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Human Estrogen Sulfotransferase (hEST1) Activities and Its mRNA in Various Breast Cancer Cell Lines. Effect of the Progestin, Promegestone (R-5020)

G. Chetrite,[1] E. Le Nestour[2] and J. R. Pasqualini[1]*

[1]*Steroid Hormone Research Unit, Institut de Puériculture, 26 Boulevard Brune, 75014 Paris, France and* [2]*Laboratoires Cassenne, 1 Terrasse Bellini, 92800 Puteaux, France*†

Using reverse transcriptase-polymerase chain reaction amplification it was possible to detect the presence of type 1 human estrogen sulfotransferase (hEST1) mRNA in the hormone-dependent: MCF-7 and T-47D, and hormone-independent: MDA-MB-231 and MDA-MB-468, human breast cancer cells. The expression of this mRNA is significantly higher in the MDA-MB-468 cells and a correlation of this mRNA expression with the enzymatic activity was observed. The progestin promegestone (R-5020) at a low concentration (5×10^{-7} M) can significantly increase the estrogen sulfotransferase activity and its mRNA in the hormone-dependent MCF-7 and T-47D cells. As estrogen sulfates are biologically inactive, the stimulatory effect on sulfotransferase by promegestone may open attractive possibilities in the control of estradiol in human breast cancer. **© 1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

At present, it is very well established that estrogens, particularly in the form of sulfates, are concentrated in breast cancer tissues and the levels of these conjugates are particularly high in post-menopausal women [1, 2]. Information from different laboratories has clearly established that breast cancer tissues have all the enzymes necessary for the synthesis of estrogens and to conjugate to the form of sulfates (for a recent review see [3]). Estrogen sulfates are very important because they can be utilized as reserve material for the biosynthesis of active estradiol through the action of endogenous sulfatases. Secondly, estrogen sulfates can be one of the ways to protect the hormones as it is well demonstrated that estrogen sulfates are not biologically active. Estrogen sulfatase activities are well documented in breast cancer tissues as well as in isolated breast cancer cells [4–6]. Breast cancer tissue is very active in sulfotransferase for the formation of sulfates [7, 8]. It was also demonstrated that the breast cancer cell line MDA-MB-468, contains very high sulfotransferase activities [9].

*Correspondence to J. R. Pasqualini; Fax: 33-1-4542 6121
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The superfamily of steroid-sulfotransferases (ST) includes three categories according to the nature of the substrate metabolized and the type of regulation: estrogen-ST (E.C. 2.8.2.4: EST), hydroxy-ST (HST) (e.g.: dehydroepiandrosterone-ST), and phenol-ST (PST) (e.g.: aryl sulfotransferase) which is divided into a phenol sulfating form (P-PST) and a monoamine sulfating form (M-PST) [10–12]. Sulfonation of estrone is specifically made by EST at nanomolar concentrations, but P-PST and HST can also act at micromolar concentrations [12]. EST c-DNA from various sources, as well as genes of human EST (hEST) and PST, have been cloned and reveal great homology between them [13–18]. Type 1-hEST (hEST1) corresponds to the first human cDNA and gene of EST cloned by Bernier *et al.* [15, 16] using a probe derived from bovine placenta EST sequence. In fact, the hEST1 gene was named *STM* by the Hugo Nomenclature Committee since the coding region and the 3′-untranslated region are identical to the cDNA encoding M-PST [19]. However, these two cDNAs are coming from different mRNA species since they differ in the 5′-non coding region. The analysis of the hEST 1 (or *STM*) gene sequence revealed that a single gene can express at least three

mRNA species (e.g.: those of hEST1, M-PST, and a human brain aryl-ST [HAST3]), by alternative promoters using two separate exon 1 (1a and 1b) [20].

In these studies we explore the expression of the hEST1 mRNA in different breast cancer cell lines and compare this messenger with sulfotransferase activities. The effects of the progestin promegestone (R-5020) on the activity and mRNA level of hEST1 in T-47D and MCF-7 cells are also presented.

MATERIALS AND METHODS

Chemicals

[6,7-^{3}H(*N*)]-Estrone (^{3}H-E_1), (sp. act. 49 Ci/mmol), [6,7-^{3}H(*N*)]-estradiol (^{3}H-E_2) (sp. act. 48 Ci/mmol), [4-^{14}C]-estradiol (^{14}C-E_2) (sp. act. 57 mCi/mmol) and [4-^{14}C]-estrone (^{14}C-E_1) (sp. act. 51 mCi/mmol) were purchased from New England Nuclear Division (DuPont de Nemours, Les Ulis). The purity of the radioisotopes was assessed by thin-layer chromatography (TLC) in the appropriate system before use. Unlabeled E_1, E_2, estrone-3-sulfate (E_1S) and estradiol-3-sulfate (E_2S) were obtained from Sigma-Aldrich Chimie, (St Quentin Fallavier). The progestin promegestone (R-5020: 17α, 21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione) was a gift from Cassenne Laboratories, Puteaux. All other chemicals were of the highest purity available.

