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Involvement of DNA Methylation in the Control of the Expression of an Estrogen-Induced Breast-Cancer-Associated Protein (pS2) in Human Breast Cancers

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Abstract *pS2* gene has been used to investigate the relationship between alterations of DNA methylation patterns in human tumors and gene expression. The expression of *pS2,* which is transcriptionally controlled by estrogens in breast cancer cell lines, is restricted to estrogen-receptor-rich human breast tumors. We found that the CCGG site within the promoter/enhancer sequence of *pS2* was hypomethylated in estrogen-receptor-rich breast tumors expressing this gene. The amount of DNA molecules unmethylated at this site was related to the amount of *pS2* mRNA detected in the samples. The demethylation of this region, which contains the estrogen responsive element, was confirmed by genomic sequencing. Transient expression of functional human estrogen receptors stimulated the expression of the endogenous *pS2* in HeLa cells, but failed, in BT-20 cells, to stimulate expression of this gene. Since the promoter/enhancer region of *pS2* is unmethylated in HeLa cells and methylated in BT-20 cells, these data also support the hypothesis that DNA methylation might be involved in the control of *pS2* expression. J. Cell. Biochem. 65:95–106. © 1997 Wiley-Liss, Inc.

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In mammalians, the methylation of DNA is accomplished by enzymes which catalyze the transfer of methyl groups from S-adenosylmethionine to the carbon-5 of deoxycytidine at CpG sites [Adams, 1990]. This epigenetic modification seems to have an essential role in normal mammalian development since it has been shown that targeted mutation of the DNA methyltransferase gene results in embryonic lethality [Li et al., 1992]. DNA methylation has been implicated in several cellular processes, including chromosome instability, hot spots for somatic mutations, and genomic imprinting, and in the last 15 years evidence of an involvement of DNA methylation in control of gene expression has been accumulated [for review see Adams, 1990; Razin and Cedar, 1991]. Numerous studies have correlated, in vitro and in vivo, the hypomethylation of the 5′ region of viral or cellular genes [Doerfler, 1983] with their expression, and several experiments have shown that methylation can repress gene transcription [Bird, 1992]. Although global genomic and gene-specific hypomethylation are generally observed in tumor tissues [Gama-Sosa et al., 1983; Feinberg and Vogelstein, 1983; Ribieras et al., 1994], simultaneous occurrence of CpG islands hypermethylation also has been reported in several cell lines and human tumors [for review see Laird and Jaenisch, 1994]. Taken together, these findings suggest that alterations of DNA meth-

Abbreviations used: bp, base pair; CAT, chloramphenicol acetyl transferase; E_2, 17β-estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase pair; RT-PCR, reverse transcription–polymerase chain reaction; SV 40, simian virus 40; WCE, whole cell extract.

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ylation may play a variety of roles in carcinogenesis [Counts and Goodman, 1995] and that some specific alterations of DNA methylation patterns might be involved in the expression of tumor-specific genes.

In order to gain further insights into this question, we analyzed the DNA methylation patterns of the estrogen-induced breast-cancer-associated protein (*pS2*) gene in normal and tumoral human breast tissues. This gene was chosen for two reasons: *pS2* expression is restricted to subclasses of human breast cancers [Rio et al., 1987], and the promoter/enhancer sequence is not associated with a CpG island. This model therefore offers the possibility of investigating the relationship between gene activation and hypomethylation in human cancers.

pS2 is not transcribed in normal breast tissue and is predominantly expressed in estrogen-receptor (ER)-positive breast carcinomas [Rio et al., 1987]. In the MCF-7 mammary cell line, *pS2* expression is specifically controlled at the transcriptional level by estradiol [Masiakowski et al., 1982], and in human mammary tumors its expression is indicative of a favorable response to hormonotherapy [Schwartz et al., 1991]. However, in some other carcinomas and benign tumors the presence of *pS2* transcripts or proteins is not correlated with the presence of estrogen receptors [Dante et al., 1994; Rio et al., 1988; Welter et al., 1992; Wysocki et al., 1990]. This gene is physiologically expressed in stomach and in regenerative tissues surrounding ulcerous lesions of the human gastrointestinal tract. It has been recently suggested that pS2 protein might be important in stimulating gastrointestinal repair [Playford et al., 1996], since transgenic mice overexpressing the human pS2 protein have an increased resistance to intestinal damage. In addition, disruption of the *pS2* gene by homologous recombination indicates that its expression is essential for normal differentiation of the antral and pyloric gastric mucosa and that it may function as a gastric-specific tumor suppressor gene [Lefebvre et al., 1996].

In fact, the 5′ flanking region of *pS2* contains a complex promoter/enhancer region (from position −428 to position −332) responsive to estrogens, EGF, the phorbol ester tumor promoter, c-Ha-*ras* oncoprotein, and c-*jun* protein [Nunez et al., 1989]. The estrogen responsive element (ERE) has been characterized and localized in the 5′ flanking sequences from position −405 to position −393 [Berry et al., 1989]. In contrast to the other known ERE, this ERE is an imperfect palindromic sequence of 13 bp (GGTCACGGTG-*G*CC). Although several in vitro [Berry et al., 1989; Nunez et al., 1989] and in vivo [Rio et al., 1987] studies indicate that *pS2* is, in breast tumoral tissues, controlled by estrogen receptors, additional factors seem to be involved in the control of its expression [Dante et al., 1994; Rio et al., 1987; Zajchowski and Sager, 1991].

