[CANCER RESEARCH 61, 632–640, January 15, 2001]

# Expression of Estrogen Receptor (ER) Subtypes and ERβ Isoforms in Colon Cancer[1]

**Martha Campbell-Thompson,[2] I. Jeanette Lynch, and Bhavna Bhardwaj**

*Department of Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610*

## ABSTRACT

**Colon cancer incidence and mortality rates are lower in females compared with males, and numerous epidemiological studies suggest that estrogen replacement therapy (ERT) reduces cancer risk in postmenopausal women. Two estrogen receptor (ER) subtypes, ERα and ERβ, mediate genomic effects in target cells. The aim of this study was to determine the relative mRNA expression levels for ER subtypes and ERβ isoforms in colon tumors, normal colonic mucosa, and colon cancer cell lines. ERα and ERβ isoform mRNA levels were investigated in paired samples of colon tumors and normal mucosa from 26 patients using comparative reverse transcription-PCR and then Southern analyses. Constitutive steroid hormone receptor mRNA levels were determined for five colon adenocarcinoma cell lines using reverse transcription-PCR, and ERβ levels were further studied in Caco-2 cells using Northern and Western analyses. ERβ mRNA steady-state levels (relative to glyceraldehyde-3-phosphate dehydrogenase mRNA) were significantly decreased in colon tumors compared with normal mucosa in female patients. ERβ1 and ERβ2 isoform mRNA levels were significantly decreased in tumors from female patients, and ERβ1 mRNA levels were also significantly lower in tumors from female patients compared with tumors from males. ERα mRNA levels were much lower than ERβ levels and were similar between normal mucosa and tumor samples in both genders. ERβ mRNA was detected in Caco-2, T84, and SW1116 cell lines and all lines were essentially negative for ERα mRNA. Caco-2 cells coexpressed ERβ1, ERβ2, and ERβ5 mRNA, though a single protein transcript was observed. ERβ protein was detected in normal colonic superficial epithelium, vascular smooth muscle and endothelium, and enteric neurons by immunohistochemistry. These data show that ERβ is the predominant ER subtype in the human colon and that decreased levels of ERβ1 and ERβ2 mRNA are associated with colonic tumorigenesis in females. This information suggests that activation of ERβ-mediated processes in the superficial colonic epithelium may have a role in the preventive effects observed for female gender and ERT usage.**

## INTRODUCTION

Colorectal (colon) cancer is the second most common cause of cancer death in the United States (1). It is predicted that ~130,000 new cases of colon cancer will be diagnosed and about ~50,000 people will die each year from colon cancer. Mortality rates for colon cancer have fallen during the past 20 years because of early detection from increased screening. Colon cancers include nonhereditary and hereditary types. Sporadic colon cancers comprise the vast majority of cases, and incidence rates increase logarithmically after age 40. Hereditary colon cancers include familial adenomatous polyposis and HNPCC.[3]

Human colon cancers undergo a multistage carcinogenesis pathway from adenomatous polyps to carcinoma (reviewed in Refs. 2 and 3). A number of genetic events have been characterized and include alterations in "tumor suppressor" and susceptibility genes that normally encode for proteins regulating cell cycle progression and programmed cell death (4). The *adenomatous polyposis coli* gene and *mismatch repair* genes are altered early in the neoplastic process, either as inherited or somatic mutations. Additional somatic mutations in the transforming growth factor β receptor, the K-*ras* oncogene and the deleted in colon cancer and *p53* tumor suppressor genes may occur during further progression. The probability that a normal colonic epithelial cell will acquire a somatic gene alteration is low, but ~50% of the population at 70 years of age will develop a polyp of which ~5% are expected to develop into adenocarcinomas (4). Patients with HNPCC develop polyps that often progress to cancers because of defective DNA *mismatch repair* genes that result in increased mutation rates.

Given the high incidence of colon cancer in the aging population and high mortality rates for advanced disease, new prevention strategies are needed. A possible protective effect for estrogens on colon cancer risk has been suggested by numerous epidemiological and experimental studies. At all ages, women are less likely than men to develop colon cancer (5–7). Male rodents have higher aberrant crypt or tumor formation rates compared with females in several colon cancer models (8–10). The protective effects of female hormones are also evident in families with HNPCC, because the lifetime risk of developing colon cancer is significantly lower in females than in males (30% *versus* 74%, respectively; Ref. 11). Preliminary data have been reported as well in the mouse colon cancer model for familial adenomatous polyposis that show reduced tumor numbers in intact females compared with ovariectomized females (12).

ERT (alone or in combination with progestins) is estimated to reduce colon cancer risk by 30–40% in postmenopausal women (13–17). In a recent review of 30 studies including case-controls or cohorts with 3 meta-analyses, 23 studies reported a protective effect, whereas only 1 study reported an adverse effect of ERT (18). The majority of studies from 1995 or later showed protective effects, and these studies tended to control for more confounding variables such as aspirin use or smoking. The risk reductions were generally similar among recent ERT *versus* those who were on ERT for >5 years. The protective effects of ERT on incidence and size of polyps have also been reported (19–21). Some investigators attribute the greater decline in colon cancer mortality rates in aged women compared with men to the increased use of ERT since 1990 (16, 17).

Estrogens modulate sexual development and reproductive functions in addition to effecting the cardiovascular and central nervous systems and bone (reviewed in Ref. 22). Genomic effects of estrogens are mediated by at least two related members of the steroid receptor superfamily, ERα and ERβ. ERs act as ligand-activated transcription factors and modulate gene expression by interactions with promoter response elements or other transcription factors (23). Studies are uncovering a diversity of functions for each ER subtype. With either ER subtype, transactivation at an estrogen response element is similar between 17β-estradiol and the antiestrogens tamoxifen and raloxifene. At an activating protein 1 response element, 17β-estradiol increases

Received 3/24/00; accepted 11/14/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a grant from the American Cancer Society, Florida Division (to M. C-T.). Preliminary results were presented at annual meetings for the American Gastroenterological Association (see Gastroenterology, *116*: A595, 1999) and the American Association for Cancer Research (see Proc. Am. Assoc. Cancer Res., *41*: 139, 2000).

[2] To whom requests for reprints should be addressed, at Box 100214 JHMHC, Department of Medicine, College of Medicine, University of Florida, Gainesville, FL 32610. Phone: (352) 392-9621; Fax: (352) 392-3618; E-mail: thompmc@medicine.ufl.edu.

[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; ER, estrogen receptor; ERT, estrogen replacement therapy; GI, gastrointestinal; UF, University of Florida; NCI, National Cancer Institute; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; TBS, Tris-buffered saline.

reporter activity with ERα but inhibits it with ERβ. However, with the antiestrogens, transactivation via the activating protein 1 element is decreased with ERα and increased with ERβ. These interactions are further compounded by the ability of ER subtypes to form homodimers (α/α, β/β) or heterodimers (α/β) and by cell-type specific expression of ER coactivators and corepressors (24–29).

To date, few studies have examined expression of ERβ in the GI tract. Studies from this laboratory using Northern analysis showed that ERβ mRNA was expressed as multiple transcripts and in greater abundance than ERα in the rat upper GI tract (30). Both ERα and ERβ mRNA were detected in the epithelium of the stomach and upper intestine by RT-PCR. Enmark *et al.* (31) reported that ERβ mRNA was detected by RT-PCR throughout the human GI tract including colon. By *in situ* hybridization, ERβ mRNA was localized in the GI epithelium, whereas the muscle layers were devoid of staining. In the midgestational human fetus, ERα and ERβ mRNAs were coexpressed in stomach and colon with lower levels in small intestine, as determined using RT-PCR (32). Moore *et al.* (33) described five ERβ isoforms with different COOH-terminal domains attributable to differential splicing at the exon 7–8 junction. Some nucleotide sequences were homologous between ERβ2, ERβ4, and ERβ5 isoforms in the 3′ region of exon 8. In normal human colon, ERβ1, ERβ2, and ER5 mRNA were detected (33). In contrast, three colon cancer cell lines expressed only ERβ2 and ERβ5 mRNA. ERβ3 and ERβ4 mRNA were detected only in testis. Fiorelli *et al.* (34) reported expression of ERβ1 mRNA in colon cancer cell lines and ERβ2–5 mRNA in HCT8 and HCT116 lines. Arai *et al.* (35) also reported that colon cancer cell lines express ERβ mRNA but did not study isoform levels.

The potential coexistence of ER subtypes and ERβ isoforms increases the degree of complexity for determining ER-mediated functions. Analyses for ER subtypes in normal colon would be important for understanding mechanisms for potential protective effects of estrogens on colon cancer risk. Given the scarcity of information regarding ER mRNA expression levels in human colon, this study was performed to determine the relative mRNA levels of ER subtypes and ERβ isoforms between paired samples of normal human colonic mucosa and colon tumors. ERβ mRNA and protein levels were also analyzed in several colon cancer cell lines to determine whether ERβ expression patterns were similar to normal colon. Immunohistochemistry was used to localize cell-specific distribution for ERβ protein in normal colon.

