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14. ABSTRACT Estrogen stimulates proliferation of breast cancer cells whereas antagonists oppose their action. To study the molecular mechanisms of ligand-dependent regulation of transcription, we generated a cell line derived from a parent cell line containing an integrated tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Ha-ras) reporter and a vector for cherry red fluorescence-estrogen receptor (ER)pbox mutant which recognizes glucocorticoid response elements (GREs). We observed ERpbox binding to the tandem array in an estradiol (E2)-dependent manner. We also observed concurrent transcription by RNA fluorescent in situ hybridization (FISH). RNA transcription correlated with ERpbox signal on the tandem array until steady state levels were reached. Chromatin immunoprecipitation (ChIP) assays showed recruitment of ERpbox to MMTV promoter and endogenous serum- and glucocorticoid-regulated protein kinase (Sgk), promoter. These studies in live cells demonstrate ERpbox binding to the MMTV promoter and transcription in an E2-dependent manner. Moreover, they demonstrate that ER pbox fusion protein also can bind to the GRE of an endogenous target gene. Live cell imaging using ERpbox and the MMTV tandem array, in combination with ChIP and RNA FISH, are powerful techniques to visualize the mechanisms of transcriptional regulation by E2 and selective estrogen receptor modulators (SERMs) used for treatment of breast cancer. This cell line also may be a rapid and useful tool for drug screening of novel SERMs.					
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Introduction:

Estrogen stimulates proliferation of breast cancer cells. Antagonists such as tamoxifen can have the opposite effect and have been used clinically to treat breast cancer. In the nucleus, liganded ERs bind to estrogen response elements (EREs) in the promoters of target genes and regulate their transcription (1). Estrogen-bound ERs recruit co-activator complexes which contain steroid receptor co-activator (SRCs), CBP/P300, and the histone acetyl transferase, p/CAF which, in turn, increase histone acetylation. Other complexes such as vitamin D receptor-interacting protein/ TR-associated proteins (DRIP/TRAPs) which contain yeast Mediator-like subunits, help recruit RNA polymerase II (RNA pol II), and thus activate transcription. Antagonist-bound ERs recruit co-repressor complexes that have histone deacetylase activity and decrease transcription. Recently, Hager and colleagues generated a cell line (3134) containing an integrated tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Ha-ras) reporter (2). The array contained about 200 copies of the long terminal repeat (LTR) or approximately 800 to 1200 glucocorticoid response elements (GREs) to which glucocorticoid receptors (GRs) could bind. They then created another cell line (3617) from 3134 cells which expressed green fluorescent protein/GR (GFP-GR). Confocal microscopy was used to study the kinetics GFP-GR binding to the tandem array in live 3617 cells. We planned to generate a vector expressing cherry red fluorescent protein-estrogen receptor p-box mutant (CRFP-ERpbox) which recognizes glucocorticoid response elements (GREs). and activates transcription via the MMTV-LTR with estrogen when co-transfected with the appropriate reporter plasmid. We then planned to use the parental cell line 3134 to generate permanently-transfected cell lines with this chimera to study ERpbox and co-factor recruitment to the promoter in the presence of estrogen and ER antagonists.

Body:

In collaboration with Dr. Gordon Hager (NCI), we generated a cell line (6444) derived from a parent HeLa cell line containing an integrated tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Ha-ras) reporter which expressed CRFP-ERpbox which recognizes glucocorticoid response elements (GREs). ERpbox contains a three amino substitution in the first zinc finger of the DNA-binding domain that changes DNA-binding specificity from an estrogen response element (ERE) to a GRE. This cell line allowed us to visualize directly ERpbox binding to the MMTV tandem array within 15 minutes after E2 addition.