DNA methylation patterns of *pS2* were analyzed by Southern blot and genomic sequencing experiments in normal and tumoral breast tissues, normal endometrium, and stomach. The effect of DNA methylation on the inducibility of *pS2* expression was investigated, in cell lines, by transient expression of the human estrogen receptors.

MATERIALS AND METHODS

Specimens and Cell Lines

A total number of two normal breast tissue samples, 29 breast primary adenocarcinomas, two normal total stomach tissue samples, and five normal endometrium tissue samples were analyzed. After excision, a portion of the tissue was snap-frozen and subsequently stored in liquid nitrogen. For both normal and pathological samples, the remainder of the tissue was examined at the pathology laboratory. Specimens were fixed in formalin, paraffin-embedded, sectioned, and stained with hemalun-eosin-safran. HeLa, MCF-7, and BT-20 cell lines were grown as described [Nunez et al., 1989]. For all breast tumors, estrogen receptor status was determined by ligand binding assays. Samples containing less than 10 fmol of ER per milligram of total protein were considered to be ER-negative.

RT-PCR Assay

Total RNAs were extracted from cell lines and tissue samples and simultaneous amplification of *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) and *pS2* transcripts were performed as previously described [Dante et al., 1994]. The RT-PCR products were analysed on 2% agarose gel, and the ratio between *pS2* and *GAPDH* signals was determined. Detection of estrogen receptor RNA by RT-PCR was performed as described elsewhere [Fuqua et al., 1990] but using GAPDH primers as internal control instead of β-actin primers.

Southern Blot Analysis

High molecular weight DNA was extracted from cell lines and frozen tissues by standard procedures [Schwartz et al., 1991]. DNA were cleaved with *Taq*I, and methylation status at CCGG sites was determined by the isoschizomeric restriction enzymes *Msp*I and *Hpa*II, as previously described [Ribieras et al., 1994]. All filters were hybridized with a probe which reveals a *Taq*I fragment without CCGG sites of the 3′ region of the human G-γ globin gene [Ribieras et al., 1994], in order to control the loading on each lane, and with a probe which reveals the unmethylated CCGG sites of the exon 1 and the 5′ sequence of the human catalase gene [Ribieras et al., 1994], to ensure that partial cleavages observed for the *pS2* are not due to an artefactual inhibition of *Hpa*II. Hybridization probes 1 and 2 were purified from genomic cloned DNA fragments pS2(429/86) pG1 [Berry et al., 1989] and pS2-pBR322 [Jeltsch et al., 1987]. Probe 1 is from nucleotides −428 to −97, and probe 2 is from nucleotides −87 to +325, according to the published sequence [Jeltsch et al., 1987].

Genomic Sequencing Analysis of 5-Methyl dCyd

Determination of the methylation status of dCyd was performed essentially as previously described [Martin et al., 1995]. Briefly, alkali-denatured DNA was incubated in 3 M $NaHSO_3$ and 5 mM hydroquinone for 16 h at 50°C. This chemical treatment converts all cytosines to uracil, while the methylated cytosines remain unmodified. Aliquots were used for the amplification of the region of interest using strand specific primers and a two-step PCR method [Martin et al., 1995]. PCR products were then cloned in pT7blue-T Vector (Novagen, Madison, WI). After screening, plasmids were sequenced by the dideoxy method. The frequency of cytosine methylation in the samples analyzed was calculated for each CpG by the number of clones methylated at this site divided by the number of clones analyzed.

Computer Analysis

Multiple components analysis was performed using the ADE program library [Thioulouse et al., 1994].

Transfections and CAT Assays

Expression vectors used in these experiments were pS2-CAT [Kumar et al., 1987], an expression vector containing the 5′ flanking sequences of *pS2* (position ∼−1100 to +10), HEO, an expression vector encoding the human estrogen receptor [Kumar and Chambon, 1988], HEGO, an expression vector encoding the wild-type human estrogen receptor [Tora et al., 1989], and pU-lacZ, an expression vector containing the bacterial β-galactosidase gene driven by the SV40 promoter.

Plasmids were introduced into HeLa and BT-20 cells by the calcium phosphate precipitation method [Sambrook et al., 1989]. Twenty-four hours before transfection, cells were seeded (2.5×10^5/6 cm diameter or 1.5×10^6/10 cm diameter dishes) in a medium containing 10 nM of 17β-estradiol (E_2). Cell extracts were prepared 48 h after transfection.

For the determination of pS2-CAT activity, cells grown in 6 cm diameter dishes were cotransfected with 5 μg of pS2-CAT plasmid, 2 μg of vector encoding the estrogen receptor HEO, and 2 μg of control vector encoding the bacterial β-galactosidase gene (pU-LacZ) as internal standard. The CAT enzyme activity was determined from cellular extracts, and nonacetylated and acetylated [^{14}C] chloramphenicol products were analyzed by thin layer chromatography followed by autoradiography [Sambrook et al., 1989].

For gel retardation experiments, whole cell extracts (WCE) were prepared from 10 cm diameter HeLa cell dishes transfected with an expression vector encoding the human estrogen receptor HEGO and with pU-LacZ plasmid DNA as an internal control.

Immunohistology

One day after transfection, HeLa cells were seeded into eight chamber tissue culture slides (Lab-Tek; Nunc, Naperville, IL) at a density of 2×10^4 cells/chamber and grown for 2 days in fresh medium supplemented with E_2 (10 nM). After washing, cells were fixed for 10 min with paraformaldehyde (4% in phosphate buffered saline [PBS]) and permeabilized for 5 min with Triton X-100 (0.02% in PBS). After an abundant rinsing with PBS, fixed cells were incubated for 1 h with one 1/50 dilution of pS2 monoclonal antibody p2802 [Rio et al., 1988] in fetal calf serum 10% PBS. Then cells were rinsed with fetal calf serum 10% PBS and incubated for 30 min in the dark with a 1/100 dilution of anti-mouse rhodamine–conjugated secondary antibodies (Immunotech, France). After a final rinsing with PBS, slides were examined using an

epifluorescent microscope. Controls were run in parallel.

