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MIPR NO: 93MM3504

TITLE: IS SEX HORMONE BINDING GLOBULIN LOCALLY PRODUCED IN BREAST CANCER

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REPORT DATE: October 15, 1993

TYPE OF REPORT: Midterm Report

PREPARED FOR: U.S. Army Medical Research and Development Command, Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	15 October 1993	Midterm Report (10/2/92-9/30/93)

4. TITLE AND SUBTITLE
Is Sex Hormone Binding Globulin Locally Produced in Breast Cancer

5. FUNDING NUMBERS
MIPR No.
93MM3504

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Madigan Army Medical Center
Department of Clinical Investigation
Tacoma, Washington 98431-5000

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Sex hormone binding globulin (SHBG) is a high affinity binding protein for androgens and estrogens. This protein is normally produced in the liver, released into the blood and functions to regulate the amount of free androgen or estrogen available for action at target organs. Recently, receptors for SHBG have been identified on steroid responsive tissues, including prostate carcinoma and endometrium. The goal of this series of experiments was to determine if mRNA for SHBG is expressed in breast cancer cell lines and tumor tissue. Two estrogen receptor positive cell lines were used, the ZR-75-1 and MCF-7. Initial experiments in which total RNA was extracted from the cells, followed by Northern blot analysis using a 500 bp 3' SHBG cDNA probe revealed SHBG mRNA in the ZR-75-1 cells, but no detectable message from the MCF-7 cells. The ZR-75-1 cells were used for studies investigating the transcriptional regulation of SHBG mRNA, which indicated that thyroxine may increase levels of SHBG mRNA, and estrogen and insulin may reduce levels. When MCF-7 cells were re-examined for SHBG RNA using PCR, specific message could be detected. Also, evidence of alternative splicing of the SHBG mRNA in breast cancer cells was found. Finally, amplification of RNA extracted from breast tumor tissue by PCR revealed the presence of SHBG mRNA in estrogen receptor positive tumors.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Sex Hormone Binding Globulin, PCR, ZR-75-1 Cells	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Kathleen H Moore Ph.D. 15 October 1993

Principal Investigator's Signature Date

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Introduction

The normal growth and development of breast tissue is regulated by estrogen with modulation by progesterone (1). The transformation of normal tissue to a cancerous tissue is now considered to be a multistage process which may involve gene activation (2), mutation of genes, or the loss of suppressor genes (3). While estrogen receptor is found in greater than 50% of breast cancers, only 60% of these respond to hormone manipulation. The concept has developed that steroid mediated growth of cancer cells involves locally produced (autocrine and paracrine) growth factors. A cascade of growth factors is necessary for the breast cell to begin abnormal growth, including platelet derived growth factor (PDGF), transforming growth factor α (TGFα), fibroblast growth factor (FGF), and insulin-like growth factors (2).

Oncogenes and their expressed protein product, oncoproteins, are associated with the abnormal growth of cancerous tissue. Essentially, these are genes and proteins which are associated with cellular growth or differentiation (4). The protein of interest in this work, sex hormone-binding globulin (SHBG), may also function as an oncoprotein. SHBG is normally produced in the liver, and in its functional dimeric form, acts as a serum transport protein for androgens and estrogens (5, 6). The mRNA for SHBG had been previously identified in cell lines developed from another steroid responsive cancer, prostate cancer (7). Up to this time, however, mRNA for SHBG has not been detected in either breast cancer cell lines or from tumor tissue. Others have reported the presence of membrane receptors for SHBG on breast cancer cell lines (8, 9, 10), suggesting that SHBG and its ligand, estrogen, may be internalized by receptor-mediated endocytosis (8).

The objective of this project is to determine if mRNA for SHBG can be detected in breast

transcription of SHBG in a breast cancer cell line by common growth factors such as estrogen, insulin, epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).

Methods

Cell culture Two estrogen receptor positive cell lines, MCF-7 and ZR-75-1, were obtained from the American Type Culture Collection (Rockville, MD), and maintained in tissue culture (75 cm^2 flasks). The MCF-7 cells are maintained in DMEM with 10% fetal bovine serum (FBS), the ZR-75-1 cells in RPMI with 10% FBS. Media for both cell lines was supplemented with Pen G (0.2 units/ml), Streptomycin (0.2 μg/ml) and L-glutamine (2 mM).

