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17-β-Estradiol-Dependent Regulation of Somatostatin Receptor Subtype Expression in the 7315b Prolactin Secreting Rat Pituitary Tumor *in Vitro* and *in Vivo**

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ABSTRACT

In the present study, we have investigated the role of estrogens in the regulation of somatostatin receptor subtype (sst) expression in 7315b PRL-secreting rat pituitary tumor cells *in vitro* and *in vivo*. sst were undetectable in freshly dispersed cells of the transplantable 7315b tumor. When 7315b cells were cultured in medium containing 10% FCS, the number of high affinity sst increased with prolonged culture time. However, when the medium was supplemented with 10% horse serum (HS) instead of FCS, no sst were detectable on 7315b cells even after three weeks of culturing. In contrast to HS, FCS contains high E_2-levels (HS, 8 pM; FCS, 134 pM).

The antiestrogen tamoxifen (0.5 μM) significantly inhibited the sst number to 50.5% of the value of untreated FCS-grown cells, suggesting that E_2 stimulates sst expression in 7315b rat pituitary tumor cells. E_2 (10 nM) induced a rapid increase in sst number in HS-grown 7315b cells. Octreotide (1 μM) significantly inhibited PRL release and the intracellular PRL concentration of 7315b cells that were cultured in medium supplemented with FCS or with HS + 10 nM E_2 but not in HS alone. This indicates that the sst present on these cells are biologically active. RT-PCR analysis revealed that none of the five currently known sst subtypes were present in freshly dispersed 7315b pituitary tumor cells. The expression of sst_2- and sst_3- messenger RNA (mRNA) was unequivocally correlated to the presence of E_2 because these sst subtypes were detected only in cells that were cultured for 7 and 14 days in medium supplemented with FCS or with HS + 10 nM E_2. sst_1, sst_4 and sst_5 messenger RNA could not be detected.

The 7315b tumor itself synthesizes and secretes huge amounts of PRL. The high PRL levels in tumor-bearing rats inhibit the ovarian E_2-production. No detectable E_2 levels could be measured in the serum of 7315b tumor-bearing rats. The sc administration of 20 μg/day E_2-benzoate normalized the circulating E_2 levels in 7315b tumor-bearing rats. Moreover, E_2-treatment indeed induced sst expression *in vivo* as shown by ligand binding studies using membrane homogenates and [^{125}I-Tyr^3]-octreotide as radioligand and by autoradiography on tissue sections. In agreement with the *in vitro* studies, the expression of the sst_2 subtype was established by RT-PCR analysis in 7315b tumors of E_2-treated rats. However, in contrast to the *in vitro* studies, E_2-treatment did not effectuate the expression of the sst_3 subtype, suggesting that the *in vitro* stimulus of E_2 is stronger.

In conclusion: 1) sst_2 and sst_3 expression in the 7315b rat prolactinoma model is primarily dependent upon the presence of estrogens; 2) the antihormonal action of octreotide in 7315b tumor cells *in vitro* is mediated via the sst_2 and/or sst_3 subtypes; 3) the absence of sst expression *in vivo* can be explained by the hormonal environment of the 7315b tumor cells. The 7315b tumor cells *in vivo* may down-regulate their own receptor status via their host, because of the ensuing hyperprolactinemia results in a hypo-estrogenic state. (*Endocrinology* **138:** 1180–1189, 1997)

SOMATOSTATIN (SS) is a regulatory peptide in a number of organ systems (brain, pituitary, gastrointestinal tract, pancreas). It inhibits the release of various hormones and may act as a neurotransmitter in the central nervous system (1). These actions of somatostatin are mediated via specific, high affinity, G protein-coupled membrane receptors (sst's). Recently, five sst subtypes, code named sst_{1-5}, have been cloned (2).

The clinical introduction of SS analogs, like octreotide, has opened new opportunities for the medical therapy of patients with sst-positive tumors. The drug, for example, effectively controls hormonal hypersecretion from most GH- and TSH-secreting pituitary tumors but also from carcinoids and pancreatic islet cell tumors (3). *In vivo* visualization of such tumors is possible after the injection of an isotope-labeled SS analog (3). Apart from visualization, these studies also demonstrated a considerable uptake of radioactivity by most sst-positive tumors. Preliminary studies suggest that radiotherapy with isotope-labeled octreotide can be successful in patients with inoperable metastasized sst-positive cancers (4). These developments suggest that manipulation of the expression of sst on tumors may have both diagnostic and therapeutic consequences. Also, a transient up-regulation of sst expression might improve the success of this form of radiotherapy.

In the present study, we evaluated whether sst expression can be manipulated in the model of the transplantable rat PRL-secreting pituitary tumor 7315b. This pituitary tumor originated via *in vivo* dedifferentiation from the PRL/ACTH-secreting pituitary tumor 7315a (5, 6). Previous studies have shown that sst are undetectable on freshly dispersed 7315b pituitary tumor cells, whereas the number of sst increases when 7315b cells are cultured in medium supplemented with 10% FCS (7). When 7315b pituitary tumor cells were cultured in medium that was supplemented with 10% horse serum (HS), sst remained undetectable, even after several weeks of

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culturing. This suggests that FCS harbours important stimulatory factor(s) for sst expression.

It was subsequently hypothesized that the very low E_2-levels in rats bearing the 7315b tumor (a high PRL level caused by the tumoral PRL hypersecretion inhibits ovarian E_2-production) were responsible for the absence of sst *in vivo*. Somatostatin was previously shown to inhibit PRL release *in vivo* and *in vitro* (8, 9). Studies by Gooren *et al.* (10) and Kimura *et al.* (11) have shown that E_2 is involved in the induction of the inhibitory effect of somatostatin on PRL release. Our previous studies showed that somatostatin barely inhibits PRL release in normal rat anterior pituitary cells that had been cultured in 10% estrogen-stripped FCS, whereas the addition of 1 nM E_2 induced a dose-dependent inhibition of PRL release by somatostatin (12). Indeed, the number of sst sites on normal lactotrophs increases after E_2-pretreatment (11). E_2 also affects the numbers of other anterior pituitary receptors. Reduction of receptor content after E_2-treatment has been described for dopamine as well as for angiotensin II receptors (13, 14). Taken together, the above data suggest that estrogens are important in the regulation of sst expression.

In the present study, we have therefore investigated the role of estrogens in the regulation of sst subtype expression on 7315b PRL-secreting rat pituitary tumor cells *in vitro* and *in vivo*.

