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Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor β2 promoter in breast cancer cells

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Retinoic acid (RA)-resistance in breast cancer cells has been associated with irreversible loss of retinoic acid receptor β, *RARβ*, gene expression. Search of the causes affecting *RARβ* gene activity has been oriented at identifying possible differences either at the level of one of the *RARβ* promoters, *RARβ2*, or at regulatory factors. We hypothesized that loss of *RARβ2* activity occurs as a result of multiple factors, including epigenetic modifications, which can pattern *RARβ2* chromatin state. Using methylation-specific PCR, we found hypermethylation at *RARβ2* in a significant proportion of both breast cancer cell lines and primary breast tumors. Treatment of cells with a methylated *RARβ2* promoter, by means of the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza-CdR), led to demethylation within *RARβ2* and expression of *RARβ* indicating that DNA methylation is at least one factor, contributing to *RARβ* inactivity. However, identically methylated promoters can differentially respond to RA, suggesting that *RARβ2* activity may be associated to different repressive chromatin states. This supposition is supported by the finding that the more stable repressive *RARβ2* state in the RA-resistant MDA-MB-231 cell line can be alleviated by the HDAC inhibitor, trichostatin A (TSA), with restoration of RA-induced *RARβ* transcription. Thus, chromatin-remodeling drugs might provide a strategy to restore *RARβ* activity, and help to overcome the hurdle of RA-resistance in breast cancer. *Oncogene* (2000) **19,** 1556–1563.

Keywords: breast cancer; DNA methylation; chromatin remodeling; retinoic acid receptor (RAR)β

Introduction

Retinoic acid (RA) controls fundamental developmental processes, induces terminal differentiation of myeloid progenitors and suppresses cancer and cell growth (Smith *et al*., 1992; Gudas *et al*., 1994). RA activity is mediated by nuclear receptors, the retinoic acid receptors, RARs, that act as RA-dependent transcriptional activators in their heterodimeric forms with retinoid X receptors, RXRs (Chambon, 1996). RARs induce local chromatin changes at level of target genes, containing responsive RA elements (RAREs) by recruiting multiprotein complexes with histone acetyltransferase (HAT) activity and histone deacetylase (HDAC) activity, that dynamically pattern chromatin modification and regulate gene expression (see for review Chambon, 1996; Minucci and Pelicci, 1999).

RARs and RXRs, when disrupted, result in severe developmental defects and neoplastic transformation (Smith *et al*., 1992; Gudas *et al*., 1994; Chambon, 1996). In breast cancer cells, the expression of one member of the *RAR*s family, *RARβ* is found consistently downregulated or lost (Roman *et al*., 1992; Shao *et al*., 1994; Swisshelm *et al*., 1994; Li *et al*., 1995; Widshwendtner *et al*., 1997; Xu *et al*., 1997; Liu *et al*., 1997). *RARβ* downregulation can be reversed by RA in estrogen receptor (ER)-positive, but not in ER-negative breast carcinoma cell lines, believed to represent more advanced forms of tumors (Liu *et al*., 1997). Loss of RA-induced *RARβ* expression is considered a crucial step in the development of RA-resistance in breast carcinogenesis. A complex regulatory region, with two promoters regulates *RARβ* gene expression. Only one promoter, *RARβ2*, containing several RA-response elements, including a canonical and an auxiliary RA response element, βRARE (de The' *et al*., 1990; Valcarel *et al*., 1994) is active in human mammary epithelial cells (HMEC). The transcription of the *RARβ2* promoter is mediated by multiple RARs including, RARα and RARβ itself (Chiba *et al*., 1997) able to recruit coactivator and corepressor protein complexes with HAT/HDAC activities, respectively (Chambon, 1996). To understand why *RARβ* activity is downregulated, or lost, in breast cancer, intense search has been oriented at identifying possible alterations affecting either the *RARβ2* promoter, or regulatory factors (Seewaldt *et al*., 1995; Widschwendtner *et al*., 1997; Xu *et al*., 1997; Tsou *et al*., 1998; Folkers *et al*., 1998).

DNA methylation is an epigenetic change that induces chromatin modifications and repression of transcription via a methyl CpG binding protein MeCP2, and recruitment of a Sin3A/HDAC corepressor complex (Nan *et al*., 1998; Wade *et al*., 1998; Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). For this reason, we decided to investigate whether *RARβ2* promoter was affected by DNA methylation. Indeed, we found hypermethylation at the *RARβ2* promoter both in breast carcinoma cell lines, and a significant proportion of primary breast tumors. Treatment with the methyltransferase inhibitor

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5-Aza-CdR partially reversed the DNA methylation state, and restored *RARβ* transcription, thus indicating that DNA methylation is at least one factor contributing to *RARβ* inactivity. However, the available data indicate that DNA methylation is only a component of the observed *RARβ* gene inactivity. Very likely, RA-inducibility of *RARβ* gene is influenced by modifications altering *RARβ2* chromatin, produced by the nuclear receptors that act at βRARE (*RARα* and the same *RARβ*), as well as DNA methylation.

Results

The RARβ2 *promoter is methylated in breast cancer cell lines independently of their ER status and RA-inducibility*

RARβ transcription was first tested in a panel of breast cancer cell lines grown in the absence of exogenous RA, by reverse transcriptase-PCR (RT–PCR), using primers encompassing exons 5 and 6 (de The' *et al*., 1990; van der Leede *et al*., 1992; Toulouse *et al*., 1997). Under these conditions, only one cell line, Hs578t, produced a detectable 256 bp RT–PCR product (Figure 4a). Thus, we confirmed previous reports that *RARβ* gene expression is down regulated/lost in breast cancer cell lines. Growing cells in the presence of RA can assess the distinction between downregulation and loss. As previously reported (Swisshelm *et al*., 1994; Liu *et al*., 1997; Shang *et al*., 1999), we observed induction of *RARβ* expression and growth inhibition in T47D, MDA-MB-435, MCF7 and ZR75-1 cell lines treated for 48 h with 1 μM RA, but not in the MDA-MB-231 and MDA-MB-468 cell lines.

To see whether the *RARβ2* methylation status correlated with the ER status, we examined the methylation status at *RARβ2* in a panel of ER-positive (MCF7, T47D, ZR75-1) and ER-negative (Hs578t, MDA-MB-231, MDA-MB-435, MDA-MB-468) cell lines.