SHBG mRNA expression under hormonal and growth factor treatment To study the effect of different hormones and growth factors on SHBG expression in breast cancer, ZR-75-1 cells were used. These cells were chosen as mRNA for SHBG could be detected with a 3'cDNA probe for SHBG (11) (covering 550 bp of the 3' end of the message) in conventional Northern analysis. Six different compounds were tested; dihydrotestosterone (DHT) at 10^{-6}M and 10^{-10}M, epidermal growth factor (EGF) at 10^{-8}M and 10^{-12}M, estradiol at 10^{-6}M, 10^{-8}M and 10^{-10}M, insulin-like growth factor I (IGF) at 10^{-8}M and 10^{-10}M, insulin at 10^{-6}M and 10^{-10}M and thyroxine (T4) at 10^{-8}M and 10^{-12}M. Cells were grown to confluence and then the media was changed to phenol red free RPMI with 10% charcoal stripped FBS, to remove exogenous steroids. Cells were maintained in this steroid poor environment for 8 days with media changed at day four. Twenty-four hours before addition of test compounds, cells were changed to serum free, phenol red free RPMI. Growth factors and hormones were added twice daily for three days, and total RNA harvested on day four.

Extraction of RNA from cultured cells and Northern analysis RNA was extracted from

adherent cells by the guanidinium thiocyanate method (12). Recovered RNA was resuspended in DEPC treated water, and recovery estimated by measuring the OD 260.

The mRNA for SHBG in the stimulation experiments was detected using conventional Northern analysis. 25 μg of total RNA was electrophoresed on a 1.5% agarose gel, blotted by capillary transfer to a nylon membrane and probed with a ^{32}P labeled 3'SHBG cDNA probe (11). The blot was exposed to X-ray film for two weeks at -70°C and bands quantitated by a Shimadzu scanning densitometer. The blot was then stripped and probed with a ^{32}P labeled probe for 18S ribosomal RNA to correct for RNA loading of each lane of the gel.

Polymerase Chain Reaction (PCR) "Reverse" PCR was used to detect mRNA for SHBG in MCF-7 cells and from RNA extracted from formalin fixed tumor tissue (GeneAmp RNA PCR kit, Perkin Elmer Cetus), using a GeneAmp PCR System 9600 (Perkin Elmer). RNA extracted from ZR-75-1 was used as a positive control, as SHBG mRNA could be detected in these cells by conventional Northern analysis. The primers used were: forward primer 5'ACTCAGGCAGAATTCAATCTC3', reverse primer 5'CTTTAATGGGAAGCGTCAGT3'. The primers were synthesized using a Milligen Cyclone DNA synthesizer, and purified by butanol extraction (13). The reverse primer was used in the RNA reverse transcription step. These primers were projected to amplify the SHBG mRNA from base 556 to 1065 of the complete cDNA as reported by Hammond et al (11). The reverse transcriptase step was done at 42°C for 30 minutes, followed by 99°C for 5 min and cooling to 5°C for 5 min. A three temperature PCR program was used for amplification of DNA: 30 sec at 94°C, 30 sec at 56°C and 45 sec at 72°C for 35 cycles. Total volume in the reverse transcriptase reaction was 20 μl, and in the PCR amplification, 100 μl. Ten μl of PCR product was electrophoresed on a 4%

agarose gel and bands were visualized by ethidium bromide stain. The gel was transferred using Southern's procedure, including denaturing the DNA in the gel, to a nylon membrane (magnagraph, MSI), and the nucleic acids UV crosslinked to the membrane. The PCR product from the two breast cancer cell lines studied, ZR-75-1 and MCF-7, were analyzed using the 3'cDNA SHBG probe, labeled with digoxigenin-11-dUTP using the random primer method, and chemiluminescent detection, using the Genius 1 chemiluminescent kit (Boehringer-Mannheim). Hybridization temperature was at 65°C, and washes were also performed at 65°C. In replicate experiments, bands could be detected with as little as 10 seconds of exposure of the membrane to the x-ray film. The PCR product from formalin fixed tumor tissue was analyzed using the 3'SHBG cDNA as probe, labeled with ^{32}P by the random primer method, and exposure to x-ray film for up to 2 weeks at -70°C.