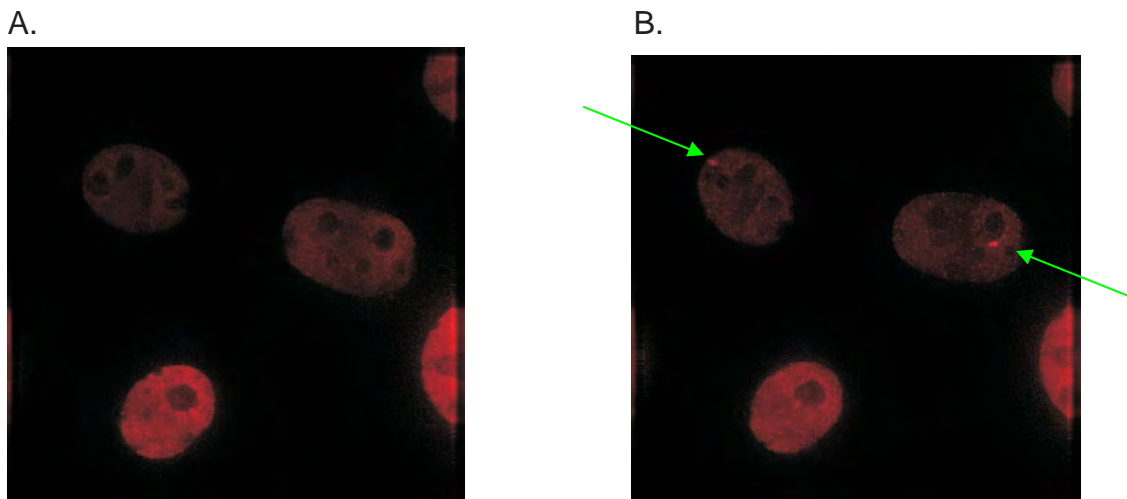


Figure 1. Living 6444 cells with integrated virus expressing cherry-ERpbox. Live cell imaging showing the same cells before A) and B) 15 minutes after addition of E2. The ERpbox mutant loads the MMTV tandem array in an E2-dependent manner.

We then examined the colocalization of ER recruitment to the MMTV promoter and RNA transcription. We observed concurrent transcription of v-ras transcripts from the MMTV promoter by RNA fluorescent *in situ* hybridization (FISH).

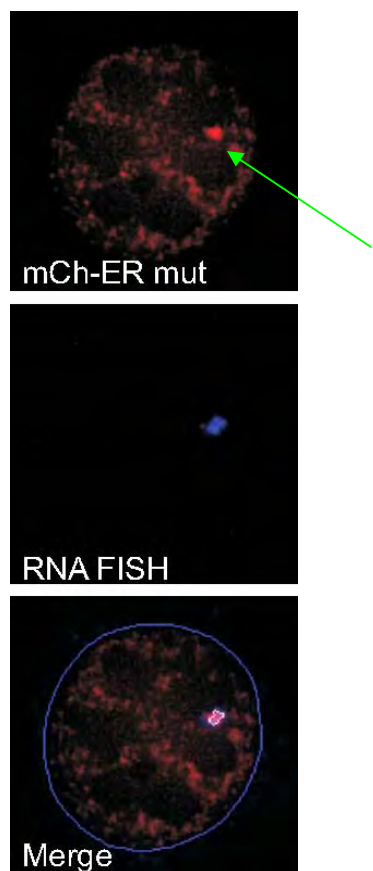


Figure 2. Image of a 6444 cell nucleus after treatment and preparation from MMTV RNA FISH. The ERpbox colocalizes with RNA FISH signal. The blue and yellow outlined regions of interest (ROIs) in the merge image were automatically defined by automated image analysis algorithm. This analytical microscopy and the algorithm is described in (3). The blue outline is the edge of the nucleus and the yellow outline is the ROI defining the array.

Additionally, the amount of RNA transcription correlated with ERpbox signal on the tandem array until steady state levels were reached at 30 minutes.. We observed a time-dependent increase in ERpbox recruitment and transcription from the MMTV tandem array

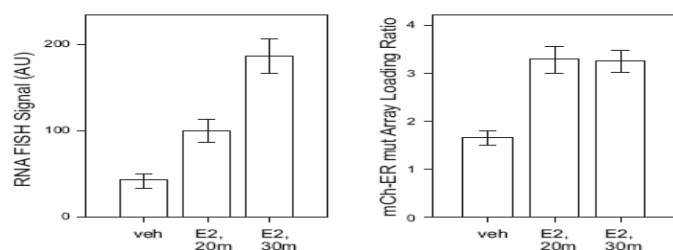


Figure 3. Automated image analysis measuring MMTV FISH signal and a steady state association of ERpbox with the MMTV array. These are mean values ($n > 100$ cells) \pm SEM error bars. The measurements were made using the ROIs exemplified in Figure 2.

We next performed chromatin immunoprecipitation (ChIP) assays to examine recruitment of ERpbox to MMTV promoters on the tandem array and an endogenous serum- and glucocorticoid-regulated protein kinase (Sgk), promoter. We observed that ERpbox rapidly bound to both promoters in an estrogen-dependent manner.

