gene into four breast tumor cell lines: MCF7, MDA-MB-231, MDA-MB-435, and BT 474. Transduces clones from all four lines exhibited growth arrest upon culture in the presence of retinoic acid. **The full report on results from two of the lines has been recently published (*Cell Growth and Differentiation* 6:1077-1088, September 1995) and a copy of the paper can be found in the appendix.** RARß+ clones from one cell line studied in detail, MCF-7, not only showed growth suppression, but also underwent programmed cell death or apoptosis by three different assays. Our results offer the exciting possibility that the RARß gene can be considered as possible tumor suppressor gene. We will next be assessing this possibility by studying these RARß cells in nude mice.

3) **Determination of genes expressed as a consequence of culture in retinoic acid (Task 3):** We hae begun our studies to detect differences in gene expression in cells cultured in the differentiating agent, retinoic acid. We have used the method of differential display (DD) of mRNA [see recent publication:[17]]. This methodology is very powerful as it can detect transcripts/genes which are differentially regulated in either a positive or negative fashion. The table below shows our progress with this task.

Table 1

Number of primer combinations tested	25
Bands eluted from DD gel	24
Gene candidates cloned	9
Candidates to be cloned	4
Gene candidates sequenced	3
Sequenced candidates for continued study	1
Candidates still under investigation (for cloning or sequencing potential)	10

We have elected to further study one candidate, which exhibits approximately 2-4-fold increased expression in MCF7 cells cultured in 1 uM retinoic acid for 48 hours. This gene encodes for a protein related to a ubiquinol-cytochrome C reductase 9.5 kD polypeptide.

This peptide is likely to be part of a inner mitochondrial space complex responsible for electron/proton pumping, and ultimately the energy metabolism of cells. It is likely to be a nuclear protein. Future studies will be aimed at cloning the full length clone from a cDNA library and complete sequence analysis.

4) **Initiation of studies to determine quantitative and qualitative expression of the RARß gene in vivo (Task 4).**

a) We have begun to study quantitative differences between the expression of RARα, ß and γ by RT-PCR analysis of RNA from tumor and normal cell lines/strains using the primers described by Ferrari et al. [18]. This method was chosen since it will allow the use of small tissue samples (next stage) and amplification of the three RAR types from a single cDNA preparation, avoiding a major variable, which is the yield of the reverse transcription reaction (RT). We have initiated standardization of quantitative RT-PCR of tumor cell line Hs578T and normal HMEC cell line, AG11134. PCR amplification of the expected RARα and ß products has been accomplished from the internal standard generated by Ferrari et al. Amplification of RARα from both cell lines has been accomplished.

b) For the determination of qualitative expression of the RARß gene in tumor sections, we have begun to determine parameters for *in situ* hybridization to tumor cell lines MCF7 (negative control) and Hs578T (positive control). Cell pellets have been formalin fixed, embedded in paraffin and sections of 10 microns prepared. Biotinylated sense and antisense RNA probes for the human RARs in the eukaryotic expression vectors pSG5 or pTL1 (Pierre Chambon) are prepared according to the manufacturers suggestion (Boehringer Mannheim). The signal is amplified with a mouse antibiotin antibody and goat antimouse antibody and detected by horseradish peroxidase conjugated streptavidin and chromogen application. The slides are counterstained with sefronin orange and acridine orange and scored for the presence or absence of bluish black signal.

We have also collected and stored breast biopsy specimens from over 100 individuals.

Conclusions:

1. We have demonstrated that two additional breast cancer cell lines, Hs578T and MDA-MB-231 exhibit differential protein complexes for the ßRARE, compared to complexes seen for normal cells.

2. We have identified a set of mutations in the RARß promoter of the ER- breast tumor cell line, MDA-MB-231, which could be responsible for partial suppression of expression of the gene.

3. We have demonstrated that introduction of the human RARß gene into four human breast tumor cell lines elicits growth suppression when cells are cultured in the presence of retinoic acid; and that the ER+ cell line, MCF7, undergoes programmed cell death.

4. We have isolated a novel gene, related to ubiquinol cytochrome C reductase, which is upregulated by retinoic acid in MCF7 breast cancer cells.

In summary, our hypothesis that RARß is a tumor suppressor for breast cancer is supported by the evidence that growth is suppressed when the gene is placed into tumor lines and by the finding specific high molecular weight binding of protein complexes for

the ßRARE in 3/3 tumor cell extracts tested to date in comparison to absence of this high molecular weight binding in 3/3 normal HMECs tested to date.

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Expression of Retinoic Acid Receptor β Mediates Retinoic Acid-induced Growth Arrest and Apoptosis in Breast Cancer Cells[1]

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Abstract

The expression of the retinoic acid receptor β (RARβ) mRNA is absent or down-regulated in most human breast cancer cell lines. To investigate the role RARβ may have in regulating the proliferation of breast cancer cells, we used retroviral vector-mediated gene transduction to introduce the human RARβ gene into two RARβ-negative breast tumor cell lines, MCF-7 and MDA-MB-231. RARβ-transduced clones underwent growth inhibition associated with G_1 arrest when treated with 1 μM all-*trans*-retinoic acid (RA). Moreover, the MCF7-RARβ transduced clones also underwent apoptosis after 4 to 6 days of RA treatment. The RA-induced growth arrest in MDA231-RARβ transduced cells is associated with c-myc mRNA down-regulation, whereas the RA-mediated apoptosis of MCF7-RARβ transduced cells is not associated with c-myc down-regulation. These observations suggest a critical role for RARβ in mediating growth arrest and apoptosis in breast cancer cells.

Introduction

Retinoids and their receptors are involved in regulating normal epithelial cell growth and differentiation (1) and may play a key role in breast cancer chemoprevention (2). Understanding the mechanisms by which retinoids and their receptors interact with cellular regulatory pathways and prevent malignant transformation is important in developing novel treatment and preventive strategies.

The biological effects of RA[3] are mediated through a complex array of nuclear RARs belonging to the steroid/thyroid hormone superfamily of transcription factors (3–7). Multiple RARs have been identified; among these are RARα, β, and γ. RARα is expressed ubiquitously in adult tissue, and RARγ is primarily expressed in skin. RARβ is unique because it is primarily restricted to epithelial cells and exhibits induced expression in response to RA that is mediated by an enhancer element, the retinoic acid response element, within its promoter (8, 9). Although normal cultured human mammary epithelial cells express RARβ mRNA, most breast cancer cell lines fail to express this gene. Furthermore, RARβ mRNA expression is up-regulated in senescent human mammary epithelial cells, suggesting that this gene may be involved in regulating terminal differentiation of mammary epithelium (10).

Since RARβ may be involved in mediating normal cellular differentiation, the loss of its expression could contribute to malignant transformation. The lack of expression of RARβ has been demonstrated by Northern analysis in several solid tumor types including lung carcinoma (11, 12), squamous cell carcinoma of the oral pharynx (13), and epithelial ovarian cancer (14). This raises the possibility that RARβ is a general regulator of cellular proliferation and that its selective inactivation allows cells to bypass one of the major pathways for growth control. This hypothesis is supported by Houle *et al.* (15), who report that RARβ inhibits metastasis and growth of epidermoid lung cancer cell lines in a nude mouse model.

The RARs may also be involved in mediating apoptosis (programmed cell death) of certain cells. Apoptosis (16–18) plays an important role in embryogenesis (19, 20), in normal tissue involution (21, 22), and in regulating the death of terminally differentiated cells (23, 24). There is evidence that retinoid-mediated truncation defects of the embryonic limb are caused by apoptosis mediated by the RAR-β2 nuclear receptor (25). Apoptosis also appears to play an important role in mediating the action of antineoplastic agents such as ionizing radiation (26, 27) and many antitumor drugs (28–30). Tumor response to therapy may depend on the ability of individual malignant cells to undergo apoptosis (31–33).

