

# The Increased Expression of Peroxisome Proliferator-Activated Receptor- $\gamma$ 1 in Human Breast Cancer Is Mediated by Selective Promoter Usage

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## ABSTRACT

**Peroxisome proliferator-activated receptor- $\gamma$ 1 (PPAR $\gamma$ 1) is transactivated by a wide range of ligands in normal human mammary epithelial and breast cancer cells. Although transactivation of PPAR $\gamma$  mediates the expression of genes that are markers of differentiation, its overexpression in cancers of the breast, thyroid, colon, and lung suggests its dysregulation may play a role in oncogenesis, cancer progression, or both. We report the overexpression of PPAR $\gamma$  is caused by the use of a tumor-specific promoter in breast cancer cells that is distinct from the promoter used in normal epithelia. Thus, the increase in PPAR $\gamma$  expression seen in breast cancer cells results from promoter recruitment, providing new insights into the expression and actions of PPAR $\gamma$  in breast cancer.**

## INTRODUCTION

Breast cancer in women in the United States accounts for more deaths than any other malignancy (1). The majority of these breast tumors originate from the ductile epithelia, and infiltrating ductal carcinomas account for >70% of all of the breast cancers (2). Current chemotherapies are accompanied by significant toxicity and benefit only a limited number of patients. The limited number of therapeutic options and the high degree of prevalence have stimulated the search for new and more selective molecular targets for the management of breast cancer. Cancer is the result of dysregulation of differentiation and apoptosis. The peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) as an important mediator of terminal differentiation in adipocytes (3, 4) has led to the examination of its role in mediating similar programs in breast adenocarcinomas (5–7).

PPAR $\gamma$  is a member of the nuclear hormone receptor superfamily and plays critical roles in adipogenesis (3, 4, 8), insulin-mediated glucose homeostasis (9), and development (10). Ligands for PPAR $\gamma$  include 15-deoxy $\Delta$ 12,14 prostaglandin J<sub>2</sub> (PGJ<sub>2</sub>), dietary fatty acids, and the thiazolidinedione class of hypoglycemic drugs (11–14). PPAR $\gamma$  is expressed in normal human mammary epithelial cells (HMECs) (6) and established breast cancer cell lines and is functionally responsive to ligand-mediated transactivation (15). Although PPAR $\gamma$  is thought to mediate differentiation in most tissues, its role in tumor progression or suppression, however, is poorly understood. In some tissues, it has been shown that a reduction in the expression of PPAR $\gamma$  can increase carcinogenesis. In these studies, PPAR $\gamma$  heterozygous (+/-) knockout mice have a much greater risk of developing colon tumors following exposure to azoxymethane, an inducer of colorectal cancer (16). Similarly, following chemical induction, these animals also develop more mammary tumors (17). By contrast, constitutive overexpression of PPAR $\gamma$  in animal studies increases the risk of breast cancer in mice already susceptible to the disease (18). It has been suggested that this paradox may be resolved by careful dose-response studies, in which the level of PPAR gene expression

and transactivation are carefully controlled (19). This implies that the level of expression may play a critical role in determining the physiologic outcome of transactivation in a cell-specific context.

Two isoforms of PPAR $\gamma$  protein, termed  $\gamma$ 1 and  $\gamma$ 2, have been identified in humans and rodents (20, 21), which differ by the addition of 28 or 30 N-terminal amino acids, respectively. The additional amino acids on  $\gamma$ 2 are coded for by a distinct first exon. Other untranslated first exons have been reported in humans (22, 23), mice (21), and monkeys (24). This suggests that the expression of PPAR $\gamma$  is under complex regulatory mechanisms. Because benign breast ducts express lower levels of PPAR $\gamma$  protein compared with infiltrating carcinoma cells (5) and because the expression of PPAR $\gamma$  is positively correlated with breast cancer metastasis (25), we have sought to examine the mechanism that underlies the changes in expression that accompanies tumor progression. Here we report the identification of additional untranslated first exons that mediate the changes in expression seen between normal mammary epithelial cells and breast cancer cells. These data show that distinct promoters mediate the expression between these cell types and that promoter switching accompanies the changes in expression levels. Genomic analysis reveals that this is a large gene, spanning >150 kb, with seven or more transcriptional start sites, and that tissue-selective and tumor-specific promoters mediate expression. Finally, in light of the complexity of the gene and the cross-usage of nomenclature to identify promoter usage and protein structure, we propose nomenclature to distinguish these differences.