Materials and Methods

Animals and treatment

Female Buffalo rats (140–160 g) were housed, two animals per cage in a light/dark cycle (lights on 1000 h; lights off 2200 h) throughout the experiments. Food and water were available *ad libitum*. 7315b PRL-secreting rat pituitary tumor cells were grown on female Buffalo rats, as described previously (5). Rats were distributed randomly between the following experimental groups: control rats (n = 3) injected daily sc with 100 μl sesame oil (Fluka); control rats (n = 3) treated sc with 20 μg/day E_2-benzoate (15) in oil (Mycofarm Nederland BV; de Bilt); rats inoculated with 7315b tumor cells and injected daily sc with 100 μl sesame oil (n = 9) and rats inoculated with 7315b tumor cells and treated daily sc with 20 μg/day E_2-benzoate in oil (n = 9). Blood samples were taken, under anesthesia, from the orbital plexus at approximately 10.30 am on days 1, 5, 9, 12, and 15. Animals were killed after 8 (n = 3) and 15 (n = 6) days. Tumor tissue was frozen in liquid nitrogen and stored at −80 C until analysis.

Animals were kept, treated, and cared for in accordance with the guidelines approved by the European Community on November 24, 1986.

Cell culture

7315b PRL-secreting rat pituitary tumor cells were grown on female Buffalo rats (5). 7315b pituitary tumor cells were isolated by mechanical dispersion (6). The cells were used either directly after isolation or were precultured in MEM with Earle's salts, supplemented with nonessential amino acids, sodium pyruvate (1 mmol/liter), penicillin (10^5 U/liter), fungizone (0.5 mg/liter), L-glutamine (2 mmol/liter), sodium bicarbonate (2.2 g/liter final concentration), and either 10% FCS or 10% HS as indicated. The pH of the medium was adjusted to 7.4. The cells were cultured in a humid incubator at 37 C under 5% CO_2.

7315b Cells for binding studies were plated at a density of 10^6 cells/flask (Costar, Cambridge, MA) in 10-ml culture medium. Cells were reseeded every 5–7 days of culture at a concentration of 10^6 cells/flask. E_2 (Sigma Chemical Co., St. Louis, MO) and tamoxifen (Sigma) were added at concentrations as mentioned in the tables and figure legends. Cells were collected at different time points, washed twice with saline, and stored as pellets at −80 C until analysis.

In incubation studies without or with octreotide (Sandoz Pharma, Basel, Switzerland) and E_2, 40,000 cells (precultured for 7 days as mentioned in the figure legends) were seeded per well in 1 ml culture medium in 24-well plates (Costar). After 7 days of incubation, the cells, which did not attach to the wells, were harvested. The media plus cells were centrifuged for 5 min at 600 × *g*. Subsequently, the media were collected and stored at −20 C until analysis. The remaining cell pellets were washed with an ice-cold saline solution, centrifuged again, and the resulting cell pellets were extracted with 300 μl 1 M ammonia solution, 0.2% (vol/vol) Triton X-100 by sonification during 5 sec at amplitude 15 (Soniprep 150; Beun-de Ronde BV, Abcoude, The Netherlands). Thereafter, 2 ml assay buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.0) was added. The cell extracts were stored at −20 C until analysis of the PRL concentrations.

Assays

Rat PRL concentrations in the culture media, cell extracts, and blood samples were determined by a double antibody RIA, as described previously (16). Materials and protocols were supplied by NIDDK, as well as the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD).

E_2-levels in FCS, HS, and blood samples were determined by RIAs as described by Fauser *et al.* (17). Materials for E_2-measurements were supplied by Diagnostic Products Corporation (Los Angeles, CA).

Somatostatin receptor binding studies

The method of membrane isolation and the reaction conditions were the same as those described by Reubi (18). The radioligand used in the binding studies was the ^{125}I-labeled SS-analog Tyr^3-octreotide (SMS 204–090; Sandoz, Basel, Switzerland). Briefly, membrane preparations (corresponding to 30–50 μg protein) of frozen tumor samples, freshly dispersed tumor cells and of cells cultured in MEM with 10% FCS or 10% HS without or with E_2 were incubated in a total volume of 100 μl at room temperature for 60 min with 40,000 cpm (approximately 0.15 nM) radioligand and increasing concentrations of unlabeled Tyr^3-octreotide in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/liter bacitracin, pH 7.6) containing 0.2% BSA (Boehringer Mannheim B.V., Mannheim, Germany). After the incubation, 1 ml ice-cold HEPES buffer (pH 7.6) was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in an Eppendorf microcentrifuge (7). The remaining pellet was washed twice with ice-cold HEPES buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled Tyr^3-octreotide.

RT-PCR

Poly A^+ messenger RNA (mRNA) was isolated from frozen cell pellets using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). Approximately 10^6 cells were lysed during 1 min on ice in a buffer containing 10 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 5 mM KCl, 1% Triton X-100, 2–5 U/100 μl RNAsin (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 45 sec. 2× binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA, 0.4% LiDS) and 50–100 μl prewashed Dynabeads Oligo $(dT)_{25}$ were added to the supernatant. The mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed two times with 10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS and two times with a similar buffer in which LiDS was omitted. Poly A^+ mRNA was eluted from the beads in 30 μl of a 2 mM EDTA pH 7.5 solution during 2 min at 65 C.

Frozen tumor tissue (~200 mg) was grinded in a metal homogenizer that was cooled by liquid nitrogen. Total RNA was extracted from 100 mg frozen powdered tumor tissue using 2 ml TRIzol reagent (GIBCO BRL, Renfrewshire, Scotland, UK) according to the manufacturer's instructions. To remove DNA contamination, 30 μg total RNA was treated for 30 min at 37 C with 3 U RNase-free, DNase-I (Stratagene Cloning Systems, La Jolla, CA) in a buffer containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM $CaCl_2$ (final volume 25 μl). DNase-I activity was blocked by incubation with approximately 10 μg proteinase K (Boehringer Mannheim B.V.) for 60 min at 37 C in a final volume of 30 μl, in which the $CaCl_2$ concentration was adjusted to 5 mM. Poly A^+ mRNA

TABLE 1. Effects of tamoxifen on sst-expression in 7315b FCS cultured cells

Treatment	K_d (nM)	% increase in sst-number[a]
Control	0.80 ± 0.07	100
Tamoxifen (0.5 μM)	0.70 ± 0.0	50.5 ± 1.1

[a] The increase in receptor number between days 7 and 14 of culture was taken as 100% reference. Values are the means of two independent experiments ± SEM.

was isolated from 30 μg DNase-I pretreated total RNA, using Dynabeads Oligo$(dT)_{25}$ according to the manufacturer's instructions. For each poly A^+ mRNA isolation, 100 μl of the oligo-(dT)-beads suspension was used in a volume of 500 μl and the poly A^+ mRNA was finally eluted in 20 μl 2 mM EDTA (pH 8.0).