RESULTS

The methylation status of CCGG sites of the 5′ region of *pS2* was investigated on Southern blots using the pair of the isoschizomeric restriction endonucleases, *Hpa*II and *Msp*I. *Msp*I yielded fragments resulting from cleavage of all CCGG sites, whereas *Hpa*II cleaved only CCGG sites with unmethylated internal cytosine. In order to precisely map these sites, we also cleaved all DNAs with *Taq*I, and filters were successively hybridized with several probes. Within the *pS2* region analyzed, which included the 5′ flanking sequences, exon 1, and part of intron 1, *Taq*I and *Msp*I digestion did not show any polymorphism. In contrast, *Hpa*II polymorphisms were observed in practically all samples analyzed.

Correlation Between the Methylation Status of CCGG Sites of the 5′ Region of *pS2* and Its Expression in Human Breast Cell Lines

The methylation status of CCGG sites of *pS2* was first investigated in two human breast cancer cell lines: the MCF-7 cell line, which expresses *pS2* and contains estrogen receptors, and the BT-20 cell line, which is an estrogen-receptor-negative and *pS2*-negative cell line [Rio et al., 1987].

Hybridization with probes 1 and 2 (Fig. 1C), which map the 3 CCGG sites at positions −354 (M1), −84 (M2), and −20 (M3), respectively, revealed that these three sites were unmethylated in DNA extracted from MCF-7 cells, since *Msp*I patterns were identical to *Hpa*II patterns (Fig. 1A,B; *Msp*I digest (lanes 2) compared to *Hpa*II digest [lanes 3]). In DNAs extracted from BT-20 cells, the Taq1-Taq2 1.06 kb (Fig. 1A, lane 4) band, which contains the M1, M2, and M3 sites, was only partially cleaved to shorter fragments by *Hpa*II; a consistent part of the DNA molecules was unmethylated at M2 sites (visualized with probe 1 by the 0.65 kb band in Fig. 1A, lane 4) and at M3 sites, as indicated by the 0.71 kb band in Fig. 1A (lane 4) and the 0.34 and 0.4 kb bands in Fig. 1B (lane 4). However, M1 sites were found to be heavily methylated in these cells, since the 0.27 and 0.38 kb bands corresponding to unmethylated M1 sites were not detected (Fig. 1A, lane 4).

In intron I, the CCGG sites were methylated in BT-20 cell lines, and in MCF-7 the M5 site was partially methylated (data not shown). Taken together, these results suggest that the methylated status of *pS2* is associated with its expression. In addition, the analysis of the 3 CCGG sites M1, M2, and M3 within the 5′ regulatory sequences further suggests that the methylation status of M1 sites might be correlated with *pS2* expression. This hypothesis was further investigated in human breast tissues.

Correlation Between the Methylation Status of CCGG Sites of the 5′ Region of *pS2* and a Reduced Level of Expression in Human Breast Cancers

The amount of *pS2* mRNA was evaluated by RT-PCR assays as previously described [Dante et al., 1994]. Immunohistochemical studies have shown that the percentage of pS2-positive tumor cells varied greatly between tumors [Henry et al., 1991; Rio et al., 1987], and the percentage of 3–5% of pS2-positive tumor cells has been commonly adopted as the threshold for pS2-positivity [Henry et al., 1991]. In order to evaluate the sensitivity of the RT-PCR method used, pS2 and GAPDH transcripts were amplified from samples containing RNA extracted from the pS2-positive MCF-7 cells and from the pS2-negative BT-20 cells mixed in various proportions. In the conditions used in these experiments, pS2 transcripts were not detected in samples containing less than 5% of MCF-7 RNA, and the pS2/GAPDH ratios were dependent on the amount of MCF-7 RNA. From these data and the analysis of a few samples in which pS2 mRNA were quantitated by Northern blot experiments [Dante et al., 1994], samples were classified on a three point scale: negative sample (0), pS2/GAPDH ratio lower than 0.2; low level of expression (1+), 0.2 ≤ pS2/GAPDH ratio ≤0.3 (≥5% to ≤25% of pS2-positive RNA); and strong expressing samples (2+), pS2/GAPDH ratio >0.3.

As expected from the data obtained by the Southern blot analysis of cell lines, the CCGG sites (M1, M2, and M3) located at the 5′ end of *pS2* were hypomethylated in DNAs extracted from breast tumors expressing this gene at a high level (7/7 cases of pS2 2+ analyzed; three representative samples are shown in Fig. 1A, B, lanes 7–9). In DNAs extracted from ER-rich tumors (>10 fmol of ER per milligram of total protein) exhibiting a low level of *pS2* expression (1+), these sites were also hypomethylated but to a lesser extent (two representative samples