## MATERIALS AND METHODS

**Cell Culture.** MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). HepG2 cells were a gift from Dr. Hollie Swanson (University of Kentucky College of Medicine). MDA-MB-231 cells were cultured in IMEM (Biofluids, Rockville, MD) containing 2% TCH serum replacement medium (Celox, St. Paul, MN), and MCF-7 cells were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 2% TCH and 0.5% fetal bovine serum (Hyclone, Logan, UT). Normal HMECs (Cambrex, East Rutherford, NJ) were cultured in MEGM BulletKit. In all of the cases, cells were grown in medium lacking phenol red at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were grown in T-75 flasks before transferred to 12-well plates (Corning, Corning, NY) in preparation for transfection.

**Western Blot Analysis.** Cells were collected by trypsinization and centrifugation and suspended in 300  $\mu$ l lysis buffer on ice for 10 min. They were spun at 13,500 rpm for 15 min at 4°C. Supernatants were transferred to a new tube, and protein concentration was determined by BCA assay (Pierce, Rockford, IL). One hundred twenty  $\mu$ g protein with loading buffer were heated to 95°C for 5 min and then electrophoresed on 10% SDS-PAGE gel with protein size standard (Bio-Rad, Hercules, CA). Protein was transferred to 0.45  $\mu$ m nitrocellulose membrane (Bio-Rad, Ready Gel Blotting Sandwiches), followed by blocking in 5% nonfat milk at room temperature for 1 h. After blocking, the membrane was incubated with anti-PPAR $\gamma$  (1:200 dilution, sc-7196; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After washing the membrane at room temperature three times, 5 min each, the membrane was incubated with secondary horseradish peroxidase-conjugated antibodies (1:2,000 dilution, sc-2004; Santa Cruz Biotechnology) at room temperature for 1 h. Detection was carried out by enhanced chemiluminescence (Supersignal; Pierce) for 5 min at room temperature and quantitated using ChemImage 400

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imaging system (Pittsburgh, PA). To assess loading,  $\beta$ -actin was used as internal control.

**Plasmids for Analysis of Promoter Fragment Activity.** pRL-TK vector (Promega, Madison, WI) was used as internal control reporter in all of the transient transfection assays. pGL3-pA13kb (pGL3- $\gamma$ 1 p3kb), pGL3-pB (pGL3- $\gamma$ 2 p1kb), and pGL3-pA2 (pGL3- $\gamma$ 3 p800bp) are expression mammalian vectors that have 3 kb, 1 kb, and 800 bp from the 5'-flanking region of exon A1, exon B, and exon A2, respectively, and were a gift from Dr. Johan Auwerx (Louis Pasteur, Illkirch, France). Construction of the pA3-luciferase reporter was made from a BAC clone, RP11-335i9/pBACe3.6, purchased from BACPAC Resources Center (Children's Hospital Oakland Research Institute, Oakland, CA). GenBank accession no. for RP11-335i9 is AC091492, which included a partial segment of the PPAR $\gamma$  gene. The 3-kb fragment flanking the 5' end of A3 was cloned by PCR. The primers used for the amplification are forward primer, 5'-CTCTGCAAAGAGAGCCAG-3'; and reverse primer, 5'-CAGAGGACGAACCAGAGAGC-3'. PCR conditions were 95°C, denature for 3 min and then for 1 min at 95°C, 1 min at 53°C, and 3 min at 72°C for 30 cycles, followed by 72°C for 10 min for the elongation.