To further investigate the identity of the RNA products amplified by PCR, specific oligonucleotide probes were prepared for SHBG exons 6 (5'CAGGCAGCAGGCTCAGGCCACCT3'), 7 (5'GTCTTCAGCC AAGCGTCGAAGATG3') and 8 (5'CAAGGTCAGAGGCTGGATGTGGAC3'). As was done with the primers used above, probes were synthesized using a Milligen Cyclone DNA synthesizer, and purified by butanol extraction (13). Integrity of the oligonucleotides was verified by ion-exchange HPLC using a mono-P column (Pharmacia). The oligonucleotides were labeled with digoxigenin-11-dUTP using the 3'tailing reaction using a oligonucleotide tailing kit (Genius 6, Boehringer Mannheim). Three duplicate lanes of PCR product were prepared on a 4% agarose gel, transferred to a nylon membrane, and the membrane then cut into three identical strips. Each membrane strip was probed with one of the exon specific probes. Membranes were hybridized at 55°C for 2.5 hr,

washed twice at room temperature, and twice at 45°C. Bound probes were detected by chemiluminescence (Genius 1 kit).

Results

Figure 1a, b: The mRNA for SHBG could be detected in the breast cancer cell line ZR-75-1 by conventional Northern analysis. Shown in figure 1a is the Northern blot for the experiment to investigate factors which may alter transcription of the message. In the lower panel is the autoradiograph of the same blot stripped of the SHBG probe, and re-hybridized with a probe against 18s ribosomal RNA, to quantitate the relative RNA load on each lane. Bands at 1.8 kB and 3.3 kB were detected with the SHBG 3' cDNA probe, which is consistent with the sizes of bands observed by others for SHBG mRNA isolated from prostate cancer cell lines (14).

The intensity of the bands was quantitated by scanning densitometry (figure 1b), and when normalized to 18s RNA to correct for differential loading of the lanes, it appears that DHT did not influence the level of expression of SHBG mRNA in these cells, however estradiol may suppress expression in a dose dependent manner. Insulin, when used at 10^{-6}M appears to inhibit the production of SHBG mRNA. The higher levels of the other two protein growth factors tested, 10^{-8}M EGF and IGF appear to inhibit the transcription of SHBG, while the lower levels used, 10^{-12}M EGF and 10^{-10}M IGF may stimulate production of SHBG mRNA. Consistent with other biological systems (15), thyroxine appears to stimulate the production of SHBG mRNA.

Figure 2: The polymerase chain reaction (PCR) was used to further investigate whether mRNA for SHBG could be found in breast cancer cell lines. Total RNA from ZR-75-1 (positive control) and MCF-7 cell lines were used. The ZR-75-1 cell line was used as a positive control

for the PCR procedure as mRNA had previously been identified by northern analysis, however mRNA for SHBG could not be detected in the MCF-7 cell line. As shown in figure 2, bands were easily detectable in the PCR product from RNA isolated from MCF-7 cells and ZR-75-1 cells. Two major bands were detected with the 3'cDNA probe, one at approximately 500 bp (the expected product) and another at approximately 300 bp. Shown is a representative experiment, the PCR amplification has been conducted five times by three different technicians. In some experiments, a larger band, as shown in this figure is found, at 900 to 1000bp, and a very small band of 100 bp is also found. The two major bands, at 500 bp and 300 bp, have been consistently amplified in all experiments using RNA isolated from these two cell lines. The difference in size between these two major bands is consistent with the size of one of the exons comprising the region amplified. The region of RNA amplified begins in exon 5 and ends in exon 8. Exon 6 is 137 bp, which is smaller than the difference between the two bands, however, exon 7 is 208 bp which is close to the difference in size of the two bands.

Figure 3: To further characterize the two major bands amplified by PCR, exon specific probes were used. Both the 500 bp and 300 bp bands are labeled with exon probes 6 and 8, however, only the 500 bp band is detected when the probe for exon 7 is used. This indicates that the 500 bp band contains exons 5 (site of the 5' PCR primer), 6, 7 and 8, and the 300 bp band contains exons 5, 6, and 8, but is missing exon 7, or at least the sequence of exon 7 which is complimentary to the probe.

Figure 4: To date, RNA has been isolated from tumor tissue collected from three different patients, with two samples (left breast biopsy [2A], right breast sample following modified radical mastectomy [2B]) from the same patient. SHBG mRNA was detected in both the

samples from patient 2. Interestingly, the modified message (300 bp) was identified in the sample from the left breast biopsy (2A), and the full length product (500 bp) was amplified from the tumor sample from her right breast (2B). No SHBG PCR product was detectable in the tumor samples from the other two patients (patients 1 and 3). The tumor tissue in which SHBG message was detected (patient 2) had 176 femtomoles (fm) progesterone receptor and 183 fm estrogen receptor per milligram (mg) tissue, which is considered positive. Patient 1 had 1 fm of estrogen and progesterone receptor per mg of tissue, which is considered receptor negative. Patient 3 had 118 fm/mg progesterone receptor, but much lower estrogen receptor of 23 fm/mg protein.