In the present study, we describe the use of retroviral vector-mediated gene transduction to introduce the RARβ gene into two breast epithelial tumor cell lines, MCF-7 and MDA-MB-231, which do not express RARβ mRNA (10). We observe in these RARβ-transduced breast cancer cell lines that RA induces apoptosis in ER-positive MCF-7 cells and includes marked growth inhibition, without apoptosis, in the ER-negative MDA-MB-231 cells. Moreover, we note that the RA-induced growth arrest in MDA231-RARβ transduced cells is associated with c-myc mRNA down-regulation relative to RA-treated vector controls. In contrast, the RA-induced apoptosis of MCF7-RARβ transduced cells is not associated with c-myc down-regulation.

Results

Retroviral-mediated Transduction of RARβ into Breast Cancer Cell Lines. As detailed in "Materials and Methods," we constructed an LXSN-based retroviral vector harboring the coding sequence of the human RARβ gene (Fig. 1). This

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[3] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; ER, estrogen receptor; TdT, terminal deoxynucleotidyl transferase; PI, propidium iodide; FACS, fluorescence-activated cell sorting.

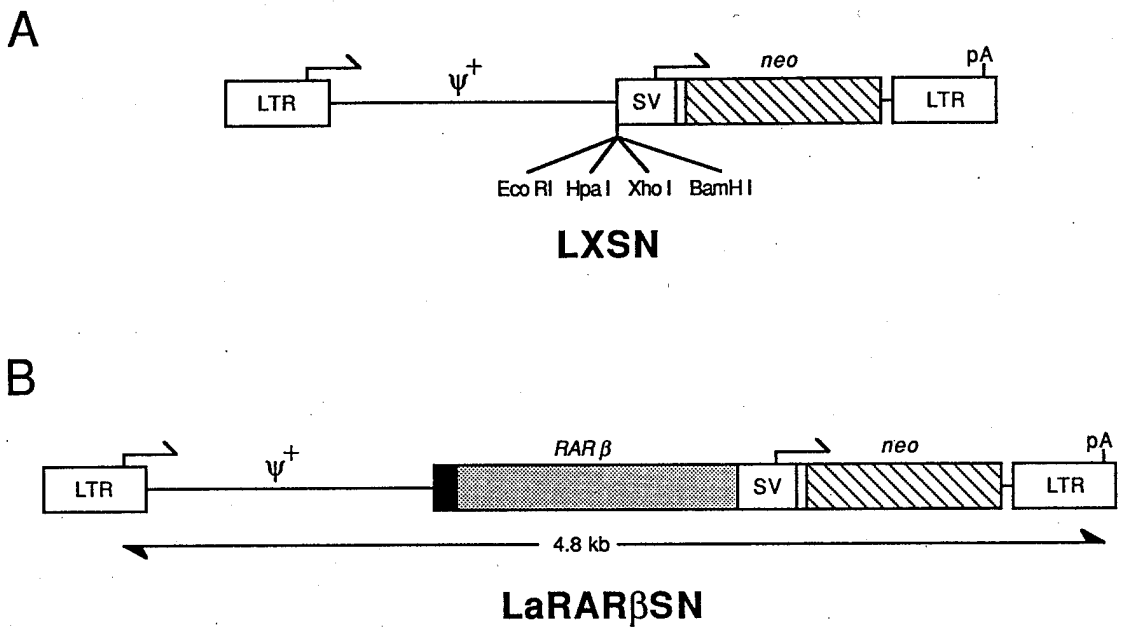

Fig. 1. Structure of the retroviral control vector LXSN (*A*) and the vector LaRARβ-SN containing the RARβ coding sequence (*B*). The LaRARβSN vector consists of the RARβ cDNA inserted into the LXSN cloning site. Construction is detailed in "Materials and Methods." The *black region* in the LaRARβSN vector corresponds to 94 bp of 5′ untranslated adenosine deaminase sequence.

vector includes a 94-bp sequence of 5′ untranslated adenosine deaminase sequence to place the cDNA in an optimum translational context (34), as well as the neomycin phosphotransferase selectable marker. This RARβ retroviral construct is designated LaRARβSN.

The cell lines MCF-7 (ER positive) and MDA-MB-231 (ER negative), which do not express RARβ mRNA (10), were infected with the LaRARβSN retroviral vector, and individual G418-resistant clones were isolated (see "Materials and Methods"). These transductants are designated MCF7-RARβ and MDA231-RARβ, respectively. Vector control clones were obtained by infecting the same cell lines with the retroviral vector LXSN, which does not contain the RARβ gene (Fig. 1). These controls are designated MCF7-SN and MDA231-SN.

Northern and Western blots were performed on the viral transduced cells to determine the levels of RARβ mRNA and protein expression, respectively. The LTR-initiated 4.8-kb RARβ mRNA was observed in the LaRARβSN-infected cells but not in the parental cells nor vector controls (Fig. 2). Nine of 10 MCF7-RARβ clones and 9 of 9 MDA231-RARβ clones that were tested expressed the transduced RARβ mRNA. There was no change in RARβ mRNA expression when RARβ transduced cells were treated with 1 μM RA. Endogenous RARβ mRNA was not expressed by vector control or parental cells, nor was it reactivated by the expression of the exogenous RARβ (data not shown).

The level of RARβ protein expression was determined in transduced clones by Western blotting using an RARβ-specific polyclonal antiserum (35). The expected single M_r 53,000 band was detected in cellular lysate from MCF7-RARβ and MDA231-RARβ transduced clones but not in the lysate of vector controls or the parental cell line (Fig. 3).

Northern analysis was performed on parental cell lines, RARβ transduced clones, and vector control cells treated with and without RA to determine whether the expression of RARβ alters the expression of RARα. The level of RARα mRNA expression in all MCF-7 cells (parental cells, vector controls, and RARβ transduced cells) was uniformly higher (10-fold) than what was observed for all MDA-MB-231 cells (parental cells, vector controls, and RARβ transduced cells). There was, however, no increase in RARα mRNA expression in RA-treated or untreated RARβ transduced clones relative to parental cells or vector control clones (data not shown).

Growth Inhibition of RARβ Transduced Breast Cancer Cells in Response to Retinoic Acid. Steady state, log phase growth rates of the retroviral vector-infected clones were determined in the presence or absence of 1 μM RA (Table 1). Doubling times were obtained for MCF-7 transduced clones at 4.5 to 5.5 days after RA treatment and for MDA-MB-231 transduced clones 3 to 5.5 days after RA treatment. Both MCF7-RARβ and MDA231-RARβ clones exhibited a significant RA-induced growth suppression as evidenced by an increase in doubling times relative to RA-treated vector control clones. In general, the MCF7-RARβ clones exhibited greater RA-mediated growth inhibition than the MDA231-RARβ clones. All RA-treated MCF7-RARβ clones exhibited a significant increase (2-fold to complete growth arrest) in doubling time, with two clones (MCF7-RARβ6 and MCF7-RARβ11) undergoing complete growth arrest after treatment (Table 1). All RA-treated MDA231-RARβ transductants exhibited a 1.8- to 3.3-fold increase in their doubling times but did not undergo complete growth arrest. In contrast, RA treatment of three MCF7-SN and three MDA231-SN vector control clones resulted in no growth inhibition (Table 1). The increased doubling times exhibited by untreated MCF7-RARβ7 and MCF7-RARβ9 transduced clones relative to vector controls likely represent an increased sensitivity of these two clones to endogenous retinoids present in the FCS in the cell culture media. When these two clones and vector controls were