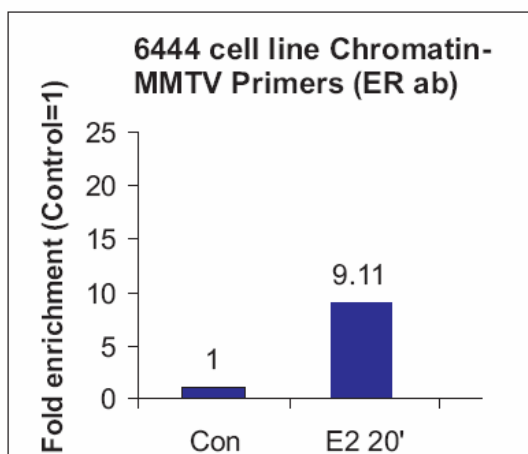
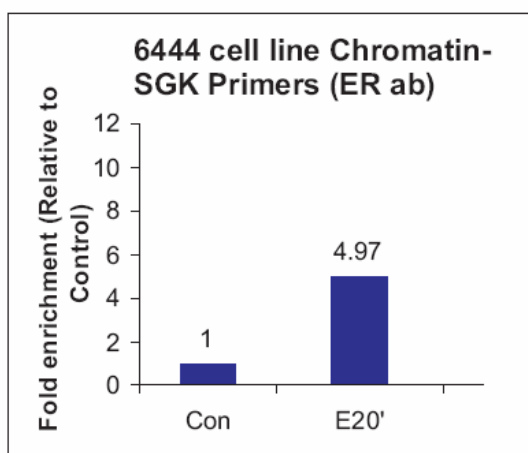


Figure 4. ChIP assays measuring steady state binding of ERpBox to the MMTV array and to the established GREs in the endogenous SGK gene.



These studies in live cells demonstrate ERpbox binding to the MMTV promoter and transcription in an E2-dependent manner. Moreover, they demonstrate that ERpbox fusion protein also can bind to the GRE of an endogenous target gene, and may be a novel way to modify hormonal regulation of glucocorticoid-regulated target genes. Live cell imaging using ERpbox and the MMTV tandem array, in combination with ChIP and RNA FISH, are powerful tools to visualize the mechanisms of transcriptional regulation by E2 and antagonists.

We currently are extending these studies to ER antagonists such as tamoxifen and ICI 182, 780. We also have generated GFP-constructs of co-activators such as SRC-1 and SRC-3, and CBP which will enable us to examine ligand-dependent co-recruitment of these co-factors in live cells.

Key Research Accomplishments:

- 1) Development of a novel cell line (6444) derived from a parent HeLa cell line containing an integrated tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Ha-ras) reporter which expressed CRFP-ERpbox which recognizes and activates MMTV promoter.
- 2) This cell line enables visualization of ERpbox binding to the MMTV tandem array in live cells. Concurrent RNA transcription also can be visualized by RNA FISH. The recruitment of ERpbox to the array which is measured by fluorescent intensity using confocal microscopy correlates with CHIP assays of ER recruitment to the array.
- 3) ERpbox also can bind to the promoter of an endogenous target gene that contains a GRE allowing a change in the repertoire of genes regulated by glucocorticoids to now be regulated by estrogens.
- 4) This system will enable visualization and study of co-expressed GFP-coactivators and CRFP-ERpbox in the same cell.
- 5) This system also will enable visualization of ERpbox binding to promoters in the presence of agonists and antagonists, and provide insight into their mechanism(s) of action. It also may be a useful tool for screening selective estrogen receptor modulators (SERMs) that bind to ER and promote ER binding to DNA.

Reportable Outcomes:

We plan to submit our findings as an abstract to the upcoming Endocrine Society Meeting in San Francisco in June 2008. We plan to perform further studies using ER antagonists before preparing and submitting a manuscript.

Conclusion:

These studies in live cells demonstrate ERpbox binding to the MMTV promoter and transcription in an E2-dependent manner. Moreover, they demonstrate that ERpbox also can bind to the GRE of an endogenous target gene thus changing the entire repertoire of genes regulated by a given hormone. Live cell imaging using ERpbox and the MMTV tandem array, in combination with CHIP and RNA FISH, are powerful tools to visualize the mechanisms of transcriptional regulation by E2 and antagonists.