## MATERIALS AND METHODS

**Patient Samples and Cell Lines.** Matched surgical samples of normal colonic mucosa and colonic tumors were obtained from 26 patients during 1995–1999 (Table 1). Tumor samples included 3 polyps and 23 adenocarcinomas (14 moderately differentiated). The normal mucosa was harvested adjacent to the tumor or from distal resection margins. All patients gave informed consent in accordance with the University of Florida Institutional Review Board or other institutions using Declaration of Helsinki guidelines. Samples were provided by the University of Florida Cancer Tissue Bank (UF, $n = 14$) or purchased from the National Cancer Institute Cooperative Human Tissue Network (NCI, $n = 12$). Patients were randomly selected from each source by gender without exclusion for race. Medical histories were not obtainable, however; only one female patient was 34 years of age (potentially premenopausal), whereas the remainder were >52 years of age. One male patient was 38 years of age and the remainder were >48 years of age. After dissection by the pathologist, portions of tumor and the normal mucosa were frozen promptly and stored in liquid nitrogen. Ten samples from UF were stored for 2–3 years before assay, whereas other samples were stored for 2–8 months before assay. Histopathology confirmed that the tumor samples were comprised of ~90% tumor cells, and that the normal mucosa samples did not contain pathology.

Human adenocarcinoma cells lines from colon (HT-29, Caco-2, T84, SW1116, and SW48) and breast (MCF-7) were obtained from the American Type Culture Collection. Cell lines were maintained in DMEM/Ham's F-12 (1:1) media supplemented with 10 mM glutamine, antibiotics (penicillin, streptomycin), and 5–10% fetal bovine serum (Hyclone) in a humidified atmosphere of 95% $O_2$-5% $CO_2$ at 37°C. Cells were grown to 80–90% confluency in phenol red-free DMEM media supplemented with 5% dextran charcoal-stripped fetal bovine serum for at least 48 h before RNA preparation.

Table 1 *Patient demographics (n = 26) and tumor classification by polyp or adenocarcinoma with differentiation status*

| | Female | Male |
|---|---|---|
| No. of patients | 15[a] | 11[b] |
| Age | | |
| Mean ± SD | 67.7 ±14 | 62.9 ±14 |
| Range | 34–89 | 38–91 |
| Race | | |
| Caucasian | 11 | 9 |
| African-American | 2 | 0 |
| Unknown | 2 | 2 |
| Tumor classification | | |
| Polyp | 3 | 0 |
| Adenocarcinoma (differentiation) | | |
| Poor | 2 | 2 |
| Moderate | 7 | 7 |
| Mucinous | 1 | 0 |
| Unknown | 2 | 2 |

[a] Sources, UF = 8, NCI = 7
[b] Sources, UF = 6, NCI = 5

**Oligonucleotide Primers.** Oligonucleotide primer pairs were designed for ERα, ERβ, PR, VDR, and GAPDH using published literature or sequence information contained in the National Center for Biotechnology Information GenBank database with OLIGO 4.0 software (National Biosciences, Plymouth, MN; Table 2). Oligonucleotide primers were tested using BLAST software to confirm gene specificity and to determine exon locations (36). ERβ primer sets were designed to detect a region of the $NH_2$ terminus that is shared by all isoforms or to detect specific exon 8 sequences to differentiate ERβ1 from ERβ2, ERβ4, and ERβ5 isoforms. To compare mRNA levels on a semiquantitative level, the ER primer pair efficiencies were first tested using full-length human cDNA as templates. PCR amplifications were performed in parallel using 10-fold dilutions of each respective template. Linearity of the integrated density signal for patient sample ER and GAPDH was tested using a range of cDNA template amounts. PCR primer pairs were selected that showed linear amplification rates using 30 (GAPDH) or 45 (ER) amplification cycles at a 55°C annealing temperature. PCR products from two to three tissue samples were cloned to verify sequence identity. Nucleotide sequences were determined by automated sequencing at the University of Florida Interdisciplinary Center for Biotechnology Research.

**RNA Isolation and Northern Analysis.** Total RNA was prepared using a modified guanidine thiocyanate-phenol-chloroform extraction method with two precipitations in isopropanol and ethanol as described previously (37). Patient samples (~0.5 g) were pulverized in a mortar and pestle cooled with liquid nitrogen and homogenized in guanidine thiocyanate. Cancer cells were washed with PBS and guanidine thiocyanate was added to the culture dish. Poly(A)$^+$-select mRNA was prepared from Caco-2, HT29, and MCF-7 cells and normal colon samples using poly-dT conjugated magnetic beads (PolyATtract kit, Promega) as described (30). RNA concentration and quality were assessed by spectrophotometric readings at 260 and 280 nm. Samples of total RNA or poly(A)$^+$mRNA were fractionated on a 6% formaldehyde-1.2% agarose gel and photographed after ethidium bromide staining. The RNA samples were transferred to nylon membranes using overnight capillary blotting in 20× SSC and were covalently cross-linked to the membrane with UV light.

Membranes were prehybridized for 15 min at 62°C in a solution containing 62 mM Na $PP_i$ (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA in a rotating oven (38). Human cDNA fragments were isolated by restriction enzyme digestion and gel electroelution before labeling with $^{32}$P-dATP by random primer extension (Decaprime, Ambion). The probes were denatured by boiling for 10 min and then added to the prehybridization solution. The membranes were hybridized overnight at 62°C and washed twice in 40 mM Na $PP_i$ (pH 7.2), 1 mM EDTA, and 5% SDS for 15 min at 65°C. Autoradiography was performed

Table 2 *Oligonucleotide primer sequences for comparative RT-PCR*

| Gene | Primer | Primer Sequence (5′–3′ nucleotide numbers) | Exon | Expected size in bp (exons) | Sequence accession no. |
|---|---|---|---|---|---|
| *ERα* | Sense | ATGTGGGAGAGGATGAGGAG (1135–1154) | 4 | | X03635 |
| | Antisense | AACCGAGATGATGTAGCCAGCAGC (1664–1641)[a] | 7 | 530 | |
| *ERβ* | Sense | TAGTGGTCCATGGCCAGTTAT (684–704) | 1 | | AF051427 |
| | Sense | GGTCGTGTGAAGGATGTAAGG (909–929) | 2 | | |
| | Antisense | GGGAGCCACACTTCACCAT (1076–1058) | 4 | 393 (1–4) | |
| | Sense | TGCTTTGGTTTGGGTGATTGC (1723–1743)[b] | 7 | | |
| *ERβ1* | Antisense | TTTGCTTTTQCTGTCCTCTGC (1981–1961)[b] | 8 | 259 (7–8) | |
| *ERβ2*[c] | Antisense | ATGAGGTGAGTGTTTGAG (1856–1836)[d] | 8 | 134 (7–8) | AF051428 |
| *ERβ2*[e] | Antisense | TGCTCCATCGTTGCTTCAGGC (1902–1882) | 8 | 925 (2–8) | |
| | | | | 179 (7–8) | |
| *PR* | Sense | GCATGTCAGTGGGCAGATGCT (3762–3781)[f] | 5 | | X51730 |
| | Antisense | AACTTCAGACATCATTTCCGG (4190–4173)[f] | 7 | 432 | |
| *VDR* | Sense | CTGACCCTGGAGACTTTGACC (32–52) | 2 | | AF026260 |
| | Antisense | TTTTGGATGCTGTAACTGACC (719–699) | 6 | 688 | |
| *GAPDH* | Sense | TCATCATCTCTGCCCCCTCTG (3751–3771) | 5 | | J04038 |
| | Antisense | GCCTGCTTCACCACCTTCTTG (4474–4454) | 7 | 439 | |

[a] PCR primer adapted from Ref. 70.
[b] PCR primer adapted from Ref. 71.
[c] Homologous with regions in ERβ4 (AF061054). See also Fig. 3.
[d] PCR primer adapted from Ref. 71.
[e] Homologous with regions in ERβ4 and ERβ5 (AF061055). See also Fig. 3.
[f] PCR primer adapted from Ref. 72.

at −75°C with an intensifying screen for 0.5–5 days. Membranes were stripped with 0.5× SSC-0.5% SDS at 95°C between hybridizations.

**Comparative RT-PCR and Southern Analysis.** To compare the relative abundance of specific mRNAs, equal amounts of total RNA from groups of 5–9 patients or from cell lines were transcribed into cDNA in parallel to insure similar conditions. Random hexamer-primed cDNA synthesis was performed using 2 μg total RNA in a final volume of 20 μl as described (30). Reverse transcription reactions were carried out at 42°C for 1 h and inactivated at 99°C for 5 min.

Comparative PCR was performed as described previously, with modification (30, 39). GAPDH mRNA was first amplified at a low cycle number as an internal standard before gel electrophoresis and photography. If needed, cDNA dilutions were adjusted and GAPDH levels reamplified with the aim to produce similar intensities for GAPDH signals between samples. A master PCR solution was made consisting of diluted Taq polymerase, buffer, and deoxynucleotide triphosphates (Idaho Technology, Idaho Falls, ID). Aliquots of the master PCR solution were added to microfuge tubes, on ice, containing sufficient volumes of sample cDNA and water to create a master sample solution for amplification of four to five genes. After mixing, 8-μl aliquots from each master sample solution were placed in cold tubes and 2 μl of primers were added (final, 5–10 pM). The cDNAs were amplified by PCR in a DNA air thermo-cycler programmed to heat to 94°C for 30 s, and then 45 cycles (94°C for 0 s, 55°C for 0 s, 72°C for 30 s). The reaction tubes for GAPDH were removed at 30 cycles and stored at −20°C until analysis. Aliquots (8.5 μl) of the PCR reactions were analyzed by electrophoresis in a 3% agarose gel and photographed. PCR products were transferred to a membrane, as described, for Northern analysis. Membranes were hybridized with $^{32}$P-labeled human cDNAs before autoradiography. Control amplification reactions were run concurrently. Positive controls were performed using reverse transcription reactions from cell lines or plasmid-derived cDNA inserts as templates. Negative control reactions for each primer pair contained the master PCR solution with water substituted for cDNA template.