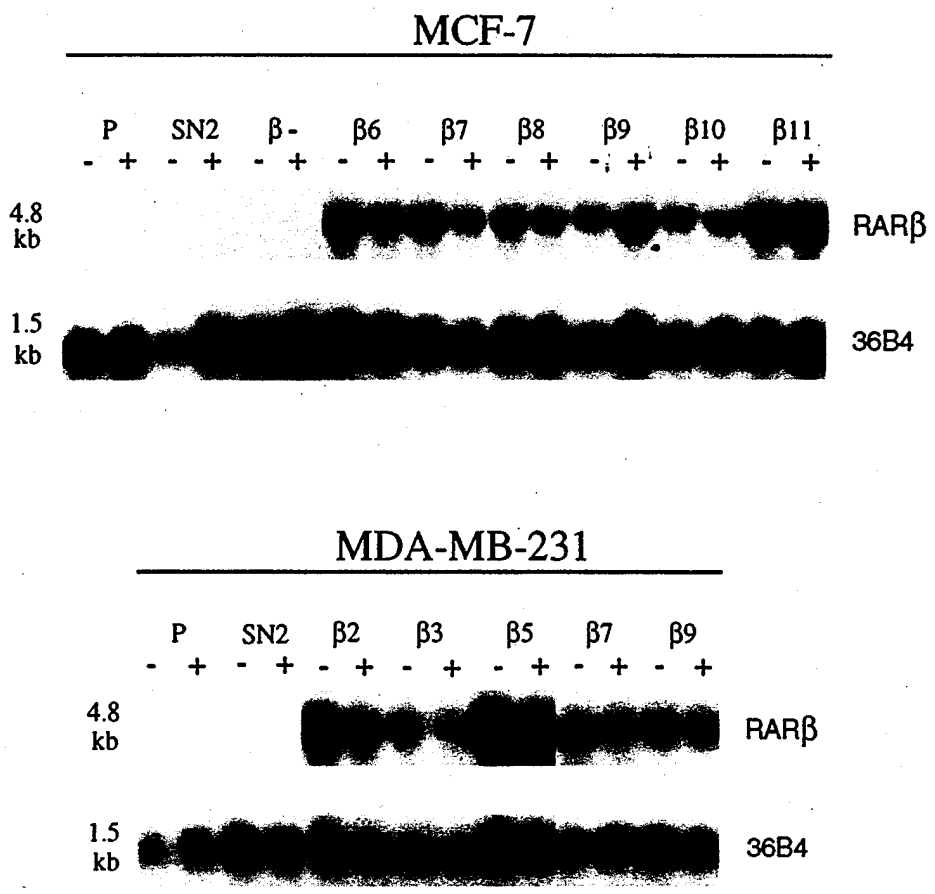

Fig. 2. Expression of RARβ mRNA in transduced breast cancer lines. Northern analysis of parental (*P*), RARβ transduced breast cancer cell lines (β), and vector controls (*SN*) treated for 48 h with (+) and without (–) 1 μM RA, demonstrating the expression of the 4.8-kb RARβ retroviral vector mRNA. MCF7-β⁻ is a RARβ transduced clone that does not express RARβ. Ten μg RNA were loaded per lane. 36B4 is a 1.5-kb mRNA expressed uniformly in all breast cell lines and, therefore, serves as a loading control (70).

grown in media containing charcoal/dextran-stripped FCS, the doubling times of these two RARβ transduced clones and vector controls were all approximately 36 h (data not shown).

Cell Cycle Changes Observed in RARβ Transduced Clones. To determine more precisely the effect of RA on the growth inhibition of breast cancer cell lines, flow cytometric analysis of isolated nuclei stained with PI was performed on RARβ-transduced and control cells treated with or without 1 μM RA for 4 days (36). We observed that both the RA-treated MCF7-RARβ and RA-treated MDA231-RARβ clones exhibited a marked increase in the percentage of cells in G_1 and a concomitant decrease in the percentage of cells in S phase, indicating that RA induced a G_1 arrest in the RARβ transduced cells (Table 2). In contrast, vector control clones (MCF7-SN13 and MDA231-SN1) exhibited only a slight decrease in the percentage of cells in S phase when treated with RA (Table 2). This cell cycle analysis indicates that RA-mediated G_1 arrest may account for the growth inhibition induced by RA in the RARβ transduced breast cancer cell lines (Table 1).

Morphological Changes Observed in RA-treated RARβ Clones. In addition to the above noted growth arrest, we observed that four of four ER-positive MCF7-RARβ transduced clones (MCF7-RARβ6, MCF7-RARβ7, MCF7-RARβ9, and MCF7-RAR β11) underwent morphological changes suggestive of, but not specific for, apoptosis beginning 4 days after treatment with RA. These changes included nuclear condensation, cytoplasmic vacuolization, loss of adherence, and cell shrinkage (Fig. 4). These changes were not observed when the ER-negative MDA231-RARβ transduced clones (MDA231-RARβ2, MDA231-RARβ3, MDA231-RARβ5, MDA231-RARβ7, and MDA231-RARβ9) were treated with RA (data not shown). Untreated MCF7-RARβ transduced cells exhibit modest cytoplasmic shrinkage relative to vector controls. This morphological change likely reflects the presence of endogenous calf retinoids present in the FCS found in the tissue culture medium. Untreated MCF7-RARβ transduced cells do not exhibit nuclear condensation, cytoplasmic vacuolization, or loss of adherence. Rare MCF7-SN vector control cells exhibited cellular changes suggestive of apoptosis when treated with RA.

Flow Cytometric Evidence of Apoptosis in RA-treated MCF7-RARβ Transduced Clones. To confirm that apoptosis was indeed induced by RA in MCF7-RARβ transduced cells, we performed flow cytometric analysis of isolated nuclei after 6 days of treatment with RA when the above noted morphological changes were most evident (36). As described above (Table 2), MCF7-RARβ transduced cells undergo G_1 arrest after 4 days of treatment with RA, a time point when morphological changes consistent with apoptosis are first evident. After 6 days of RA exposure, MCF7-RARβ transduced cells demonstrate a hypodiploid "sub-G_1" peak (Fig. 5), which is characteristic of apoptosis and represents endonuclease cleavage of DNA at specific linker intervals (37, 38). Five of five MCF7-RARβ transduced

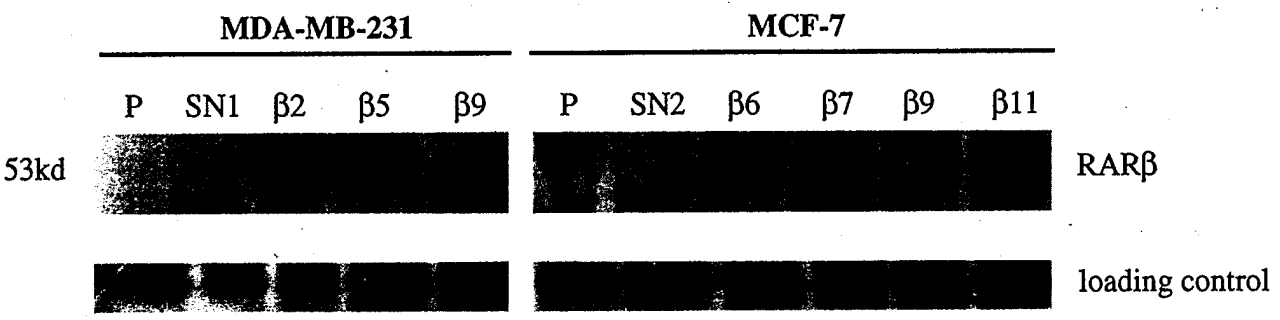

Fig. 3. Protein expression of RARβ in the MCF-7 RARβ and MDA-MB-231 transductants. Western blot of RARβ transduced MCF-7 and MDA-MB-231 clones (β), parental line (*P*), and vector control (*SN*). One hundred μg protein were loaded per lane. The blot was incubated with a 1:50 dilution of RARβ-specific polyclonal antiserum (35) and detected by chemiluminescence using a biotin/avidin detection system. RARβ transduced cells express the expected single M_r 53,000 band. The protein gel was stained with Coomassie blue, and an unidentified M_r 45,000 protein, which was present in both cell lines, was used as a loading control.