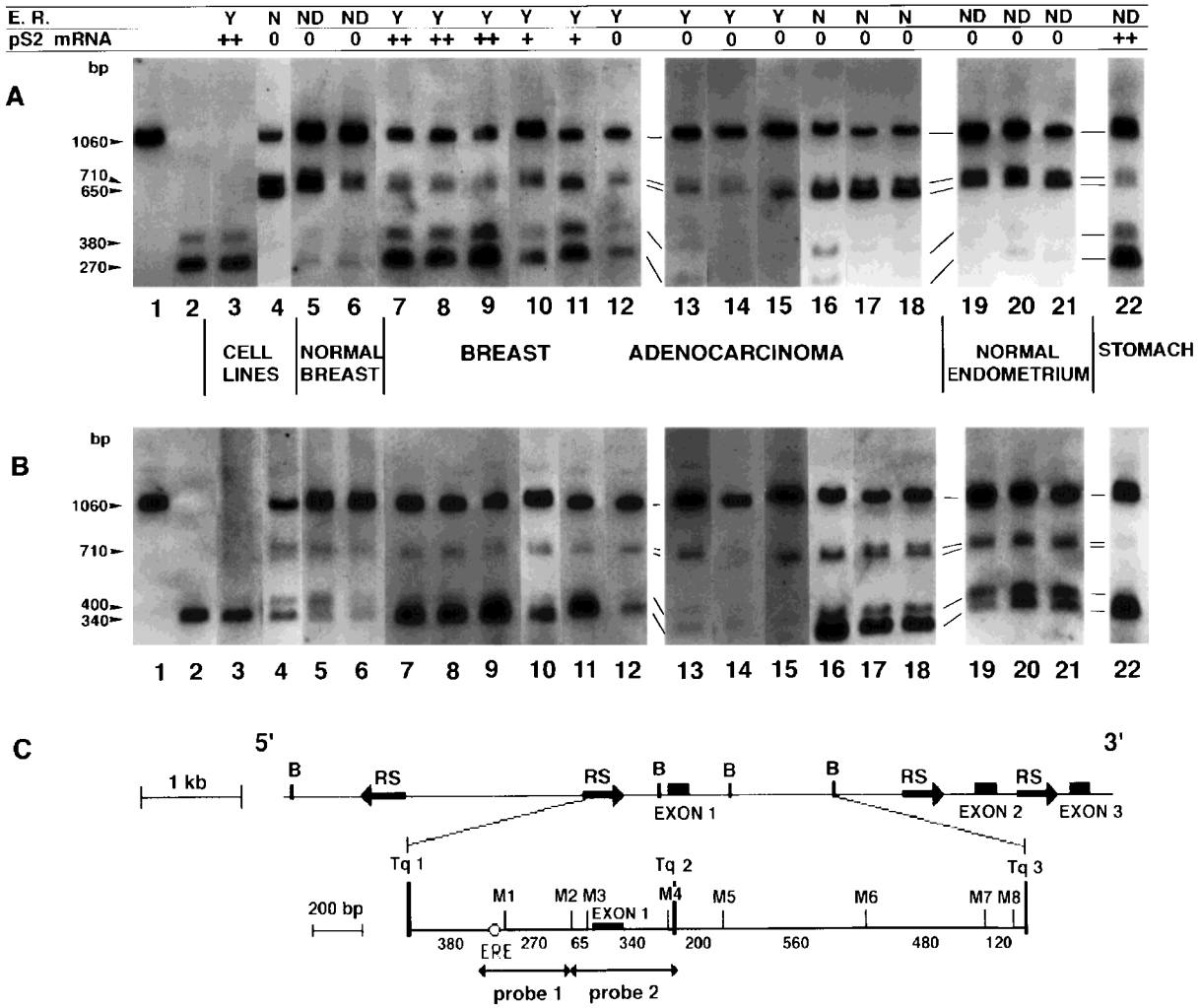

Fig. 1. DNA methylation patterns of the 5′ region of *pS2* in cell lines and human tissues. Southern blot analysis; DNA methylation patterns shown are representative of those observed in normal and tumoral tissues studied. Detection of estrogen receptors (E. R.) in each sample is indicated by Y (yes), N (no), or ND (not determined), and the amount of pS2 mRNA by 0 (pS2-negative samples), + (low level of expression), and ++ (strong expressing samples). DNA were cleaved with *Taq*I (*lanes 1*), *Taq*I-*Msp*I (*lanes 2*), and *Taq*I-*Hpa*II (*lanes 3–22*). **A:** Hybridization of DNA digests with probe 1 (see C). DNA were cleaved with *Taq*I (lane 1), *Taq*I-*Msp*I (lane 2), and *Taq*I-*Hpa*II (lane 3–22). Human breast cancer cell lines (lanes 3,4, MCF-7 and BT-20, respectively), normal breast (lanes 5,6), breast adenocarcinomas (lanes 7–18), normal endometrium (lanes 19–21), and normal stomach (lane 22). **B:** Hybridization of the same filters with probe 2 (C). **C:** Schematic representation of the *pS2* gene, with positions of the three exons (*black boxes*), repeated sequence elements (*black arrows*), and *BamH*I (B) sites. The studied region is presented on an expanded scale, with the positions of *Taq*I (Tq) and *Msp*I (M) sites and restriction map analysis. The sizes of *Taq*I-*Msp*I and *Msp*I-*Msp*I fragments are indicated in base pair. The ERE is indicated by a *white circle.*

out of the eight cases of pS2 1+ analyzed are shown in Fig. 1A,B, lanes 10,11). In contrast, in pS2-negative samples, two different DNA methylation patterns were observed at the 5′ end of *pS2*.

In DNAs extracted from the two normal breast samples (pS2-negative) the M1, M2, and M3 sites in the promoter/enhancer region were heavily methylated (Fig. 1A,B, lanes 5,6). These sites were also methylated in six out of eight cases of ER-rich/pS2-negative breast tumor samples analyzed; in two cases these CCGG sites were slightly demethylated (four representative samples are shown in Fig. 1A,B, lanes 12–15).