**Western Analysis.** Cells were grown in dishes and washed with PBS. Cell lysates were harvested in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; and 1 μg/ml aprotinin]. Human colon and rat ovaries were pulverized and homogenized as described (40). Protein concentration was determined by bicinchoninic acid analysis (Pierce) using BSA as a standard. Protein samples were boiled in Laemmli sample buffer containing β-mercaptoethanol (Bio-Rad, Hercules, CA) for 5 min before electrophoresis by 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. After staining with Ponceau S dye, the membrane was blocked with 5% nonfat dry milk in TBS [10 mM Tris-HCl (pH 7.4), 150 mM NaCl] for 2–4 h at room temperature and incubated with affinity-purified rabbit polyclonal antihuman ERβ antisera (PA1–311, 1 μg/ml; Affinity Bioreagents, Golden, CO; 06-629-MN, 2 μg/ml; Upstate Biotechnology, Lake Placid, NY) diluted in TBS overnight at 4°C. The antisera were produced with synthetic peptides representing a similar region of the $NH_2$ terminus that is conserved between human and rat. After washing in TBS, the membrane was incubated with peroxidase-conjugated donkey antirabbit antisera (1:10,000 in TBS-2% nonfat dry milk; Amersham, Piscataway, NJ) and binding was detected by autoradiography using enhanced chemiluminescence (Amersham). Duplicate membranes were probed by substitution of the primary antibody solution with either normal rabbit serum (1:2500) or with TBS-1%BSA buffer to detect nonspecific staining.

**Immunohistochemistry.** Formalin-fixed paraffin blocks for paired normal mucosa and moderately differentiated colon tumors for three female patients were obtained from the National Cancer Institute. Sections were processed and analyzed by immunohistochemistry using the PA1.311 antisera (10 μg/ml) and the microwave antigen retrieval method, as described (41). Controls included substitution of the primary antibody with normal rabbit serum and preabsorption of the antisera with peptide antigen as described by the supplier (PEP-011, Affinity Bioreagents).

**Data and Statistical Analyses.** Autoradiograms or photographs were scanned at 150-dpi resolution using Adobe Photoshop 3.0, and integrated densities were determined using NIH Image 1.61 software (PC version, Scion Corporation, MD). To confirm that GAPDH steady-state mRNA levels were similar between tumors and normal mucosa, relative densities for GAPDH mRNA transcripts (Northern analysis) were expressed as ratios to the sample 18S rRNA densities (ethidium bromide staining). Steroid hormone receptor levels were expressed as ratios of integrated density for steroid hormone receptor to GAPDH products. Statistical analyses for ANOVA and correlations were performed using Crunch statistical package 3.0 (Crunch Software, Oakland CA). Repeated-measures ANOVA was used, treating gender as a between-subject factor and sample type (tumor and normal mucosa) as a within-subject factor to test whether there were statistically significant differences in ER mRNA levels between tumors and normal mucosa (*post hoc t* tests; $P < 0.05$).

## RESULTS

**Validation of Comparative RT-PCR Method.** A comparative PCR assay was developed with the aim of comparing relative mRNA expression levels of ER subtypes and ERβ isoforms between paired samples of normal mucosa and tumors. Several procedures were used to normalize cDNA template concentrations between patient samples. First, four patient groups were processed in parallel to ensure comparable conditions within groups for all steps. Second, Northern analysis confirmed the quality and concentration of total RNA in each sample before the reverse transcription step. Third, amplification of the constitutively expressed *GAPDH* gene was performed at low cycle

numbers to monitor efficiency of the reverse transcription step and sample cDNA template concentration. GAPDH PCR products were amplified in all but two samples of normal mucosa from male patients, and no differences in mRNA levels were observed between samples assayed within 2 months of collection compared with 2 years. To confirm that GAPDH mRNA steady-state levels were similar between tumors and normal mucosa, GAPDH mRNA transcript densities were determined by Northern analysis, and mRNA levels were compared with the respective 18S rRNA densities from gel photography. GAPDH mRNA signal densities were similar between tumors and normal mucosa (integrated density ratio: GAPDH mRNA/18S rRNA: tumor, 1.27 ± 0.07; mucosa, 1.41 ± 0.23; $n = 6$ pairs).

Preliminary studies determined the optimal primer pairs and PCR cycle conditions resulting in a linear relationship between ER and GAPDH mRNA levels. GAPDH PCR products showed concentration-dependent amplification at 30 cycles (Fig. 1), and GAPDH mRNA steady-state levels determined by RT-PCR were similar between sample types [integrated density: tumor, 0.053 ± 0.001 ($n = 26$); mucosa, 0.047 ± 0.004 ($n = 24$)]. Several primer pairs for ERβ cDNA were tested as shown in Table 2. The PCR primer pair for ERβ exons 1–4 reliably produced a PCR product that was visible by ethidium bromide staining and thus was chosen for initial sample screening for overall ERβ mRNA levels. To compare the mRNA levels of ERα and ERβ on a semi-quantitative level, the amplification efficiencies of the respective primer pairs were tested using full-length cDNAs as templates. Similar signal densities of the resulting PCR products were detected for each primer pair with detection over a 2-log scale (Fig. 1). Differences in primer efficiency for the ERβ1 or ERβ2 exon 7–8 primer pairs were also tested using full-length ERβ1 or ERβ2 cDNA as templates. Amplification efficiency was similar between the isoform pairs over a 2-log detection scale, although positive reaction products by Southern analysis required ~100-fold greater template concentrations. To confirm the data for ERβ2 and to detect coexpression of Eβ5, another primer was designed for a 3′ terminal region of exon 8 that is 100% homologous between ERβ2, ERβ4, and ERβ5 isoforms (Table 2). This second primer set reliably produced PCR products for ERβ2 and ERβ5 from patient samples that were visible by ethidium bromide staining.

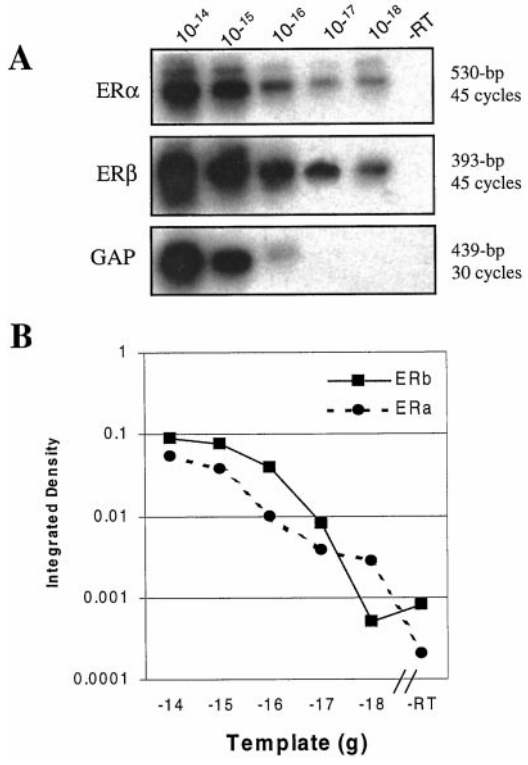


Fig. 1. PCR-Southern analysis of ER subtype primer efficiencies. PCR was performed in parallel for ERα, ERβ, and GAPDH primers using full-length human cDNA as templates in 10-fold decreasing amounts/reaction (g of cDNA). Samples were separated by gel electrophoresis and blotted to membranes. Membranes were hybridized with $^{32}$P-labeled cDNA probes derived from sequenced PCR products. *A*, the autoradiograms of PCR products illustrate decreasing signal intensities with decreasing template amounts. *B*, integrated density analysis shows a similar range of template detection limits for ER subtype primers.