Table 1 Growth rates of RARβ-transducted clones in the presence or absence of 1μM RA

Cells (5×10^4) were seeded per 35-mm Petri dish and treated with or without 1 μM RA. Cells were counted in duplicate every 24 to 36 h. Final growth rates were calculated for MCF-7 transduced clones at 4.5 to 5.5 days during RA treatment and for MDA-MB-231 transduced clones at 3 to 5.5 days during RA treatment. Control cells were in steady-state log phase growth during this time period.

Clone	Doubling time without RA (h)	Doubling time with RA (h)
MCF7-SN2	36	38
MCF7-SN 8	36	36
MCF7-SN 13	34	36
MCF7-RARβ6	38	Complete growth arrest
MCF7-RARβ7	70	135
MCF7-RARβ9	60	180
MCF7-RARβ11	38	Complete growth arrest
MDA231-SN 1	22	23
MDA231-SN 2	24	23
MDA231-SN 3	27	28
MDA231-RARβ2	24	80
MDA231-RARβ5	31	80
MDA231-RARβ7	24	56
MDA231-RARβ9	31	56

clones examined (MCF7-RARβ3, MCF7-RARβ6, MCF7-RARβ7, MCF7-RARβ9, and MCF7-RARβ11) demonstrate this peak. RA-treated MCF7-SN vector control clones exhibit a broadening of the G_1 peak and a small sub-G_1 shoulder peak, which demonstrate a RA effect but are not characteristic of apoptosis. In contrast, a hypodiploid sub-G_1 peak is not observed for MDA231-RARβ transduced clones (MDA231-RARβ2, MDA231-RARβ5, and MDA231-RARβ9) after 5, 6, or 7 days of RA treatment (data not shown).

Confirmation of Apoptosis in RA-treated MCF7-β Transduced Clones. To further document that MCF7-RARβ transduced cells undergo apoptosis when treated with RA, we used two confirmatory assays to detect the DNA fragmentation that occurs specifically in apoptosis and distinguishes apoptosis from other modes of cell death such as necrosis (17, 39).

The diphenylamine assay measures endonuclease-fragmented (solubilized) DNA in the cytoplasm of apoptotic cells (40). Time points were obtained at 0, 2, 4, and 6 days after treatment of the target cells with RA. Four of four MCF7-RARβ transduced clones treated with RA demonstrate increased DNA fragmentation. In contrast, MCF7-SN vector control clones demonstrated only a sight increase in fragmented DNA on day 6 (Fig. 6). The percentage of fragmented DNA for the MCF7-RARβ transduced clones on day 6 was 38% for MCF7-RARβ6, 37% for MCF7-RARβ7, 22% for MCF7-RARβ9, and 36% for MCF7-RARβ11 (average of two experiments). In contrast, no significant increase in DNA fragmentation was observed in three of three MDA231-RARβ transduced clones when treated with RA (Fig. 6). The percentage of fragmented DNA on day 6 was 8% for MDA231-RARβ2, 5% for MDA231-RARβ5, and 5% for MDA231-RARβ9 (average of two experiments).

The TdT method was also used to detect apoptotic strand breaks. In this assay, 3' hydroxyl termini of apoptotic-induced strand breaks are labeled with biotin-dUTP by exogenous TdT and can be detected *in situ* or by flow cytometry after staining with avidin-conjugated FITC (39). Incorporation of labeled dUTP was seen in four of four MCF7-RARβ transduced clones (MCF7-RARβ6, MCF7-RARβ7, MCF7-RARβ9, and MCF7-RARβ11) treated with 1 μM RA for 6 days. This was not observed in three of three RA-treated MCF7-SN vector control clones nor in RA-treated MDA231-RARβ transduced clones (MDA231-RARβ2, MDA231-RARβ5, and MDA231-RARβ9; Fig. 7).

Differential c-myc Expression in RARβ Transduced Breast Cancer Cells. Our observations indicate that RARβ is a regulator of cellular proliferation and is important for the induction of apoptosis in MCF7-RARβ transduced cells. To better understand how RA and RARβ may act in breast cancer cells, we investigated c-myc mRNA expression in RA-treated RARβ transduced cells and vector controls. The c-*myc* proto-oncogene is a transcription factor that participates in opposing cellular fates of proliferation and apoptosis (41–44). We observed that the RA-mediated growth arrest of the ER-negative MDA231-RARβ transduced cells is associated with c-myc down-regulation (58 to 89% decrease; average of 71% decrease) relative to RA-treated controls (26–36% decrease; average of 31% decrease; Fig. 8). In contrast, the RA-induced apoptosis of ER-positive

Table 2 Effects of RA on the breast cancer cell cycle

Adherent RARβ transduced cells (–RARβ) and vector controls (–SN) were incubated for 4 days in the presence or absence of all- *trans*-retinoic acid (1 μM). The distribution of cells in the various phases of the cell cycle was determined by flow cytometry.

Clone	% G_0-G_1		% change	% S		% change
	–RA	+RA		–RA	+RA	
MCF7-SN13	48	51	+6	38	33	–13
MCF7-RARβ6	42	65	+55	42	16	–62
MCF7-RARβ7	39	56	+44	43	18	–58
MCF7-RARβ9	44	53	+21	34	19	–44
MCF7-RARβ11	43	52	+21	39	25	–36
MDA231-SN1	48	49	+2	38	34	–11
MDA231-RARβ2	44	58	+32	48	30	–38
MDA231-RARβ9	26	38	+46	51	38	–25