Discussion

This is the first report which can document the expression of mRNA for SHBG in breast cancer cell lines and tumor tissue. RNA species at 1.8 kb and 3.3 kb were detected. This is similar to the sizes of RNA detected with the same probe from prostate cancer cell lines (7). This size distribution of RNA for SHBG in breast cancer cells, however is different from what is detected in a hepatoma cell line, Hep G2, in which only one transcript for SHBG is found at 1.6 kb. The larger band at 3.3 kb may represent incompletely processed mRNA for SHBG, as the predicted size for the SHBG message with the introns is approximately 3.3-3.5 kb (16).

Estradiol appears to suppress expression of SHBG mRNA in the ZR-75-1 breast cancer cells. This is similar to the action of estradiol on the expression of SHBG mRNA from Hep G2 cells (17). Insulin also demonstrated a dose related suppression of the expression of SHBG mRNA, which is consistent with other observations that insulin can reduce the secretion of SHBG into conditioned media from Hep G2 cells (15). Thyroxine appears to increase levels of

SHBG mRNA in these breast cancer cells, which also is consistent with in-vivo observations that serum concentrations of SHBG increase in hyperthydrodism (18). The regulation of the transcription of SHBG mRNA in breast cancer cells appears to be similar to its regulation in other cell systems, indicating that promoter regions are intact in the ZR-75-1 cell line. What will be the subject of further investigation is whether SHBG mRNA can be detected in estrogen receptor negative cancer cell lines.

Two major PCR products are routinely amplified from the ZR-75-1 and MCF-7 breast cancer cells. These different sized products are consistent with alternative splicing of the SHBG mRNA in these cells. Alternative splicing of SHBG and its testis analog, androgen binding protein, has been reported in other tissues, including fetal rat liver (19), human testis (16) and human liver (20). Common features of these alternative transcripts include excision of exons 1 and 7 of SHBG and substitution of an alternative exon 1 at the 5' end of the message. The predicted protein sequence of these alternative transcripts is different from SHBG due to a frame shift in the coding sequence approximately half way through the sequence and may have a different function. It remains to be determined if similar alternative transcripts of SHBG are found in breast cancer cells as in liver and testis.

In addition to identifying SHBG RNA in breast cancer cell lines, we also have analyzed by PCR for the presence of SHBG message in formalin fixed tumor tissue. Thus, this gene is expressed in actual disease and not just in cell lines. The sample in which SHBG message was detected had the highest concentration of estrogen and progesterone receptors (sample 2a/b). Sample 1 (SHBG negative) was estrogen and progesterone receptor negative. Sample 3, also SHBG negative, had approximately the same level of progesterone receptor as sample 2, but a

much lower level of estrogen receptor. We are analyzing additional tumor tissue to further investigate the relationship between the presence of SHBG mRNA, the level of steroid receptor and histological tumor type expressing SHBG mRNA. We also are analyzing normal tissue and premalignant (hyperplastic) breast tissue to determine if SHBG mRNA is present before malignant disease is detectable.

Thus the SHBG gene is expressed in tumor tissue from patients, and in breast cancer cell lines. Based on a small number of patients, the expression of SHBG seems to be associated with elevated estrogen receptor levels.

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Figure legends:

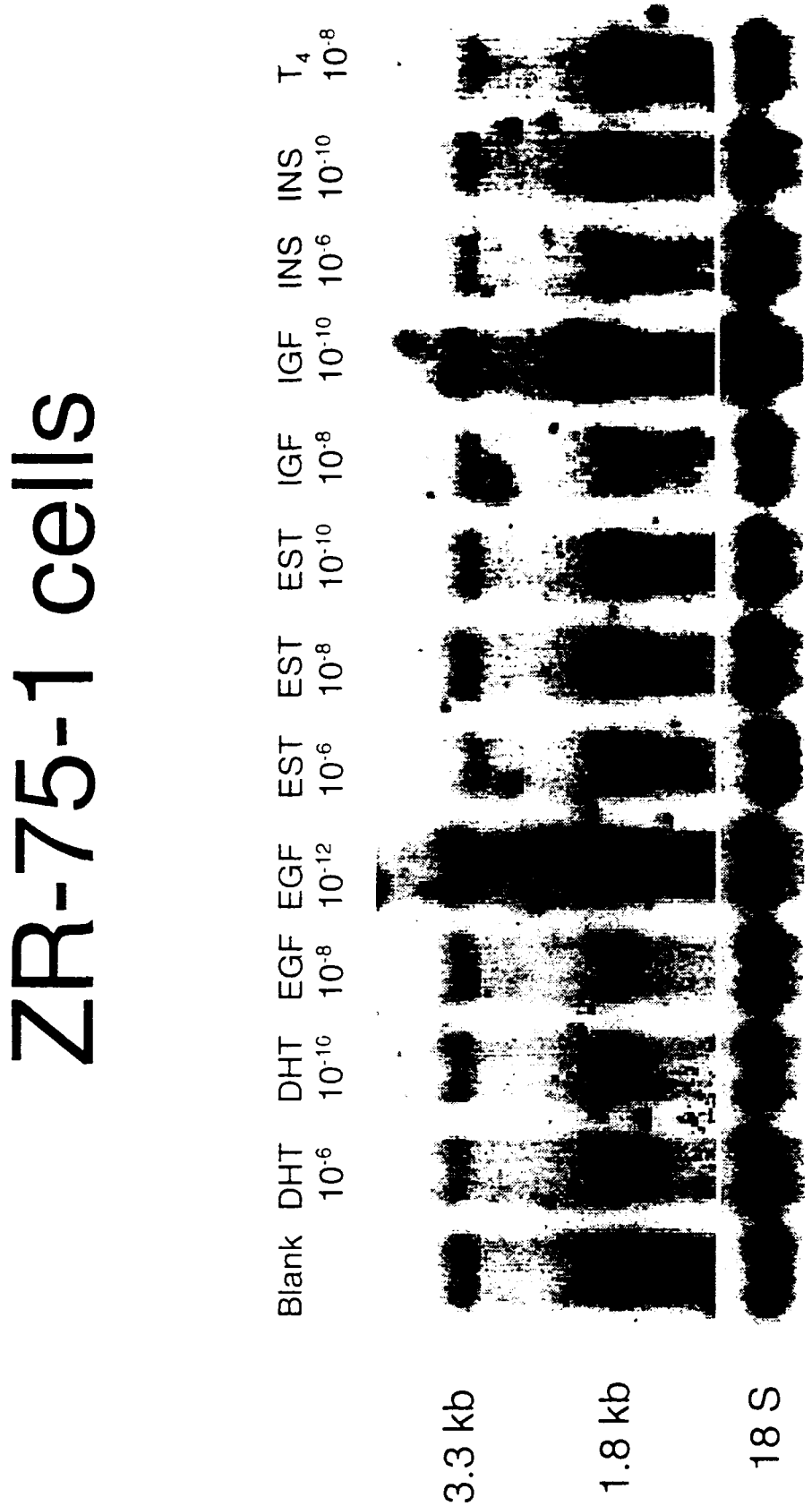
Figure 1: a) Northern blot analysis of expression of SHBG mRNA from ZR-75-1 breast cancer cells. Blot was probed with a ^{32}P 500 bp 3'cDNA for SHBG (8). Two bands were detected with this probe, at 3.3 kB and 1.8 kB. The same blot was stripped and probed with an oligonucleotide for 18S ribosomal RNA, also labeled with ^{32}P. Cells were treated with different growth factors and hormones, as indicated, the molar concentration of the different factors also is indicated. b)Densitometry of the bands detected by Northern analysis. All measurements were normalized with the density of their corresponding 18s ribosomal RNA band, and the ratio of the density of the SHBG bands from the treated cells to the control is plotted. The control culture (blank) is given a value of 1. The ratio of the upper band refers to the 3.3 kB band, and the lower band, the 1.8 kB band.

Figure 2: Amplification of SHBG mRNA by reverse PCR. 500 ng of RNA from ZR-75-1 and MCF-7 was used. 10 μl of the PCR product (100 μl total volume) was electrophoresed on a 4% agarose gel, transferred to a nylon membrane and probed with the 3'cDNA probe labeled for chemiluminescent detection. A 123 bp ladder was also run on the gel for estimation of the sizes of the detected products (not shown). Sizes of the amplified products are indicated.

Figure 3: The product from PCR of ZR-75-1 mRNA was probed with oligonucleotides targeted against exons 6, 7, or 8. The exon 6 probe detected bands at 500 and 300 bases, and the exon 8 probe detected an additional band at 100 bases. The exon 7 probe labeled a band at 500 bases only, indicating that the 300 bp band is missing at least some of the bases that comprise exon 7.

Figure 4: PCR product from RNA isolated from formalin fixed tissue, probed with the 500 bp 3'cDNA of SHBG. ZR-75-1 RNA was included as a positive control, the negative control contained SHBG primers, but no template. 1000 ng of RNA was added to the PCR reaction for all patient samples.