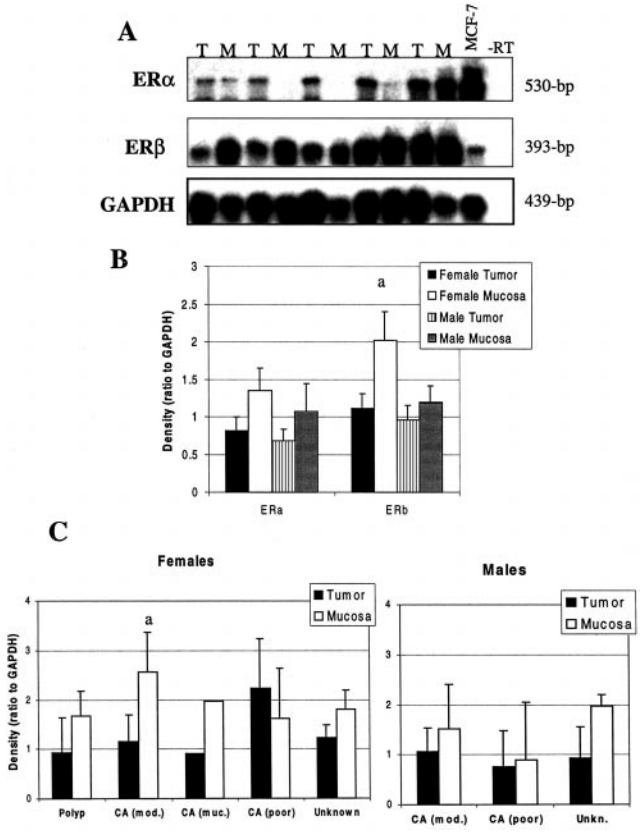


Fig. 2. Expression pattern of ER subtype mRNA in colon tumors and normal mucosa. Expression of ER subtypes was detected by comparative RT-PCR-Southern analysis as described in "Materials and Methods." PCR products were separated by agarose gel electrophoresis, transferred, and identified by hybridization to $^{32}$P-labeled cDNA probes. Detection of GAPDH mRNA levels at 30 cycles was used as an internal control in parallel experiments. *A*, representative autoradiograms are illustrated for female patients after 30-min exposures for ERβ and GAPDH and after 2-h exposure for ERα. *B*, integrated density analysis of ERα and ERβ mRNA levels (ratio to GAPDH) show a significant difference between normal mucosa and tumors in female patients ([a] $P < 0.02$; ANOVA). Mean ± SE; $n = 9$–14 samples. *T*, tumor; *M*, normal mucosa; *MCF-7*, human breast cancer cell line; *−RT*, negative control. *C*, density analysis of ERβ mRNA levels (ratio to GAPDH) in female and male patients by histological type [[a] $P < 0.05$; ANOVA for moderate differentiation; mean ± SD as $n = 2$ for poorly differentiated and unknown tumor types (Table 1)]. *CA*, carcinoma; *mod.*, moderate; *muc.*, mucinous.

**ER Subtype and ERβ Isoform mRNA Levels in Normal Colon Compared with Colon Tumors.** ERβ mRNA was detected in normal mucosa and tumors in 14 of 15 females and in 9 of 11 males. ERβ mRNA steady-state levels in tumors were ~55% of that in normal mucosa in female patients ($P < 0.02$; Fig. 2). In male patients, ERβ levels in tumors were ~80% of that in normal mucosa (Fig. 2). ERβ levels in normal mucosa from females were generally higher than in normal mucosa from males [2.0 ± 0.4 (females) *versus* 1.2 ± 0.21 (males); $P = 0.07$].

In patient samples, ERα mRNA steady-state levels were much lower than ERβ mRNA levels because PCR products were usually not visible by ethidium bromide staining, and autoradiography exposure times for Southern analysis were longer. ERα mRNA steady state

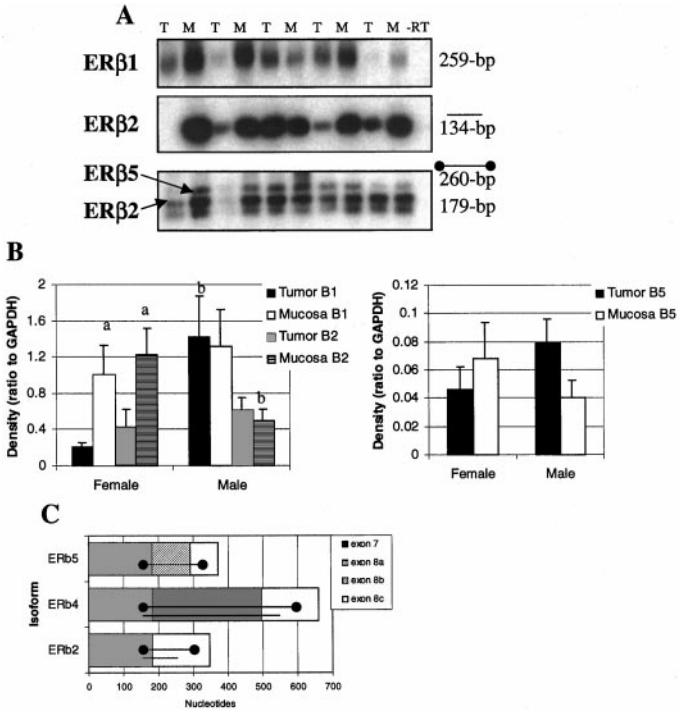


Fig. 3. ERβ isoforms in human colon tumors and normal mucosa. Expression of ERβ isoform mRNA was detected in tumors and paired normal mucosa by comparative RT-PCR-Southern analysis as described in "Materials and Methods." PCR products were separated by agarose gel electrophoresis and identified by hybridization with ERβ1 or ERβ2 cDNA probes. *A*, a representative analysis is shown for female patients. The ERβ2 antisense primer (*middle row*) is homologous with a region in ERβ4, and a second ERβ2 antisense primer (*bottom row*) is homologous with regions in ERβ4 and ERβ5 (see *C*; Table 2). ERβ4 products (expected size, 494 bp) were not identified. *B*, integrated density analysis of patient ERβ1, ERβ2, and ERβ5 mRNA levels (ratio to GAPDH mRNA) illustrate that ERβ1 and ERβ2 mRNA levels were decreased in tumors compared with normal mucosa in females ($^a P < 0.05$). Significant differences were found between genders for tumor ERβ1and normal mucosa ERβ2 mRNA levels ($^b P < 0.05$). Mean ± SE; $n = 10$ females, 6–7 males. *C*, bar graph representation of exon 7 and 8 regions of homology between ERβ2, ERβ4, and ERβ5 isoforms. *Exons 7* and *8c*, 100% homologous. *Horizontal lines*, the nucleotide regions amplified for two ERβ2 primer sets. *−RT*, negative control.

levels were not significantly different between tumor and mucosa in either males or females, although tumor levels were generally lower than in normal mucosa (Fig. 2). In an adenocarcinoma from a male patient, an ERα splicing variant was cloned that contained an exon-5 deletion and a single conservative nucleotide substitution. Correlations between ER subtypes and age were not observed for either gender.

To determine mRNA expression patterns of ERβ isoforms and whether the decrease in ERβ mRNA levels in female tumors was specific for a given ERβ isoform, ERβ1 and ERβ2/ERβ4/ERβ5 mRNA levels were analyzed. In female patients, ERβ1 and ERβ2 mRNA steady-state levels were both significantly decreased in tumors compared with normal mucosa (Fig. 3). In 1 of 10 females, ERβ1 and ERβ2 mRNA levels in a tumor were higher than in normal mucosa (Fig. 3). In male patients, ERβ1 and ERβ2 mRNA levels were not different between tumor and normal mucosa (Fig. 3). ERβ2 RT-PCR results were similar using either exon-8 primer. Between genders, ERβ1 mRNA steady-state levels were significantly lower in tumors, and ERβ2 levels were significantly higher in the normal mucosa of females compared with the corresponding samples in males (Fig. 3). ERβ5 mRNA levels were less abundant than for ERβ2, with no significant differences between samples (Fig. 3). Several PCR products with higher molecular weights were detected using ERβ2 antisense primers in patient samples. Additional ERβ1 PCR products using the exon 1–4 primer set were also detected at smaller molecular sizes, indicating possible detection of exon-deletion splicing variants. However, all these products were detectable at much lower intensity than the expected PCR product.

To determine whether there was a trend in overall ERβ mRNA levels by tumor differentiation, data were also analyzed by tumor differentiation (Fig. 2). Moderately differentiated tumors comprised the majority of samples for both females and males, and the ERβ mRNA levels were significantly decreased in female tumors ($P < 0.05$; ANOVA). Three polyps from female patients (63, 68, and 81 years of age) were analyzed, and overall ERβ mRNA levels were slightly lower, with greater variability, when compared with normal mucosa. ERβ1 mRNA levels were also lower [0.77 ± 0.034 (polyp) *versus* 1.57 ± 0.60 (mucosa)], yet ERβ2 and ERβ5 mRNA levels were not different in polyps.

**Human Colon Adenocarcinoma Cells Caco-2 Differentially Express Steroid Hormone Receptor mRNA Levels.** ERβ mRNA was detected in Caco-2, T84, and SW1116 colon cancer cell lines (Fig. 4). A fainter signal for ERβ mRNA was detected in HT-29 and SW48 cells. As expected, ERα mRNA was readily detected in the human breast carcinoma line MCF-7 (Fig. 4). Four colon cell lines were negative for ERα mRNA expression, with extremely low levels detected in SW1116 cells compared with MCF-7 cells. PR mRNA

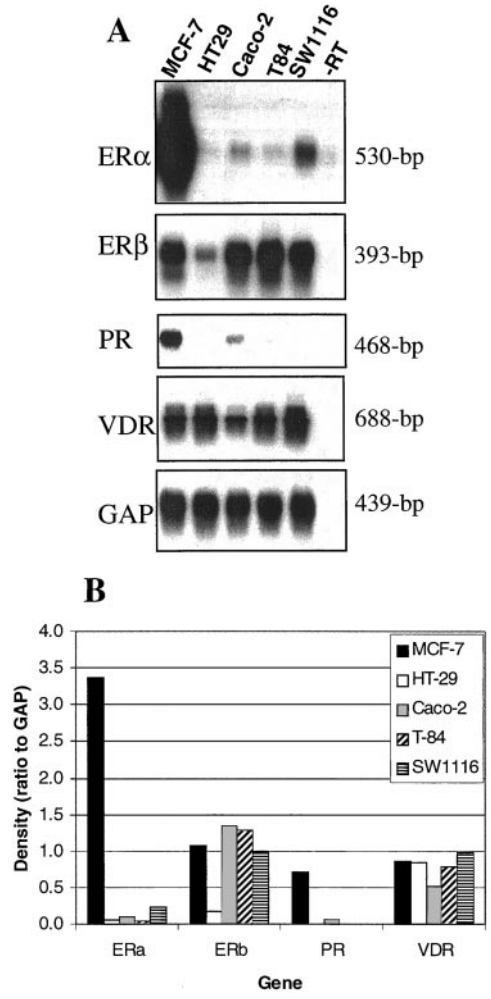


Fig. 4. Steroid hormone receptor mRNA steady state levels in human colon and breast (MCF-7) cancer cell lines. Cells were grown as described in "Materials and Methods," and total RNA levels were determined by comparative RT-PCR-Southern analysis. *A*, the autoradiogram for ERα PCR products was overexposed to detect mRNA levels in SW1116 cells. *B*, integrated density units for each gene (ratio to GAPDH) by cell line. *−RT*, negative control.