In estrogen-receptor-negative samples (<10 fmol of ER per milligram of total protein) which were also pS2-negative adenocarcinoma samples (5/5 cases; three representative samples are shown in Fig. 1A,B, lanes 16–18), *Hpa*II patterns were similar to those obtained with DNA extracted from the BT-20 cell line. Hypomethylation of M2 sites (Fig. 1B, lanes

16–18, 0.4 kb band) and M3 sites (Fig. 1B, lanes 16–18, 0.34 kb band) were observed, while M1 sites were heavily methylated in these samples, since only very faint bands or none were detectable at 0.27 kb and 0.38 kb with probe 1 (Fig. 1A, lanes 16–18) and at 0.71 kb with probe 2 (Fig. 1B, lanes 16–18).

No correlation between the methylation status of CCGG sites located in intron I and *pS2* expression was observed. In addition, the upstream region of the promoter/enhancer was methylated in all samples analyzed, cell lines included (data not shown). Taken together, these results show that the methylation status of M1 sites is correlated with the expression of *pS2* in breast tissues and that the amount of DNA molecules possessing unmethylated M1 sites is related to the amount of pS2 mRNA detected in the samples by RT-PCR.

The correlation between the hypomethylation of the 5′ end of *pS2* and its expression is also observed in nonpathological samples. In normal stomach tissue (2/2 cases analyzed), which physiologically express *pS2* but do not possess estrogen receptors, the M1, M2, and M3 sites of the 5′ region of *pS2* were strongly cleaved by *Hpa*II (Fig. 1A,B, lanes 22). In contrast, in normal endometrium (5/5 cases), which do not express *pS2* but possess estrogen receptors, the *Hpa*II patterns (Fig. 1A,B, lanes 19–21), indicated that M1 sites were methylated in these tissues. The hypomethylation of the promoter/enhancer sequences of *pS2* seems, therefore, associated with its expression rather than the presence of estrogen receptors.

Determination of the Methylation Pattern of *pS2* From Individual Chromosomes

In the region studied, only the hypomethylation of the M1 site (located in the promoter/enhancer sequences) is specifically associated with *pS2* expression, whereas M2 and M3 sites are also hypomethylated in ER-negative and pS2-negative tumors. These data suggest that the methylation status of CpGs located downstream of this sequence might be not associated with the methylation status of the M1 site or that the demethylation of M2 and M3 sites might represent the first step in the demethylation process leading to the demethylation of the 5′ flanking sequences of *pS2*. To answer this question, we analyzed DNA methylation patterns of individual chromosomes in a series of breast tumors.

The methylation status of the CpGs (from nt −427 to nt +46) of the upper strand of *pS2* was investigated using a "bisulphite genomic sequencing method" [Frommer et al., 1992; Martin et al., 1995]. This method was radically different from the other methods based on a random and base-specific cleavage of DNA molecules. In single-strand DNA, after bisulphite and alkali treatment, the cytosines were deaminated to uridin, while the methylated cytosines were converted to thymine at a very slow rate. DNA methylation patterns were determined by sequencing cloned DNA molecules obtained after PCR amplification of the region of interest. This method provides, therefore, a positive identification of methylated cytosines of individual DNA molecules. Data obtained from the analysis of 53 cloned DNAs obtained from two ER-rich tumors expressing pS2 at high level (P2 and P3), two ER-negative/pS2-negative tumor (P4 and P5) molecules, and from a normal breast tissue sample (P1) are summarized in Figure 2A.

CpGs sites very frequently contain cytosines resistant to the deamination induced by bisulphite (about 50%). Unmodified cytosines at other sites seem to be randomly distributed and are observed at a very low rate (less than 1%). In addition, no contamination by plasmid DNA was observed, since the plasmid-specific methylation at the *Eco*RII site [Martin et al., 1995] was not detected. Within the region analyzed, DNA methylation occurred, therefore, at CpG sites.

Southern blot experiments using *Hpa*II/*Msp*I restriction enzymes have shown that in the

Fig. 2. Genomic sequencing and methylation analysis of clones derived from the *pS2* promoter/enhancer sequence. **A:** Methylation status of 12 CpGs at the 5′ end of *pS2*. DNA molecules obtained from individual chromosomes were analyzed using a genomic sequencing method (see Materials and Methods). CpG position is as follows: *a,* −400; *b,* −388; *c,* −370; *M1,* −354; *e,* −301; *M2,* −84; *g,* −56; *M3,* −20; *i,* −13; *j,* −9; *k,* +10; and *l,* +16. *Black circles* indicate unmethylated cytosines and *open circles* methylated cytosines. P1, seven clones from a normal breast tissue sample; P2 and P3, 9 and 14 clones from two ER-rich/pS2-positive (2+) breast tumor samples; P4 and P5, 14 and 9 clones from two ER-negative/pS2-negative breast tumor samples. **B:** Representation of methylated and unmethylated CpGs of the 5′ end of *pS2* arising from MCA (see Results). Since in this analysis the methylation status is a qualitative datum, each site is represented, according to its methylation status, by + (methylated) and − (unmethylated). To underline the clusters of points, circles are drawn around these clusters. *F1* and *F2* axes represent the two main components of the variance vector (69.6% and 14.4%, respectively).