Complementary DNA (cDNA) was synthesized using 5 μl of poly A^+ mRNA and 5 μl oligo(dT) primer (GIBCO-BRL) in a buffer containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM DTT, 10 mM $MgCl_2$ and 1 mM of each deoxynucleotide triphosphate (final volume 20 μl). This mixture was incubated for 5 min at 65 C and cooled to 41 C. RNAsin (0.5 U) and AMV Super reverse transcriptase (2 U; HT Biotechnology Ltd., Cambridge, UK) were added, whereafter the mixture was incubated for 1 h at 41 C.

Two microliters from the cDNA reaction were used for amplification. The 50 μl reaction mixtures consisted of 2 μl cDNA template, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% Triton X-100, 50 μM of each deoxynucleotide triphosphate, 5 pmol oligonucleotide primers specific for each rat sst subtype, and 0.1 U of Super *Taq* DNA polymerase (HT Biotechnology Ltd). Primer pairs for the amplification of the rat sst_1, sst_2, sst_3 and sst_4 subtypes were those described by Wulfsen *et al.* (19), of which the sst_2 primers were adapted to the rat sequence. The human sst_5 primer sequences described by Kubota *et al.* (20) were adapted to the rat sequence to amplify rat sst_5. Primer pairs for rat actin were identical as described (21).

The PCR reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus Instruments, Gouda, the Netherlands). After an initial denaturation at 95 C for 5 min, the samples were subjected to 35 cycles of denaturation at 94 C for 1 min, annealing for 2 min at 59 C and extension for 1 min at 72 C. After a final extension for 10 min at 72 C, 10 μl aliquots of the resulting PCR products were analyzed by electrophoresis on 1% agarose gels. The calculated sizes of the PCR products are: $rsst_1$ 318 bp; $rsst_2$ 332 bp; $rsst_3$ 323 bp; $rsst_4$ 311 bp; $rsst_5$ 226 bp. All PCR products were sequenced with the sequenase PCR products sequencing kit (USB Amersham, Buckinghamshire, UK) to confirm their identity.

To ascertain that no DNA was present in the poly A^+ mRNA preparations, the cDNA reactions were also performed once without reverse transcriptase and amplified with primers specific for sst_3. Amplification of the cDNA samples with the β-actin specific primers served as a positive control for the quality of the cDNAs. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with the cDNA samples. As a positive control for the PCR reactions plasmid DNA containing the sst_{1-5} genes were amplified in parallel with the cDNA samples.

Receptor autoradiography

Receptor autoradiography was carried out as described by Reubi *et al.* (22). Briefly, the tissue samples were cut on a cryostat (Jung CM3000, Leica, Germany) in 10 μm sections. The sections were mounted onto precleaned gelatin coated microscope slides, and stored at −80 C. To wash out endogenous somatostatin, the sections were preincubated at room temperature for 10 min in 170 mM Tris-HCl pH 7.4. Thereafter, the sections were incubated for 60 min at room temperature in 170 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1% BSA, 40 μg/ml bacitracin in the presence of [^{125}I-Tyr^3]-octreotide (0.16×10^6 dpm/ml; about 80–160 pmol). Nonspecific binding was determined in a sequential section in the presence of excess unlabeled Tyr^3-octreotide (1 μM). The incubated sections were washed twice for 5 min in incubation buffer containing 0.25% BSA and once in incubation buffer without BSA. After a short wash with distilled water to remove salt, the sections were air dried and exposed to Hyperfilm-^{3}H (Amersham) for 1 week in x-ray cassettes. Histology was performed on hematoxylin-azophloxine stained sequential cryosections.

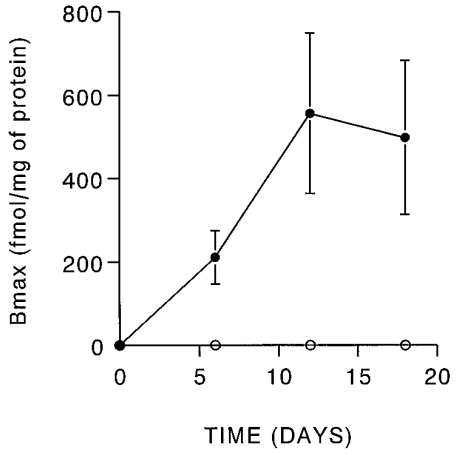

FIG. 1. Induction of sst expression in 7315b cells grown in MEM + 10% HS. 7315b Tumor cell-membranes (approximately 30 μg) were incubated with 0.15 nM [^{125}I-Tyr^3]-octreotide for 60 min at room temperature in the presence (+) or absence (-) of varying concentrations of unlabeled [Tyr^3]-octreotide. Receptor numbers were determined by Scatchard analysis. The figure shows the time course of the number of specific [^{125}I-Tyr^3]-octreotide binding sites measured on membranes of 7315b tumor cells that were grown for 6, 12, and 18 days in MEM + 10% HS (○) or in MEM + HS + 10 nM E_2 (●).

Analysis of data

Somatostatin receptor binding data were analyzed by the method of Scatchard (23). Statistical analysis of the data was performed by analysis of variance. When significant overall effects were obtained by analysis of variance, multiple comparisons were made using the Newman-Keuls test (24). All experiments reported in this study were carried out at least twice with similar results.

Results

Effects of E_2 on sst subtype expression in vitro

Our previous studies have shown that sst are undetectable on freshly dispersed 7315b pituitary tumor cells, whereas the number of sst increases when 7315b cells are cultured in medium supplemented with 10% FCS (7). To investigate the potential role of E_2 in this increase in sst number during culture, initial studies were performed by culturing the cells in charcoal-treated FCS. However, the 7315b tumor cells did not survive in this serum. Apparently, too many factors, essential for the growth of these cells, were extracted by charcoal-treatment of the FCS. On the other hand, the 7315b tumor cells grew well in medium supplemented with HS. In contrast to the increase in sst number by culturing the cells in MEM + 10% FCS, no binding of [^{125}I-Tyr^3]-octreotide could be measured when 7315b cells were cultured in MEM supplemented with 10% HS. Even after three weeks of culturing, no specific binding could be demonstrated (n = 5 experiments). When [^{125}I-Tyr^{11}]-SS14 was used as a radioligand also no specific binding was observed in HS-cultured cells, in contrast to FCS-cultured cells in which specific binding was found (data not shown). Measurement of E_2 levels in these sera showed a major difference between FCS and HS. FCS contains a 17 fold higher E_2-level compared with HS (FCS, 134 pM; HS, 8 pM).