By Southern blotting we analysed the CpG island of the *RARβ2* promoter within a 7.5 kb *Xba*I DNA fragment encompassing the TATA box, the βRARE, the transcriptional start site (TS) and the 5′ untranslated region of exon 5 (Figure 1a). In this region we can identify nine *Hpa*II sites (Shen *et al*., 1991; Baust *et al*., 1996). The DNA methylation status was analysed by using the methylation-sensitive enzyme, *Hpa*II (Figure 1b). *Msp*I, the isoschizomer of *Hpa*II, insensitive to methylation, was used as a positive control. The PCR probe spans the βRARE and the TATA box regions (Figure 1a). The same 7.5 kb region was previously analysed in a colon carcinoma cell line, and the size of all the possible fragments relative to the most 3′*Hpa*II site were reported (Cote' and Momparler, 1997). A representative blot is shown in Figure 1b. Genomic DNA from the ER-positive, RA-inducible cell line T47D is digested to completion, indicating that it is not methylated at any of the *Hpa*II sites. In contrast, DNA from the ER-positive, RA-inducible ZR75-1 cell line and DNA from the ER-negative, RA-resistant MDA-MB-231 cell line showed to be differentially methylated at the methylation-sensitive sites. (Figure 1b). Using methylation-specific PCR (MSP), we further analysed a 616 bp long *RARβ2* region from nucleotide 481 to nucleotide 1096 (Shen *et al*., 1991) in all the cell lines. MSP entails the modification of genomic DNA by sodium bisulfite that converts all unmethylated, but not methylated, cytosine to uracil (Herman *et al*., 1996). The distribution of CpGs expected after Na bisulfite modification and the four MSP primers (1–4) is reported in Figure 2a. The genomic DNAs from four breast cancer cell lines ZR751, MCF7, MDA-MB-231, MDA-MB-468 showed partial to complete methylation of the promoter region (Figure 2b). The human mammary epithelial cell (HMEC) strain 48R, expressing *RARβ* and three breast cancer cell lines, the *RARβ*-positive Hs578t and the RA-inducible MDA-MB-435 and T47D, revealed only the (U) unmethylated PCR products (Figure 2b).

These results indicate that hypermethylation of the *RARβ2* promoter occurs in breast cancer cell lines irrespective of the ER status, and can be detected in both RA-inducible, and RA-resistant breast cancer cells.

RARβ2 *is unmethylated in both mortal and immortalized HMEC, but is methylated in primary breast tumors*

Next, we asked whether hypermethylation of *RARβ2* promoter in cell lines has correlates in clinical breast cancer. As a normal control we analysed the HMEC mortal strains (48R, 172R), that are the closest representation of normal mammary epithelial cells available. We also analysed two immortal mammary epithelial strains (184A1 and 184B5). The DNA of these strains was found to be unmethylated (Figure 2c). Consequently, methylation of *RARβ2* may be an event in the progression of breast cancer, following immortalization. Genomic DNAs from three paraffinated samples of breast tumors, two ER-positive (T1, T2) and one ER-negative (T3), estimated to contain more than 90% tumor cells, were analysed with all MSP primer pairs, and shown to be partially methylated (Figure 2d). Both microdissected breast stroma, and microdissected normal epithelial cells were found unmethylated at *RARβ2* (our unpublished observations), making it very likely that the U products in the tumor samples were amplified either from residual normal epithelial cells, or stromal cells mixed to tumor cells. DNAs from matching histologically tumor free lymph node samples (N1–N3), were similarly analysed and produced only the unmethylated PCR products (Figure 2d). The DNA of additional 21 tumors was performed using two sets of primer pairs (U3/M3 and U4/M4). Fifteen (7 ER-positive and 8 ER-negative) of the 24 tumors presented methylation at the *RARβ2* promoter. With the same primer sets hypermethylation at *RARβ2* was detected in the DNA of ten out of 39 primary breast tumors collected, and analysed independently, at the Johns Hopkins University.

The overall data indicate that hypermethylation at *RARβ2* promoter occurs in approximately one third of primary breast tumors, and that the *RARβ2* methylation state is independent of the ER status of the tumor.

5-Aza-CdR induces partial demethylation at the RARβ2 *CpG island and reactivation of* RARβ *gene expression*

In order to determine whether DNA methylation is affecting, at least in part, *RARβ* gene expression, we treated all the cell lines showing methylation at the *RARβ2* promoter with the DNA methyltransferase inhibitor, 5-Aza-CdR. Treatment of cells with either