Cell culture

The hormone-dependent MCF-7 and T-47D and the hormone-independent MDA-MB-231 and MDA-MB-468 human mammary cancer cell lines were kindly provided by Dr Clarke (Georgetown University, Washington). The cells were routinely grown in Eagle's Minimal Essential Medium (MEM) buffered with 10 mmol/l HEPES (pH 7.6), supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin–streptomycin (A.T.G.C., Noisy-Le-Grand) and fetal calf serum (FCS) (D.A.P., Vogelgrun) (10% (v/v) for MCF-7 cells and 5% (v/v) for the three other cell lines), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Media were changed twice a week. The cells were passed every 10–12 d and replated in 75 cm^2 flasks (A.T.G.C.) at 3×10^6 cells/flask for MCF-7 and T-47D cells and at 1.5×10^6 cells/flask for MDA-MB-231 and MDA-MB-468 cells, or in 150 cm^2 flasks for the RNA extraction experiments. Four days before the experiments, the cells were transferred to MEM containing 5% steroid-depleted treated FCS. The FCS had been treated overnight at 4°C with dextran-coated charcoal (DCC) (0.1–1% w/v, DCC-FCS) to remove endogenous steroids. For the evaluation of the sulfotransferase mRNA, 24 h before the experiments, the cells were rendered quiescent by removing the serum from the medium. Cells were washed twice with ice-cold Hank's buffered saline solution (HBSS, calcium– magnesium-free) (A.T.G.C.) then cultivated for 6 h in MEM-DCC-FCS. The T-47D and MCF-7 cells were treated with the progestin promegestone (R-5020) at a concentration of 5×10^{-5} and 5×10^{-7} M for 6 h.

RNA extraction

Total RNA was extracted by the guanidinium isothiocyanate–phenol–chloroform single-step extraction protocol [21]. Briefly, cells were lysed directly in the culture flask by addition of 5 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M β-mercaptoethanol solution and RNA was extracted by centrifugation at $10{,}000 \times g$ at 4°C for 20 min, with a mixture of 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of phenol and 1 ml of chloroform-isoamyl alcohol (49:1, v/v). RNA was precipitated twice with isopropanol and washed in 75% ethanol. The pellet was dissolved in diethyl pyrocarbonate treated water. The integrity of the purified RNA was confirmed by formaldehyde-denaturing agarose gel electrophoresis. The quality and the concentration of RNA was determined by measurement of the A_{260}/A_{280} nm ratio.

Northern blot

RNA (10 μg) samples were electrophoresed on 2.4% agarose gel (Agarose NA, Pharmacia, Guyancourt) for 4 h at 4.5 mA/cm in MOPS buffer and transferred to a nylon membrane (Hybond-N membrane, Pharmacia) for 16 h with $20 \times$ SSC buffer ($1 \times$ SSC: 0.15 M NaCl, 0.015 M citric acid, pH 7.0). Hybridization was carried out for 24 h at 42°C in a Robbins Scientific oven (Bioprobe, Montreuil-sous-bois) using a solution of 50% formaldehyde, $6 \times$ Denhart's, $4 \times$ SSPE ($1 \times$ SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4), 250 μg/ml salmon sonicated DNA, 1% SDS (sodium dodecyl sulfate), containing the hEST1 c-DNA probe (2–3×10^6 cpm/ml; 15×10^6 cpm/μg c-DNA), labelled by random priming with ^{32}P (Amersham, Les Ulis) using a Pharmacia kit. The membrane was then washed at 30°C, first for 15 min with a $2 \times$ SSC and 0.5% SDS solution, followed by 15 min with a $2 \times$ SSC and 0.1% SDS solution. An X-ray film (Hyperfilm MP, Amersham) was exposed for 24 h to obtain an autoradiograph of the membrane and the mRNAs were quantified by densitometry (LKB Ultroscan, Model XL).

Reverse transcription and polymerase chain reaction (RT-PCR)

c-DNA was synthesized by reverse transcription of 1 μg of total extracted RNA in 15 μl containing 45 mM Tris (pH 8.3), 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg/ml bovine serum albumin, 1.8 mM of each deoxynucleotide triphosphate

(dNTP), 15 mM of random hexanucleotide primer, and 22 UI of cloned pure murine reverse transcriptase (Pharmacia). The reverse transcription reaction was carried out for 1 h at 37°C, followed by 1 min at 4°C, then 5 min at 95°C to inactivate the reverse transcriptase. The PCR reaction mixture added to the sample was composed of 1 UI of *Thermus aquaticus* (Taq) polymerase (Perkin-Elmer, Norwalk, CO) and 150 or 100 pmol of forward and reverse 5′ and 3′ type 1 human estrogen sulfotransferase (hEST1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers respectively. GAPDH was added as an internal standard to control for RNA quantity and amplification efficiency. The synthetic sense and antisense oligonucleotide primer sequences (Bioprobe) are listed in Table 1 and their positions indicated by reference to a sequence published by Bernier *et al.* [16] for human placental EST1 cDNA. We have standardized conditions and used a number of cycles, so that the amplification of both hEST1 and GADPH are each in the linear range of amplification. A negative control in which cDNA was replaced by water was systematically added in each run. The PCR reaction was performed in a thermal cycler (Model 480: Perkin-Elmer) for 45 cycles with denaturation for 1 min at 94°C, annealing for 30 s at 52°C and extension for 1 min at 72°C, each. The last cycle was performed with a final elongation step at 72°C for 10 min.

Electrophoresis

Aliquots of PCR products were mixed with loading buffer and electrophoresed, with DNA markers, on 4% agarose gel (3% Newsieve Agarose, Tebu, Le Perray-en-Yvelines) and 1% NA Agarose (Pharmacia) containing ethidium bromide (Bioprobe) in Tris/acetate-EDTA buffer. The electrophoresis was performed for 4 h at 4.5 mA/cm. The gel was exposed to UV illumination for 1 min to obtain a negative in standardized conditions (Polaroid, Cambridge, MA, film 55 positive/negative) and the strands visualized were quantified by laser densitometry.