The effect of the antiestrogen tamoxifen on sst expression was investigated by binding studies on membrane preparations using [^{125}I-Tyr^3]-octreotide. 7315b Tumor cells were

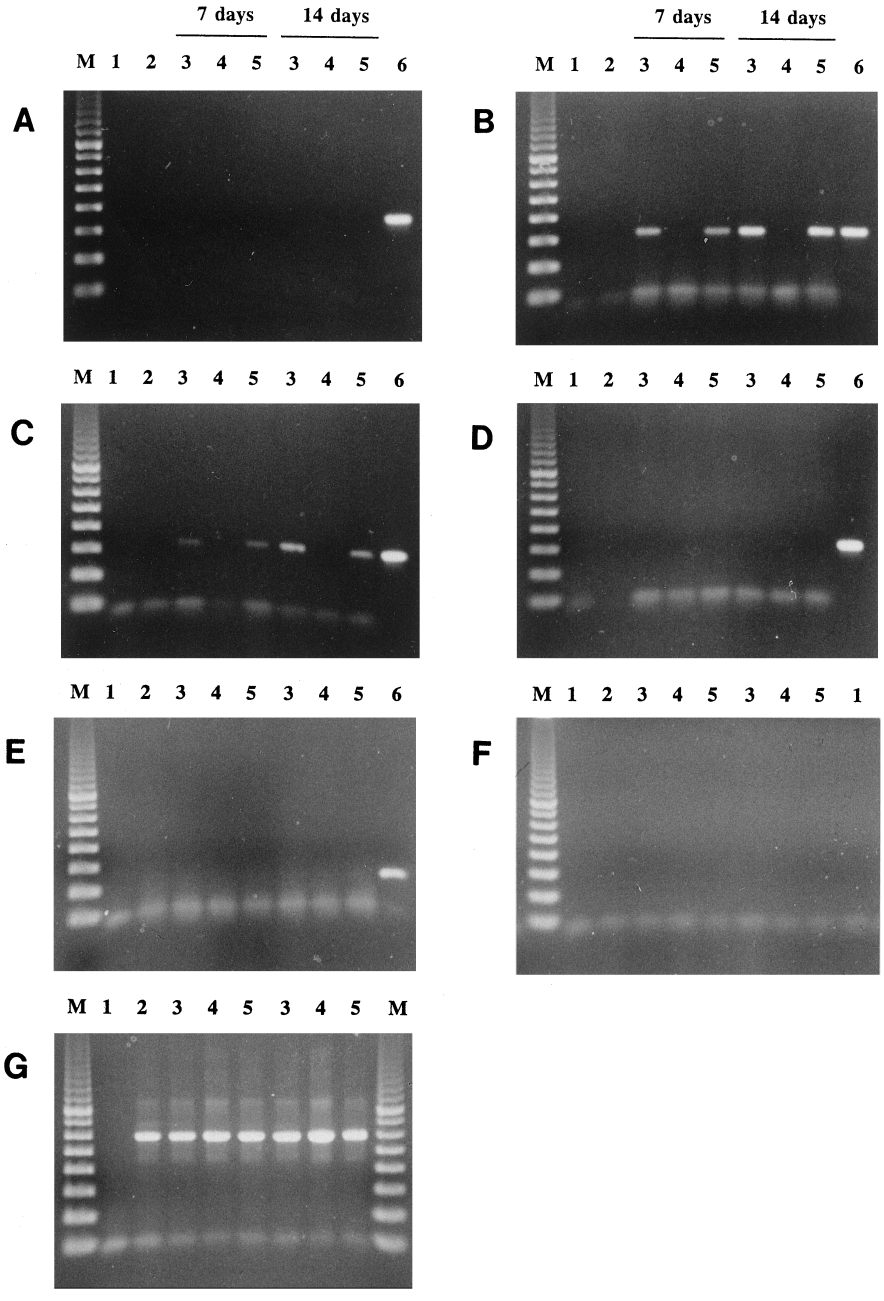

FIG. 2. E_2 induces the expression of the sst_2- and sst_3-subtypes in cultured 7315b prolactinoma cells. Poly A^+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the rat sst_{1-5} subtypes were separated on a 1% agarose gel and stained with ethidium bromide. A, $rsst_1$; B, $rsst_2$; C, $rsst_3$; D, $rsst_4$; E, $rsst_5$; F, cDNA reactions without reverse-transcriptase; G, rat β-actine. M, 100-bp ladder; 1, negative control (H_2O); 2, freshly dispersed 7315b tumor cells; 3, FCS cultured cells; 4, HS cultured cells; 5, HS + 10 nM E_2-cultured cells; 6, plasmid control.

cultured for 7 days in MEM + 10% FCS. After a medium change on day 7 of culture, the 7315b cells were reseeded at a concentration of 10^6 cells/flask and incubated without or with tamoxifen (0.5 μM) from days 7 to 14. Administration of 0.5 μM of the antiestrogen tamoxifen significantly inhibited the increase in sst number in FCS cultured cells from days 7 to 14 to 50.5% of the value of control cells that were cultured during this period in MEM + 10% FCS alone (Table 1). The dissociation constant was not significantly affected by this treatment. These results suggest that sst expression in 7315b rat pituitary tumor cells is stimulated by estrogens.

In Fig. 1, the effect of E_2 on sst expression in 7315b tumor cells grown in MEM + 10% HS is shown. 7315b Rat pituitary tumor cells were cultured in MEM + 10% HS without or with E_2 (10 nM) as indicated. While no sst were detectable in HS-grown cells, the addition of E_2 resulted in a significant, time-dependent increase of the number of sst. K_d values were not significantly different between the time-points studied and amounted 1.1 ± 0.3, 1.9 ± 0.5, and 1.2 ± 0.5 nM on days 6, 12 and 18, respectively.

Identification of sst subtype expression in cultured 7315b tumor cells

We determined the sst subtypes expressed in freshly dispersed and in cultured 7315b tumor cells by RT-PCR analysis. Poly A^+ mRNA was isolated from freshly isolated tumor cells and from 7315b cells cultured for 7 or 14 days in

MEM + 10% FCS; MEM + 10% HS; and in MEM + 10% HS + E_2 (10 nM). None of the five sst subtypes could be identified in freshly dispersed 7315b tumor cells (Fig. 2, panels A–E, no. 2), as was expected from the ligand-binding studies. Figure 2 (panels B and C, nos. 3 and 5) shows the specific expression of sst_2- and sst_3-mRNA in cells grown for 7 and 14 days in the presence of E_2, *i.e.* MEM + 10%FCS and MEM + 10%HS + E_2 (10 nM). sst_1, sst_4 and sst_5 mRNA were not detectable in the cultured 7315b cells (Fig. 2, A, D, and E). None of the known sst subtypes could be amplified in 7315b cells cultured in MEM + HS, which is in agreement with the results of the ligand-binding studies (Fig. 2, A–E, no. 4). These results point to an E_2-dependent expression of sst_2 and sst_3 in 7315b tumor cells.

All PCR products were sequenced to confirm their identity. As a negative control, the PCR was carried out with water instead of cDNA (Fig. 2, A–E, no. 1). To exclude the possibility that genomic DNA was amplified, a cDNA reaction was performed without reverse transcriptase. The subsequent PCR with primers for the rat sst_3 subtype is shown in Fig. 2F. As a positive control for the PCR reactions, plasmid DNA containing the sst_{1-5} genes were amplified in parallel with the cDNA samples (Fig. 2, A–E, no. 6). The rat actin cDNA (Fig. 2G) was amplified as a control to show the integrity of the cDNA reaction.