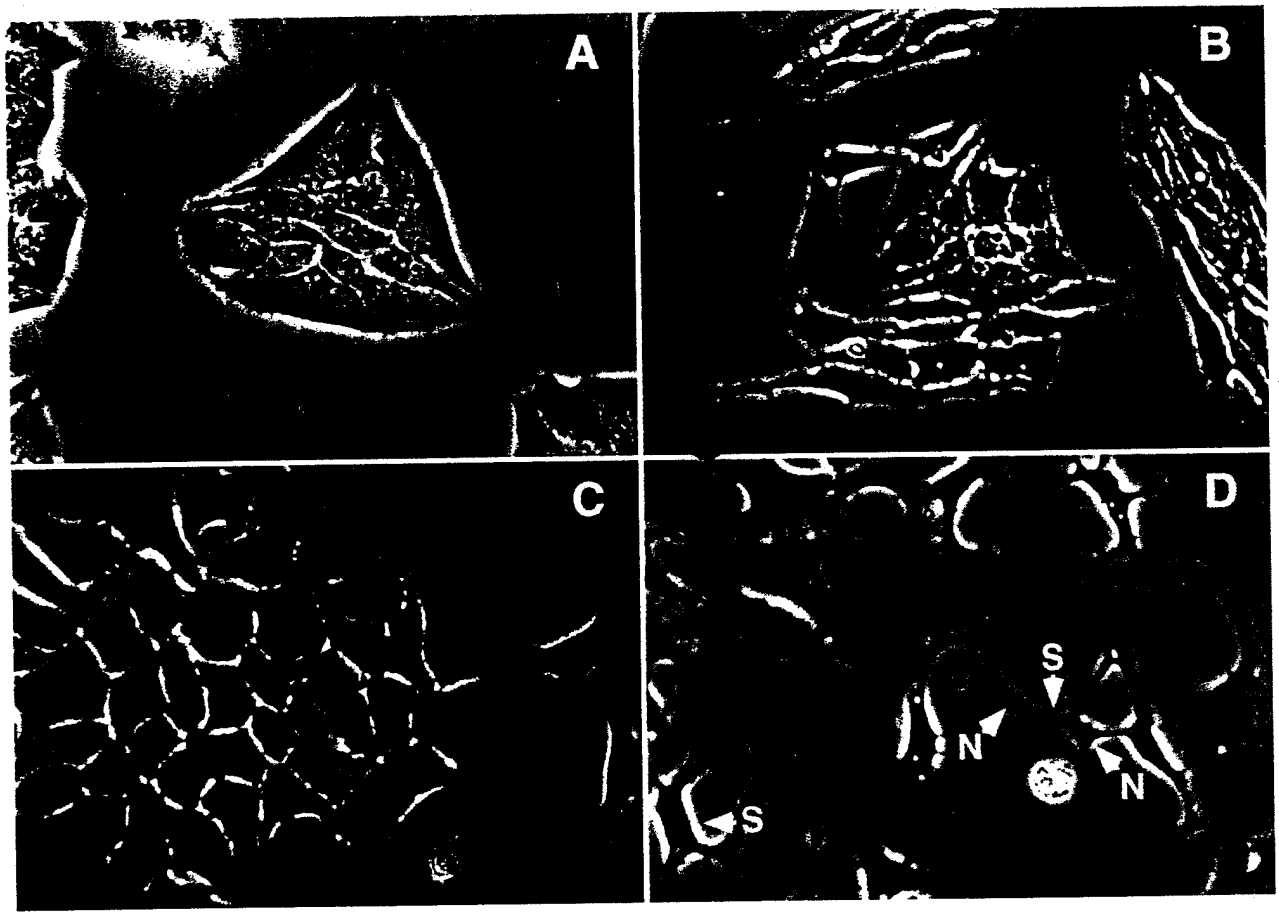

Fig. 4. Morphological effects of RA acid on RARβ transduced MCF-7 cells and vector controls. Representative MCF7-SN vector controls (*A* and *C*) and MCF7-RARβ transduced cells (*B* and *D*) were incubated with (*C* and *D*) and without (*A* and *B*) 1 μM all-*trans*-retinoic acid for 6 days. MCF7-RARβ transduced cells treated with RA exhibited nuclear condensation (*N*) and cell shrinkage (*S*).

MCF7-RARβ transduced cells is not associated with c-myc mRNA down-regulation (Fig. 8).

Discussion

Retinoids and their receptors are important regulators of embryogenesis, cell differentiation, and growth. The biological activity of retinoids is thought to be mediated through a number of closely related nuclear receptors that possess discrete DNA and ligand binding domains and act to regulate transcription of specific target genes (8, 9, 45). These RARs exhibit tissue-specific expression, suggesting that they may possess distinct biological activity. Targeted

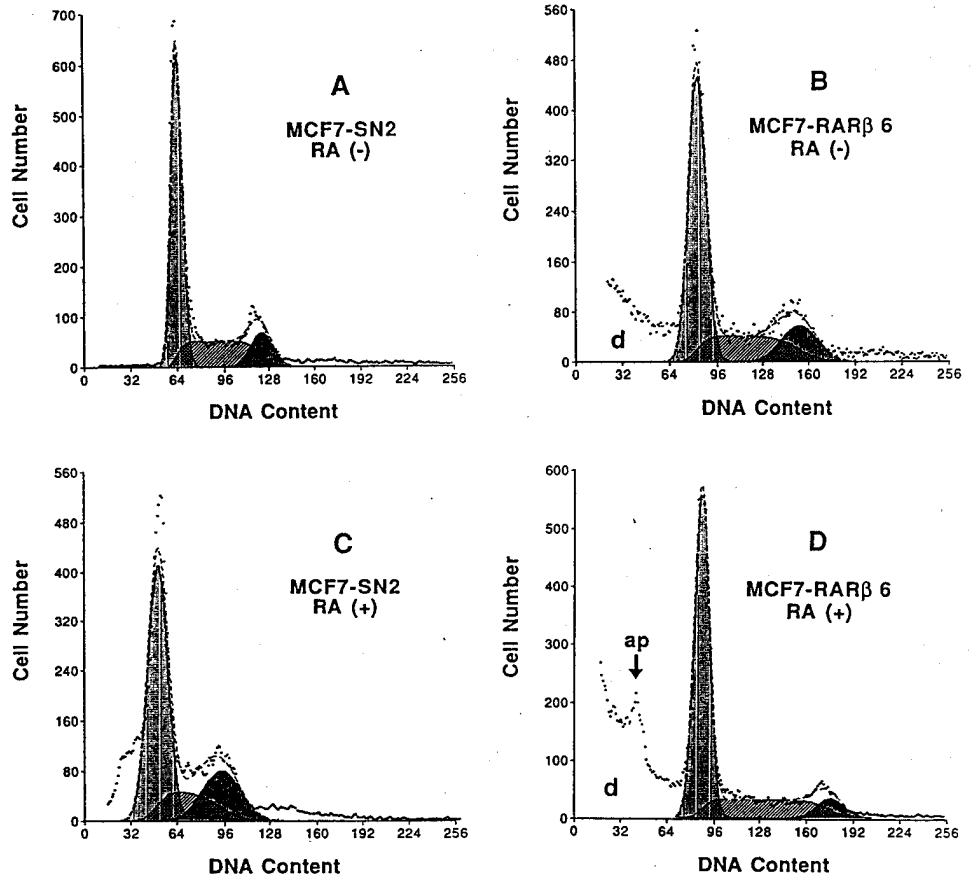

Fig. 5. Flow cytometric evidence of DNA fragmentation in RA-treated MCF7-RARβ transduced cells. Representative cell cycle histogram of MCF7-RARβ transduced cells (*B* and *D*) and MCF7-SN vector controls (*A* and *C*) treated with (*C* and *D*) and without (*A* and *B*) 1 μM all-*trans*-RA for 6 days. RARβ transduced clones treated with RA exhibit a hypodiploid sub-G_1 peak consistent with apoptosis (*ap*). Cellular debris (*d*) was observed in both RA-treated and untreated MCF7-RARβ transduced cells.

disruption of the mouse RARβ2 isoform does not interfere with the normal development or viability of animals (46), indicating that other members of the RAR family or other RARβ isoforms can compensate for its loss of activity. We do not know, however, whether selective inactivation of the RARβ gene in target tissue of adults (mouse of human) can lead to loss of growth control. RARβ is primarily expressed in normal epithelial tissue and is likely to be a critical mediator of retinoid action in epithelial cells (1). Reduced or absent expression of RARβ mRNA has been demonstrated in many malignant epithelial cell lines including non-small cell carcinoma of the lung (11, 12), epithelial ovarian cancer (14), squamous carcinoma of the oral pharynx (13), and breast cancer (10). *In situ* analyses of normal epithelial tissue and primary dysplastic and malignant lesions of the lung and aerodigestive tract demonstrate progressive loss of RARβ expression (47). Thus, the specific loss of RARβ function may be an important event in solid tumor carcinogenesis.

In the present study, we have investigated the role of RARβ in the pathogenesis of breast cancer by using retroviral vector-mediated gene transduction to introduce the RARβ gene into the breast cancer cell lines MCF-7 and MDA-MB-231, which do not ordinarily express RARβ mRNA. We observe that both cell lines transduced with RARβ exhibit RA-induced growth inhibition associated with G_1 arrest. In addition to growth arrest, we observed RA-mediated apoptosis in the MCF7-RARβ transduced cells but not in MDA231-RARβ transductants. In contrast, the vector control-infected cells MCF7-SN and MDA231-SN, which do not express RARβ, do not exhibit RA-induced growth inhibition and demonstrate little evidence of G_1 arrest (Tables 1 and 2). The slight decrease in the percentage of cells in S phase in the RA-treated control cells noted in Table 2 might reflect RA acting through a second receptor such as RARα which, in contrast to RARβ, is frequently expressed by breast cancers and has been shown recently to mediate retinoid-induced growth inhibition in certain breast cancer cell lines (48).

Our observations suggest how loss of RARβ expression might contribute to the development of breast cancer. If the normal function of RA and RARβ involves inducing growth arrest and/or apoptosis in normal breast epithelial cells, then loss of this function might disrupt this normal cycle of breast epithelial cell proliferation/differentiation/apoptosis, leading to an expanded epithelial cell population. Further mutations of cellular oncogenes or tumor suppressor genes in this expanded population of cells might lead to the development of overt breast cancer. This model predicts that loss of RARβ expression might be a relatively early event in the pathogenesis of breast cancer.