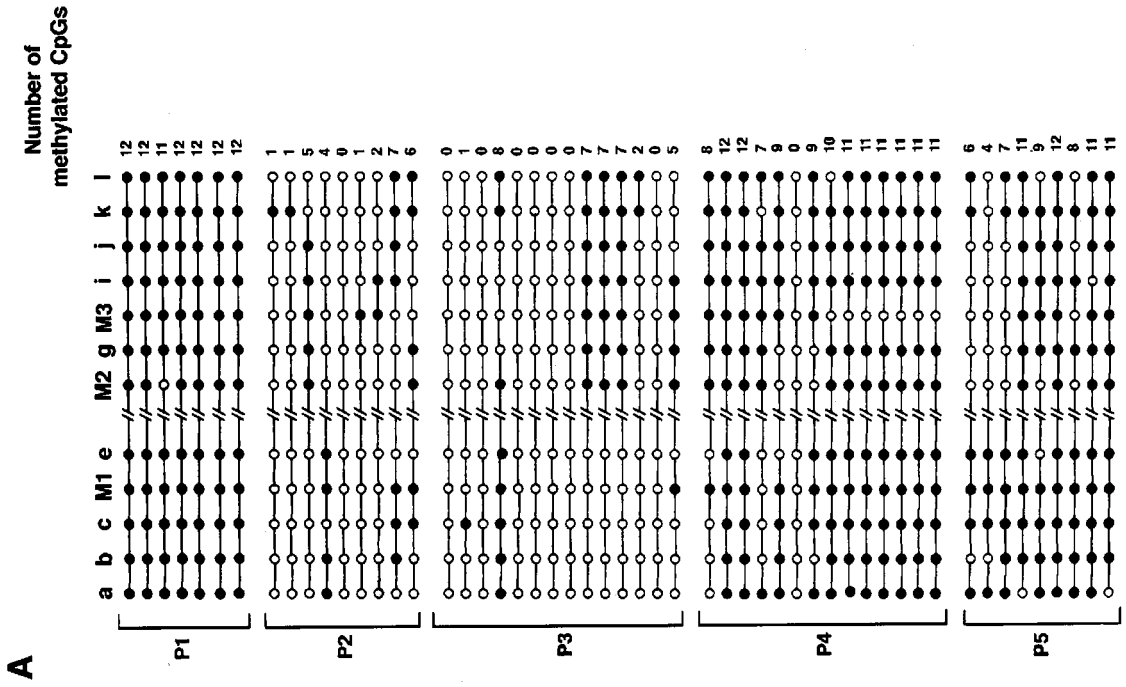
A
Number of methylated CpGs
a b c M1 e M2 g M3 i j k l
P1
P2
P3
P4
P5
B
F2
F1
+M3
+M2
+i +g +j
+l
+k
+M1 +b
+c +a +e
-c
-M1 -a -e
-b
-k
-l
-M2
-j -g -i
-M3

Figure 2.

ER-negative/pS2-negative tumors the M1 site (position −354) was largely methylated and the M2 and M3 sites (position −84 and −20, respectively) were hypomethylated. In the great majority of the clones (22/23) obtained from these DNA samples (P4 and P5), the cytosines (M1) at position −354 were found to be unchanged, and the −84 (M2) and −20 (M3) sites were deaminated in 8 out of 23 clones and 12 out of 23 clones, respectively, indicating a good correlation between the data obtained from genomic sequencing and Southern blot experiments. This correlation was also observed for ER-rich/pS2-positive samples. The three CCGG sites (M1, M2, and M3) were hypomethylated in these samples, and the great majority of the DNA molecules cloned from these samples did not exhibit methylated cytosines at these sites. However, in both subclasses of breast tumors, the demethylation of M1 sites is not always associated with the demethylation of M2 and M3 sites. These data, therefore, indicate that the demethylation of M2 and M3 sites is not the first step leading to the demethylation of the 5′ end of pS2 gene.

Although mosaic patterns of DNA methylation were observed in pathological samples, the subclasses of tumors can be distinguished by their extent of global methylation levels. Clones obtained from ER-rich/pS2-positive tumors (P2 and P3) are significantly less methylated than ER-negative/pS2-negative tumors (Student's *t*-test, $P < 0.001$ for both samples), and ER-negative/pS2-negative tumors (P4 and P5 samples) are less methylated than clones (P1) obtained from the normal breast sample (Student's *t*-test, $P = 0.058$ and $P = 0.013$, respectively).

Computerized Analysis of the Methylation Patterns of Individual Chromosomes Indicate Specific Associations Between CpGs in the 5′ Region of *pS2*

The patterns determined by genomic sequencing of the 5′ region of *pS2* were analyzed with the aid of the multiple component analysis method (MCA). MCA is a computational technique suitable for studying the pattern of distribution of objects characterized by more than two sets of categories [Lebart et al., 1984]. It is therefore a method of choice to process qualitative data and to show associations between results. Starting from the data matrices constructed from cloned DNA molecules, the position of the sites, and the methylation status of each site, variables are defined by vectors. The graphical representation was obtained by the projection in a plane of these vectors. The results of MCA are presented in the graph (Fig. 2B) that represents the positioning of the CpGs, in projection planes, according to the clone and its methylation status. These data have a symmetrical part in the analysis. In this analysis the variance vector is broken down into different components corresponding to fractions of the variance. These components are classified in decreasing order. In our case, the two axes *F1* and *F2* drawn in this plane represent 69.6% and 14.4% of the variance explained by these components.

Since in this analysis it is legitimate to interpret the relative positions of the points obtained by the projection of these vectors, the association between the CpGs can be deduced. According to the first component symbolized by the *F1* axis (Fig. 2B), we observe an association between, on the one hand, the methylated CpGs and, on the other hand, an association between the unmethylated CpGs. This analysis indicates, as expected, that some clones possess a majority of unmethylated CpGs and that other clones are essentially methylated.