Figure 1a


ZR-75-1 cells
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DHT 10^{-10}
EGF 10^{-8}
EGF 10^{-12}
EST 10^{-6}
EST 10^{-8}
EST 10^{-10}
IGF 10^{-8}
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INS 10^{-10}
T_4 10^{-8}
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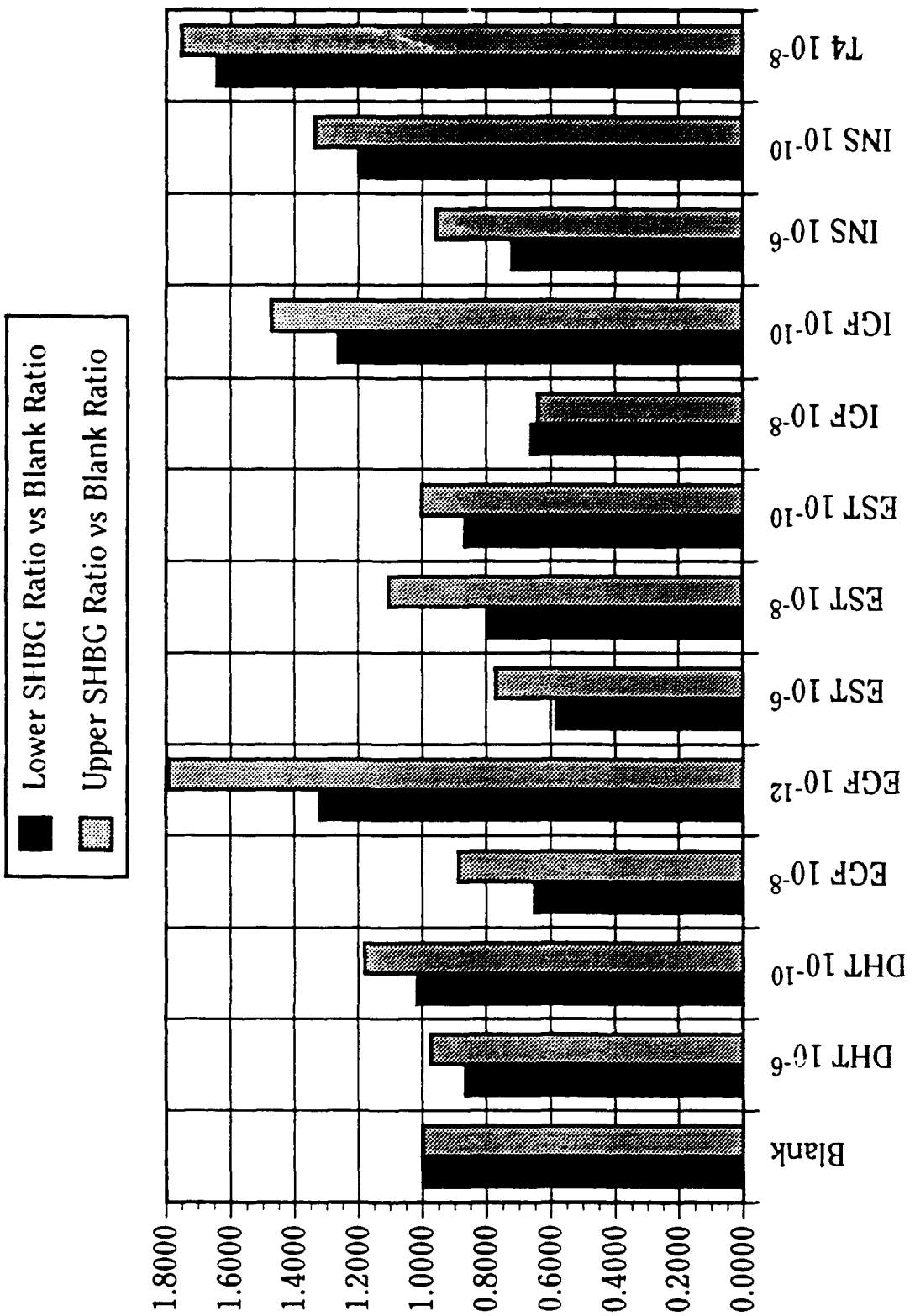
1.8 kb
18 S

Figure 1b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Upper SHBG Ratio vs Blank Ratio
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1.6000
1.4000
1.2000
1.0000
0.8000
0.6000
0.4000
0.2000
0.0000
Blank
DHT 10-6
DHT 10-10
EGF 10-8
EGF 10-12
EST 10-6
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IGF 10-8
IGF 10-10
INS 10-6
INS 10-10
T4 10-8