Determination of estrogen sulfotransferase activity

Cells near confluence were cultivated in MEM-DCC-FCS with the addition of 5×10^{-9} mol/l of [^{3}H]-E_1 alone or in the presence of promegestone (R-5020) in a concentration range of 5×10^{-7}–5×10^{-5} M. 24 h later, the medium was removed, the cells washed twice with ice-cold HBSS and harvested with 15 ml HBSS by scraping with a rubber policeman. After centrifugation, the pellet and culture medium were separately precipitated by 80% ethanol and the radioactivity was extracted for at least 24 h at −20°C. The cellular radioactivity uptake was determined in the ethanolic supernatant and the DNA content in the remaining pellet was evaluated according to Burton [22]. The unconjugated and conjugated estrogens present in the ethanolic extracts (cell compartment and culture medium) were carried out after isolation by TLC on silica gel 60F_{254} plates (Merck, Darmstadt), which were developed with either chloroform–ethylacetate (4:1, v/v) for the unconjugated estrogens or with ethylacetate/methanol/ammonium hydroxide (75:25:2, v/v) for the separation of steroid sulfates. [^{14}C]-E_1 or [^{14}C]-E_2 (5,000 dpm) was added to monitor analytical losses and unlabeled E_1, E_2, E_1S or E_2S (50 μg) were used as carriers and reference indicators. After visualization under UV, the appropriate areas were cut off into small pieces, placed in liquid scintillation vials with ethanol (0.3 ml) and allowed to extract for 30 min. Three ml of Opti-fluor (Packard, Rungis) were added and the vials were analyzed for ^{3}H and ^{14}C contents. The quantitative evaluation was calculated as a percentage of the total radioactivity associated with the cells or the medium and then expressed as pmol/mg DNA.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM) values. Student's *t*-test was used to assess the significance of the differences between means; *p* values $\leq$0.05 were considered significant.

RESULTS

Expression of human mRNA estrogen sulfotransferase in breast cancer cells

Type 1 human mRNA estrogen sulfotransferase (hEST1) of the various hormone-dependent (MCF-7, T-47D) and hormone-independent (MDA-MB-231, MDA-MB-468) human breast cancer cell lines is

Table 1. Oligonucleotide primers

	Sequence primer	Product size (bp)
	Type 1 human estrogen sulfotransferase (hEST1):	
F: [−52 to −28]	5′ CAG CCC CTG CAG GCA AGG AGA GAA C 3′	355
R: [+276 to +256]	5′ GAT CCA GGG GAA CCC TCA GGG 3′	
	GAPDH:	
F:	5′ TCC CAT CAC CAT CTT CCA 3′	762
R:	5′ GTC CAC CAC CCT GTT GCT 3′	

F: sense; R: antisense oligonucleotides.
GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

expressed in very low amounts, often below the limits of detection by Northern hybridization. Consequently, mRNAs were only detected if a c-DNA was prepared previously by reverse transcription of total RNA and then amplified by polymerase chain reaction (RT-PCR) with appropriate specific primers. The synthetic oligo primer pairs used (Table 1) are located at a specific 5′-untranslated region (forward primer, sense) and at the end of exon 3 and the beginning of exon 4 (reverse primer, antisense) of human placental EST1 c-DNA according to the sequence published by Ref. [16]. A number of 45 cycles of PCR was determined by optimization of the method and a 355-bp fragment of the hEST1 mRNA was amplified (Fig. 1(A)). Figure 1(B) indicates the relative expression of hEST1 mRNA in the four breast cancer cells studied. The hormone-independent MDA-MB-468 cells show the highest hEST1 mRNA expression. The hormone-dependent T-47D and MCF-7 cells present similar values of hEST1 mRNA expression, 70 and 66% respectively, in relation to the value of MDA-MB-468 cells which was considered as 100%. The hormone-independent MDA-MB-231 cells express the weakest amount of hEST1 mRNA (42%).

Correlation of the human estrogen sulfotransferase activity and its mRNA expression in breast cancer cells

When [^{3}H]-estrone (5 nmol/l) is incubated for 24 h with the different cells, the presence of estrogen sulfates (ES) is exclusively detectable in culture medium. Although EST is a soluble cytosolic enzyme, it is observed that ES appear only in the culture medium (Tables 2 and 3). We have previously studied the conversion of estrogens to estrogen sulfates by these breast cancer cells in function of time [9] (and unpublished data). Maximal values in the culture medium are obtained after 3 h incubation and remain rela-