The second component (Fig. 2B, *F2* axis) indicates an association between unmethylated CpGs (from a–e) at the promoter/enhancer sequences and methylated CpGs (M2, g, i, and j) at the 3′ region of this sequence. The reverse situation is also observed, since Figure 2B also indicates an association between methylated a–e CpGs and unmethylated M2, g, i, and j CpGs. From this representation it can be concluded that, in some cloned DNA molecules, the methylation status of the CpGs (a–e) located at promoter/enhancer sequences is not related to the methylation status of the CpGs (M2, g, i, and j) located downstream of this sequence. In addition, some CpGs, M3, k and l, (Fig. 2B), do not seem to be associated with these two groups of CpGs.

These data show an association between, on the one hand, the CpGs (a–e) located in the promoter/enhancer region and, on the other hand, between some CpGs (M2, g, i, and j) located in the 3′ region, thereby suggesting that the demethylation of the promoter/enhancer of *pS2* is not dependent on the surrounding sequences.

Transient Expression of Human ER Stimulates the Expression of Endogenous *pS2* Gene in HeLa Cells But Not in BT-20 Cells

In the course of this study, we observed that in HeLa cells the 5′ region of *pS2* was hypomethylated at the M1, M2, and M3 sites and methylated at the other CCGG sites (data not shown). This cell line was estrogen-receptor-negative and *pS2*-negative (Fig. 3A, lane 1), and pS2 proteins were undetectable by an immunohistochemical method (data not shown). We took this opportunity to investigate the effect of the estrogen receptors on the expression of the endogenous *pS2*.

Expression vector encoding the human estrogen receptor (HE0) was transfected into HeLa cells together with the pU-LacZ plasmid used as an internal standard. RT-PCR analysis of RNA extracted from HeLa cells transfected with the HE0 vector indicated that the transient expression of this vector (Fig. 3A, lane 4) induced the expression of the endogenous *pS2* (Fig. 3A, lane 3). In addition, immunohistochemical analysis (Fig. 3B) of the transfected HeLa cells showed that *pS2* mRNAs were efficiently translated, since the percentage (about 15%) of cells expressing *pS2* was equivalent to the percentage of cells expressing the β-galactosidase gene.

In contrast, expression of the vector encoding human estrogen receptor failed to induce the endogenous *pS2* in BT-20 cells. Control experiments showed that BT-20 cells were able to express the chimeric expression vector containing the 5′ flanking sequences of *pS2* fused to the CAT gene when cotransfected with the vector encoding the human estrogen receptor (Fig. 3C). The absence of expression of the endogenous *pS2* in transiently transfected BT-20 cells was therefore due neither to the absence of expression of ER nor to a nonfunctional cellular mechanism. In addition, in HeLa and in BT-20 cell lines, restriction maps of the *pS2* gene, obtained from genomic DNAs or from PCR fragments, were identical to those obtained from the MCF-7 cell line or pS2-pBR322 plasmid, and no mutation was detected in the 5′ region (nt −447 to nt +66) of *pS2* in the BT-20 cell line.

Since in HeLa cells the promoter/enhancer sequences were demethylated and the downstream region was methylated, whereas in BT-20 cells the promoter/enhancer sequences and the downstream region were methylated, these data are, therefore, strongly in favor of an involvement of the methylation of the 5′ region in the control of *pS2* expression.

DISCUSSION

Numerous studies have suggested that alterations of DNA methylation may play a variety of roles in carcinogenesis. However, the data