Our observations also suggest how certain retinoids may function as chemopreventive agents. Retinoids appear to be important for the chemoprevention of solid tumors and are used both to treat dysplastic lesions of the oral cavity (49) and to prevent secondary primary tumors in patients who have been treated for squamous cell carcinoma of the head and neck (50). There is also epidemiological evidence to

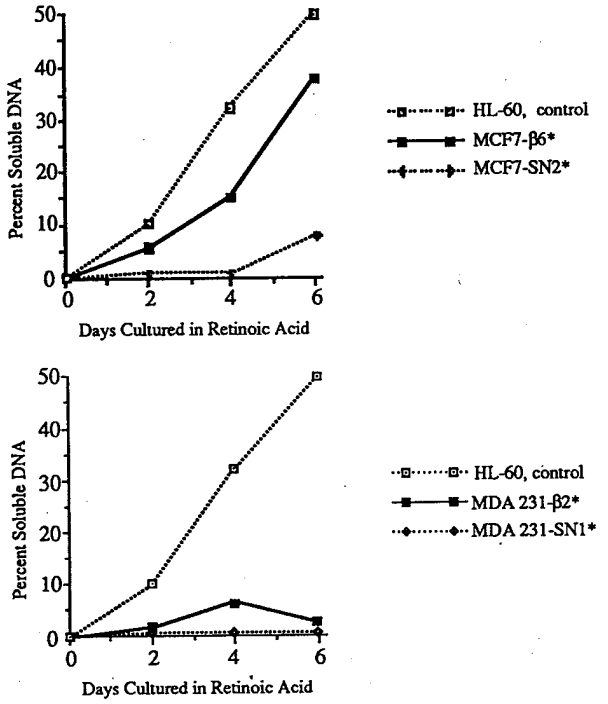

Fig. 6. Cytoplasmic DNA fraction in RA-treated RARβ-transduced breast cancer cells. The indicated cells were treated for 0, 2, 4, and 6 days with 1 μM all-*trans*-RA. Cytoplasmic DNA was determined by the diphenylamine assay as described in "Materials and Methods." *, average of two experiments. HL-60 cells exhibit RA-induced apoptosis (72) and serve as a positive control.

support the importance of retinoids in the chemoprevention of breast cancer. The risk of breast cancer is increased for women with a lower dietary intake of vitamin A and β carotene but not for those with dietary deficiencies of vitamins C or E (2). For these reasons, efforts have focused recently on using the synthetic retinoid, fenretinide, for the prevention of contralateral breast cancer (51, 52). Fenretinide has been demonstrated to preferentially accumulate in breast tissue and inhibits the formation of chemically induced mammary carcinomas in rats (53). One mechanism by which retinoids might act as chemopreventive agents is to increase the transcriptional expression of RARβ through the retinoic acid response element that is present in the RARβ promoter region (8, 9). If loss or decreased expression of RARβ is a critical event in breast tumor development, then agents such as RA that enhance RARβ expression might delay or prevent the development of overt malignancy.

It is interesting that while RA induces G_1 arrest in both MCF7-RARβ and MDA231-RARβ transductants, we observe RA-induced apoptosis in only MCF7-RARβ transduced cells. Why does RA induce apoptosis in one RARβ transduced cell line but not the other? What are the critical regulatory molecules that are involved? We observed that RARα mRNA is expressed at uniformly higher levels in all MCF-7 cells (parental cell line, vector controls, and RARβ transduced clones) relative to all MDA-MB-231 cells (parental cell line, vector controls, and RARβ transduced clones). Although the expression of transduced RARβ in MCF-7 and MDA-MB-231 clones or the subsequent treatment of these clones with RA does not increase the expression of RARα mRNA, perhaps the higher levels of RARα mRNA present in all MCF-7 cells (relative to all MDA-MB-231 cells) is directly or indirectly associated with the ability

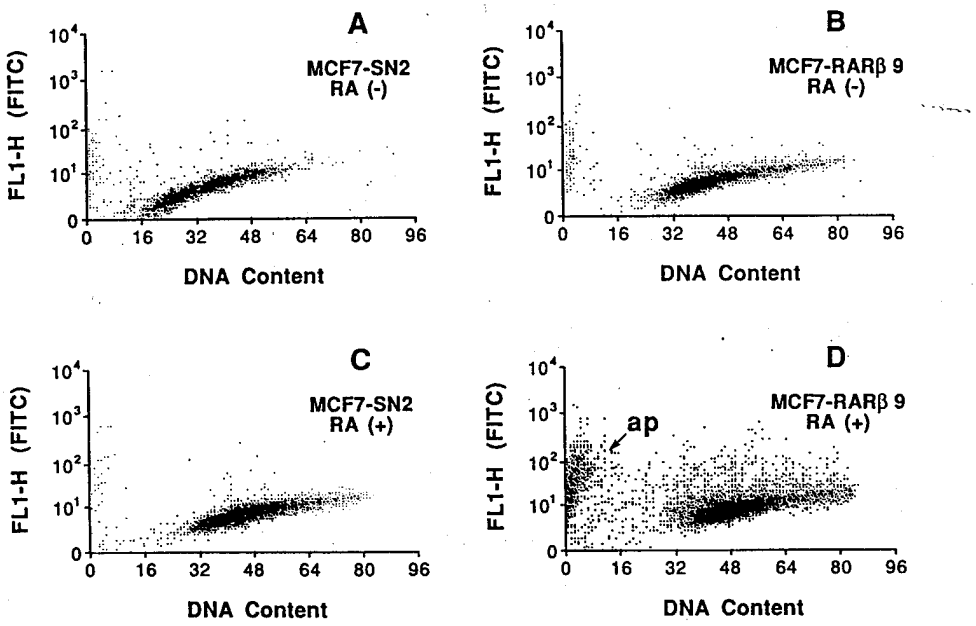

Fig. 7. Fluorescence labeling of apoptotic strand breaks by the TdT method in RA-treated MCF-7 cells. Representative MCF7-RARβ transduced cells (*B* and *D*) and MCF7-SN vector controls (*A* and *C*) treated with (*C* and *D*) or without (*A* and *B*) 1 μM RA for 6 days. Cells were incubated with TdT in the presence of biotin-labeled dUTP (Boehringer Mannheim). Detection was with avidin-conjugated FITC as described in "Materials and Methods." MCF7-RARβ transduced cells treated with RA demonstrated apoptotic strand breaks (*ap*).