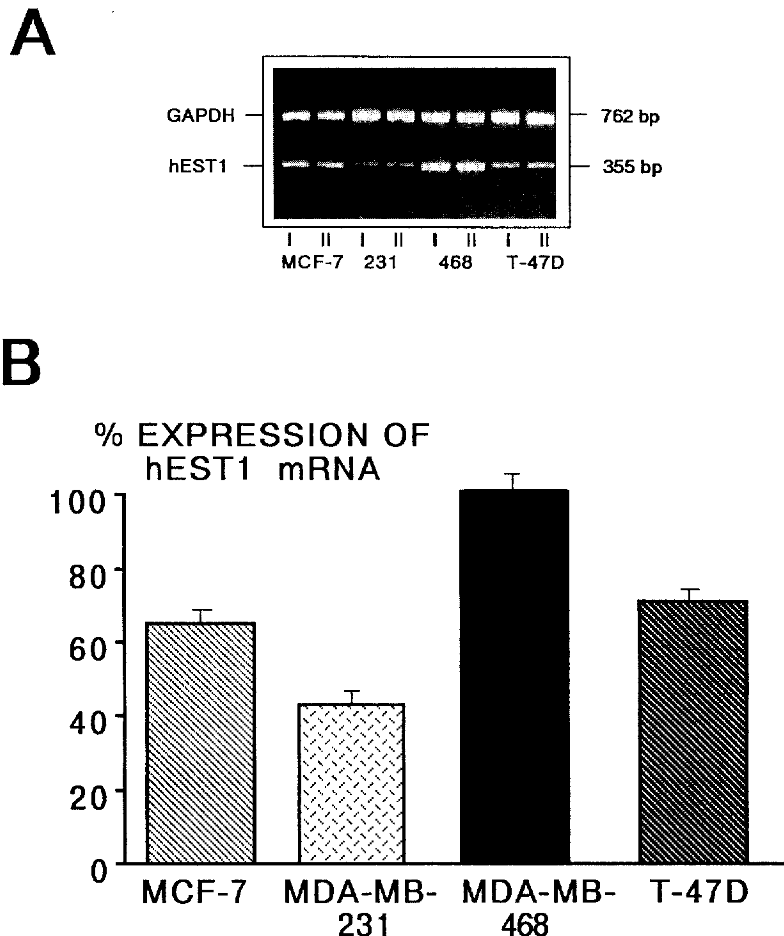

Fig. 1. Expression of type 1 human estrogen sulfotransferase (hEST1) mRNA in various breast cancer cell lines using RT-PCR amplification. (A) Electrophoresis of the expression of hEST1 mRNA in various mammary cancer cell lines. 1 μg of the total RNA was processed for RT-PCR as indicated in Materials and Methods. The data correspond to 2 experiments, I and II, using hormone-dependent: MCF-7, T-47D, and hormone-independent: MDA-MB-231 (231) and MDA-MB-468 (468), breast cancer cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as a marker of the mRNA amplification. (B) Relative expression of the hEST1 mRNA in different breast cancer cell lines. The values were calculated by laser densitometry (Ultroscan LKB). The value 100% was assigned to the expression of the hEST1 mRNA in MDA-MB-468 cells. The data represent the mean ± S.E.M. of 4 experiments

Table 2. Relative sulfotransferase activity and human estrogen sulfotransferase (hEST1) mRNA expression in the hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231, MDA-MB-468) human mammary cancer cells

Cell lines	Estrogen sulfates formed (%)	Relative hEST1 mRNA expression (%)
T-47D	13.4	66
MCF-7	11.6	70
MDA-MB-231	5.2	42
MDA-MB-468	100.0	100

Preconfluent cells were incubated for 24 h at 37°C in MEM-DCC-FCS with 5 nM of [^{3}H]-estrone. The estrogen sulfates found were only detectable in culture medium and were quantified as described in Materials and Methods. Expression of mRNA hEST1 was analyzed by RT-PCR amplification as indicated in Materials and Methods. The value of 100% was assigned to the activity and the mRNA expression of hEST1 in the MDA-MB-468 cells.

tively constant until 24 h. Table 2 shows that the quantity of ES formed correlates to their respective hEST1 mRNA expression. It is observed that the higher hEST1 activity is associated with MDA-MB-468 cells. The hEST activity in the other cells: T-47D, MCF-7 and MDA-MB-231, represent 13.4, 11.6 and 5.2% respectively, in relation to the value of MDA-MB-468 cells considered as 100%.

Metabolism of [^{3}H]-estrone in breast cancer cells

Table 3 gives the quantitative values of the transformation of E_1 with the various cells studied. It is observed that in the hormone-dependent T-47D and MCF-7 cells, E_2 is the major metabolic product: 73.8 and 64.3% respectively of the total radioactivity incubated. For the hormone-independent MDA-MB-231 cells, most of the E_1 remains untransformed. These results confirm that the reductive orientation of the 17β-hydroxysteroid dehydrogenase (E_1 to E_2) is predominant in the hormone-dependent cells, whereas in the MDA-MB-231 hormone-independent cells this transformation is in the oxidative direction (E_2 to E_1) [23]. Sulfotransferase activity is very high in the MDA-MB-468 cells, where the ES account for 85.3% of the total radioactivity in the medium. The values of these conjugates for T-47D, MCF-7 and MDA-MB-231 cells are 9.2, 8.0 and 3.6 respectively.

Effect of promegestone (R-5020) on the mRNA and the activity of human estrogen sulfotransferase in T-47D and MCF-7 breast cancer cells

The T-47D is a well-known hormone-dependent breast cancer cell line which is characterized by a very great concentration of progesterone receptor (PR: 10–20 pmol/mg DNA). In these cells, it was demonstrated that various progestins (e.g. promegestone (R-5020), nomegestrol acetate or medroxyprogesterone acetate) inhibit cell proliferation [24–26]. In previous work, we have shown that R-5020 can significantly decrease the expression of the estrone sulfatase mRNA in T-47D and MCF-7 cells [1,27]. In order to explore the effect of R-5020 on the mRNA expression of hEST1, we incubated T-47D and MCF-7 breast cancer cells with [^{3}H]-E_1 alone (5 nmol/l) or associated with the progestin promegestone (R-5020). At a concentration of 5×10^{-7} mol/l of R-5020, the hEST1 mRNA expression increased by 35% and 75% in T-47D and MCF-7 cells respectively, in relation to the control value ([^{3}H]-E_1 alone). However, at a concentration of 5×10^{-5} mol/l, the hEST1 mRNA level decreased by 25% and 36% in T-47D and MCF-7 cells respectively (Fig. 2(A) and (B)). A similar phenomenon was observed for the hEST activity in T-47D cells, which were stimulated (+26%) at a concentration of 5×10^{-7} mol/l of R-5020 and inhibited (−41%) at 5×10^{-5} mol/l (Table 4). An identical effect is observed with MCF-7 cells (data not shown).