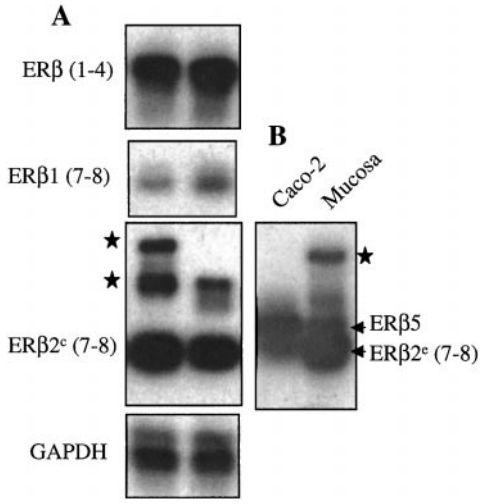


Fig. 5. RT-PCR-Southern analysis for ERβ isoforms in Caco-2 cells. PCR primer pairs were developed to amplify regions of ERβ common to all isoforms (exons 1–4) and for unique exon-8 regions for ERβ1 and ERβ2 (Table 2). *A*, two independent RNA preparations were analyzed by RT-PCR-Southern analysis. ERβ1 and ERβ2 mRNA were detected in Caco-2 cells. *B*, ERβ5 mRNA was detected in Caco-2 cells with levels greater than ERβ2. ERβ2 mRNA levels were generally in greater abundance than ERβ5 in normal human colonic mucosa (Fig. 3). Higher-molecular weight PCR products were detected using either ERβ2 primer pair (*stars*).

was detected in MCF-7 cells, with low levels detected in Caco-2 cells (Fig. 4). VDR mRNA levels were comparable among cell lines except for lower levels in Caco-2 cells (Fig. 4). Coexpression of ERβ isoforms in Caco-2 cells was studied using RT-PCR, and ERβ1, ERβ2, and ERβ5 mRNA were detected (Fig. 5). Several smaller-sized ERβ2 PCR products were also detectable using the sense exon 2 and antisense exon 8 primers, but levels were extremely low.

Poly(A)$^+$select mRNA was analyzed for ERβ mRNA levels to compare transcript size and relative abundance between Caco-2 cells and normal colon. Detection of ERβ mRNA transcripts in human colon samples required at least 6 μg poly(A)$^+$select mRNA, whereas transcripts were detected in Caco-2 cells using 2 μg samples. A major ERβ mRNA transcript at ~1.7 kb was detected in Caco-2 and MCF-7 cells and in normal human colon (Fig. 6). A faint signal at ~7.2 kb was also detected in MCF-7 cells after longer exposure times. On the basis of relative amounts of mRNA analyzed by Northern analysis, ERβ mRNA transcripts were most abundant in Caco-2 cells, and ERα mRNA transcripts were detected only in MCF-7 cells (Fig. 5).

Western analysis was performed to determine the molecular weight of ERβ protein in Caco-2 cells. An ERβ-immunoreactive signal was detected at $M_r$ ~60,000 in Caco-2 using the PA1.311 antisera (Fig. 7). A tissue sample from rat ovary was included as a positive control (42), and a doublet was detected at $M_r$ ~58,000 with a fainter band at $M_r$ ~73,000 (Fig. 6). ERβ protein in archival normal female samples was not detected by immunoblot analysis using either primary antibody source. Replicate membranes for each immunoblot were incubated without primary antibody or with normal rabbit serum, and immunoreactive bands were not observed.

**ERβ Protein Is Expressed in Superficial Epithelial and Vascular Cells and Neurons in Colon.** In the colonic epithelium, ERβ immunoreactivity was detected only in superficial epithelial cells with both nuclear and cytoplasmic staining (Fig. 8). Enteric neurons in submucosa and myenteric plexi were also immunopositive for ERβ (Fig. 8). In the submucosa, ERβ immunoreactivity was detected in the nuclei of smooth muscle and in the endothelial cells of large-sized arterioles (Fig. 8). Slight ERβ immunoreactivity was detected in superficial epithelium in one tumor, but the surface epithelium was difficult to discern in two tumors because of sample orientations. Formalin-fixed paraffin sections from rat uterus, processed in parallel with the colon specimens, showed the expected cellular distribution for ERβ protein in glandular and luminal epithelium (Fig. 8). Substitution of the primary antisera with normal rabbit serum resulted in trace positive-staining in the superficial epithelium and occasional mononuclear cells in the lamina propria (Fig. 8).

## DISCUSSION

A comparative RT-PCR technique was developed with the aim of determining relative ER subtypes and ERβ isoform mRNA levels between colon tumors and normal mucosa. In normal colonic mucosa, coexpression of ERα and ERβ mRNA was observed, with ERβ mRNA levels in greater abundance than ERα, as previously reported (31, 33). ERβ mRNA steady-state levels were

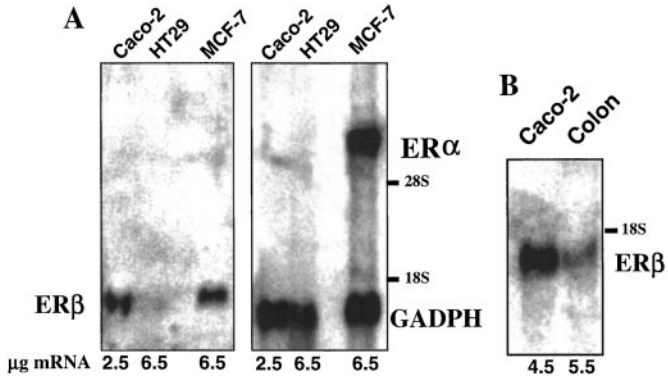


Fig. 6. Northern analysis of ERβ mRNA steady-state levels in human colon and breast cancer cell lines and in human colonic mucosa. *A*, Poly(A)$^+$ mRNA was isolated (*bottom of lanes*; μg/lane) from colon (Caco-2 and HT-29) and breast (MCF-7) cells grown in estrogen-depleted media. The membrane was hybridized with a full-length human ERβ1 cDNA probe, stripped, and rehybridized sequentially with human ERα and GAPDH probes without stripping. ERβ mRNA transcripts were detected in Caco-2 and MCF-7, whereas an ERα mRNA transcript was detected only in MCF-7 cells. Exposure times were 48 h for ERβ and 24 h for ERα and GAPDH. *B*, Poly(A)$^+$ mRNA was isolated from total RNA from pooled samples of normal human colonic mucosa and analyzed in comparison with Caco-2 cells. The membrane was hybridized as above, and the autoradiogram was exposed for 24 h. ERβ mRNA transcripts were expressed at similar sizes in Caco-2 cells and colon with greater abundance in the cell line. Relative positions of 28S and 18S rRNA bands are illustrated on the *right*. Results are representative of two to three separate mRNA preparations for each cell line and normal colon.

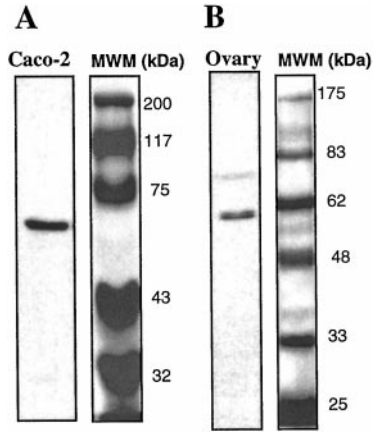


Fig. 7. Western analysis of ERβ protein expression. Proteins (*A*, 40 μg; *B*, 20 μg) were separated by 10% SDS-PAGE and transferred to membranes. The membranes were incubated with rabbit antihuman ERβ antibodies (PA1.311) and binding was detected by enhanced chemiluminescence. Molecular weights of bands were estimated from logarithmic plots of protein standard relative migration rates. *A*, a single immunoreactive band was detected at $M_r$ ~60,0000 in Caco-2 cells. *B*, a doublet at $M_r$ ~58,000 with a $M_r$ ~73,000 band of lower intensity were detected in rat ovary. The migration of prestained molecular weight standards is illustrated to the *right* (*A*, MWM; Bio-Rad Rainbow; or *B*, BioLabs). Results are representative of three independent preparations from Caco-2 cells.