Figure 2

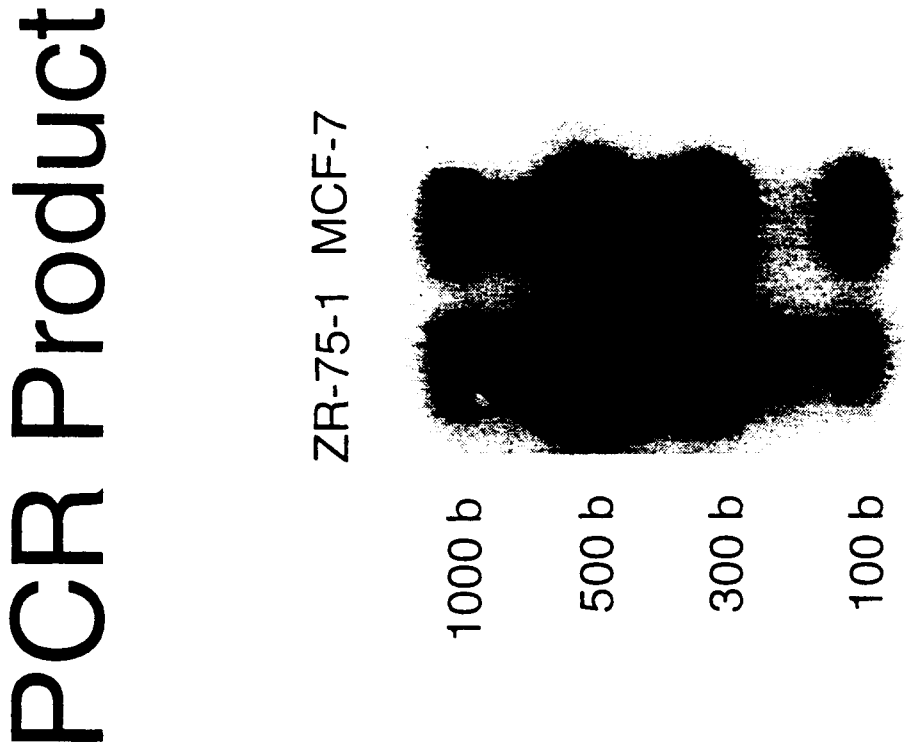
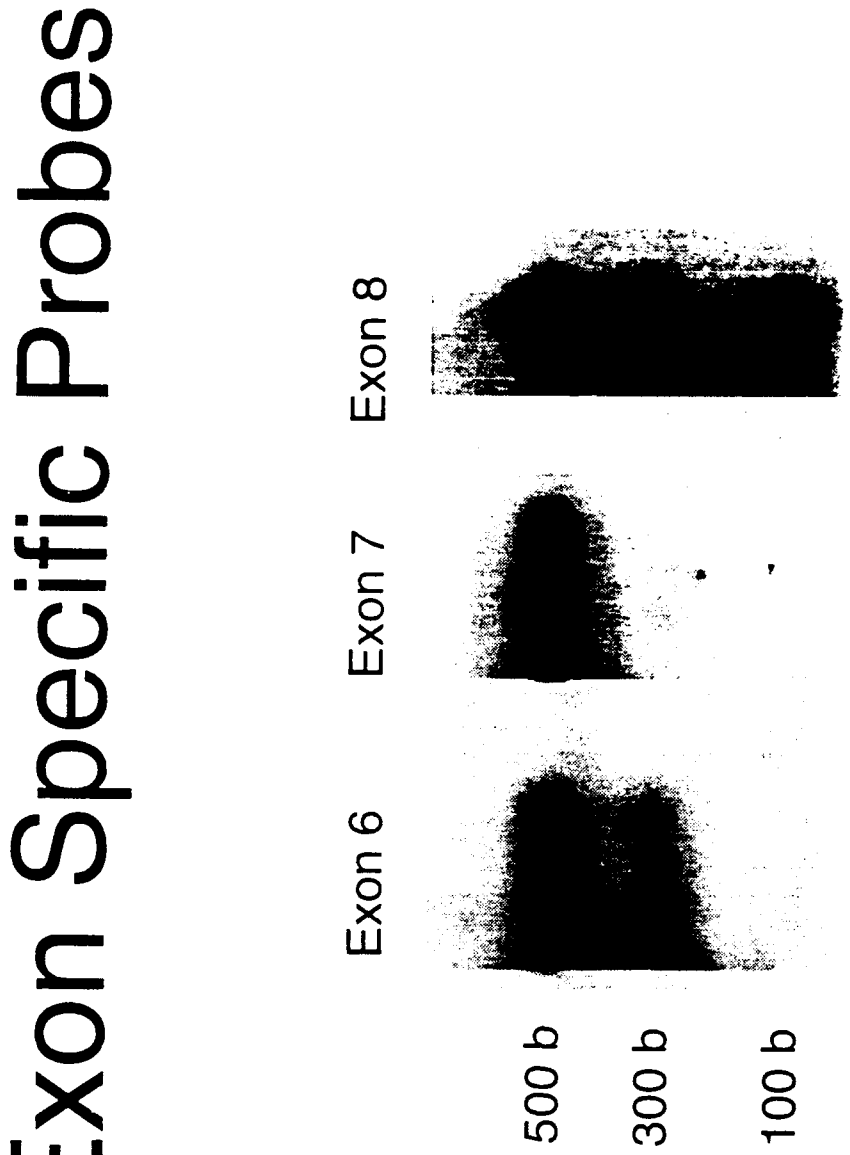