Effects of octreotide on PRL release and intracellular PRL concentrations of 7315b tumor cells

The effect of octreotide on PRL release and the intracellular PRL concentration by cultured 7315b tumor cells was studied in different culture conditions: 1) MEM + 10% FCS; 2) MEM + 10% HS; and 3) MEM + 10% HS + 10 nM E_2. 10 nM E_2 maximally stimulated PRL release by 7315b cells, as determined in a previous study (25). 7315b Rat pituitary tumor cells were precultured for 6 days in the above outlined culture media. The effects of a maximal inhibitory concentration of 1 μM octreotide (7) were investigated in 7-day incubation experiments without or with E_2 (Fig. 3). From days 6–13 of culture, octreotide significantly inhibited PRL release and intracellular PRL concentrations of 7315b cells cultured in MEM + FCS and MEM + HS + E_2 but not of cells cultured in HS alone. From these experiments, it becomes evident that estrogens influence the sensitivity of 7315b tumor cells to the SS-analog octreotide. It also shows that the increase in sst during culture in FCS or in HS + E_2 which we observed in the experiments described above reflects an increase in functional sst.

Effects of E_2 on sst subtype expression in vivo

As already described, cells isolated from the 7315b tumor are sst-negative, whereas the 7315b cells that were cultured in medium containing E_2 express a high number of functional high affinity sst. To study the differences between the *in vivo* and *in vitro* sst expression in more detail, the effect of reimplantation of precultured 7315b sst-positive cells in rats was examined. 95% of the sst had disappeared 9 days after inoculation of sst-positive cells *in vivo*, when the tumor was just palpable. sst had completely disappeared from the 7315b tumor 18 days after its inoculation (data not shown). From

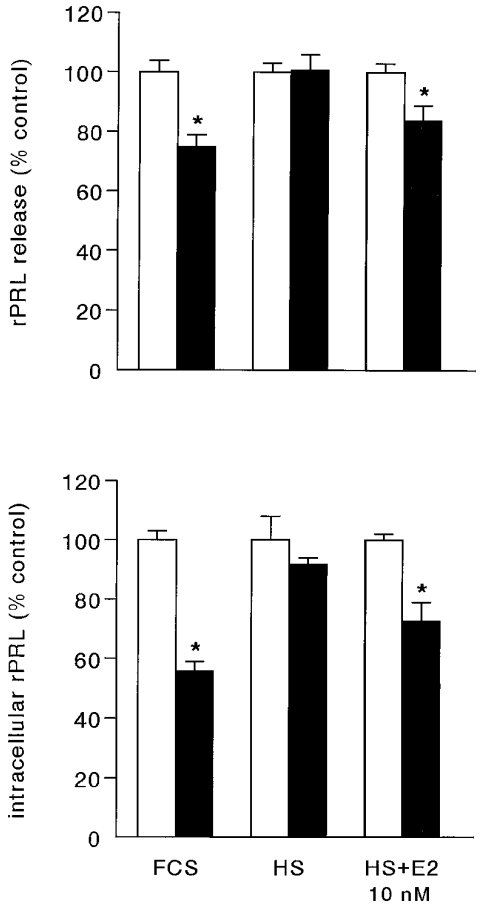

FIG. 3. Expression of functional sst's in 7315b tumor cells *in vitro*. Effect of octreotide (1 μM) on PRL release (*top*) and intracellular PRL concentration (*bottom*) of cultured 7315b rat pituitary tumor cells. 7315b Cells were cultured for 6 days in flasks in MEM supplemented with the on the x-axis mentioned additives. Thereafter, the cells were harvested and reseeded in 24-well plates with 40,000 cells/well per 1 ml, and subsequently incubated for 7 days in MEM with the aforementioned additives and with (*black bars*) or without (*open bars*) octreotide (1 μM). Cells without octreotide treatment were taken as 100% reference, *i.e.* PRL release 100% values correspond to FCS (992 ± 36); HS (335 ± 10); HS+E_2 (577 ± 19) ng/well per 7 days. Intracellular PRL concentration 100% values correspond to FCS (116 ± 4); HS (24 ± 2); HS + E_2 (52 ± 1) ng/well. Values are the mean ± SEM (n = 4 wells/group). *, $P < 0.01$ *vs.* control.

this experiment, it can be concluded that the *in vivo* environment inhibits sst expression in transplantable 7315b PRL-secreting pituitary tumors.

Because E_2 *in vitro* influences sst expression in 7315b tumor cells, it is hypothesized that the low circulating E_2-levels *in vivo* (the PRL hypersecretion of the 7315b tumor inhibits ovarian E_2-production) were responsible for the absence of sst on 7315b tumors *in vivo*. When tumor cells were inoculated in female rats, the serum E_2-level indeed dropped below the detection level in all rats (n = 6), 9 and 15 days after inoculation of tumor cells, whereas the PRL levels were high at that time (Table 2). Moreover, progesterone levels were also increased in 7315b tumor-bearing animals.

Additionally, we performed an experiment in which we treated rats daily with E_2-benzoate to supplement this low E_2-level. The sc administration of 20 μg/day E_2-benzoate to

TABLE 2. Serum PRL, E_2, and progesterone (prog) levels in 7315b tumor-bearing rats without or with E_2-treatment, 9 and 15 days after inoculation of tumor cells

	PRL (μg/liter)		E_2 (pmol/liter)		prog (nmol/liter)	
	9 days	15 days	9 days	15 days	9 days	15 days
7315b control	238 ± 67	1045 ± 67	Not detectable	Not detectable	162 ± 17	293 ± 24
7315b + E_2 (20 μg/day)	271 ± 54	709 ± 117	756 ± 65	657 ± 119	238 ± 13	167 ± 22

Serum hormone levels in control animals: PRL: 146 ± 53, E_2: 136 ± 61, prog: 46 ± 13. Not detectable means below the E_2-assay detection limit, which amounted 12 pmol/liter.