DISCUSSION

The present report shows that expression of type 1 human estrogen sulfotransferase (hEST1) mRNA can be detected by reverse transcriptase-polymerase chain reaction amplification in hormone-dependent (MCF-7, T-47D) and in hormone-independent (MDA-MB-231, MDA-MB-468) human breast cancer cells. The placental hEST1 (or *STM*) gene consists of 9 exons and 8 introns with approximately 7.7 kb in length,

Table 3. Transformation of estrone (E_1) in the cell compartment and in the culture medium after incubation with the hormone-dependent (MCF-7, T-47D) and hormone-independent (MDA-MB-231, MDA-MB-468) human mammary cancer cells

	Estrogens obtained (in pmol/mg DNA)					
	in the cells			in the culture medium		
Cell lines	estrone	estradiol	estrogen sulfates	estrone	estradiol	estrogen sulfates
T-47D	1.5 ± 0.4	4.5 ± 1.0	N.D.	23.6 ± 3.6	101.5 ± 12.1	13.2 ± 1.6
MCF-7	0.30 ± 0.05	3.7 ± 0.8	N.D.	39.7 ± 2.6	89.0 ± 7.8	11.4 ± 1.8
MDA-MB-231	4.1 ± 1.0	0.44 ± 0.01	N.D.	116.5 ± 14.6	16.3 ± 2.7	5.1 ± 0.6
MDA-MB-468	0.85 ± 0.01	0.05 ± 0.01	0.01 ± 0.007	13.7 ± 2.5	2.5 ± 0.6	120.4 ± 11.6

Preconfluent cells were incubated for 24 h at 37°C in MEM-DCC-FCS with 5 nM of [^{3}H]-E_1. The radioactive material was analyzed and quantified as described in Materials and Methods. The data represent the mean ± S.E.M. of three to five duplicate determinations.
N.D.: Not Detectable.