**Transient Transfection Analysis.** Cells were transiently transfected with 2  $\mu$ g of a luciferase reporter vector and 0.5  $\mu$ g pRL-TK (Promega) per plate using ESCORT (Sigma, St. Louis, MO). After 18–24 h, cells were lysed in 100  $\mu$ l passive lysis buffer and treated according to manufacturer's instructions (Promega). Luminometry was performed on a Berthold Technologies Lumat (LB 9507; Bad Wildbad, Germany), and data were calculated as raw luciferase units divided by raw renilla units (RLUs). When data are presented as a single, typical experiment, this is as the mean fold induction. These values were obtained by dividing the RLU data from each treatment well by the mean of the control values ( $\pm$ SE) as shown.

**5'-Rapid Amplification of cDNA End Assay.** The 5'-rapid amplification of cDNA end (RACE) assay was performed per manufacturer's instructions (GeneRacer; Invitrogen, Carlsbad, CA). RNA isolation was made using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was dissolved in diethyl pyrocarbonate-treated water before using the GeneRacer Kit. The human PPAR $\gamma$  gene (accession no. L40904)-specific primers (GSPs) used with the GeneRacer Kit are GSP, 5'-GGCTCTTCATGAGGCTTATTGTAGAGC-3' (451–477); and GSP-nested, 5'-AGGCTCCACTTTGATTGCAC-3' (398–418). All of the clones were verified by sequence analysis.

**Affymetrix Microarray Analysis.** Total RNA was recovered from cells following 3 h of treatment using the Qiagen RNeasy. The University of Kentucky Microarray Core Facility verified the total yield by gel electrophoresis and processed the total RNA samples to produce labeled cDNA samples. The cDNA samples then were hybridized to the Affymetrix (Santa Clara, CA) HG-U133A GeneChip and stained via an Affymetrix Fluidics Station 400. Finally, the facility collected the resultant microarray data using an Affymetrix GeneArray Scanner and a computer workstation running Affymetrix Microarray Suite (MAS 5.0). The raw data from each GeneChip scan were saved and exported into Microsoft Excel spreadsheets (Redmond, WA). Raw signal intensity data collected were aggregated into a single Excel spreadsheet using the probe set IDs, signal intensity values, and signal detection flag for each sample and probe set description. Mean signal intensity values ( $n = 3$  per cell type) were reported along with SE as an appropriate estimation of error.

**Real-Time Quantitative PCR.** A one-step real-time reverse transcription-PCR technique was used to determine relative expression levels of PPAR $\gamma$  mRNA using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). For analyses from cell culture, the RNeasy Mini Kit (Qiagen) was used to isolate the total RNA. The primers and probe kit for PPAR $\gamma$  were purchased from Applied Biosystems labeled with FAM reporter fluorescent dye, assay-on-demand number Hs00234592\_m1. For the internal control, 18S, a primer and probe kit also was used, assay-on-demand number Hs99999901\_s1. A one-step reaction mixture provided in the TaqMan one-step reverse transcription-PCR Master Mix Reagents kit (Applied Biosystems) was used for all of the amplifications.

Cycle parameters for the one-step reverse transcription-PCR included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. The housekeeping gene 18S was used for internal normalization.

Analysis methods as outlined in the ABI Prism 7700 Sequence Detection System User Bulletin 2 (October 2001) were performed using the relative  $C_t$  method. Briefly, this method uses the expression  $2^{-\Delta\Delta C_t}$  to estimate the

relative expression based on a calibrated sample,  $\Delta\Delta C_t = \Delta C_{t,x} - \Delta C_{t,calibrator}$ , and the gene target of interest normalized to the expression of an endogenous housekeeping gene like 18S,  $\Delta C_t = C_{t,PPAR\gamma} - C_{t,18S}$ . The mean ( $n = 4$  per cell line) values were reported along with the SE of the  $\Delta C_t$  because an appropriate estimation of error was calculated from the SDs of the  $C_t$  values for PPAR $\gamma$  and 18S through the formula  $SE = s/n$  where the SD,  $s = \sqrt{[(s_{C_t,PPAR\gamma})^2 + (s_{C_t,18S})^2]}$ .