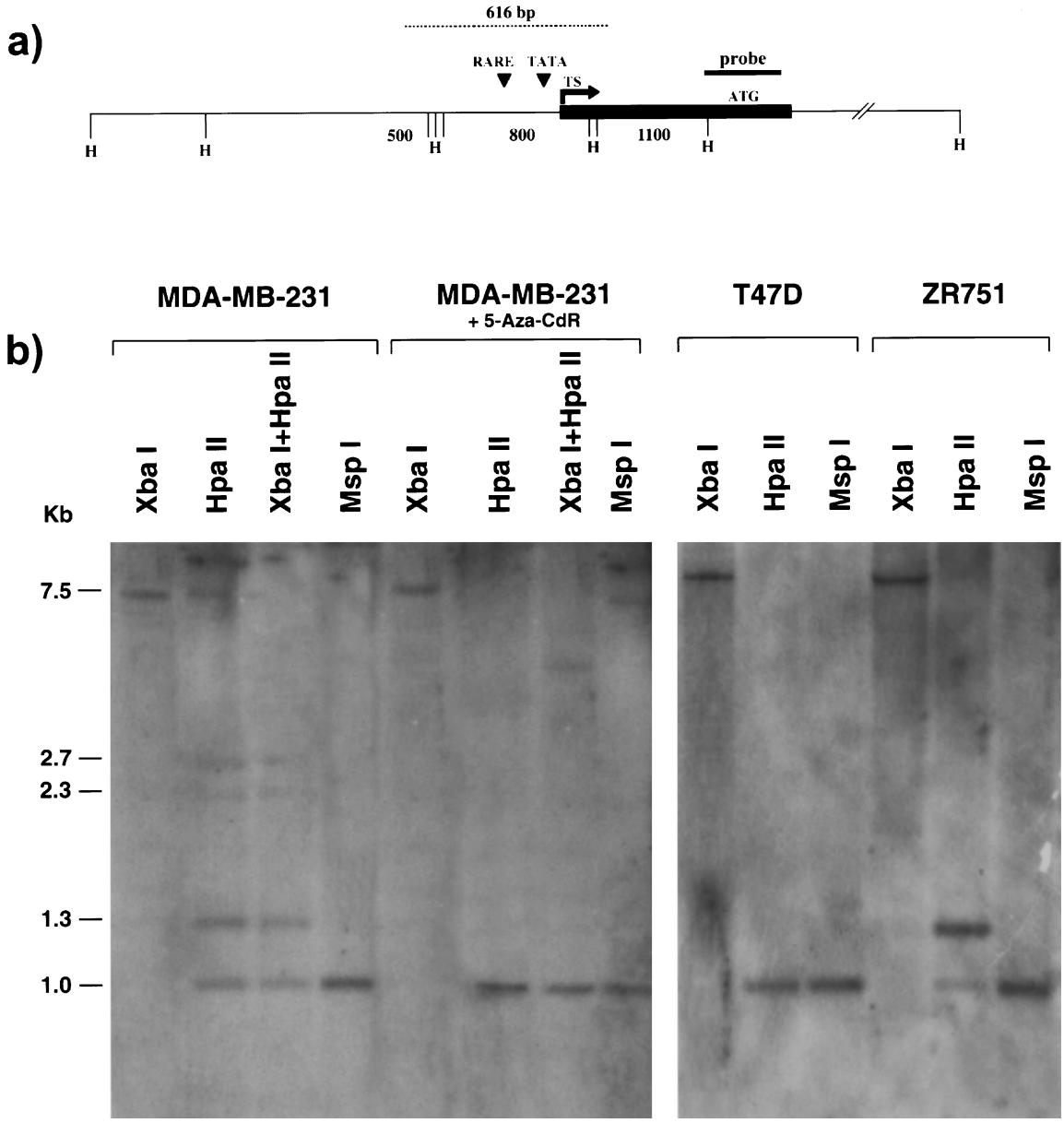

Figure 1 Methylation sensitive Southern blotting of the *RARβ2* promoter. (**a**) Genomic map of the *RARβ2* promoter-exon 5 region indicating the position of *Hpa*II sites (H) relative to βRARE, TATA, transcription start site (TS) and the ATG. (**b**) Southern analysis of : T47D and ZR571 DNAs digested with *Xba*I, *Hpa*II (right) and *Msp*I and MDA-MB-231 cells before, and after treatment with 0.8 μM 5-Aza-CdR for 3 days (left)

0.4 or 0.8 μM 5-Aza-CdR for 3 days, led to partial demethylation of the CpG rich *RARβ2* region. This was evident both by Southern analysis in the MDA-MB-231 cell line (Figure 1b, left panel), and by MSP in all cell lines (Figure 3). Moreover, 5-Aza-CdR treatment resulted in reactivation of gene expression both in RA-inducible MCF7 and ZR75-1, and RA-resistant MDA-MB-231 and MDA-MB-468 cells (Figure 4b). We asked whether reactivation of *RARβ* expression by 5-Aza-CdR A-resistant cells could be enhanced by RA. By using non-quantitative RT–PCR, we could not appreciate a difference in the level of *RARβ* transcription in MDA-MB-231 cells treated with 0.4 μM 5-Aza-CdR alone, or in combination, with 1 μM RA (Figure 4c). In this experiment, 5-Aza-CdR alone, or in combination with RA, produced 63 and 96% growth inhibition respectively. In the same experiment, treatment with 1 μM RA alone produced a negligible effect on growth inhibition (<2%). A synergistic effect of the two drugs on cancer cells was previously reported (Cote' and Momparler, 1997; Bovenzi *et al*., 1999).

These data indicate that DNA methylation is, at least, one factor influencing the downregulation/loss of *RARβ* transcription in breast cancer cell lines with a methylated *RARβ2* promoter. Cells treated with 5-Aza-CdR alone, or in combination with RA, showed re-expression of *RARβ*, which may have contributed, along with the toxic 5-Aza-CdR, to the observed growth inhibition.

The HDAC inhibitor TSA can reactivate RARβ *expression in RA-resistant cells; demethylation of the* RARβ2 *promoter is not an absolute requirement for* RARβ *reactivation*

The chromatin status at a given locus can be dynamically influenced by the degree of acetylation/

deacetylation due to HAT/HDAC activities. Absence of *RARβ* regulatory factors, like RARα, as well as DNA-methylation, can contribute to pattern chromatin modifications at *RARβ* promoter in RA-resistant cell lines. One of these cell lines, MDA-MB-231, lacks RA-inducible *RARα* activity (Shao *et al*., 1994) and displays a *RARβ2* methylated promoter. We decided to probe indirectly whether the level of HDAC at *RARβ2* can influence *RARβ* expression, by testing the effect of TSA, a HDAC inhibitor on MDA-MB-231 cells (Yoshida *et al*., 1995). Cells were treated for 2 days, in the presence or absence of 100 ng/ml TSA alone, or in combination, with 1 μM RA. By using RT–PCR, it was clear that, unlike cells treated with RA alone, cells treated with a combination of RA and TSA re-expressed *RARβ* mRNA (Figure 4d). Under the same experimental conditions, 100 ng/ml TSA alone, or in combination with 1 μM RA, produced 77

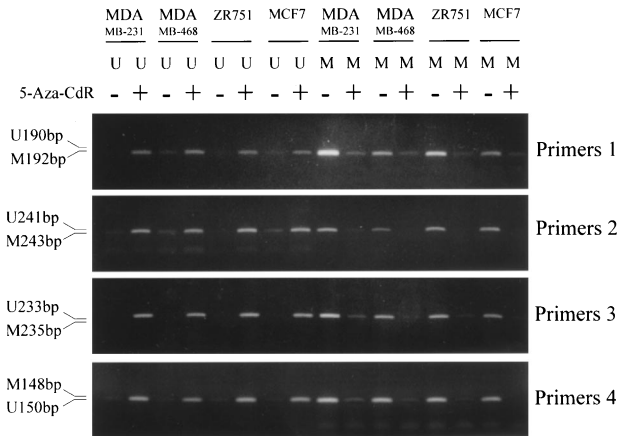

Figure 3 Treatment with 5-Aza-CdR induce partial de-methylation. MSP analysis of DNA of four breast cancer cell lines before and after treatment for 3 days with 0.8 μM 5-Aza-CdR