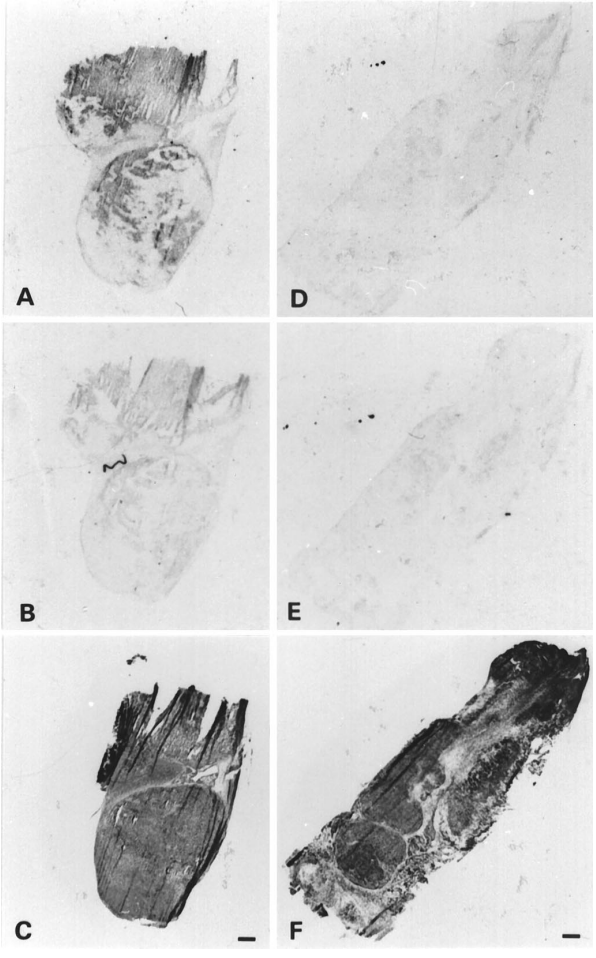

FIG. 4. sst distribution in 7315b tumors *in vivo* in E_2-treated rats. Representative autoradiograms showing total binding of [^{125}I-Tyr^3]-octreotide to a tumor section of an E_2-treated 7315b tumor-bearing rat (A) and the absence of binding in a section of a 7315b tumor of a control rat (D). B and E, Autoradiograms showing the nonspecific binding (in the presence of 1 μM Tyr^3-octreotide). C and F, Hematoxylin-azophloxine stained sections. *Bar*, 1 mm.

rats with the 7315b tumor revealed a relative constant circulating E_2-concentration of approximately 600 pmol/liter was measured in all six rats (Table 2). Rats treated with E_2-benzoate without the 7315b tumor showed a similar increase of the serum E_2-level.

The administration of E_2-benzoate to 7315b tumor-bearing rats induced sst expression in the tumors. Tumors isolated after 15 days, from the six animals that were treated with E_2-benzoate, showed specific [^{125}I-Tyr^3]-octreotide binding (see Fig. 4, A–C). The six 7315b tumor rats that were injected

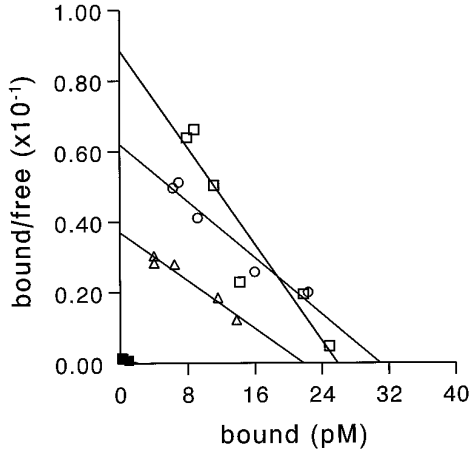

FIG. 5. E_2 induces sst expression *in vivo* in 7315b prolactinoma tumor-bearing rats. Scatchard analysis of [^{125}I-Tyr^3]-octreotide binding to 7315b tumor cell-membranes. This graph depicts binding data on membranes isolated from three E_2-treated 7315b tumors (□, ○, △). The K_d values of the E_2-treated rats are 0.5, 0.6 and 0.3 nM, respectively. No displacement could be detected on tumor cell-membranes isolated from a 7315b tumor-bearing control rat (■).

with sesame oil instead of E_2-benzoate showed no specific octreotide binding (Fig. 4, D–F). In an additional experiment, 7315b tumors were removed 8 days after inoculation when the tumors were palpable. Specific octreotide binding was already present in the E_2-treated animals (n = 3). No specific octreotide binding could be seen in rats treated with sesame oil during 8 days (n = 3). Histology of adjacent cryostat sections did not show necrosis in the tumors that were isolated after 8 days, whereas some necrotic foci were present in the large 15 days old tumors. This clearly demonstrates that the sst expression of the 7315b pituitary tumor *in vivo* is dependent upon the presence of E_2.

Binding of [^{125}I-Tyr^3]-octreotide to tumor cell-membranes isolated from three E_2-treated 7315b tumor-bearing rats was specific and Scatchard analysis of the binding data revealed a K_d value of 0.5 ± 0.1 nM and a number of 81 ± 12 fmol/mg protein. In contrast, no specific binding could be detected on tumor cell-membranes isolated from a 7315b tumor-bearing control rat (Fig. 5).

Identification of sst subtype expression in the 7315b tumors in vivo

7315b Tumors were isolated 8 (n = 3) and 15 (n = 6) days after inoculation from animals treated with E_2-benzoate and from 7315b tumor-bearing control rats. cDNA was synthesized from DNaseI pretreated poly A$^+$ mRNA and amplified

with specific primer pairs for the five rat sst subtypes. The results of the RT-PCR analysis are shown in Fig. 6. Panel B shows the specific expression of the sst_2-subtype in tumors, isolated 8 days after inoculation, from three E_2-treated animals (Fig. 6B, samples 5–7). The 7315b tumor-bearing control rats (low E_2-levels) do not express this sst_2-subtype (Fig. 6B, samples 2–4). None of the other subtypes could be amplified in the 7315b tumors *in vivo* (Fig. 6, A, C–E). Tumors isolated 15 days after inoculation showed a similar expression pattern (data not shown). Figure 6D, lane 4, shows a very weak sst_4 band. However, in none of the other tumors of 7315b tumor-bearing animals, without or with E_2-treatment, sst_4 expression was found. Therefore, expression of the sst_2 subtype in the 7315b tumor *in vivo,* is in agreement with E_2-stimulated 7315b cells *in vitro*, E_2-dependent.