Figure 3



Formalin Fixed Tissue

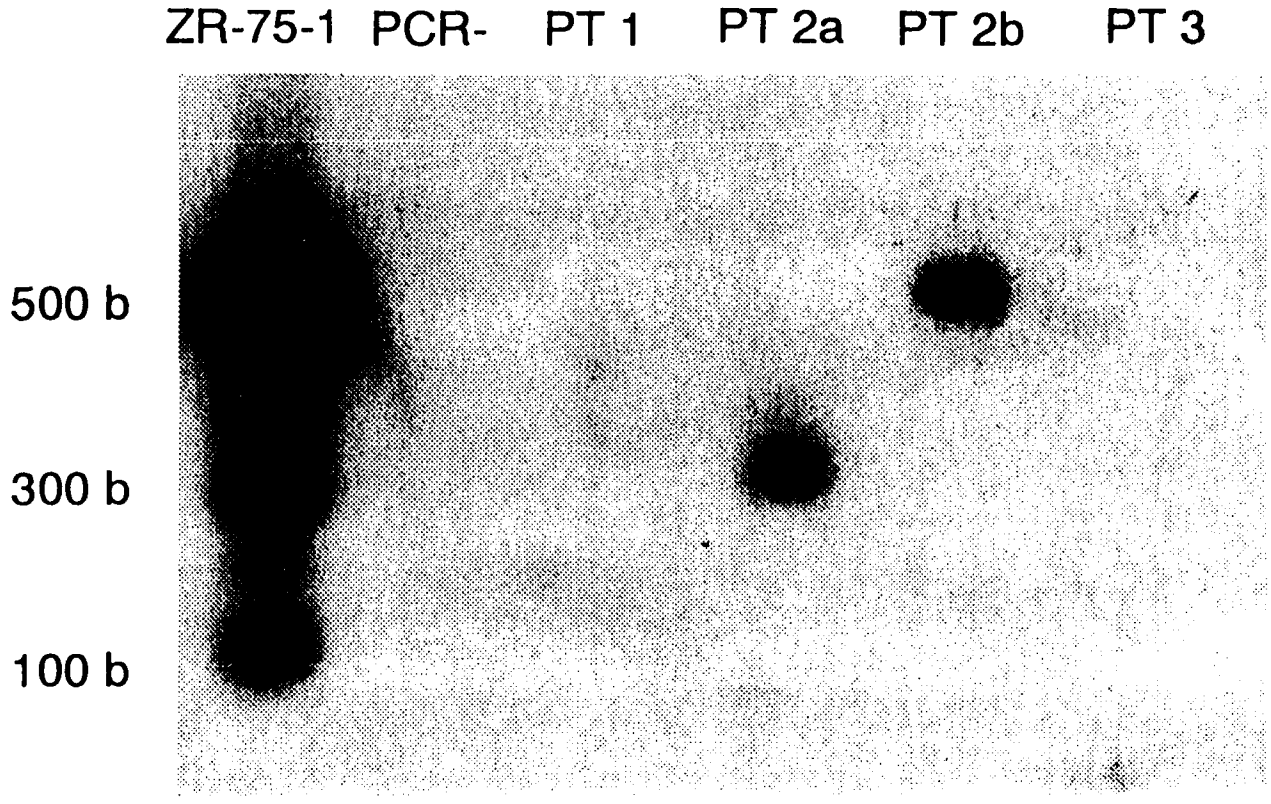


Figure 4