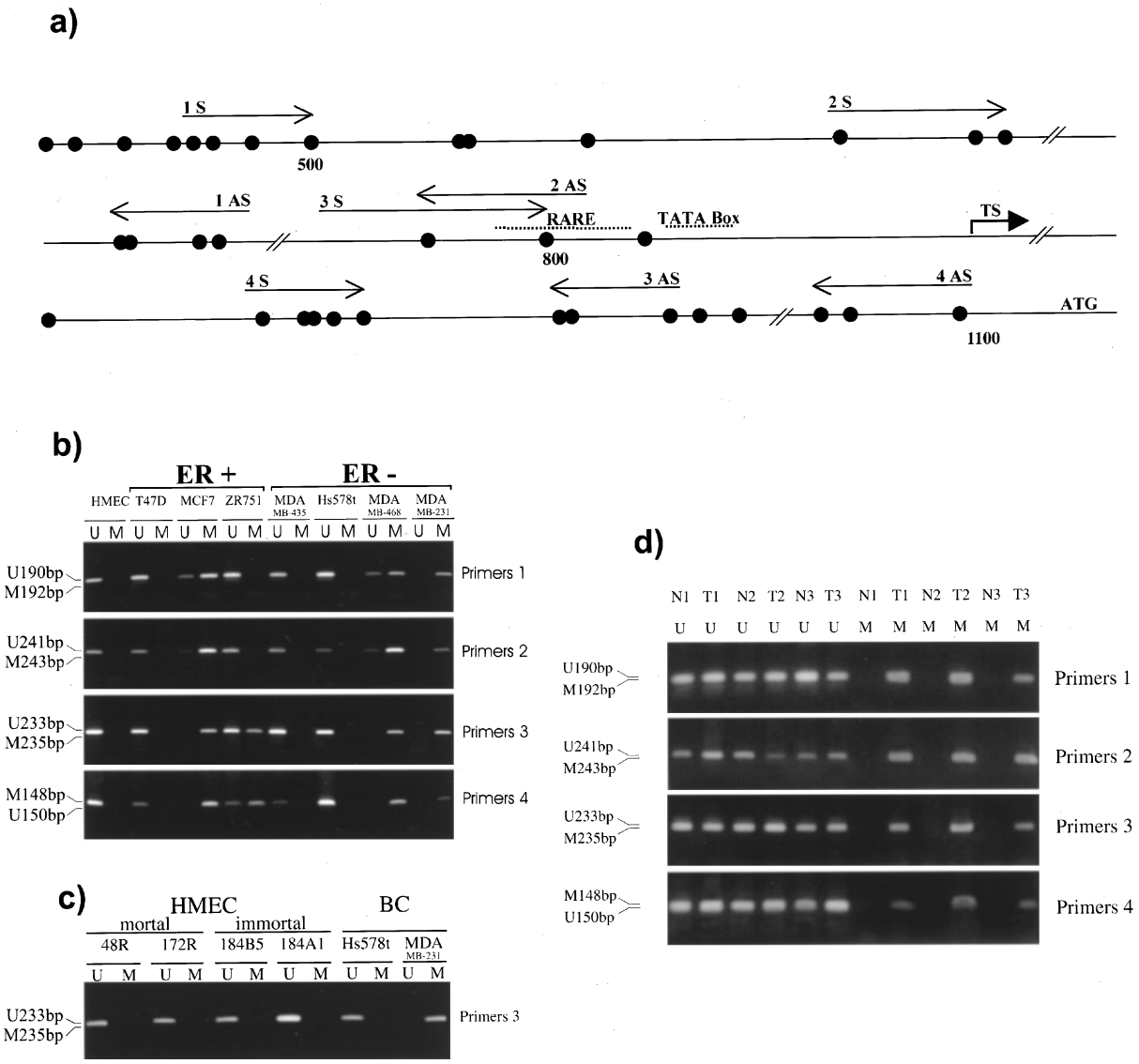

Figure 2 MSP analysis of DNA from cell lines and primary tumors. (**a**) Distribution of the methylated CpGs (filled circles) in the *RARβ2* promoter region spanning nt 498 to nt 1096 and position of the MSP primers. (**b**) MSP analysis of a panel of breast carcinoma cell lines. U and M products amplified with the four sets of MSP primers in ER-positive and -negative cell lines and the mortal HMEC (48R) strain. (**c**) MSP analysis of two mortal (48R and 172R) and two immortal (184A1 and 184B5) HMEC strains. (**d**) MSP analysis of three breast tumors (T1–T3) and matching tumor cell free lymph nodes (N1–N3)