Discussion

In the present study, we have investigated the regulation of sst subtype expression in the model of the rat PRL-secreting pituitary tumor 7315b. Initial studies revealed that 7315a and 7315b tumor cells, isolated from transplanted tumors in

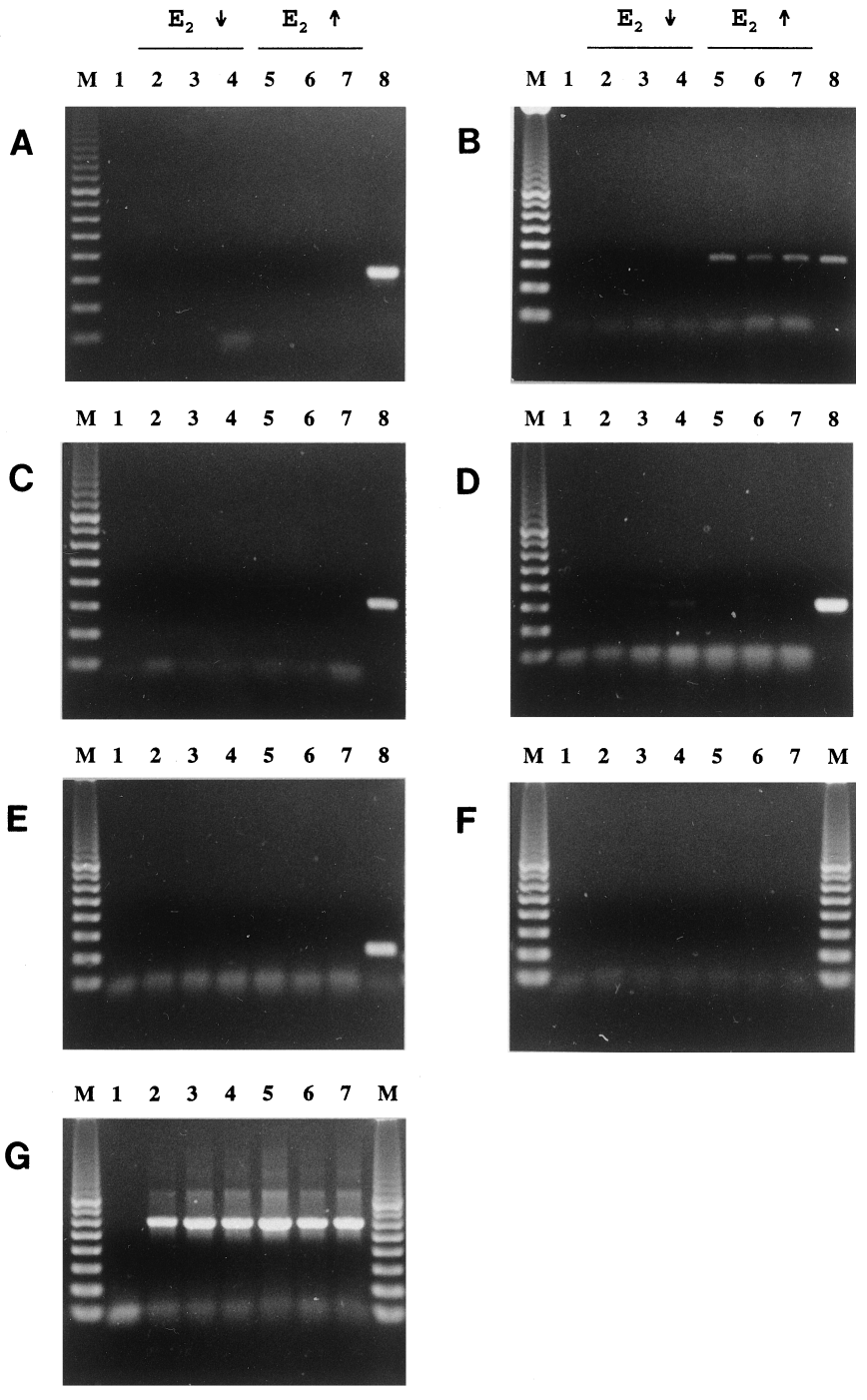

FIG. 6. E_2 induces the expression of the sst_2-subtype in 7315b prolactinoma cells *in vivo*. Poly A^+ mRNA was isolated from 7315b tumors and reverse transcribed. The resulting cDNA was amplified by PCR. PCR products of the rat sst_{1-5} subtypes were separated on a 1% agarose gel and stained with ethidium bromide. A, $rsst_1$; B, $rsst_2$; C, $rsst_3$; D, $rsst_4$; E, $rsst_5$; F, cDNA reactions without reverse-transcriptase; G, rat β-actine. M, 100 bp ladder; 1, negative control (H_2O); 2–4, 7315b tumor-bearing control rats; 5–7, E_2-treated 7315b tumor-bearing rats; 8, positive plasmid control.

Buffalo rats, were sst-negative (5). This *in vivo* observation contrasted with *in vitro* studies in which 7315b cells were cultured for 7 days or longer in medium that was supplemented with FCS: under these conditions sst became detectable (7). At that point, it was unclear which factor(s) induced sst expression *in vitro* or inhibited *in vivo* expression. Additional observations from the present study demonstrate that no sst expression occurs when 7315b rat pituitary tumor cells are cultured in medium supplemented with HS instead of FCS. A major difference was found between the E_2 level in both serum types, suggesting that this steroid may play an important role in the regulation of sst expression in 7315b tumor cells.

To obtain further evidence for this hypothesis, we studied the effects of pharmacological concentrations of the antiestrogen tamoxifen on sst expression in 7315b cells grown in FCS. The fact that the antiestrogen inhibited sst expression indirectly provides evidence for a stimulatory effect of estrogens on sst expression. The influence of estrogens on sst expression was further demonstrated in the reverse experiment, in which E_2 was added to 7315b rat pituitary tumor cells which were cultured in medium with HS. A physiologically normal level of estrogen in the culture medium was at all times accompanied with expression of sst in 7315b rat pituitary tumor cells. From these data, it can be concluded that sst expression in 7315b rat pituitary tumor cells is primarily dependent upon the presence of estrogens.

The *in vitro* model system of the PRL-secreting 7315b tumor, in which the number of sst can be manipulated, enabled us to study sst function. We found, in agreement with our earlier experiments (7), a close relation between the absence or presence of sst and the appearance of a biological effect. The functionality of these receptors was substantiated in experiments in which it was demonstrated that octreotide inhibits PRL release and the intracellular PRL concentration only from 7315b cells that were cultured in medium that was supplemented with FCS or with HS in combination with E_2. The RT-PCR data suggest that the sst_2- and/or sst_3-subtypes are involved in the antihormonal action of this SS-analog.

Other studies also support the concept of E_2 as an important regulator of sst expression. An E_2-dependent sst subtype was characterized in normal rat anterior pituitary cells by Kimura *et al.* (11, 26). Their studies revealed that E_2 regulates the sensitivity of hormone secretion by lactotrophs to SRIF by increasing the number of sst on these cells. Recently it was shown that E_2 up-regulates the expression of $sst_{1,2,3}$ in GH_4C_1 cells, although the authors did not study the expression of the sst_4 and sst_5 subtypes (27).

The results of our RT-PCR studies are in agreement with the aforementioned concept. First, none of the currently known five sst subtypes was present in the isolated 7315b tumor cells ($t = 0$). The rapid appearance of both sst_2- and sst_3-mRNA, in 7315b tumor cells that were cultured in medium containing E_2 point to a combination of receptor subtypes instead of a particular sst. It is likely from our experiments that *de novo* synthesis is the mechanism behind the E_2-induced up-regulation of sst number in 7315b rat pituitary tumor cells instead of receptor recruitment from an intracellular storage compartment to the plasma membrane (28). Further studies are needed to determine the precise mechanism of regulation of sst expression by E_2.