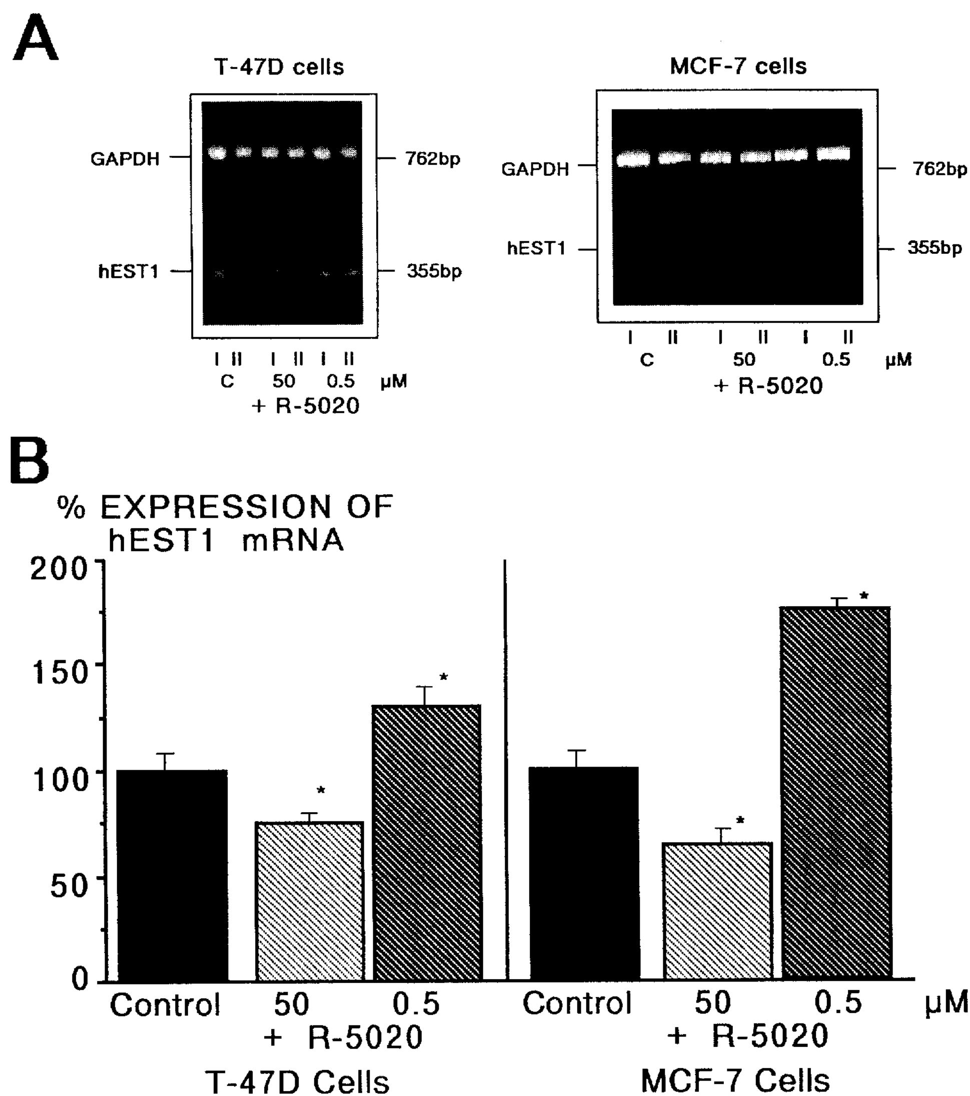

Fig. 2. Effect of the progestin promegestone (R-5020) on the expression of type 1 human estrogen sulfotransferase mRNA (hEST1) in T-47D and MCF-7 breast cancer cell lines using RT-PCR amplification. (A) Electrophoresis of the expression of hEST1 mRNA in the MCF-7 and T-47D non-treated cells (c) and in R-5020 (0.5–50 µM) treated cells. 1 µg of the total RNA of the non-treated, and the cells treated during 6 h, were processed for RT-PCR as indicated in Materials and Methods. GADPH RNA was used as a marker of the mRNA amplification. The data correspond to 2 experiments, I and II, using MCF-7 and T-47D cells. (B) Relative expression of mRNA hEST1 in T-47D and MCF-7 cells non-treated (control) and treated with R-5020 (0.5 and 50 µM) for 6 h. The values were calculated by densitometry (Ultroscan LKB). The control values were assigned 100%. The data represent the mean ± S.E.M. of 3 independent experiments. $*p < 0.05$ vs control values

and the expressed enzyme was able to transform estrone to estrone sulfate at nanomolar concentrations [16]. It was demonstrated that a single gene, assigned to chromosome 16, can transcribe at the same time brain phenol sulfotransferase (PST or HAST3), M-PST, and human placental EST1 mRNAs by alternate exon 1a and exon 1b promoters, respectively [16, 20]. Alternative splicing and/or alternative promoters are thought to confer tissue-specific expression. The coding and 3′-untranslated sequence of the hEST1 cDNA are identical to those of the mRNAs of HAST3 and M-PST, but the 5′-untranslated sequence (exon 1) is different. This strength similitude between placental hEST1 and brain hPST (or HAST3) is also demonstrated since a 95.6% sequence homology was found [15, 16, 28]. This fact, as well as the existence of several isoforms for each type of STs [29–31], makes the separation of hEST and hPST difficult. A second hEST, named type II, has been isolated from human liver cDNA library by Askoy *et al.* [14] and Falany *et al.* [32], but the cDNA sequence shares only 49% homology with the hEST1 sequence. However, the specific primers used in this study came from the noncoding exon 1b (promoter of hEST1), corresponding to the 5′ untranslated region (sense primer) and a part of the

Table 4. Effect of the progestin, promegestone (R-5020) on the estrogen sulfotransferase (hEST1) activity and on its mRNA expression using RT-PCR amplification in the hormone-dependent T-47D human mammary cancer cell line

	Estrogen sulfates formed	Relative hEST1 mRNA
$[^3H]$-E_1 alone (5 nM)	13.7 ± 1.8	100
+R-5020: 5×10^{-7} M	17.2 ± 1.5	135
+R-5020: 5×10^{-5} M	8.1 ± 1.0	75

The EST activity and mRNA expression were analyzed and quantified as described in Materials and Methods. The value of 100% was assigned to the mRNA expression of hEST1 in non-treated cells ($[^3H]$-E_1 alone).
The data represent the mean ± S.E.M. of 3–4 duplicate determinations.

coding exon 3-exon 4 sequence (antisense primer) of human placental EST1 cDNA. Consequently, the transcripts 355 bp produced by RT-PCR correspond specifically to the expression of mRNA hEST1 in breast cancer cells.

As indicated in Fig. 1, the level of hEST1 mRNA is different for the cells tested. The hormone-dependent MCF-7 and T-47D cells express similar values of mRNA hEST1; however, those of hormone-independent cells are very different. MDA-MB-468 cells show the highest mRNA expression of the various cells studied and MDA-MB-231 cells the lowest.

EST is an unstable enzyme and is known to consist of a number of isoenzyme forms which may be regulated differently in various tissues, as well as within the same tissue or in the various periods of the cell cycle [13, 33–35].

Using specific substrates and immunoblot techniques, Falany *et al.* [35, 36] found that only a human phenol-sulfating form of PST, and not EST, can sulfonate E_2 in the cytosol of hormone-dependent breast cancer cells, as well as in the hormone-independent BT-20 breast cancer cell line. However, these authors found no sulfonation in MDA-MB-231 and MDA-MB-468 cells. Other investigators, using biochemical or immunological methods, have detected the presence of EST and HST activities in the cytosol of MCF-7 and ZR 75-1 cells or in mammary tumors [4, 37, 38]. These discrepancies can be explained by various factors including: cell origin, cell passage numbers, culture conditions, the usage of different sera batches, the condition of the enzymatic assays: cytosol fractions or monolayer cells, the presence and the concentrations of the cofactor 3-phosphoadenosine-5′-phosphosulfate.

It is remarkable that a relatively significant correlation between hEST1 mRNA expression and the ST activities exists in these breast cancer cells, suggesting that the sulfonation of E_1 can be carried out through hEST1. A similar relative correlation between the enzyme activity and the mRNA expression in these breast cancer cells has been demonstrated for the estrone sulfatase [27].