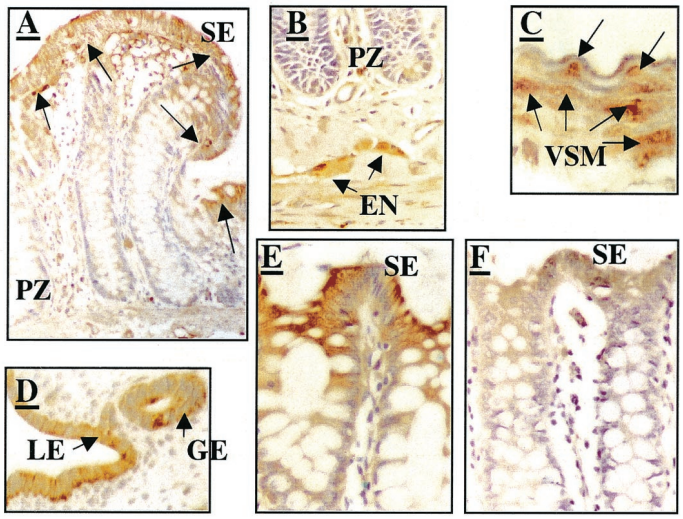


Fig. 8. Immunolocalization of ERβ protein in normal colon. The sections were incubated with polyclonal antibodies recognizing ERβ (*A*, *B*, *C*, *D*, and *E*) or normal rabbit serum (*F*). Antibody localization was detected using peroxidase-labeled secondary antibodies and diaminobenzidine chromogen (*brown*). Nuclei were counterstained with hematoxylin. *Arrows*, immunopositive signals. ERβ staining was detected in superficial epithelial cells, whereas crypt cells were negative (*A* and *E*). Enteric neurons (*B*) and vascular smooth muscle cells and endothelium of arterioles (*C*) were also stained for ERβ. *D*, rat uterine sections illustrated ERβ immunolocalization in luminal and glandular epithelium. *F*, incubation of the sections with normal rabbit serum illustrated trace cytoplasmic staining.

significantly decreased in colonic tumors compared with normal mucosa in female patients, whereas in male patients, ERβ mRNA levels were not different between samples. Although medical histories for ERT usage were not obtainable, ERβ mRNA levels in normal mucosa were similar regardless of age in females. Nothing is known regarding *in vivo* hormonal regulation of ERβ expression in humans, although up-regulation of ERβ mRNA levels by 17β-estradiol or down-regulation by progestin treatments have been observed in human breast cancer cell lines (43, 44).

Differences in overall ERβ mRNA levels between tumors and normal mucosa were paralleled by alterations in ERβ1 and ERβ2 mRNA levels. Interestingly, ERβ1 mRNA levels in female tumors were significantly lower than in male tumors, emphasizing a possible gender-specific role for the ERβ1 isoform in colon tumorigenesis. ERβ2 mRNA levels were more abundant in normal mucosa of females compared with males with decreased ERβ2 mRNA levels in tumors indicating that this isoform could also contribute to estrogen-mediated functions in colon. To date, one group has reported functional studies for ERβ2 in human pituitary cells (45). In comparison to ERβ1, ERβ2 is truncated at the COOH-terminus but has 26 unique amino acids because of alternative splicing. Transfection experiments with ERβ2 showed a lack of ligand- and estrogen response element-binding and preferential dimerization with ERα. A dominant negative activity was demonstrated only against ERα transactivation. Important physiological differences could result in cells that coexpress ERβ isoforms depending on constitutive ERα levels. In tissue such as the colon, where ERβ isoforms predominate, each ERβ isoform needs to be evaluated for ligand-dependent -independent effects on cell growth, development, or death. Very low levels for ERβ1 and ERβ2 splicing variants were also detected by RT-PCR-Southern analysis. Other ERβ1 splicing variants, including exons 2, 3, 5, 6, and 5+6 deletions, have been reported in breast and pituitary cancers (46–48). The overall functional effect of these ERβ variants would be expected to be minimal because of low expression levels and presence in both normal mucosa and colon tumors.

A trend between adenoma-carcinoma progression and ERβ mRNA levels was shown only for moderately differentiated carcinomas, because sample sizes were too low in the other categories. Alterations in ERβ mRNA expression may occur after the initiation phase of colonic carcinogenesis secondary to somatic mutations, hypermethylation of promoter regions, or decreases in cell types that express ERβ. Successive alterations for several genes have been well-characterized in the adenoma-carcinoma sequence. Hypermethylation of CpG islands in the promoter region of genes, including *ERα*, has been observed during aging and tumor progression in the human colon and results in decreased gene expression (49). This study shows that terminally differentiated colonocytes express ERβ protein, whereas other studies indicate that ERα protein may be localized in the submucosa (31, 50, 51). Progressive loss of differentiated cell types during cancer progression would be expected in both genders, so alternative mechanisms need to be considered for the gender differences.

Foley *et al.* (52) recently reported that ERβ protein levels were decreased in colon cancer patients. Paired samples from 11 patients (5 males and 6 females) were studied using RT-PCR and immunoblot analysis. Although decreases in ERβ protein levels were detected in both genders, alterations in ERβ mRNA levels were not observed. Our studies show that decreased levels of ERβ mRNA, including ERβ1 and ERβ2 isoforms, can be detected in female cancer patients. Assay conditions, such as total amount of RNA transcribed, enzyme sources, primer sensitivities, or sample populations, could account for the differences. The signal intensities for ERβ protein levels were remarkably high given that ERβ mRNA transcripts have not been detectable using standard amounts of poly(A)$^+$ select mRNA by Northern analysis (31, 45, 53). By immunoblot analysis, we were unable to detect specific bands for ERβ protein in the cell lines using the antisera (Upstate Biotechnology) and protein isolation methods cited in the study by Foley *et al.* (52), nor was ERβ protein detected in colon samples using two antibody sources. This study does show that ERβ protein was detected in the superficial epithelium rather than in crypt regions, so sample differences could account for the discrepancies.

In our study, ERα mRNA was expressed in lower abundance than ERβ mRNA, with no difference in ERα mRNA levels between tumors and normal mucosa in both genders. Previous studies report conflicting results regarding human colonic ERα expression depending on the detection method. 17β-Estradiol ligand binding studies could detect either ER subtype and cDNA probes, PCR primers, or antibodies directed against the ligand and hormone-binding domains may cross-react between ER subtypes because of sequence homology in these regions. One study suggested that survival of patients with ERα-positive normal mucosa was longer than patients with ERα-negative normal mucosa, whereas the ERα status of tumors had no prognostic value (54). In another study using ligand-binding assays, ERα-was detected in similar amounts in normal mucosa and in colon tumors, and levels did not vary by the sex or the age of patients or by the histopathological grade of the tumor (55). Several other studies showed no correlation in ERα levels between tumor and normal mucosa by various methods (50, 51, 56–59).

Issa *et al.* (60) showed that ERα-promoter hypermethylation increased as a direct function of age in human colon regardless of gender and suggested that ERα is a tumor suppressor gene in human colon. ERα mRNA expression was detected in normal mucosa but not in tumors and cell lines (RT-PCR), and ERα over-expression suppressed growth in the RKO colon cancer cell line. Our results show that ERα mRNA can be detected by RT-PCR, albeit in very low levels, in human colon tumors. Differences in PCR primer efficiency or other technical considerations may account for the difference in

results. Paradoxically, overexpression of wild-type ERα in ERα-negative cell lines and treatment with estrogens can lead to antiproliferative effects and increased differentiation (reviewed in Ref. 61). It has recently been proposed that ERβ functions as a negative regulator for ERα (22, 62). It would seem unlikely that ERβ has such a role in the colon, inasmuch as ERα mRNA levels are much less abundant than ERβ. For these reasons, we propose that ERβ may mediate estrogenic effects on colon cancer susceptibility.

Given the difficulty in identifying wild-type ERα in human colon, it is not surprising that ERα variants have not been reported. In this study, an ERα exon-5 deletion variant was cloned from a colon adenocarcinoma of a male patient. ERα variants have been reported in several normal and neoplastic tissues (63, 64). These variants include nucleotide insertions, exon duplications, point mutations, and alternative splicing resulting in exon-deleted transcripts. The ERα exon-5 deletion variant has a truncated ligand-binding domain and is coexpressed in the majority of ERα-positive tissues, however the role of this ERα variant in colon cancer is expected to be minor.

Virtually nothing is known about ERβ function in colonic epithelium. Normal human colon cell lines are not generally available, because they usually do not maintain a normal phenotype with passage. Human colon cancer cell lines that are often used as models for aggressive (HT-29) or absorptive (Caco-2) phenotypes were studied. Our data show that Caco-2 cells express mRNA for ERβ1, ERβ2, and ERβ5 isoforms. Expression of ERβ2 and ERβ5 mRNA has been reported in other colon cancer cell lines, whereas ERβ1 and ERβ4 mRNA were absent (33). Fiorelli *et al.* (34) reported expression of all five ERβ isoforms in several other colon cancer cell lines. The colon cell lines in the current study were essentially negative for ERα mRNA by RT-PCR in agreement with others, although one study has reported detection of ERα mRNA in Caco-2 cells (34, 60, 65, 66). The selection of various sublines because of culture conditions could account for differences.