Taken into account that the 7315b tumor secretes huge amounts of PRL, and the fact that ovarian estrogen production is inhibited by a high PRL level, we hypothesized that the circulating E_2-level *in vivo* might be too low to induce sst expression. We therefore manipulated the circulating E_2-level in rats bearing the 7315b tumor.

We showed that inoculation of sst positive 7315b cells in female Buffalo rats rapidly results in a disappearance of sst. As hypothesized above, a probable explanation for this phenomenon might be that the high circulating PRL concentrations (1045 ± 67 μg/liter, 15 days after inoculation of tumor cells) produced by the tumor suppress estrogen production by the ovaries of the tumor-bearing rats. These PRL data are in agreement with a previous study (5). Due to the lowered circulating estrogen levels no sst are expressed any more on the 7315b tumor *in vivo*. The E_2-concentration in serum of tumor-bearing rats indeed dropped below the detection level at 9 days after inoculation of 7315b tumor cells, whereas the E_2-level in the normal cycling female rat serum varied between 75 and 197 pmol/liter on day 1.

We indeed found that *in vivo* administration of 20 μg/day of E_2-benzoate induced sst expression in all E_2-treated 7315b tumor-bearing animals. Ligand-binding studies on membrane preparations of the tumors of E_2-treated rats revealed the presence of high affinity sst with K_d values in the nanomolar range. No specific binding could be detected in tumors of the untreated 7315b tumor bearing rats. In addition, sst could also be visualized on tissue sections of E_2-treated 7315b tumors by *in vitro* sst autoradiography.

The transplantable 7315b prolactinoma tumor is well bounded, which makes it easy to be isolated and is an advantage for the RT-PCR analysis. In this respect, it is less probable to obtain false positive mRNA signals due to surrounding normal tissue or distant blood vessels (particularly veins) as described by Reubi *et al.* (29).

The expression of the sst_2-subtype mRNA was induced by E_2-treatment of the 7315b tumor-bearing rats as determined by RT-PCR analysis. This is in analogy to the *in vitro* study. However, RT-PCR analysis of *in vitro* cultured 7315b cells (in the presence of E_2) also revealed the expression of the sst_3-subtype. It seems therefore that the *in vitro* stimulus of E_2 on sst expression is stronger than *in vivo*. The high circulating PRL concentrations *in vivo* also induce high circulating progesteron-levels (a 6-fold increase as compared with control, nontumor-bearing rats, 15 days after inoculation of tumor cells). Progesteron, however, down-regulates the nuclear estrogen receptor (30). As a result of this down-regulation, it is possible that E_2 is less effective in inducing sst expression *in vivo* compared with the *in vitro* culture conditions. Further studies are needed to elucidate this point, as well as to quantify the relative abundance of sst_2 and sst_3 mRNA levels by quantitative methods like RNase protection assay or quantitative RT-PCR.

All five sst subtypes are expressed in the normal rat pituitary (31). In the human pituitary adenomas studied so far, multiple sst subtypes are also identified (32–36). Unfortunately, the different studies report conflicting results with respect to the sst subtype expression in human prolactino-

mas. No sst_2 expression was found in prolactinomas in one study, using RNase protection assays (32), whereas other studies showed a predominant expression of this subtype by RT-PCR analysis (35, 36).

From the results of this study, it is clear that the 7315b tumor is able to indirectly manipulate (down-regulate) its own sst expression *in vivo* via its host. Figure 7 provides a model of the *in vivo* and *in vitro* events regulating sst subtype expression in the PRL-secreting rat pituitary tumor. The mechanism that the transplantable 7315b tumor regulates and suppresses its own expression of sst via its host might also occur in other tumors. For example, recent data suggest that elevated circulating cortisol levels suppress sst expression on ACTH-secreting pituitary tumor cells of patients with Cushing's disease, whereas these receptors become evident only after bilateral adrenalectomy and/or pretreatment with high doses of RU 38486 (37).

The experimental 7315b prolactinoma model might also be representative for the situation encountered in most untreated female prolactinoma patients. Octreotide was shown to be unable to suppress circulating PRL levels in patients with microprolactinomas (38). Also, *in vitro* binding studies using [^{125}I-Tyr^3]-octreotide demonstrated that four of five prolactinomas did not contain sst (39), whereas in another study using [^{125}I-Tyr]-SRIH eight out of nine prolactinomas contained only a very low number of sst binding sites (40). In line with these observations, we were unable to visualize the tumor tissue of three female microprolactinoma patients *in vivo* with sst scintigraphy (41). In analogy to the 7315b pituitary model system, lowered E_2 levels might account for the absence of sst expression on the prolactinomas of untreated female patients.

Studies on the manipulation of sst numbers might be of clinical value. A wide variety of human tumors express sst (see Refs. 2 and 42 for recent reviews). The expression of a high density of high affinity somatostatin membrane receptors on these tumors made it possible to develop the technique of *in vivo* sst scintigraphy. After injection of isotope-coupled octreotide, primary sst positive tumors, as well as their often unrecognized metastases, are visualized in most patients with neuroendocrine tumors. Currently, we evaluate the possibility to carry out radiotherapy, as well as radioisotope guided surgery in patients with sst-positive tumors (3, 42, 43). Especially for these procedures, a transient up-regulation of sst numbers on tumors might improve the efficacy of these procedures.

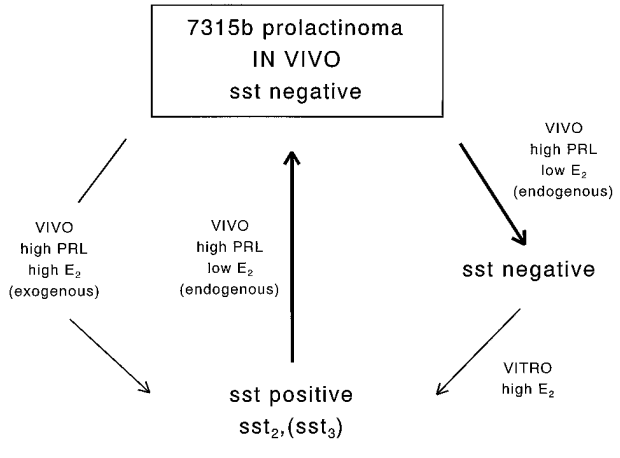

FIG. 7. Model of estrogen dependent regulation of sst subtype expression in 7315b rat prolactinoma cells *in vivo* and *in vitro*.

In conclusion, our studies have demonstrated that sst subtype expression in the 7315b rat prolactinoma tumor, both *in vitro* and *in vivo*, is dependent upon the presence of estrogens. The 7315b tumor cells *in vivo* may manipulate their own sst status via their host because the ensuing hyperprolactinemia results in a hypo-estrogenic state.

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