One of the attractive aspects of the present study is the different dose-dependent effects of the progestin, promegestone (R-5020) on the activity of type 1 hEST and its mRNA in the T-47D and MCF-7 cells. At 0.5 μM of R-5020 the increase of the mRNA hEST1 level by 35% and 75% in T-47D and MCF-7 cells respectively, is correlated with the increase of hEST activity. However, at a 100-fold concentration of R-5020 an inhibitory effect is observed in hEST activity and its mRNA in both cell lines (see Table 4 and Fig. 2). At present we do not have a clear explanation for this dual effect, but there are many examples in which a hormone or anti-hormone can produce an opposite effect; e.g.: tamoxifen at low doses stimulates proliferation of breast cancer cells and at high concentrations inhibits cell growth [39]. In relation to these findings, it is interesting to mention that progesterone induces hEST activity in Ishikawa human endometrial adenocarcinoma cells, as well as in the secretory endometrial tissue [33, 40, 41]. It was also demonstrated that E_2 at a concentration of 1 nM can increase the formation of estrogen sulfates in MCF-7 cells [34, 37].

In conclusion, the present data demonstrate that breast cancer cells express type 1 human estrogen sulfotransferase (hEST1) mRNA and that their levels are correlated to the enzymatic ST activity. The progestin, promegestone, at a low concentration, can stimulate hEST activity by increasing the level of hEST1 mRNA. As promegestone also decreases estrone sulfatase activity and its mRNA, this combined effect allows a reduction of the estrogenic stimulation in breast cancer cells and opens new therapeutic perspectives in the use of progestins in breast cancer.

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REFERENCES

1. Pasqualini J. R., Chetrite G. and Le Nestour E., Control and expression of oestrone sulphatase activities in human breast cancer. *Journal of Endocrinology* **150** (1996) S99–S105.
2. Pasqualini J. R., Chetrite G., Blacker C., Feinstein M.-C., Delalonde L., Talbi M. and Maloche C., Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer. *Journal of Clinical Endocrinology and Metabolism* **81** (1996) 1460–1464.
3. Pasqualini J. R. and Chetrite G., Activity, regulation and expression of sulfatase, sulfotransferase and 17β-hydroxysteroid dehydrogenase in breast cancer. In *Hormone-Dependent Cancer*, eds J. R. Pasqualini and B. S. Katzenellenbogen. Marcel Dekker Inc., New York, 1996, pp. 25–80.
4. Tseng L., Mazella J., Lee L. Y. and Stone M. L., Estrogen sulfatase and estrogen sulfotransferase in human primary mammary carcinoma. *The Journal of Steroid Biochemistry* **19** (1983) 1413–1417.
5. Pasqualini J. R., Gelly C. and Lecerf F., Estrogen sulfates: Biological and ultrastructural responses and metabolism in