## RESULTS AND DISCUSSION

**Selective Expression of PPAR $\gamma$  in HMECs and Breast Cancer Cells.** Transient transfection analysis indicated that MCF-7 and MDA-MB-231 breast cancer cells are more sensitive to PPAR $\gamma$  transactivation than are normal mammary epithelial cells. To determine whether this is because of the increased expression of the receptor in breast cancer cells, Western blot analysis has been performed (Fig. 1). Densitometric analysis indicates that both breast cancer cell lines express significantly higher levels of PPAR $\gamma$  than do HMECs. Furthermore, no effects of culture conditions were seen on expression when HMEC, MCF-7, and MDA-MB-231 cells were transfected with a PPAR-response element-reporter construct and cultured in each of the three media for 24 h (data not shown).

The gene encoding PPAR $\gamma$  can be translated into two proteins, which differ by the addition of 28 N-terminal amino acids on  $\gamma$ 2 compared with  $\gamma$ 1. This is accomplished through the use of distinct transcriptional start sites as seen in the mRNA structures (Fig. 2). Previous work indicated that a promoter associated with exon A1 initiates transcription of PPAR $\gamma$ 1. In mice and humans, mRNA initiated from exon A1 also contained exon A2 upstream of the first coding exon I (20, 21). In the case of the  $\gamma$ 2 protein, a distinct promoter is used that is associated with the coding exon B (20, 21). It more recently has been reported that promoters associated with exons A2 and I can mediate transcription and have been termed  $\gamma$ 3 and  $\gamma$ 4, respectively (23, 26). Because  $\gamma$ 1,  $\gamma$ 3, and  $\gamma$ 4 code for the  $\gamma$ 1 protein, merging the gene and protein structure nomenclature has created considerable confusion. Therefore, we propose to revise the nomenclature to distinguish between transcriptional start sites of the gene that is independent of the protein that the mRNA would encode. Thus, we propose that the  $\gamma$ 1,  $\gamma$ 3, and  $\gamma$ 4 promoters be called pA1, pA2, and pI, respectively, whereas the  $\gamma$ 2 promoter would be called pB. This will facilitate the discussion of additional promoters as described below.

**Identification of the Gene Structure and Promoter Usage of PPAR $\gamma$ .** To begin to determine the relative promoter usage and to identify additional potential transcriptional start sites, we have performed 5'-RACE on RNA isolated from HMECs and MCF-7 and MDA-MB-231 breast cancer cells (Fig. 2). These data show that three previously unreported exons, termed A3, A4, and A5, were detected.

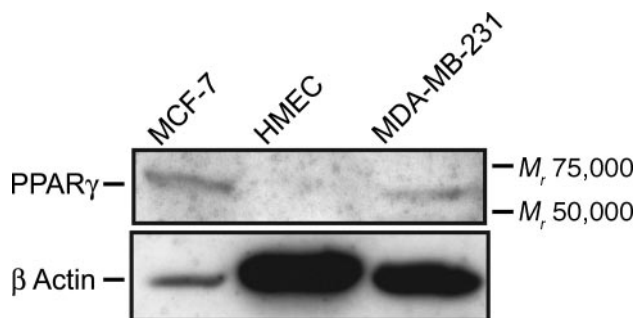
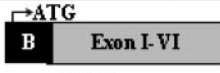
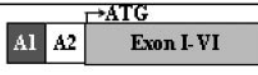
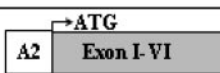
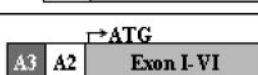
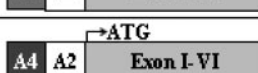
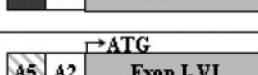
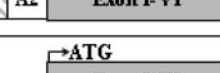


Fig. 1. Western blot analysis of PPAR $\gamma$  expression in MCF-7, MDA-MB-231, and HMEC. Proteins were extracted from untreated cells and subjected to SDS-PAGE. The  $M_r$  55,000 upper band is PPAR $\gamma$ , whereas the lower band is  $\beta$ -actin, which also was blotted as a loading control.