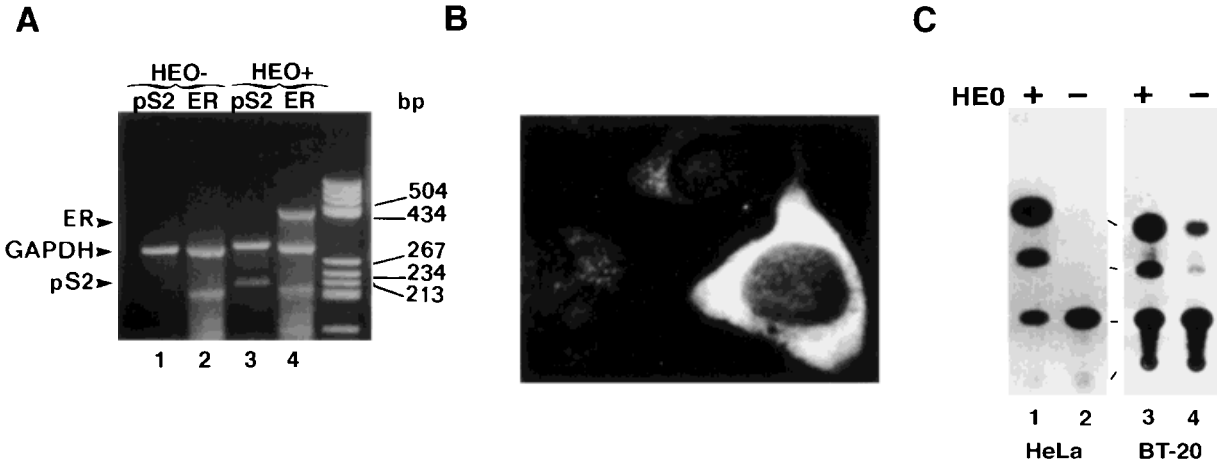

Fig. 3. Stimulation of the endogenous *pS2* gene of HeLa cells by transient expression of ER. HeLa and BT-20 cells were transiently transfected with pU-LacZ and HE0 plasmids (HE0+) or pS2-CAT plasmid (HE0−). *pS2* expression was studied by RT-PCR (A) and immunohistochemical methods (B) from the same transfection experiment (see Materials and Methods). **A:** Total RNA was extracted from HE0− (*lanes 1,2*) and from HE0+ (*lanes 3,4*) HeLa cells. ER (*lanes 2,4*) and pS2 (*lanes 1,3*) transcripts were simultaneously amplified with GAPDH transcripts as internal control. The RT-PCR specific products (pS2: 208 pb; ER: 438 pb; GAPDH: 308 pb bands), are indicated by *arrowheads.* **B:** Identification of pS2-expressing HeLa cells by immunohistochemical analysis. Shown here are a pS2-positive HeLa cell, with strong cytoplasmic staining, and two pS2-negative HeLa cells. **C:** pS2-CAT activity was determined (as described in Materials and Methods) in HeLa cells HE0+ (*lane 1*), HeLa cells HE0− (*lane 2*), BT-20 cells HEO+ (*lane 3*), and BT-20 cells HE0− (*lane 4*).

presented here are to our knowledge the first in vivo demonstration of an involvement of DNA methylation changes in the control of a hormono-regulated gene in human breast cancers.

It had been shown that the gene studied (*pS2*) is specifically expressed in subclasses of breast cancers (ER-rich tumors) and its detection is a good indicator of the presence of functional estrogen receptors. In normal breast tissue *pS2* expression is not detected [Rio et al., 1987; this report], and all the CCGG sites located at this locus are methylated.

In contrast, analysis of the methylation patterns at CCGG sites located at the 5′ end of *pS2* show some variability between samples in human breast adenocarcinomas. A short region containing the promoter/enhancer sequences [Berry et al., 1989] is hypomethylated in breast tumors expressing *pS2,* and these sequences retain a normal level of methylation in ER-rich tumors which do not express *pS2.* In breast tumors the demethylation of the 5′ end of *pS2* seems to be very specific. In ER-negative and pS2-negative tumors, M2 and M3 sites are also demethylated, while M1 (located in the promoter/enhancer sequences) remains methylated. In addition, the upstream region of the promoter/enhancer sequence was methylated in all samples analyzed, cell lines included. The hypomethylation of M1 sites is associated with *pS2* expression; however, an involvement of M2 and M3 sites cannot be ruled out. Analysis of individual chromosomes is also in favor of an involvement of the methylation in the control of *pS2* expression, since the CpGs located in the 5′ region of this gene were essentially unmethylated in the cloned DNA molecules obtained from *pS2*-expressing tumor samples. Further, computer analysis of the data obtained by the genomic sequencing method showed a correlation between the methylation status of the CpGs within the promoter/enhancer region flanking the M1 site and *pS2* expression, suggesting that the CpGs located outside the *Msp*I site are also involved in the control of *pS2* expression.

The amount of DNA molecules possessing unmethylated M1 sites is related to the amount of pS2 mRNA detected in these samples. However, a complete cleavage by *Hpa*II of the 1.06 kb band containing the promoter/enhancer sequences was never observed in the carcinoma samples analyzed. Since we have correlated the demethylation of this region with *pS2* expression, these data suggest that the samples analyzed contained cells which do not express this gene. In good agreement with this hypothesis are the observations that in pS2-positive breast tumors the percentage of tumors cells positively stained by antibodies directed against this protein varied greatly [Henry et al., 1991; Rio et al., 1987]. Examination of primary breast cancers, using immunohistochemical methods, has shown that the percentage of tumors containing more than 50% of pS2-positive tumor cells was very low (4 out of 172 cases analyzed) and that the mean percentage for positively staining tumors was 14.9% [Henry et al., 1991].

Among the 28 breast carcinoma samples analyzed, two ER-rich/pS2-negative samples exhibited hypomethylated M1 sites. The absence of *pS2* expression in these two samples might be due to the absence of another factor required for *pS2* expression in these cells or to the presence of nonfunctional ER. In this regard, the presence of nonfunctional ER in human breast cancers has been observed by several authors [Fuqua et al., 1992; Scott et al., 1991]. Moreover, it should be noted that transient expression of the human estrogen receptor in HeLa cells induces the expression of the unmethylated endogenous *pS2.*

The demethylation of the 5′ end of *pS2* does not seem to be dependent on the presence of ER as has been described for the avian vitellogenin II gene. A single injection of estradiol to immature chickens induces the demethylation of the estradiol/glucocorticoid-receptor binding sites via an active strand-specific mechanism [Saluz et al., 1986], whereas in human endometrium *pS2* is methylated and not expressed despite the presence of estrogen receptors. The analysis, by the MCA method, of the data obtained by genomic sequencing indicated an association between, on the one hand, the CpGs located in the promoter/enhancer region and, on the other hand, between some CpGs located downstream this region. These data therefore suggest that the methylation pattern of *pS2* might be regulated by two different mechanisms. The mosaic patterns of methylation does not seem to be a tumor-specific phenomenon, since mosaic patterns were also observed during development in mouse for the imprinted *Igf*2 and *H19* genes [Feil et al., 1994] and the hypoxanthine phosphoribosyltransferase gene on the inactive X chromosome [Park and Chapman, 1994]. It would be interesting therefore to determine whether an active demethylation mechanism,

as has been described during in vivo and in vitro cellular differentiation [Jost, 1993; Kafri et al., 1993], is also induced in human breast tumors.

Finally, all these data are in favor of an involvement of DNA methylation in the control of *pS2* expression. Since in these tumors the response to the estrogen defines subclasses of tumors, the data described in this report suggest that DNA methylation changes are closely linked to the neoplasic transformation of these tissues.

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