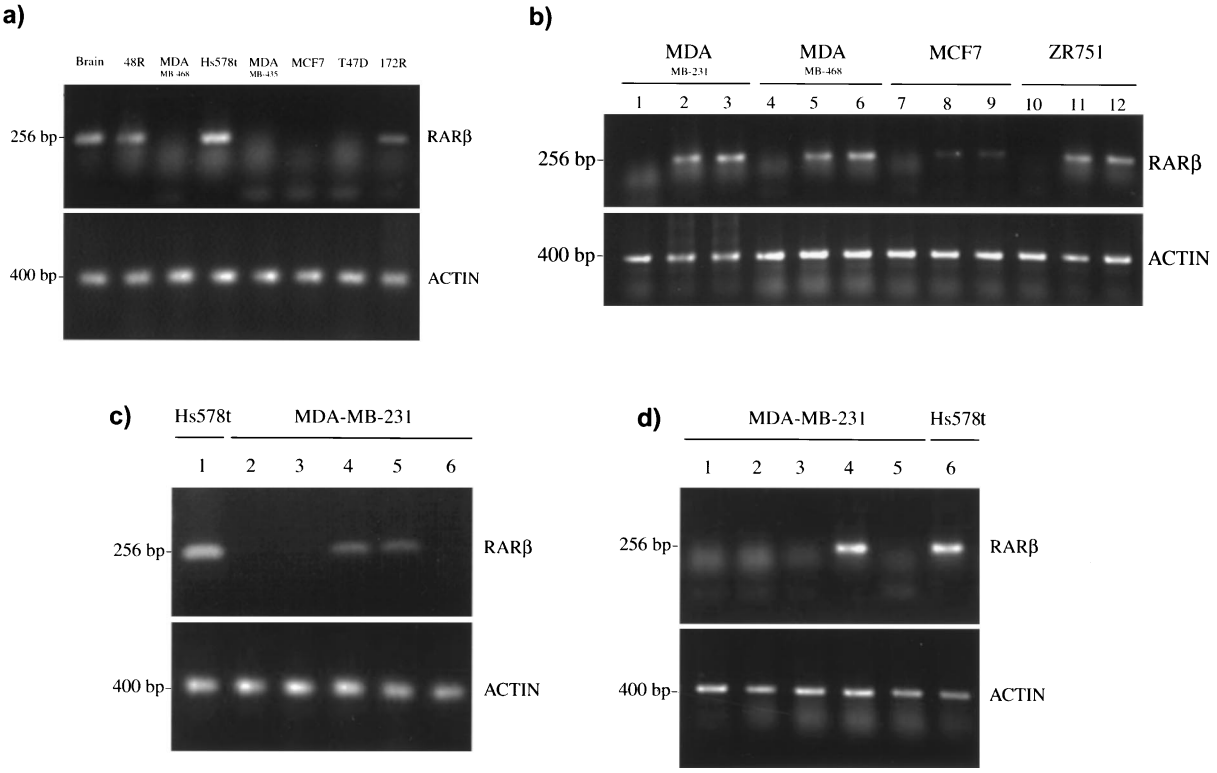

Figure 4 Treatment with 5-Aza-CdR and TSA triggers re-expression of *RARβ*. (**a**) RT–PCR of mortal HMEC strains 48R and 172R and breast cancer cell lines. Brain RNA was used as a control. (**b**) RT–PCR of breast cancer cell lines treated for 3 days with 0.4 μM (lanes 2, 5, 8 and 11) and 0.8 μM (lanes 3, 6, 9 and 12) 5-Aza-CdR and untreated cells (lanes 1, 4, 7 and 10). (**c**) RT–PCR of MDA-MB-231 cells untreated (lane 2) in comparison with cells treated for 3 days with 1 μM RA (lane 3), 0.4 μM 5-Aza-CdR (lane 4), 0.4 μM 5-Aza-CdR + 1 μM RA (lane 5). Hs578t used as positive control (lane 6). (**d**) RT–PCR of MDA-MB-231 cells untreated (lane 1), in comparison with cells treated for 48 h with 1 μM RA (lane 2), 100 ng/ml TSA (lane 3), 100 ng/ml TSA + 1 μM RA (lane 4); solvent (lane 5)

and 92% growth inhibition, respectively. Treatment with 1 μM RA alone did not affect significantly growth inhibition (<2%). By MSP analysis, we could assess that *RARβ* expression was restored in the presence of a methylated *RARβ2* promoter. The MSP profile obtained with primer set 3, spanning the βRARE region is reported in Figure 5. This finding indirectly shows that global alterations of HDAC activity, generated by TSA in MDA-MB-231 cells, involved *RARβ2* resulting in RA-induced *RARβ* expression. Further, demethylation at *RARβ2* did not seem to be an absolute requirement for *RARβ* gene expression in MDA-MB-231 cells. Noteworthy, persistence of methylation at *RARβ2* was observed also in MCF7 cells where *RARβ* transcription could be restored in the presence of RA (data not shown). Growth inhibition was observed in cells treated with TSA alone, or in combination, with RA. Very likely, *RARβ* along with TSA, a drug known to induce growth inhibition (Yoshida *et al*., 1995), contributed to the massive growth inhibitory effect that we observed.

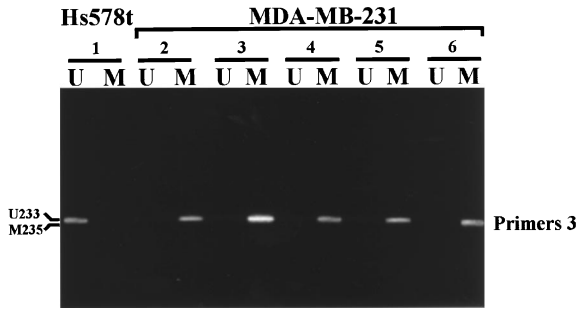

Figure 5 DNA methylation in *RARβ2* promoter of MDA-MB-231 cells expressing RA-induced *RARβ* after TSA treatment. MSP analysis of MDA-MB-231 cells using primers 3 shows that there is no demethylation of the βRARE containing region in cells treated with 1 μM RA (lane 3), 100 ng/ml TSA (lane 4), 100 ng/ml TSA + 1 μM RA (lane 5); in comparison with untreated cells (lane 2), or cells grown in the presence of solvent (lane 6). MSP of Hs578t was used as a control (lane 1)

Discussion

RARβ2 *promoter is methylated in breast cancer*

In this study, we show evidence that, in breast cancer cells, *RARβ2* promoter undergoes DNA hypermethylation, an epigenetic change known to induce chromatin modifications and influence gene expression (Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). We detected methylation of the *RARβ2* promoter region, both in breast carcinoma cell lines, and a significant proportion of primary breast tumors. *RARβ2* methylation status did not correlate with the ER status of breast cancer cells and was observed both in *in situ* lesions and invasive tumors (our unpublished observations).

It is not clear when epigenetic changes occur during breast cancer progression. However, methylation of the promoter was not detected in both mortal, and

immortal human mammary epithelial cell (HMEC) strains, as well as in normal microdissected breast epithelial cells (our unpublished observations). These results suggest that aberrant methylation of the *RARβ2* CpG island may be a later event following immortalization. Treatment of breast cancer cells presenting with a methylated *RARβ2*, with the demethylating agent 5-Aza-CdR, induced partial DNA demethylation and restored *RARβ* gene expression. This evidence clearly indicates that DNA methylation is at least a component contributing to *RARβ* downregulation/loss.