Fig. 2. PPAR $\gamma$  cDNA structures isolated from normal human epithelia and breast cancer cells. mRNA structures were determined by 5'-RACE analysis and mapped to the public genomic database. Translational start sites containing the ATG initiation codons are located in exons B and I. In breast cancer cells or normal epithelia, all of the mRNA would encode the PPAR $\gamma$ 1 protein because no transcripts containing exon B have been observed in these tissues. The detailed promoter structure depicts both exonic sizes in brackets under the exon name and intronic sizes below the genomic structure. The proposed nomenclature for each transcript is listed under *Promoter*.

Promoter	mRNA Exon usage	Protein	Expression
pB		PPAR $\gamma$ 2	Adipocytes
pA1		PPAR $\gamma$ 1	MCF-7 and MDA-MB-231
pA2		PPAR $\gamma$ 1	HepG2 cells and Human monocytes
pA3		PPAR $\gamma$ 1	Normal mammary epithelia
pA4		PPAR $\gamma$ 1	MCF-7, MDA-MB-231 and Rhesus adipocytes
pA5		PPAR $\gamma$ 1	MCF-7 and MDA-MB-231
pI		PPAR $\gamma$ 1	Adipocytes, HepG2 and Caco-2 cells

In each case, if promotion were mediated from any of these, designated pA3, pA4, or pA5, the resulting mRNAs would contain exon A2 sliced onto the common acceptor site upstream of the first coding exon I. Therefore, the use of promoters associated with any of these exons would result in the expression of PPAR $\gamma$ 1. In normal epithelia, all full-length 5'-RACE clones contained A3 spliced onto A2 and exon I. None of the normal epithelial clones contained any other first exon. By contrast, 5'-RACE clones from MCF-7 and MDA-MB-231 indicate that pA1, pA3, pA4, and pA5 are used to drive the expression of PPAR $\gamma$ 1 in breast cancer cells. In no instance has exon B appeared on any clone nor have we been able to amplify an mRNA containing

exon B, indicating that PPAR $\gamma$ 2 is not expressed in normal mammary epithelia or in breast cancer cells. RNase protection assays from MCF-7 and MDA-MB-231 cells indicate that >98% of PPAR $\gamma$ 1 mRNA contains exon A1 (data not shown) and therefore is driven by pA1.

The genomic location of coding and noncoding exons has been mapped, and their relative locations on the human genome are shown in Fig. 3. Although there is a high degree of homology between the human and mouse PPAR $\gamma$  genomic structures, the recently reported structure of the monkey *PPAR $\gamma$*  gene appears to differ significantly (24). Furthermore, the first exon reported by Greene *et al.* (22), which