Multiple ERβ mRNA transcripts have been observed in several human tissues by Northern analysis, with ERβ1 and ERβ2 mRNA transcripts reportedly expressed at ~7.2–7.5-kb and ~1.7-kb in testis (31, 45, 53). ERβ mRNA transcripts were detected at ~1.7-kb in Caco-2 and MCF-7 cell lines and in normal human colon, with a ~7.2-kb mRNA transcript detected only in MCF-7 cells. Because ERβ protein was detected in Caco-2 cells at the expected molecular weight, the low molecular-sized ERβ mRNA transcript may result from colon-specific regulatory factors on ERβ isoform expression levels or transcription start sites (67). Expression of ERβ protein in a cell line with an absorptive phenotype also supports our data showing immunolocalization of ERβ protein in superficial epithelial colonocytes.

Two other steroid hormone nuclear receptors were analyzed in this study. In particular, expression of PR was determined, because interactions with ERα are well known in some reproductive tissues. Very low levels of PR mRNA were detected in these colon cancer cell lines, suggesting that studies on the regulation of PR expression as a marker of ERβ function might be difficult. VDR mRNA was expressed with an inverse relationship between ERβ and VDR mRNA levels observed between HT-29 and Caco-2 cells. Estrogenic up-regulation of rat intestinal VDR has been observed *in vivo*, but studies using intestinal cell lines have not been reported (68). Caco-2 cells express functional aromatase, an enzyme that converts steroids such as testosterone to estradiol and estrone, so that ligand-activation of ERβ protein could result from exogenous or endogenous sources of 17β-estradiol (69).

Females have a lower lifetime risk for developing colon tumors, even in families with HNPCC, and the use of ERT reduces colon cancer risk, implying that female hormones, namely estrogens, decrease susceptibility for colon cancer. This study suggests that ERβ-mediated functions, in part, could be a potential mechanism by which estrogens alter susceptibility for colon cancers. Coexpression of ERβ isoforms increases the degree of complexity in understanding mechanisms mediated by ER ligands, whether endogenous or exogenous. Given the increased usage of ERT and selective ER modulators in women for the prevention of various diseases, investigations on ligand activation of ERβ-mediated functions in human colon are needed. Inasmuch as the numbers of elderly women are increasing and the survival rates for patients with advanced colon tumors have only modestly improved, consideration of new preventive strategies for colon cancer is also needed. The potential clinical significance of this study is that ERβ may mediate chemopreventive effects for estrogens in the colon and selective ERβ ligands might be a colon cancer prevention strategy.

## ACKNOWLEDGMENTS

We thank Drs. Leigh Murphy (University of Manitoba, Winnipeg, Manitoba, Canada) and Harry Nick (University of Florida, Gainesville, FL) for thoughtful advice; Dr. John T. Moore for supplying full-length ERβ1 and ERβ2 cDNAs (Glaxco Wellcome Research, Research Triangle Park, NC); Dr. Sally MacKay (University of Florida, Gainesville, FL) for supplying SW48 and SW1116 cells; and Vernon Nathaniel, Lissette Leon, and Charlyn Austria for excellent technical support.

## REFERENCES

1. Racial/ethnic patterns of cancer in the United States 1988–1992. *In:* B. A. Miller, L. N. Kolonel, L. Bernstein, J. L. Young, Jr., G. M. Swanson, D. West, C. R. Key, J. M. Liff, C. S. Glover, G. A. Alexander, *et al.* (eds.), NIH Pub. 96. Bethesda, MD: National Cancer Institute, 1996.
2. Gruber, S. B., Petersen, G. M., Kinzler, K. W., and Vogelstein, B. Cancer, crash sites, and the new genetics of neoplasia. Gastroenterology, *116:* 210–212, 1999.
3. Boland, C. R., Sato, J., Saito, K., Carethers, J. M., Marra, G., Laghi, L., and Chauhan, D. P. Genetic instability and chromosomal aberrations in colorectal cancer: a review of the current models. Cancer Detect. Prev., *22:* 377–382, 1998.
4. Kinzler, K. W., and Vogelstein, B. Landscaping the cancer terrain. Science (Washington DC), *280:* 1036–1037, 1998.
5. Ries, L. A., Wingo, P. A., Miller, D. S., Howe, H. L., Weir, H. K., Rosenberg, H. M., Vernon, S. W., Cronin, K., and Edwards, B. K. The annual report to the nation on the status of cancer, 1973–1997. Cancer (Phila.), *88:* 2398–2424, 2000.
6. DeCosse, J. J., Ngoi, S. S., Jacobson, J. S., and Cennerazzo, W. J. Gender and colorectal cancer. Eur. J. Cancer Prev., *2:* 105–115, 1993.
7. Levi, F., La Vecchia, C., Randimbison, L., Te, V. C., and Franceschi, S. Patterns of large bowel cancer by subsite, age, sex, and marital status. Tumori, *77:* 246–251, 1991.
8. Ochiai, M., Watanabe, M., Kushida, H., Wakabayashi, K., Sugimura, T., and Nagao, M. DNA adduct formation, cell proliferation, and aberrant crypt focus formation induced by PhIP in male and female rat colon with relevance to carcinogenesis. Carcinogenesis (Lond.), *17:* 95–98, 1996.
9. Odagiri, E., Jibiki, K., Kato, Y., Nakamura, S., Oda, S., Demura, R., and Demura, H. Steroid receptors in dimethylhydrazine-induced colon carcinogenesis. Cancer (Phila.), *56:* 2627–2634, 1985.
10. Gershbein, L. L. Action of estrogen and adrenocorticoids on adenocarcinoma induction by 1,2-dimethylhydrazine in male rats. Res. Commun. Chem. Pathol. Pharmacol., *81:* 117–120, 1993.
11. Froggatt, N. J., Green, J., Brassett, C., Evans, D. G. R., Bishop, D. T., Kolodner, R., and Maher, E. R. A common MSH2 mutation in English and North American HNPCC families: origin, phenotypic expression, and sex specific differences in colorectal cancer. J. Med. Genet., *36:* 97–102, 1999.
12. Carothers, A. M., Weyant, M. J., Mahmound, N. N., Bilinshi, R. T., and Bertagnolli, M. M. Estrogen modulates intestinal tumorigenesis *in vitro* and *in vivo*. Proc. Am. Assoc. Cancer Res., *59:* A371, 1999.
13. Paganini-Hill, A. Estrogen replacement therapy and colorectal cancer risk in elderly women. Dis. Colon Rectum, *42:* 1300–1305, 1999.
14. Nanda, K., Bastian, L. A., Hasselblad, V., and Simel, D. L. Hormone replacement therapy and the risk of colorectal cancer: a meta-analysis. Obstet. Gynecol., *93:* 880–888, 1999.
15. Grodstein, F., Newcomb, P. A., and Stampfer, M. J. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. Am. J. Med., *106:* 574–582, 1999.
16. Hebert-Croteau, N. A meta-analysis of hormone replacement therapy and colon cancer in women. Cancer Epidemiol., Biomark. Prev., *7:* 653–659, 1998.
17. Franceschi, S., and La Vecchia, C. Colorectal cancer and hormone replacement therapy: an unexpected finding. Eur. J. Cancer Prev., *7:* 427–438, 1998.