References:

1. **McDonnell DP, Norris JD** 2002 Connections and regulation of the human estrogen receptor. *Science* 296:1642-4
2. **McNally JG, Muller WG, Walker D, Wolford R, Hager GL** 2000 The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* 287:1262-5
3. **Voss TC, John S, Hager GL** 2006 Single-cell analysis of glucocorticoid receptor action reveals that stochastic post-chromatin association mechanisms regulate ligand-specific transcription. *Mol Endocrinol* 20:2641-55

Appendices:

c.v. enclosed

Supporting Data:

N/A

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Amherst College	B.A.	1973-78	Chemistry
Johns Hopkins University School of Medicine	M.D.	1978-82	Medicine

A. Positions and HonorsPositions and Employment

1982-85 Internship and Residency, Internal Medicine, University of Chicago Hospitals, Chicago, IL

1985 - 1988 National Research Service Award Fellow, National Institutes of Health, Bethesda, MD.

1988 - 1989 Clinical Staff Fellow, Endocrinology, NIH, Bethesda, MD

1989 - 1992 Research Associate, Howard Hughes Medical Institute, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

1992-1993 Instructor, Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

1994-1997 Assistant Professor, Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

1997-2003 Chief, Molecular Regulation and Neuroendocrinology Section, Clinical Endocrinology Branch, NIDDK, NIH, Bethesda, MD

2004- Associate Professor, Department of Medicine, Johns Hopkins Bayview Medical Center and Johns Hopkins University School of Medicine, Baltimore, MD

Other Experiences and Professional Memberships

1997-2001 Member, Editorial Boards, Thyroid, Molecular Endocrinology, and Endocrinology

1998- Member, Editorial Board, Reviews in Endocrinology and Metabolism

1998-2000 Member, Research Committee, American Thyroid Association

1998 Chairman, Research Committee, American Thyroid Association

1999-2002 Member, Annual Meeting Steering Committee, Endocrine Society

1999-2003 Member, Veterans Health Administration Endocrinology Merit Review Subcommittee

2000- Associate Editor, Current Drug Targets-Immune, Endocrine, and Metabolic Disorders

2001-2003 *Ad hoc* reviewer, Advanced Technology Programs, National Institutes of Science and Technology

2002- Member, Faculty of 1000, Biology Reports. Cell Signaling section.

2002-2004 Member, Planning Committee for American Thyroid Association Annual Meetings

2003 Co-organizer of Sixth International Workshop on Resistance to Thyroid Hormone, Miami, FL

Principal Investigator/Program Director (Last, First, Middle): Yen. Paul Michael

- 2005 Opening Lecture "Genomic expression profiles of thyroid hormone action" European Thyroid Association Genomics Workshop, Athens, Greece
- 2006 *Ad hoc* reviewer, Molecular and Cellular Endocrinology Study Section, NIDDK
- 2006 Reviewer, Neurogenetics and Neurogenomics Special Study Section, Molecular, Cellular, and Developmental Neuroscience Integrated Review Group, Center for Scientific Review, NIH
- 2007 NIA Consensus Study Panel on "Thyroid and Aging" Washington, D.C.
- 2007 Member, Organizing Committee, Eighth International Workshop on Resistance to Thyroid Hormone, Azores, Portugal
- 2007 Associate Editor, Molecular Mechanisms of Hormone Action Textbook, Humana Press
- 2008 Member, Planning Committee, FASEB Conference "Non-genomic action of steroid hormone receptors." Tucson, AZ

Journal Peer Reviewer:

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Cell Metabolism
Endocrine Practice
Endocrine Reviews
Endocrinology
Journal of Biological Chemistry
Journal of Cell Science
Journal of Clinical Endocrinology and Metabolism
Journal of Clinical Investigation
Journal of Endocrine Investigation
Journal of Molecular Endocrinology
Lancet
Molecular Biology of the Cell
Molecular and Cellular Biology
Molecular and Cellular Endocrinology
Molecular Endocrinology
Nature Medicine
Oncogene
Physiological Genomics
Physiological Reviews
Proceedings of the National Academy of Science
Thyroid
Trends in Endocrinology and Metabolism

Foundation Grant Reviewer:

National Science Foundation
Wellcome Trust
University of Leuven
Italian Association for Cancer Research

Honors

- 1992, 1993 AAP, ASCI, AFCR Trainee Investigator Awards
- 1992 Nichols Institute New Investigator Award (Endocrine Society)
- 1993 Boots Clinical Fellowship and Mentor Award for outstanding thyroid research (Endocrine Society)

2001 Abbott Clinical Thyroid Research Mentor Award (Endocrine Society)

Fellows in my laboratory have received Merck Senior Fellow Award (2000); Quest New Investigator Award (2001); and two Abbott Clinical Fellow Awards (2001) from the Endocrine Society.