MCF-7 human breast cancer cells. *Breast Cancer Research and Treatment* **8** (1986) 233–240.
6. MacIndoe J. H., The hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate by MCF-7 human breast cancer cells. *Endocrinology* **123** (1988) 1281–1287.
7. Godefroi V. C., Locke E. R., Singh D. V. and Brooks S. C., The steroid alcohol and estrogen sulfotransferases in rodent and human mammary tumors. *Cancer Research* **35** (1975) 1791–1798.
8. Raju U., Blaustein A. and Levitz M., Conjugation of androgens and estrogens by human breast tumors *in vitro*. *Steroids* **35** (1980) 685–695.
9. Pasqualini J. R., Steroid sulphotransferase activity in human hormone-independent MDA-MB-468 mammary cancer cells. *European Journal of Cancer* **28A** (1992) 758–762.
10. Adams J. B. and Phillips N. S., Properties of estrogen and hydroxysteroid sulphotransferases in human mammary cancer. *The Journal of Steroid Biochemistry* **36** (1990) 695–701.
11. Anderson E. and Howell A., Oestrogen sulphotransferases in malignant and normal human breast tissue. *Endocrine-Related Cancer* **2** (1995) 227–233.
12. Falany C. N., Wheeler J., Oh T. S. and Falany J. L., Steroid sulfation by expressed human cytosolic sulfotransferases. *The Journal of Steroid Biochemistry and Molecular Biology* **48** (1994) 369–375.
13. Komatsu K., Oeda T. and Strott C. A., Cloning and sequence analysis of the 5′-flanking region of the estrogen sulfotransferase gene: Steroid response elements and cell-specific nuclear DNA-binding proteins. *Biochemical and Biophysical Research Communications* **194** (1993) 1297–1304.
14. Askoy I. A., Wood T. C. and Weinshilboum R., Human liver estrogen sulfotransferase: Identification by cDNA cloning and expression. *Biochemical and Biophysical Research Communications* **200** (1994) 1621–1629.
15. Bernier F., Leblanc G., Labrie F. and Luu-The V., Structure of human estrogen and aryl sulfotransferase gene. *Journal of Biological Chemistry* **269** (1994) 28200–28205.
16. Bernier F., Lopez Solache I., Labrie F. and Luu-The V., Cloning and expression of cDNA encoding human placental estrogen sulfotransferase. *Molecular and Cellular Endocrinology* **99** (1994) R11–R15.
17. Her C., Askoy I. A., Kimura S., Brandriff B. F., Wasmuth J. J. and Weinshilboum R. M., Human estrogen sulfotransferase gene (STE): Cloning, structure and chromosomal localization. *Genomics* **29** (1995) 16–23.
18. Wood T. C., Aksoy I. A., Aksoy S. and Weinshilboum R. M., Human liver thermolabile phenol sulfotransferase: cDNA cloning, expression and characterization. *Biochemical and Biophysical Research Communications* **198** (1994) 1119–1127.
19. Dooley T. P., Probst P., Munroe P. B., Mole S. E., Liu Z. and Doggett N. A., Genomic organization and DNA sequence of the human catecholamine-sulfating phenol sulfotransferase gene (STM). *Biochemical and Biophysical Research Communications* **205** (1994) 1325–1332.
20. Luu-The V., Bernier F. and Dufort I., Steroid sulfotranferases. *Journal of Endocrinology* **150** (1996) S87–S97.
21. Chomczynski P. and Sacchi N., Single-step method of RNA isolation by acid guanidiniu, thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** (1987) 156–159.
22. Burton K., A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal* **62** (1956) 315–323.
23. Nguyen B.-L., Chetrite G. and Pasqualini J. R., Transformation of estrone and estradiol in hormone-dependent and hormone-independent human breast cancer cells. *Breast Cancer Research and Treatment* **34** (1995) 139–146.
24. Vignon F., Bardon S., Chalbos D. and Rochefort H., Antiestrogenic effect of R-5020 a synthetic progestin in human breast cancer cells in culture. *Journal of Clinical Endocrinology and Metabolism* **56** (1983) 1124–1130.
25. Sutherland R. L., Hall R. E., Pang G. Y. N., Musgrove E. A. and Clarke C. L., Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. *Cancer Research* **48** (1988) 5084–5091.
26. Botella J., Duranti E., Duc I., Cognet A. M., Delansorne R. and Paris J., Inhibition by nomegestrol acetate and other synthetic progestins on proliferation and progesterone receptor content of T-47D human breast cancer cells. *The Journal of Steroid Biochemistry and Molecular Biology* **50** (1994) 41–47.
27. Pasqualini J. R., Maloche C., Maroni M. and Chetrite G., Effect of the progestagen promegestone (R-5020) on mRNA of the oestrone sulphatase in the MCF-7 human mammary cancer cells. *Anticancer Research* **14** (1994) 1589–1594.
28. Zhu X., Veronese M. E., Bernard C. C. A., Sansom L. N. and McManus M. E., Identification of two human brain aryl sulfotransferase cDNAs. *Biochemical and Biophysical Research Communications* **195** (1993) 120–127.
29. Oeda T., Lee Y. C., Driscoll W. J., Chen H.-C. and Strott C. A., Molecular cloning and expression of a full-length complementary DNA encoding the guinea-pig adrenocortical estrogen sulfotransferase. *Molecular Endocrinology* **6** (1992) 1216–1226.
30. Wilborn T. W., Comer K. A., Dooley T. P., Reardon I. M., Heinrikson R. L. and Falany C. N., Sequence analysis and expression of the cDNA for the phenol-sulfating form of human liver phenol sulfotransferase. *Molecular Pharmacology* **43** (1992) 70–77.
31. Lee Y. C., Komatsu K., Driscoll W. J. and Strott C. A., Structural and functional characterization of estrogen sulfotransferase isoforms: Distinct catalytic and high affinity binding activities. *Molecular Endocrinology* **8** (1995) 1627–1635.
32. Falany C. N., Krasnykh V. and Falany J. L., Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. *The Journal of Steroid Biochemistry and Molecular Biology* **52** (1995) 529–539.
33. Clarke C. L., Adams J. B. and Wren B. G., Induction of œstrogen sulfotransferase activity in the human endometrium by progesterone in organ culture. *The Journal of Clinical Endocrinology and Metabolism* **55** (1982) 70–75.
34. Adams J. B., Vrahimis R. and Phillips N., Regulation of estrogen sulfotransferase by estrogen in MCF-7 human mammary cancer cells. *Breast Cancer Research and Treatment* **22** (1992) 157–161.
35. Falany J. L. and Falany C. N., Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. *Cancer Research* **56** (1996) 1551–1555.
36. Falany J. L., Lawing L. and Falany C. N., Identification and characterization of cytosolic sulfotransferase activities in MCF-7 human breast carcinoma cells. *The Journal of Steroid Biochemistry and Molecular Biology* **46** (1993) 481–487.
37. Adams J. B., Philips N. S. and Hall R., Metabolic fate of estradiol in human mammary cancer cells in culture: Estrogen sulfate formation and cooperativity exhibited by estrogen sulfotransferase. *Molecular and Cellular Endocrinology* **58** (1988) 231–242.
38. Sharp S., Anderson J. M. and Coughtrie M. W. H., Immunohistochemical localisation of hydroxysteroid sulphotransferase in human breast carcinoma tissue: A preliminary study. *European Journal of Cancer* **30A** (1994) 1654–1659.
39. Reddel R. R. and Sutherland R. L., Tamoxifen stimulation of human breast cancer cell proliferation *in vitro*: A possible model for tamoxifen tumour flare. *European Journal of Cancer and Clinical Oncology* **20** (1984) 1419–1424.
40. Tseng L. and Liu H. C., Stimulation of arylsulfotransferase activity by progestins in human endometrium *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **53** (1981) 418–421.
41. Falany J. L. and Falany C. N., Regulation of estrogen sulfotransferase in human endometrial adenocarcinoma cells by progesterone. *Endocrinology* **137** (1996) 1395–1401.