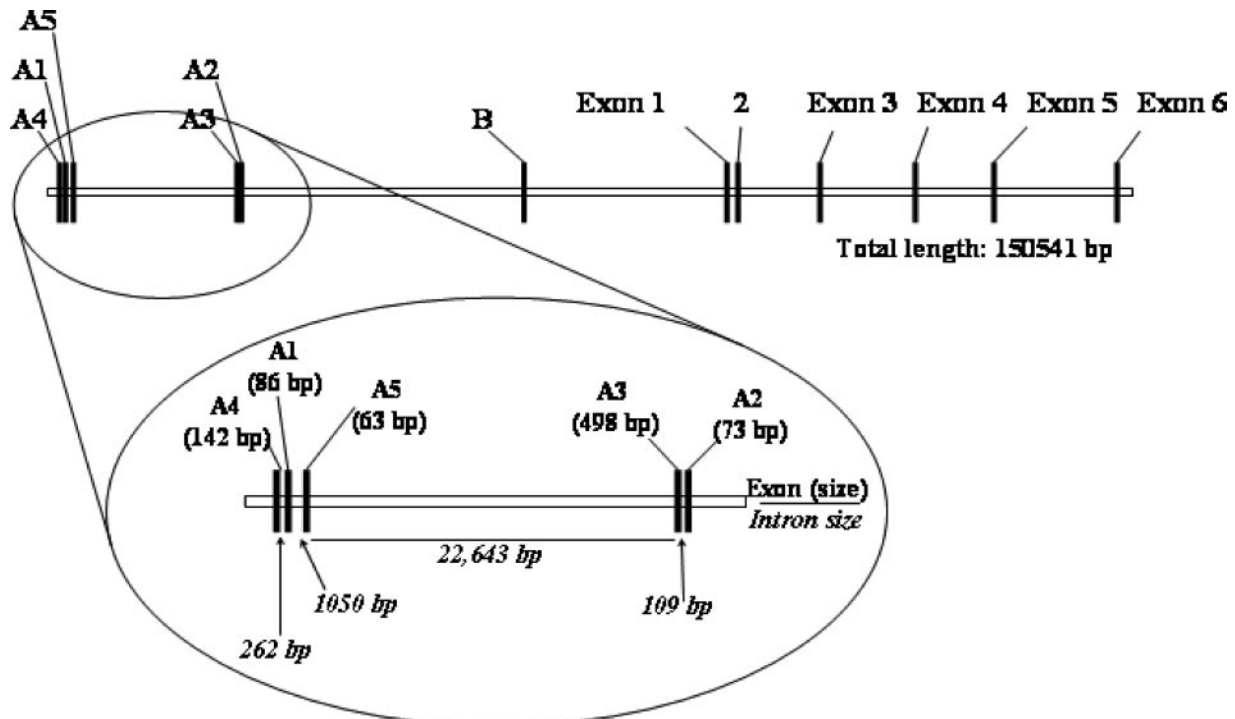


Fig. 3. The genomic structure of human and mouse PPAR $\gamma$ . The human *PPAR $\gamma$*  gene contains seven coding exons (exons B and I-VI). Noncoding exons A1 and A2 have been observed in the mouse and human, and their location relative to the coding exons is highly conserved. Unique to humans are the noncoding exons A3, A4, and A5. In the expanded map of the promoter, exon sizes are shown next to the exon name, and intronic sizes are shown below the gene structure.