RARβ2 *methylation state and RA-inducibility*

The correlation between *RARβ2* methylation and RA-inducibility in different breast cancer cell lines, indicates that DNA methylation is not the only factor influencing *RARβ* silencing. Survey of different breast cancer cell lines shows that *RARβ* is downregulated, but can be reinduced by RA both in MDA-MB-435 and T47D cells, with unmethylated *RARβ2* promoter and in MCF7 and ZR751 cells, with a methylated promoter. In contrast, in MDA-MB-231 and MDA-MB-468 cell lines the methylated *RARβ2* promoter is indifferent to RA treatment. Apparently, different degrees of repression can affect *RARβ2* promoter, and only in some cases, the ligand is sufficient to alleviate methyl-directed repression. Extinction of *RARβ* transcription must be determined by a stable repressive state in the chromatin structure determined by more than one mechanism, including DNA methylation.

DNA-methylation might be secondary to RARβ2 *promoter inactivity*

We hypothesize that low intracellular levels of RA in breast cancer cells may induce chromatin structure alterations at *RARβ2*, similar to the ones observed in the P19 embryonal carcinoma cell line (Bhattacharyya *et al*., 1997). Although the mechanism of chromatin structure alterations are not fully understood, current evidence indicates that local histone acetylation is a crucial factor (Razin, 1998). An altered chromatin environment may predispose to DNA methylation, a condition that might further affect histone deacetylation at *RARβ2* (Razin, 1998; Ng and Bird, 1999; Jones and Wolffe, 1999). The first to propose that gene inactivity 'invites' *de novo* methylation was Bird (1986). The hypothesis was further refined, after the discovery of the mechanistic link between DNA methylation and chromatin conformation mediated by the MeCP2/Sin3A/HDAC corepressor complex (Nan *et al*., 1998; Wade *et al*., 1998). According to the revisited hypothesis, Ng and Bird (1999) propose that: 'DNA methyltransferase – either independently or assisted by accessory proteins – may be capable of reading the histone acetylation pattern on the chromatin and its *de novo* methyltransferase activity can respond differentially to different states of chromatin modification. In this case, deacetylated chromatin would provoke *de novo* methylation. This self-reinforcing mechanism, supported by DNA methylation and histone deacetylation, could provide a stable state of inactive chromatin, unless overcome by other mechanisms'.

RARβ2 promoter in breast cancer might provide an ideal system to test this hypothesis, given the heterogeneous correlation between its methylation state and RA-inducibility in different breast cancer cells. Unmethylated, RA-inducible *RARβ2* promoters are expected to be associated with, either an active chromatin state, or a mild repressive state. A methylated *RARβ2* promoter is expected to be associated with a more repressive chromatin environment. As a consequence, transcription from a methylated promoter should be possible, either by recruiting consistent HAT activity, or by inhibiting excessive HDAC activity. These speculations are so far supported by compelling circumstantial evidence. Notably, RA can induce both RARα and RARβ in MCF7 cells from a *RARβ2* methylated promoter (Shao *et al*., 1994; Shang *et al*., 1999; our unpublished observations). This suggests that RA may trigger recruitment of HAT activity at *RARβ2*, sufficient to override methylation-related chromatin constraints. On the contrary, in the MDA-MB-231 cells we saw that RA-induced *RARβ* transcription is possible after treatment with TSA, a HDAC inhibitor, already known to induce chromatin alterations at *RARβ2* promoter in P19 cells (Minucci *et al*., 1997). Analysis of the DNaseI sensitivity pattern, in and around *RARβ2*, as well as the assessment of *RARβ2* histone acetylation state (Keshet *et al*., 1986; Hebbes *et al*., 1994; Eden *et al*., 1998), in both MCF7 and MDA-MB-231 cells will give us an idea of the relation between chromatin environments and RARβ transcription. Moreover, these studies are expected to shed light on the relation of histone acetylation and methylation of the *RARβ2* promoter. This issue is of particular interest since it is not yet completely clear whether DNA demethylation is indeed always required to restore transcription from genes with fully methylated promoters (Cameron *et al*., 1999; Ferguson *et al*., 1998; Razin, 1998; Ng and Bird, 1999).

In conclusion, we provide evidence that DNA-methylation at *RARβ2* promoter in breast cancer cells is affecting, at least in part, *RARβ* transcription. We argue that DNA-methylation is secondary to the inactive state at *RARβ2* promoter and may contribute to create a stable repressive *RARβ2* environment and extinction of *RARβ* transcription. Further understanding of epigenetic changes and chromatin alteration at *RARβ2* may have preventive and therapeutic implications. Changes altering *RARβ2* chromatin structure and *RARβ* transcription in breast cancer might be prevented in the presence of supraphysiological levels of RA (Minna and Mangeldorf, 1997). Knowledge of *RARβ2*-methylation state of primary breast cancers might be useful to identify tumors that are more likely to respond to RA-therapy. Finally, the possibility to re-induce *RARβ* activity in RA-resistant breast cancer cells, using both TSA and RA, a combination proven to be effective for treating leukemia (Grignani *et al*., 1998; Guidez *et al*., 1998; He *et al*., 1998; Lin *et al*., 1998; Warrell *et al*., 1998), might have therapeutic implications also in the treatment of RA-resistant breast tumors.

Materials and methods

Cell cultures

Human epithelial mammary cells (HEMC) from reduction mammoplasty including three mortal strains, 184, 48R and

172R, and two immortal strains, 184A1 and 184B5, were obtained and cultured according to the protocols designed by Dr Martha Stampfer (see the HMEC Homepage, http://www.lbl.gov/~mrgs/index.htlm) using Clonetics (Walkersville, MD, USA) reagents.

Human breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO) (Hs578t, MCF-7, MDA-MB-231 and T47D) or IMEM medium (Biofluids) (MDA-MB-435, MDA-MB-468, ZR751) with 5% fetal calf serum (FCS). For drug treatments, exponentially growing cells were seeded in 10 cm^2 plates at a density of 3×10^5 cells/plate or in 6-well plates at 1×10^5 cells/well. Cells were allowed to attach overnight before the addition of the appropriate concentration of 5-Aza-2′ deoxycytidine (5-Aza-CdR) (Sigma), Trichostatin A (TSA) (Sigma) or RA (Sigma). When reduction of retinoids was required, cells were treated in either medium with 0.5% FCS or charcoal-dextran stripped FCS (Hyclone). At the indicated time points, both attached and detached cells were harvested, counted with Trypan Blue (Life Technologies) and processed for DNA or RNA extraction. 5-Aza-CdR was dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8). Trichostatin A and all-trans-retinoic acid (RA) (Sigma) were reconstituted in absolute ethanol (solvent). The growth inhibition (%) was calculated as: $(1\text{-NT/NC}) \times 100$, where NT is the number of treated cells and NC is the number of control cells.