18. Crandall, C. J. Estrogen replacement therapy and colon cancer: a clinical review. J. Womens Health Gend. Based Med., *8:* 1155–1166, 1999.
19. Chen, M. J., Longnecker, M. P., Morgenstern, H., Lee, E. R., Frankl, H. D., and Haile, R. W. Recent use of hormone replacement therapy and the prevalence of colorectal adenomas. Cancer Epidemiol., Biomark. Prev., *7:* 227–230, 1998.
20. Platz, E. A., Martinez, M. E., Grodstein, F., Fuchs, C. S., Colditz, G. A., Stampfer, M. J., and Giovannucci, E. Parity and other reproductive factors and risk of adenomatous polyps of the distal colorectum (United States). Cancer Causes Control, *8:* 894–903, 1997.
21. Potter, J. D., Bostick, R. M., Grandits, G. A., Fosdick, L., Elmer, P., Wood, J., Grambsch, P., and Louis, T. A. Hormone replacement therapy is associated with lower risk of adenomatous polyps of the large bowel: the Minnesota Cancer Prevention Research Unit Case-Control Study. Cancer Epidemiol., Biomark Prev., *5:* 779–784, 1996.
22. Gustafsson, J. Estrogen receptor β: a new dimension in estrogen mechanism of action. J. Endocrinol., *163:* 379–383, 1999.
23. Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and estrogen receptor β. Endocrinology, *138:* 863–870, 1997.
24. Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C., and Ali, S. Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. J. Biol. Chem., *272:* 25832–25838, 1997.
25. McInerney, E. M., Weis, K. E., Sun, J., Mosselman, S., and Katzenellenbogen, B. S. Transcription activation by the human estrogen receptor subtype β (ERβ) studied with ERβ and ERα receptor chimeras. Endocrinology, *139:* 4513–4522, 1998.
26. Makela, S., Savolainen, H., Aavik, E., Myllarniemi, M., Strauss, L., Taskinen, E., Gustafsson, J. A., and Hayry, P. Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors α and β. Proc. Natl. Acad. Sci. USA, *96:* 7077–7082, 1999.
27. Jones, P. S., Parrott, E., and White, I. N. Activation of transcription by estrogen receptor α and β is cell type- and promoter-dependent. J. Biol. Chem., *274:* 32008–32014, 1999.
28. Cerillo, G., Rees, A., Manchanda, N., Reilly, C., Brogan, I., White, A., and Needham, M. The oestrogen receptor regulates NFκB and AP-1 activity in a cell-specific manner. J. Steroid Biochem. Mol. Biol., *67:* 79–88, 1998.
29. Thenot, S., Charpin, M., Bonnet, S., and Cavailles, V. Estrogen receptor cofactors expression in breast and endometrial human cancer cells. Mol. Cell. Endocrinol., *156:* 85–93, 1999.
30. Campbell-Thompson, M. Estrogen receptor α and β expression in rat upper gastrointestinal tract with regulation of trefoil family factor 2 mRNA levels in ovariectomized rats. Biochem. Biophys. Res. Commun., *240:* 247–518, 1997.
31. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, K-A. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J. Clin. Endocrinol. Metab., *82:* 4258–4265, 1997.
32. Brandenberger, A. W., Tee, M. K., Lee, J. Y., Chao, V., and Jaffe, R. B. Tissue distribution of estrogen receptor α (ER α) and estrogen receptor β (ER β) messenger RNA in the midgestational human fetus. J. Clin. Endocrinol. Metab., *82:* 3509–3512, 1997.
33. Moore, J. T., McKee, D. D., Slentz-Kesler, K., Moore, L. B., Jones, S. A., Horner, E. L., Su, J-L., Kliewer, S. A., Lehmann, J. M., and Willson, T. M. Cloning and characterization of human estrogen receptor β isoforms. Biochem. Biophys. Res. Commun., *247:* 75–78, 1998.
34. Fiorelli, G., Picariello, L., Martineti, V., Tonelli, F., and Brandi, M. L. Functional estrogen receptor β in colon cancer cells. Biochem. Biophys. Res. Commun., *261:* 521–527, 1999.
35. Arai, N., Strom, A., Rafter, J. J., and Gustafsson, J. A. Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. Biochem. Biophys. Res. Commun., *270:* 425–431, 2000.
36. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., *25:* 3389–3402, 1997.
37. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
38. Church, G. M., and Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA, *81:* 1991–1995, 1984.
39. Brass, N., Heckel, D., and Meese, E. Comparative PCR: an improved method to detect gene amplification. Biotechniques, *24:* 22–24, 26, 1998.
40. Campbell-Thompson, M. L., and McGuigan, J. E. Canine parietal cell binding by antibodies to the complementary peptide of somatostatin. Am. J. Med. Sci., *305:* 365–373, 1993.
41. Campbell-Thompson, M., Lauwers, G., Reyher, K., Cromwell, J., and Shiverick, K. 17β-estradiol modulates gastroduodenal preneoplastic alterations in rats exposed to the carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Endocrinology, *140:* 4886–4894, 1999.
42. O'Brien, M. L., Park, K., In, Y., and Park-Sarge, O. K. Characterization of estrogen receptor-β (ERβ) messenger ribonucleic acid and protein expression in rat granulosa cells. Endocrinology, *140:* 4530–4541, 1999.
43. Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., Lakins, J., and Lupu, R. Expression and regulation of estrogen receptor β in human breast tumors and cell lines. Oncol. Rep., *7:* 157–167, 2000.
44. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. Cancer Res., *59:* 529–532, 1999.
45. Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. Molecular cloning and characterization of human estrogen receptor βcx: a potential inhibitor of estrogen action in human. Nucleic Acids Res., *26:* 3505–3512, 1998.
46. Shupnik, M. A., Pitt, L. K., Soh, A. Y., Anderson, A., Lopes, M. B., and Laws, E. R., Jr. Selective expression of estrogen receptor α and β isoforms in human pituitary tumors. J. Clin. Endocrinol. Metab., *83:* 3965–3972, 1998.
47. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. Estrogen receptor-β mRNA variants in human and murine tissues. Mol. Cell. Endocrinol., *138:* 199–203, 1998.
48. Vladusic, E., Hornby, A., Guerra-Vladusic, F., and Lupu, R. Expression of estrogen receptor β messenger RNA variant in breast cancer. Cancer Res., *58:* 210–214, 1998.
49. Toyota, M., and Issa, J. P. CpG island methylator phenotypes in aging and cancer. Semin. Cancer Biol., *9:* 349–357, 1999.
50. Singh, S., Poulsom, R., Hanby, A. M., Rogers, L. A., Wright, N. A., Sheppard, M. C., and Langman, M. J. Expression of oestrogen receptor and oestrogen-inducible genes *pS2* and *ERD5* in large bowel mucosa and cancer. J. Pathol., *184:* 153–160, 1998.
51. Waliszewski, P., Blaszczyk, M., Wolinska-Witort, E., Brews, M., Snochowski, M., and Hurst, R. E. Molecular study of sex steroid receptor gene expression in human colon and colorectal carcinomas. J. Surg. Oncol., *64:* 3–11, 1997.
52. Foley, E. F., Jazaeri, A. A., Shupnik, M. A., Jazaeri, O., and Rice, L. W. Selective loss of estrogen receptor β in malignant human colon. Cancer Res., *60:* 245–248, 2000.
53. Mosselman, S., Polman, J., and Dijkema, R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett., *392:* 49–53, 1996.
54. Di Leo, A., Messa, C., Russo, F., Misciagna, G., Guerra, V., Taveri, R., and Leo, S. Prognostic value of cytosolic estrogen receptors in human colorectal carinoma and surrounding mucosa. Dig. Dis. Sci., *39:* 2038–2042, 1994.
55. Meggouh, F., Lointier, P., and Saez, S. Sex steroid and 1,25-dihydroxyvitamin D3 receptors in human colorectal adenocarcinoma and normal mucosa. Cancer Res., *51:* 1227–1233, 1991.
56. Kaklamanos, I. G., Bathe, O. F., Franceschi, D., Lazaris, A. C., Davaris, P., Glinatsis, M., and Golematis, B. C. Expression of receptors for estrogen and progesterone in malignant colonic mucosa as a prognostic factor for patient survival. J. Surg. Oncol., *72:* 225–229, 1999.
57. Oshima, C. T., Wonraht, D. R., Catarino, R. M., Mattos, D., Forones, N. M. Estrogen and progesterone receptors in gastric and colorectal cancer. Hepato-gastroenterology, *46:* 3155–3158, 1999.
58. Francavilla, A., Di Leo, A., Polimeno, L., Conte, D., Barone, M., Fanizza, G., Chiumarulo, C., Rizzo, G., and Rubino, M. Nuclear and cytosolic estrogen receptors in human colon carcinoma and in surrounding noncancerous colonic tissue. Gastroenterology, *93:* 1301–1306, 1987.
59. Korenaga, D., Orita, H., Maekawa, S., Itasaka, H., Ikeda, T., and Sugimachi, K. Relationship between hormone receptor levels and cell-kinetics in human colorectal cancer. Hepato-gastroenterology, *44:* 78–83, 1997.
60. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. Methylation of the oestrogen receptor CpG island links aging and neoplasia in human colon. Nat. Genet., *7:* 536–540, 1994.
61. Rochefort, H. Estrogen-induced genes in breast cancer and their medical importance. Bull. Acad. Natl. Med., *183:* 955–968, 1999.
62. Hall, J. M., and McDonnell, D. P. The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology, *140:* 5566–5578, 1999.
63. Murphy, L. C., Dotzlaw, H., Leygue, E., Douglas, D., Coutts, A., and Watson, P. H. Estrogen receptor variants and mutations. J. Steroid Biochem. Mol. Biol., *62:* 363–372, 1997.
64. Chaidrun, S. S., and Alexander, J. M. A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol. Endocrinol., *12:* 1355–1366, 1998.
65. Lointier, P., Wildrick, D. M., and Boman, B. M. Growth effects of tamoxifen on Lovo colon carcinoma cells and cultured cells from normal colonic mucosa. Anticancer Res., *12:* 1523–1525, 1992.
66. Jacobs, E., Watson, S. A., Hardcastle, J. D., and Robertson, J. F. Oestrogen and progesterone receptors in gastrointestinal cancer cell lines. Eur. J. Cancer, *32A:* 2348–2353, 1996.
67. Donaghue, C., Westley, B. R., and May, F. E. Selective promoter usage of the human estrogen receptor-α gene and its regulation by estrogen. Mol. Endocrinol., *13:* 1934–1950, 1999.
68. Schwartz, B., Smirnoff, P., Shany, S., and Liel, Y. Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. Mol. Cell. Biochem., *203:* 87–93, 2000.
69. English, M. A., Kane, K. F., Cruickshank, N., Langman, M. J., Stewart, P. M., and Hewison, M. Loss of estrogen inactivation in colonic cancer. J. Clin. Endocrinol. Metab., *84:* 2080–2085, 1999.
70. Fiorelli, G., Gori, F., Petilli, M., Tanini, A., Benvenuti, S., Serio, M., Bernabei, P., and Brandi, M. L. Functional estrogen receptors in a human preosteoclastic cell line. Proc. Natl. Acad. Sci. USA, *92:* 2672–2676, 1995.
71. Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor β1, β2, and β5 messenger RNAs in human breast tissue. Cancer Res., *59:* 1175–1179, 1999.
72. Knauthe, R., Diel, P., Hegele-Hartung, C., Engelhaupt, A., and Fritzemeier, K. H. Sexual dimorphism of steroid hormone receptor messenger ribonucleic acid expression and hormonal regulation in rat vascular tissue. Endocrinology, *137:* 3220–3227, 1996.