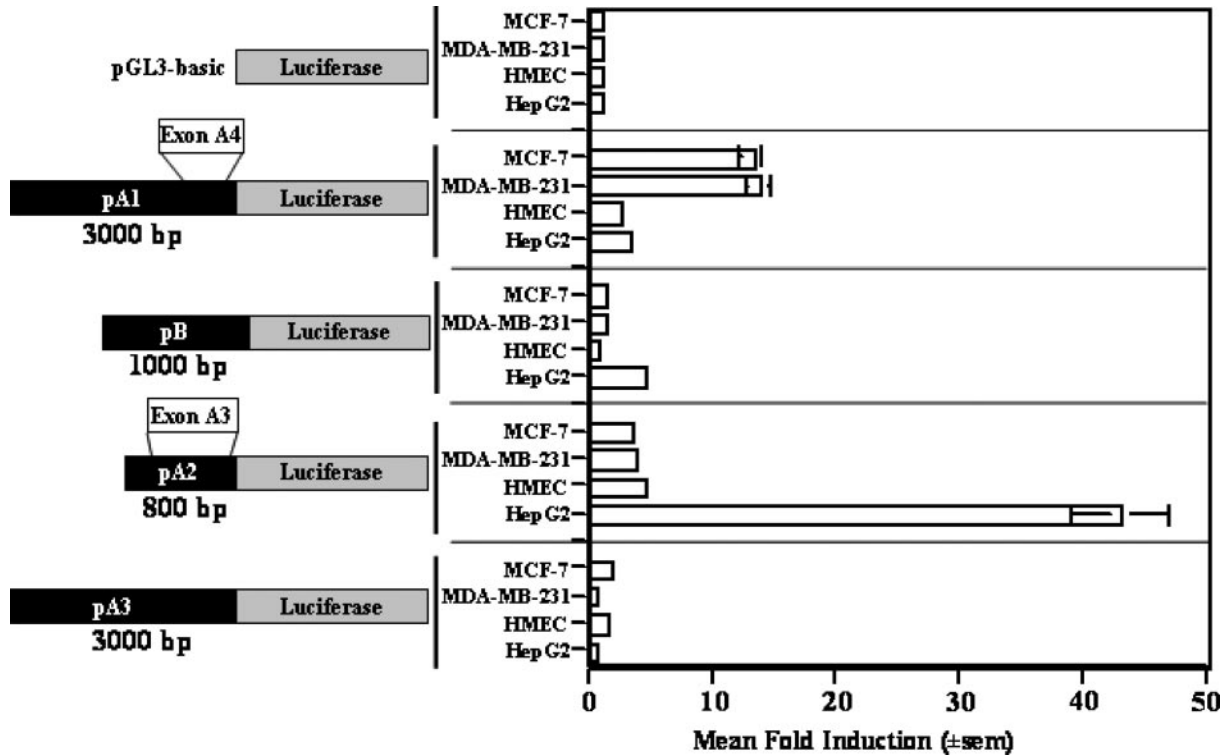


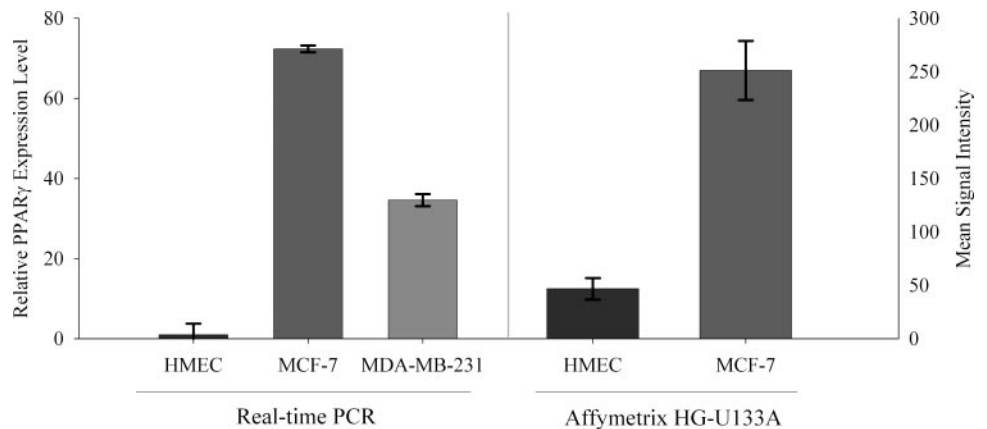
Fig. 4. Transient transfection analysis of PPAR $\gamma$ 1 promoter usage. Genomic sequences flanking the transcriptional initiation site of exons A1, B, A2, and A3 were cloned onto the luciferase reporter gene as described. Reporter expression was assessed in two breast cancer cell lines, MCF-7 and MDA-MB-231, normal mammary epithelial cells, and in the hepatocarcinoma line HepG2. Embedded within pA1 is exon A4, and exon A3 is within the 800 bp upstream of A2 as shown in Fig. 3.

was cloned from human hematopoietic stem cells (GenBank L40904), is distinct from those reported here and appears to be spliced onto a cryptic acceptor site within exon A1. Also in contrast to this report, L40904 does not contain exon A2 but is spliced directly onto exon I (22). Our efforts to map the first exon of L40904 to the human genome have been unsuccessful. The gene encoding PPAR $\gamma$  is complex, spans >150 kb, and has as many as eight distinct first exons, depending on the transcriptional start site.