Tissue samples

Normal and tumor tissues were collected from existing tumor banks (Instituto per lo Studio e la Cura dei Tumori, Milan; the Cancer Center, Rotterdam, the Johns Hopkins Breast Cancer Program, Baltimore, MD, USA). All tumor samples were obtained from excess clinical specimens and institutional guidelines for the acquisition and maintenance of such specimens were followed.

DNA and RNA extraction

Extraction of DNA and RNA from breast cancer cell lines was performed by using DNAzol and Trizol respectively (Life Technologies) according to the manufacturer's instructions. Genomic DNA was further treated with 500 μg/ml proteinase K at 55°C, extracted with phenol-chloroform-isoamylic alcohol (24 : 24 : 1) (CIA) and ethanol precipitated. Extraction of DNA from paraffinated breast cancer and lymph node tissues was essentially performed as previously described (Formantici *et al*., 1999). One to three consecutive sections estimated to contain at least 90% tumor cells were incubated at 58°C overnight in 200 μl of extraction buffer (50 mM KCl, 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, and the solution was heated at 95°C for 15 min to inactivate the proteinase K and then centrifuged at 6000 r.p.m. The DNA in the supernatant was used for analysis.

Southern blotting

Genomic DNA (7 μg) was digested overnight with 15 U/μg of *Xba*I, *Hpa*II and *Msp*I enzymes, electrophoresis on a 0.8% agarose gel and transferred to Hybond-N filter. A 227 bp probe was amplified using the sense 5′-AGA GTT TGA TGG AGT TGG GTG GAG-3′ and antisense 5′-CAT TCG GTT TGG GTC AAT CCA CTG-3′ primers, gel purified and labeled with ^{32}P-dCTP using the Megaprime DNA labeling system (Amersham). After hybridization the filters were washed and exposed to X-ray film at −80°C for autoradiography.

Methylation specific PCR (MSP)

Bisulfite modification of genomic DNA was essentially performed as described by Herman *et al*. (1996). Modified DNA was used immediately or stored in aliquots at −20°C. The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.7), 1.5 mM $MgCl_2$), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) or unmodified DNA (50 ng). Reactions were hot started at 95°C before the addition of 2.5 U of Taq polymerase (Qiagen). Amplification was carried out in a Thermal Cycler 480 Perkin Elmer for 30 cycles (1 min at 94°C, 1 min at the annealing temperature (at) selected for each primer pair, 1 min at 72°C), followed by 4 min at 72°C. Twelve μl of the PCR reaction were electrophoresed onto 1.5% agarose gels, stained with ethidium bromide and visualized under UV. Two primer pairs, W3 sense 5′-CAGCCCGGGTAGGGTTCACC-3′, W3 antisense 5′-CCGGATCCTACCCCGACGG-3′, and W4 sense 5′-CCGAGAACGCGAGCGATCC-3′ and W4 antisense 5′-GGCCAATCCAGCCGGGGCG-3′, were designed on the human *RARβ2* sequence (Shen *et al*., 1991) and used to control the Na bisulfite modification. The primer pairs selected to detect the unmethylated DNA were as follows: U1 sense 5′-GTG GGT GTA GGT GGA ATA TT-3′ and U1 antisense 5′-AAC AAA CAC ACA AAC CAA CA-3′ (at 55°C); U2 sense 5′-TGT GAG TTA GGA GTA GTG TTT T-3′ and U2 antisense 5′-TTC AAT AAA CCC TAC CCA-3′ (at 49°C); U3 sense 5′-TTA GTA GTT TGG GTA GGG TTT ATT-3′ and U3 antisense 5′-CCA AAT CCT ACC CCA ACA-3′ (at 55°C); U4 sense 5′-GAT GTT GAG AAT GTG AGT GAT TT-3′ and U4 antisense 5′-AAC CAA TCC AAC CAA AAC A-3′ (at 55°C); The sequences of the primers to detect the methylated DNA were: M1 sense 5′-AGC GGG CGT AGG CGG AAT ATC-3′ and M1 antisense 5′-CAA CGA ACG CAC AAA CCG ACG-3′ (at 63°C); M2 sense 5′-CGT GAG TTA GGA GTA GCG TTT C-3′ and M2 antisense 5′-CTT TCG ATA AAC CCT ACC CG-3′ (at 57°C); M3 sense 5′-GGT TAG TAG TTC GGG TAG GGT TTA TC-3′ and M3 antisense 5′-CCG AAT CCT ACC CCG ACG-3′ (at 64°C); M4 sense 5′-GTC GAG AAC GCG AGC GAT TC-3′ and M4 antisense 5′-CGA CCA ATC CAA CCG AAA CG-3′ (at 64°C).

The distrubution of the CpG methylated sites and the position of the primers is reported in Figure 2. M and U primers were designed in the same regions, with one or two nucleotide differences to meet annealing requirements. Fragment M3 (position 773–1007) contains the βRARE (792–808) and the transcription start site (position 844); fragment M4 (position 949–1096) contains an Sp1 element (position 1074–1081).

RT–PCR

The exon 5 (sense primer 5′-GAC TGT ATG GAT GTT CTG TCA G-3′) and exon 6 (antisense primer 5′-ATT TGT CCT GGC AGA CGA AGC A-3′) were designed on the basis of published RARβ2 transcript (de The' *et al*., 1990; van der Leede *et al*., 1992) and used to amplify 50 ng of DNase treated total RNA using the Superscript One-Step RT–PCR System (Life Technologies). RT–PCR with actin primers (sense primer 5′-ACC ATG GAT GAT GAT ATC G-3′ and antisense primer 5′-ACA TGG CTG GGG TGT TGA AG-3′ was used as an internal RNA control.

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References

Baust C, Redpath L and Schwarz E. (1996). *Int. J. Cancer,* **67,** 409–416.

Bhattacharyya N, Dey A, Minucci S, Zimmer A, John S, Hager G and Ozato K. (1997). *Mol. Cell Biol.,* **17,** 6481–6490.