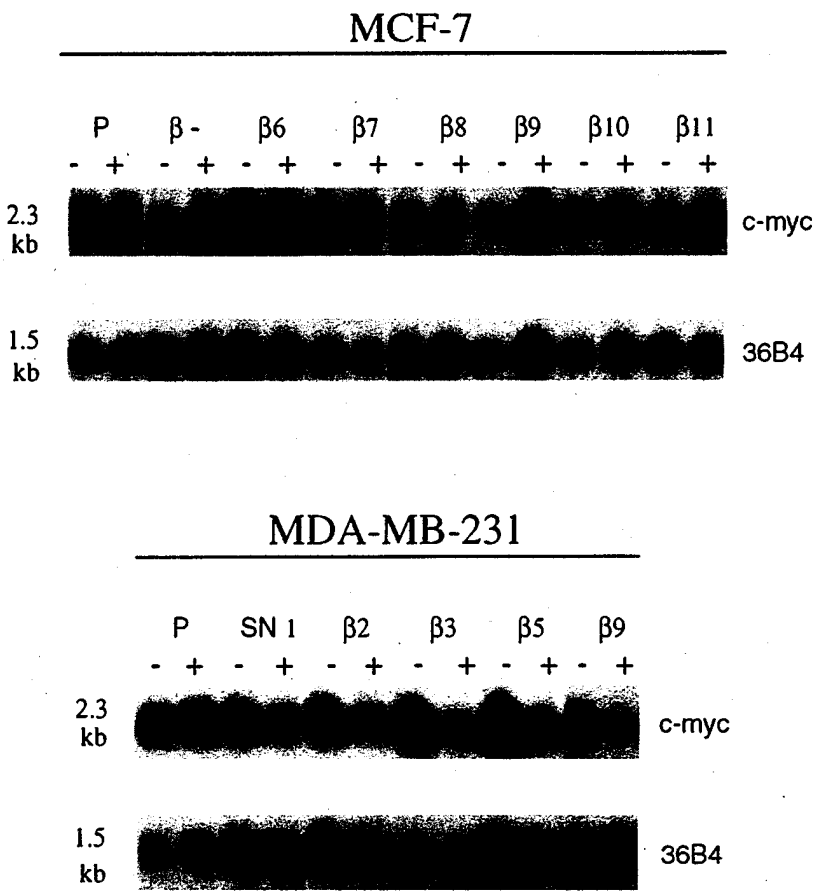

Fig. 8. Effects of RA on c-myc mRNA expression in RARβ transduced cells and vector clones. Northern blot of c-myc mRNA expression in RARβ transduced cells (β) and vector controls (*SN*) with (+) and without (–) 48 h 1 μM RA treatment. Clone MCF7-RARβ– is a RARβ transduced clone that does not express RARβ mRNA. Ten μg of RNA were loaded per lane. 36B4 is a 1.5-kb mRNA expressed uniformly in all breast cell lines and, therefore, serves as a loading control (70).

of MCF7-RARβ transduced clones to undergo RA-mediated apoptosis.

We have also noted that the RA-induced apoptosis of the ER-positive MCF7-RARβ transduced cells is associated with unchanged or slightly elevated levels of c-myc mRNA while, in contrast, the RA-induced growth arrest (without apoptosis) of the ER-negative MDA231-RARβ transduced cells is associated with a significant decrease in c-myc mRNA expression relative to controls (Fig. 8). The regulation of c-myc expression in breast cancer cells appears to be linked to ER status. In ER-positive, estrogen-dependent cell lines, c-myc mRNA expression is stimulated by estrogen, whereas in ER-negative, estrogen-independent cells, c-myc is constitutively expressed and is not regulated by estrogen (54–56). Since it has been demonstrated previously that deregulated c-myc expression leads to apoptosis upon growth factor deprivation in normal fibroblasts and hematopoietic cells (41–44), it is possible that the lack of c-myc mRNA down-regulation noted in the RA-treated ER-positive MCF7-RARβ transduced cells may be related to the RA-mediated apoptosis (and perhaps the estrogen dependence) of these cells.

Another factor that may be important in determining whether the transduced breast cancer cells undergo RA-mediated apoptosis is the p53 status of these cells. It has been postulated that p53 is a genetic switch that can mediate both G_1 arrest and/or apoptosis in cells exposed to DNA-damaging agents (57–59). Apoptosis mediated by DNA-damaging agents such as cytotoxic chemotherapy and ionizing radiation is inhibited in p53 (–/–) null cells derived from knock-out mice compared to p53 wild-type controls (57). Recently, a link between p53, cell cyclins, and growth arrest has been discovered with the cloning of the *p21/WAF1/CIP1* gene, which is directly activated by p53 (60, 61) and acts to arrest cell growth by inhibition of cyclin-dependent kinases (60–63). Since MCF-7 cells are reported to harbor an intact *p53* gene (64) whereas MDA-MB-231 cells exhibit mutated p53 (65), this reported difference in p53 status may account for the difference in RA-mediated apoptosis exhibited by these cells.

Materials and Methods

Materials. all-*trans*-RA (Sigma Chemical Co.) 1 mM stock solution was prepared in 100% ethanol. Control cultures received equivalent volumes of the ethanol solvent. Fresh RA stocks were prepared every 7 to 10 days and were used under reduced light.

Cell Lines and Media. MCF-7 and MDA-MB-231 breast tumor lines were purchased from American Type Culture Collection. Tumor lines were grown in αMEM (JRH Bioscience) supplemented with 1 mM sodium pyruvate (Sigma), 10 mM HEPES (Sigma), and nonessential amino acids (1:100 dilution of 100 × stock), 5% FCS (Atlanta), 12.5 ng/ml epidermal growth factor (Sigma), 0.5 μg/ml hydrocortisone (Sigma), and 0.5 μg/ml insulin (Sigma). The FCS used in these experiments did not undergo processing to remove endogenous calf serum retinoids. G418 media was pre-

pared by the addition of 1000 μg/ml of G418 (GIBCO) to the above standard media.

Mycoplasma testing was performed on all cell lines and transduced clones as reported previously by Russell *et al.* (66). Cells that were routinely maintained in G418 were grown in the absence of G418 for two passages (>10 days) prior to *Mycoplasma* testing.

Construction of the LaRARβSN Retroviral Vector. In a previous study, we had constructed a retroviral vector harboring RARβ but noted that this construct demonstrated reduced activity in HL-60R cells (as assessed by the differentiative response of these cells to RA) compared with similar RARα and RARγ constructs (67). To provide for a presumably better translational context for the RARβ cDNA, we constructed a new RARβ retroviral vector using an LXSN-based retroviral vector designated pLASN containing adenosine deaminase cDNA. (34). Using PCR, we amplified the 1.6-kb RARβ cDNA insert from our previously constructed LRARβSN plasmid (67) using the oligonucleotide primers 5′-ACTGGGATCCATGGTGGACTGTATGGATGTTCTGTC-3′ corresponding to the initial amino acid sequence of human RARβ and 5′-ACTGGGATCCTGGAACTGAAGGTACTGG-3′ corresponding to 3′ untranslated sequences approximately 60 bp 3′ to the RARβ stop codon. These primers placed a *Bam*HI and *Nco*I site at the 5′ end and a *Bam*HI site at the 3′ end of the amplified cDNA. The generation of the 5′ *Nco*I site necessitated making a conservative change in the second RARβ amino acid from phenylalanine (TTT) to valine (GTG). The amplified cDNA fragment was digested with *Bam*HI and ligated into the *Bam*HI site of the pVZ-1 plasmid cloning vector. This vector was subsequently digested with *Nco*I and *Bam*HI, releasing the 1.6-kb RARβ fragment. The fragment was subsequently used in a three-way ligation with a 4.3-kb *Nco*I-*Xba*I fragment and a 1.7-kb *Xba*I-*Bam*HI fragment from the retroviral vector pLASN (34). The resulting recombinant retroviral vector construct, designated LaRARβSN (Fig. 1), includes 94 bp of 5′ untranslated sequences from the human adenosine deaminase gene immediately preceding the RARβ initiator ATG.

Retroviral vectors were generated from this recombinant plasmid as described previously (68). Briefly, 10 μg of cesium chloride-banded LaRARβSN retroviral construct plasmid was transfected via $CaPO_4$ precipitation into the PE 501 murine ecotropic retrovirus packaging cell line (68). The supernatant was harvested after 2 days and used to infect the PA317 amphotropic packaging cell line. These cells were cultured in G418 (1 mg/ml), and individual foci of resistant cells were isolated by cloning rings and expanded. Producer clones expressing the appropriately sized Moloney murine leukemia virus long terminal repeat-initiated RARβ transcript (4.8 kb) were identified by Northern blots and supernates from these producers titered on NIH3T3 thymidine kinase-negative target cells. Supernate from the highest titer producer clone (5×10^4/ml) was used in all subsequent experiments.

Retroviral Transduction. The breast cancer cell lines MCF-7 and MDA-MB-231 were plated at 5×10^4 cells in 35-mm tissue culture Petri dishes (Corning) in standard medium. Transducing virions from the PA317-LaRARβSN retroviral producer line was added at a multiplicity of infection at 1:1 in the presence of 4 μg/ml Polybrene (Sigma). Cells were selected after 24 h with standard medium containing G418 1000 μg/ml. Twenty-four h later, cells were trypsinized and plated under G418 selection at serial dilutions of 1:1, 1:10, and 1:100. Clones were selected after 14 to 21 days and isolated using cloning rings. Clones were expanded under continuous G418 selection at 1000 μg/ml.