**Distinct Promoters Mediate Expression of PPAR $\gamma$  in HMECs and Breast Cancer Cells.** To examine the mechanism of differential promoter usage and to confirm the RNase protection data indicating that the primary promoter in breast cancer cell lines is mediated by pA1, we fused the 5' flanking sequences of exons A1, A2, A3, and B to a luciferase reporter plasmid. These have been used in transient transfection assays in MCF-7, MDA-MB-231, HMECs, and in the human hepatocarcinoma cell HepG2 (Fig. 4). Because of the close proximity of A3 to A2, separated by a 109-bp intron, A3 is

completely contained within the 800-bp pA2 tested here. Likewise, because A1 and A4 are separated by a 262-bp intron, A4 is contained within the 3000 bp of pA1 tested here. In all of the cases, data are normalized to the constitutive renilla reporter and compared back with the basic luciferase vector lacking the genomic promoter elements. These data indicate that pA1 is able to drive reporter activity in breast cancer cells more effectively than in normal epithelia. This agrees with the RACE data, in which no A1 promotion could be seen in normal epithelia. However, reporter activity in normal epithelia is higher in the pA1 than in the reporter lacking pA1 (basic), suggesting that normal epithelia may have the necessary transcription factors to mediate expression from this promoter in a plasmid form but not in the native chromatin structure. Portions of pA1 are GC rich, suggesting that methylation could be playing a role in promoter usage that would be distinct between a plasmid reporter and the transcriptional regulation of an endogenous gene. Although exon A4 was seen on one RACE clone, it was almost undetectable on RNase protection assays

Fig. 5. Relative expression of PPAR $\gamma$ 1. The expression of PPAR $\gamma$  as measured by real-time PCR is markedly higher in both breast cancer cell lines than in normal epithelia. These relative expression levels were confirmed by the differences in mean signal intensity seen on the Affymetrix chips.



and was present on <1% of the mRNA from MCF-7 cells. The functional significance of A4 within pA1 is not clear. Transient transfection with pB confirms our 5'-RACE data, indicating that this promoter is not used in HMEC or the breast cancer cell lines examined, commensurate with the data in Fig. 1 showing no PPAR $\gamma$ 2 expression in these cells. However, this promoter is active in HepG2 cells. The pA2 also is activated in normal and breast cancer lines and appears to be the major regulator of PPAR $\gamma$  in HepG2 cells as reported previously (23). Within the pA2 is a 107-bp intron, the entire 498-bp exon A3, and an additional 200 bp flanking the 5'-start site of exon A3. The structural significance of these in regulating expression is not yet clear. The 3000 bp flanking exon A3, pA3, are less effective in mediating reporter activity in normal epithelia and in MCF-7 and are completely ineffective in MDA-MB-231 or HepG2 cells. Because RACE data suggest that the majority, if not all, transcription in normal epithelia is initiated at exon A1, it could be that this promoter does not lie within the 3000 bp flanking the start site or that additional repressor elements also lie upstream.

**The Increase in PPAR $\gamma$  Expression Seen in Breast Cancer Cells Is Mediated by an Increase in Transcriptional Regulation.** It previously has been reported that PPAR $\gamma$  protein levels, measured by Western blot analysis or by immunohistochemistry, are higher in breast tumor samples than in the surrounding normal tissue (5). Here we show that the increased expression of PPAR $\gamma$ 1 protein seen in breast cancer cells compared with normal epithelial cells (Fig. 1) also is seen at the level of mRNA. This is shown by real-time PCR and from microarray data (Fig. 5). There also appears to be a progressive increase in expression during tumor progression in breast cancer patients. Quantitation of PPAR $\gamma$  showed a much higher level of expression in the tumors from patients relapsed with breast cancer metastatic to the lung than from primary tumors from the same patient (25). The changes in promoter usage described here could underlie the mechanism of the changes in expression levels seen during the course of breast cancer progression. Whether the increase in expression is the cause or consequence of tumor progression remains to be established, and work in our laboratory is focusing on examining these competing hypotheses. Clearly, understanding the molecular mechanisms of these changes could provide new insights in the function of PPAR $\gamma$  in these cells and could provide new insights and targets for managing, detecting, or preventing breast cancer.

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