Bird A. (1986). *Nature,* **321,** 209–213.

Bovenzi V, Le NLO, Cote' S, Sinnet D, Momparler LF and Momparler RI. (1999). *Anticancer Drugs,* **10,** 471–476.

Cameron EE, Bachman KE, Myohanen S, Herman JG and Baylin SB. (1999). *Nature Genet.,* **21,** 103–107.

Chambon P. (1996). *FASEB J.,* **10,** 940–954.

Chiba H, Clifford J, Metzger D and Chambon P. (1997). *Mol. Cell Biol.,* **17,** 3013–3020.

Cote' S and Momparler RL. (1997). *Anticancer Drugs,* **8,** 56–61.

De The' H, del Mar Vivanco-Ruiz M, Tiollais P, Stunnenberg HG and Dejan A. (1990). *Nature,* **343,** 177–180.

Eden S, Hashimshony T, Keshet I, Cedar H and Torne AW. (1998). *Nature,* **394,** 842.

Ferguson AT, Lapidus RG and Davidson NE. (1998). *Oncogene,* **17,** 577–583.

Folkers GE, van der Burg B and van der Saag PT. (1998). *J. Biol. Chem.,* **273,** 32200–32212.

Formantici C, Orlandi R, Ronchini C, Pilotti S, Ranzani GN, Colnaghi MI and Menard S. (1999). *J. Pathol.,* **187,** 424–427.

Grignani F, Dematteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, *et al.* (1998). *Nature,* **391,** 815–818.

Gudas LJ, Sporn MB and Roberts AB. (1994). In: *The Retinoids. Biology, Chemistry and Medicine.* Raven Press: New York, pp. 443–520.

Guidez F, Ivins S, Zhu J, Soderstrom M, Waxman S and Zelent A. (1998). *Blood,* **91,** 2634–2637.

He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A and Pandolfi PP. (1998). *Nature Genet.,* **18,** 126–135.

Hebbes TR, Clayton AL, Thorne AW and Crane-Robinson C. (1994). *EMBO J.,* **13,** 1823–1830.

Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 9821–9826.

Jones PL and Wolffe AP. (1999). *Semin. Cancer Biol.,* **9,** 339–347.

Keshet I, Lieman-Hurwitz J and Cedar H. (1986). *Cell,* **44,** 535–543.

Li X-S, Shao Z-M, Sheikh MS, Eiseman JL, Sentz D, Jetten AM, Chen J-C, Dawson ML, Aisner S, Rishi AK, Gutierrez P, Schnapper L and Fontana JA. (1995). *J. Cell Physiol.,* **165,** 449–458.

Lin RJ, Nagy I, Inoue S, Shao W, Miller Jr WH and Evans RM. (1998). *Nature,* **391,** 811–814.

Liu Y, Lee M-O, Wang H-G, Li Y, Hashimoto Y, Klaus M, Reed JC and Zhang X-K. (1997). *Mol. Cell Biol.,* **16,** 1138–1149.

Minucci S, Horn V, Bhattacharyya N, Russanova V, Ogryzko VV, Gabriele L, Howard BH and Ozato K. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 11295–11300.

Minna JD and Mangeldorf DJ. (1997). *J. Natl. Cancer Inst.,* **89,** 602–604.

Minucci S and Pelicci P. (1999). *Semin. Cell Dev. Biol.,* **2,** 215–225.

Ng H-H and Bird A. (1999). *Curr. Opin. Genet. Dev.,* **9,** 158–163.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN and Bird A. (1998). *Nature,* **393,** 386–389.

Razin A. (1998). *EMBO J.,* **17,** 4905–4908.

Roman SD, Clarke CL, Hall RE, Alexander IE and Sutherland RL. (1992). *Cancer Res.,* **52,** 2236–2242.

Seewaldt VI, Johnson BS, Parker MB, Collins SJ and Swisshelm K. (1995). *Cell Growth Differ.,* **6,** 1077–1088.

Shao Z-M, Sheikh MS, Chen JC, Kute T, Aisner S, Schnaper L and Fontana JA. (1994). *Int. J. Oncol.,* **4,** 849–853.

Shang Y, Baumrucker CR and Green MH. (1999). *J. Biol. Chem.,* **274,** 18005–18010.

Shen S, Kruyt FA, den Hertog J, van der Saag FT and Kruijer W. (1991). *DNA Seq.,* **2,** 111–119.

Smith MA, Parkinson Dr, Cheson BD and Friedman MA. (1992). *J. Clin. Oncol.,* **10,** 839–864.

Swisshelm K, Ryan K, Lee X, Tsou HC, Peacoque M and Sager R. (1994). *Cell Growth Differ.,* **5,** 133–141.

Toulouse A, Morin J, Pelletier M and Bradley WEC.(1997). *BBA,* **1309,** 1–4.

Tsou HC, Yao YJ, Xie XX, Ping XL and Peacocke M. (1998). *Exp. Cell Res.,* **245,** 221–227.

Valcarel R, Holz H, Garcia Jimenez C, Barettino D and Stunnenberg HG. (1994). *Genes Dev.,* **8,** 3068–3079.

Van der Leede BJ, Folkers GE, Kruyt FA and van der Saag PT. (1992). *Biochem. Biophys. Res. Commun.,* **188,** 695–702.

Wade PA, Jones PL, Vermaak D, Veenstra GJ, Imhof A, Sera T, Tse C, Ge H, Shi YB, Hansen JC and Wolfee AP. (1998). *Cold Spring Harb. Symp. Quant. Biol.,* **63,** 435–445.

Warrell Jr, RP, He LZ, Richon V, Callega E and Pandolfi PP. (1998). *J. Natl. Cancer Inst.,* **90,** 1621–1625.

Widschwendtner M, Berger J, Daxenbichler G, Muller-Holzner E, Widschwendtner A, Mayr A, Marth C and Zeimet AG. (1997). *Cancer Res.,* **17,** 4158–4161.

Xu XC, Sneige N, Liu X, Nandagiri R, Lee JJ, Lukumanji F, Hortobagy G, Lippman SM, Dhingra K and Lotan R. (1997). *Cancer Res.,* **57,** 4992–4996.

Yoshida M, Horinouchi S and Beppu T. (1995). *Bioessays,* **17,** 423–430.