B. Selected Peer-Reviewed Publications (recent publications from 74 total)

1. Sasaki S, Leson-Wood LA, Dey A, Kuwata T, Weinbraub BD, Humphrey G, Yang WM, Seto E, **Yen PM**, Howard BH, Ozato K. Ligand-induced recruitment of a histone deacetylase in the negative-feedback regulation of the thyrotropin beta gene. *EMBO J* 1999;18:5389-5398.
2. Feng X, Yuan J, Meltzer PB, **Yen PM**. Thyroid hormone regulation of hepatic genes *in vivo* detected by cDNA microarray. *Mol Endocrinol* 2000; 14:947-955.
3. Baumann CT, Maruvada P, Hager GL, **Yen PM**. Nuclear–cytoplasmic shuttling by thyroid hormone receptors: Multiple protein interactions are required for nuclear retention. *J Biol Chem* 2001; 276:11237-11246.
4. Feng X, Jiang Y, Meltzer PM, **Yen PM**. Transgenic targeting of a dominant negative corepressor to liver blocks basal repression by thyroid hormone receptor and increases cell proliferation. *J Biol Chem* 2001;276:15066-15072.
5. Ando S, Sarlis NJ, Zhang M, Krishnan J, Feng X, Oldfield E, **Yen PM**. Alternative-splicing of TR β mRNA in a TSH-secreting pituitary adenoma is a mechanism for hormone resistance. *Mol Endocrinol* 2001; 15:1529-1538.
6. Ando S, Sarlis NJ, Oldfield EH, **Yen PM**. Somatic mutation of thyroid hormone receptor β can cause a defect in negative-regulation of thyrotropin in a thyrotropin-secreting pituitary tumor *J Clin Endo Metab* 2001; 86:5572-5576.
7. Phillips SA, Rotman-Pikelny P, Lazar J, Skarulis MC, Hauser P, de Rodriguez S, Brucker-Davis F, **Yen PM**. Extreme thyroid hormone resistance in a patient with novel truncated thyroid hormone receptor mutant *J Clin Endo Metab* 2001; 86:5142-5147.
8. Parikh S, Ando S, Schneider A, Skarulis MC, Sarlis NJ, **Yen PM**. Resistance to thyroid hormone in a patient with normal thyroid hormone receptors α and β . *Thyroid* 2002; 12:81-86.
9. Rotman-Pikielny P, Hirschberg K, Maruvada P, Suzuki, K, Royaux IE, Green ED, Kohn LD Lippincott-Schwartz J, **Yen PM**. Pendrin retention in endoplasmic reticulum is a major mechanism for Pendred syndrome. *Hum Mol Genetics* 2002; 11:2625-2633.
10. Poguet AL, Feng X, **Yen PM**, Meltzer P, Samarut J, Flamant F. Cyclin D2 as a mediator of the antiapoptotic function of thyroid hormone in the cerebellum external granular layer. *Developmental Biology* 2003; 254:188-199.
11. Maruvada P, Baumann CT, Hager GL, **Yen PM**. Dynamic shuttling and intranuclear diffusion of nuclear hormone receptors. *J Biol Chem* 2003; 278:12425-12432.
12. **Yen PM**, Feng X, Flamant F, Chen YD, Samarut J, Refetoff S, Meltzer PS. Effects of hormonal status and thyroid hormone receptor (TR) isoforms on hepatic gene expression profiles in TR knockout mice. *EMBO reports* 2003; 4:581-587.
13. Maruvada P, Dmitrieva NI, East-Palmer J, **Yen PM**. Differential expression of thyroid hormone receptor during the cell cycle determines sensitivity to thyroid hormone *Mol Biol Cell* 2004; 15:1895-1903.
14. Liu Y, Ando S, Xia X, Yao R, Kim M, Fondell, **Yen PM**. p62, a TFIIH subunit, directly interacts with thyroid hormone receptor and enhances T3-mediated transcription. *Mol Endocrinol*. 2005 19:879-84
15. Liu Y, Xia X, Fondell JD, **Yen PM**. Thyroid hormone-regulated target genes have distinct patterns of coactivator recruitment and histone acetylation. *Mol Endocrinol*. 2006 20:483-90

Current Funding

R01 NIDDK 1R01DK069899-01A1. Thyroid hormone regulation of transcription 2005-2009 (\$190,000/year)

DOD: Breast Cancer Concept Award. Visualization of estrogen receptors in live cells 2006-2007 (\$75,000)

Principal Investigator/Program Director (Last, First, Middle): Yen. Paul Michael