Northern Blotting. RNA was extracted with guanidine hydrochloride and subjected to Northern blotting in formaldehyde denaturing gels as described previously (10). Ten μg RNA were loaded per lane. Molecular probes used in the Northern analysis are as follows: human RARβ probe, a 1.4-kb *Sac*I-*Bam*HI fragment (4); human RARα probe, a 1.8-kb *Eco*RI fragment (4); and human c-myc probe, a 1.8-kb *Eco*RI fragment (69). The 36B4 probe (700-bp *Pst*I fragment) was used as a loading and transfer control probe (70).

Western Blotting. Cellular lysates were prepared as follows: cells were grown to 50% confluency in 100-mm Falcon tissue culture Petri dishes in standard medium. Cells were then treated with 1 μM RA or 0.1% ethanol and harvested after 48 h. Confluency did not exceed 70%. Cells were washed twice in ice-cold PBS, scraped with a rubber policeman from the flask surface, transferred to an Eppendorf tube in 1.5 ml, and pelleted at 3000 rpm in a refrigerated microfuge for 3 min. The supernatant was removed, and the pellet was resuspended in Lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 μg/ml aprotonin, and 2.5 μM phenylmethylsulfonyl fluoride] to a final concentration of 5×10^5 cells per 10 μl buffer and incubated on ice for 30 min. The lysate was centrifuged at 6000 rpm in a refrigerated microfuge for 10 min. The supernatant was then transferred to a clean Eppendorf tube, and the lysates were stored at −20°C. The protein concentration of cellular lysates was determined by a Pierce bicinchoninic acid kit. Cellular lysates (100 μg total protein) were loaded on 10% polyacrylamide gels (71) and then electroblotted (Hoeffer) at 80 mA for 45 min onto Hybond-ECL membrane (Amersham). The membrane was blocked with 20% BSA (Sigma) in PBS overnight at room temperature and then incubated with a 1:50 dilution of anti-RARβ polyclonal antiserum for 1 h at room temperature with agitation. The RARβ-specific polyclonal antiserum was a generous gift of Pierre Chambon (Institut de Chimie Biologique, Strasbourg, France; Ref. 35). The membrane was washed three to five times at room temperature with 250 ml PBS containing 0.1% Tween and then incubated with biotin-labeled $F(ab')_2$ fragment anti-rabbit IgG (Kirkegaard and Perry) at a 1:4000 dilution for 1 h at room temperature. The blot was washed as before and incubated with avidin-conjugated horseradish peroxidase at a 1:32,000 dilution for 15 min at room temperature. The blot was washed again, and complexes were detected by using ECL Western blotting detection reagents (Amersham) as described by the manufacturer.

Cell Growth Curves. Cells were plated at 5×10^4 per 35-mm tissue culture Petri dish (Corning) and grown in standard medium containing 1000 μg/ml G418 with 1 μM RA or with an equivalent volume of ethanol solvent (0.1%). Cells were trypsinized at 24- to 48-h time intervals and counted in duplicate. Doubling times were obtained for MCF-7 and MDA-MB-231 transduced clones at 4.5 to 5.5 and 3 to 5.5 days during RA treatment, respectively, by plotting cell number on a log scale *versus* time on a linear scale. Vector control cells were in steady-state log phase growth during these time periods.

DNA Staining of Cell Nuclei with PI for FACS Analysis. Cells (5×10^5) were plated in T75 flasks (Corning) and grown in standard medium with 1 μM RA or 0.1% ethanol alone. Cells were harvested at 4 days for cell cycle analysis

(Table 2) and at 6 days for demonstration of DNA fragmentation (Figs. 5 and 7). Cells did not exceed 70% confluency. Cells (0.5 to 1.0 × 10^6) were trypsinized, washed in cold PBS, resuspended in 250 μl buffer [Tris-HCl (pH 7.5), 20 mM NaCl, and 20 mM MgCl] and incubated on ice for 5 min. Lysis bufer (250 μl; NP40 1%) was added, and the cells were vortexed and then incubated on ice for 5 min. DNase-free RNase (10 μg/ml; Boehringer Mannheim) was then added, and cells were incubated for 30 min at 37°C. Cells were transferred to 6 ml polystyrene tubes and 500 μl PI [PBS with 100 μg/ml PI (Sigma), 0.1% Triton X-100, and 1% FCS] were added. The cells were incubated in the dark at 4°C for 1 h before reading.

Diphenylamine Assay for Determination of Cytoplasmic DNA Fraction. Cells (2.5 to 5.0 × 10^5) were plated per T25 flask (Corning) and were grown in standard media. Retinoic acid stock was added directly to the media on days 0, 2, and 4 to bring the final concentration to 1 μM. Confluency did not exceed 70%. On day 6, cells were trypsinized and washed in cold PBS. Cells were pelleted and then resuspended in 100 μl lysis buffer [5 mM Tris (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100] on ice for 15 min. The lysate was spun at 12,000 rpm for 30 min in a refrigerated microfuge. The supernatant was removed and transferred to a second Eppendorf tube. Both tubes were placed on ice and 1 ml of 0.5 N perchloric acid was added to the nuclear pellet in the first tube and vortexed. Perchloric acid (0.5 ml 1N) was added to the cytoplasmic fraction in the second tube and vortexed. Both tubes were spun at 12,000 rpm for 15 min. The supernatants were then discarded, and 1 ml 0.5 N perchloric acid was added to the pellets. The tubes were heated to 70°C for 20 min to hydrolyze the DNA and then cooled to room temperature. Diphenylamine solution (1 ml) was added [diphenylamine (Aldrich) 1.5 g in 100 ml glacial acetic acid, to which was added sulfuric acid 1.5 ml. The solution was stored in the dark. On the day of use, 0.1 ml of 1.6% acetaldehyde was added.] The tubes were incubated 16 to 20 h at 30°C. Absorbance was read at 600 nm (40, 72).

TdT Assay. Cells were plated and treated with RA in the same manner as they were for PI staining. Cells (0.5 to 1.0 × 10^6) were trypsinized and washed in cold PBS. Cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 min on ice and then washed in 3 ml PBS. Cells were resuspended in 70% ice-cold ethanol and immediately transferred to −20°C.

After fixation, the cells were resuspended in 50 μl of TdT buffer [0.1 M sodium cacodylate (pH 7.0) (Sigma), 1 mM calcium chloride (Sigma), 0.1 mM DTT (Sigma), 0.05 mg/ml BSA, 10 units terminal transferase (Boehringer Mannheim), and 0.5 nM biotin-16-dUTP (Boehringer Mannheim)]. Cells were incubated in this solution for 1 h, rinsed in PBS, and then resuspended in 100 μl of staining buffer (saline sodium citrate buffer [0.6 M sodium chloride, 0.06 M] sodium citrate, 2.5 μg/ml fluoresceinated avidin (Boehringer Mannheim), 0.1% Triton X-100, and 5% nonfat dry milk (w/v)]. Following incubation in staining buffer for 30 min in the dark at room temperature, cells were resuspended in PBS containing 10 μg/ml PI and 10 μg/ml DNase-free RNase. Cells were placed on ice in the dark for 1 h. Flow cytometry was performed as described below.

FACS Analysis. Fluorescence of individual nuclei and whole cells was performed using a FACScan flow cytometer equipped with an argon-ion laser at 488 nm and 250 mW light output and Lysis II software (Becton Dickinson immunocytometry systems). The red (PI) and green (fluorescein) fluorescence emissions from each cell were separated and measured using the standard optics of the FACScan. Ten thousand events were collected in list mode fashion, stored, and analyzed on Multicycle AV software (Phoenix Flow Systems, San Diego, CA).

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