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Excessive activation of growth factor receptors can lead to the unrestrained cellular proliferation characteristic of tumors. Our objective is to determine if SNX1, a protein involved in intracellular membrane trafficking, can be used to downregulate EGF receptors in mammary gland. Our approach is to characterize the gene for SNX1 and to generate transgenic animals overexpressing SNX1 in mammary glands. We have characterized a genomic clone for SNX1 and had planned to use this clone for transgenic vector construction. However, the size of the first intron in SNX1 is too large for this approach to be used successfully. Instead, a WAP-SNX1 cDNA vector has been constructed and we are now ready to generate transgenic animals.

A Career Development Award was a second component of the application. Career development activities include: participation as reviewer on the American Cancer Society Cell Structure and Metastasis study section and participation on a search committee charged with identifying a Director for Breast Cancer Research at UAMS. In addition, the State of Arkansas Breast Cancer Research Program awarded me a one-year pilot research grant to examine the relationship between HER-2/*neu* and EGF receptors in mammary gland cell proliferation.

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FOREWORD

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 05/14/2000 Date

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INTRODUCTION

Inhibiting Tumorigenesis by Growth Factor Receptor Down Regulation Using a Sorting Nexin

The development of the mammary gland and its normal function in lactation is controlled by a variety of steroid hormones and peptide growth factors whose concentrations vary depending on the functional status of the gland. Disruption of this complex signaling network by genetic damage, environmental toxins or other factors can override normal restraints on cell growth and lead to the formation of tumors. Epidermal growth factor (EGF) levels been found to be elevated in breast tumor tissues and are an indicator of poor survivability because these tumors are generally resistant to therapies aimed at interruption of estrogen action. As a consequence of elevated EGF receptor levels, proliferative responses to growth factors are enhanced. This can result in hyperplasia and an increased probability of mutations occurring that further contribute to unrestrained tumor growth.

To prevent excessive mitogenic signaling, ligand bound receptors are removed from the cell surface in clathrin coated pits. Once inside the cell, receptor-ligand complexes are sorted away from nutritional receptors like the LDL receptor and targeted for degradation in lysosomes. The membrane trafficking events underlying lysosomal targeting involve the recognition of small amino acid "codes" by the sorting machinery. I recently discovered a protein, the sorting nexin, that recognizes the EGF receptor targeting code and stimulates its transport to the lysosome. In tissue culture cells, overexpression of sorting nexin 1 (SNX1) down regulated EGF receptors by dramatically shortening receptor half-life. As a consequence, EGF-stimulated receptor tyrosine kinase and immediate early gene activities were inhibited substantially. To test the hypothesis that SNX1 can be used *in vivo* to regulate mitogenic signaling by down regulating the EGF receptor, thereby inhibiting tumorigenesis, I propose to use an established transgenic mouse model for therapeutic intervention. In transgenic mice engineered to overexpress transforming growth factor alpha (TGFα), a ligand for the EGF receptor, mammary gland tumors invariably occur in females after 2 or 3 pregnancies. To determine if it is feasible to inhibit TGFα induced mammary gland tumorigenesis by overexpressing SNX1, the following technical objectives are proposed:

1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA.
2. Prepare and characterize a transgenic mouse line with SNX1 expression targeted to the mammary gland using the whey acidic protein (WAP) promoter.
3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

BODY

Technical Objective 1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA that I cloned.

Task 1: **Months 1-2: Plate and screen genomic phage library by hybridization with ^{32}P labeled SNX1 cDNA. Prepare plaque pure phage stocks.**

Progress: In collaboration with H. Steven Wiley, University of Utah School of Medicine, 3 bacterial artificial chromosomes (BAC) containing the human SNX1 gene were isolated. Fluorescence *in situ* hybridization was used to determine the chromosomal localization of both SNX1 and SNX2 (Figure 1). Three different P1 clones were used to deduce the chromosomal localization of SNX1 and 2 were used for SNX2. Hybridization efficiency ranged from 85 to 91%. SNX1 was localized to human chromosome 15q22 and SNX2 was localized to chromosome 5q23.

Task 2: **Months 3-4: Purify phage DNAs and characterize the phage inserts by restriction mapping and Southern hybridization**

Progress: One of the BAC clones for SNX1, labeled 6K1, has been characterized by subcloning and restriction mapping (Figure 2). Characterization of multiple clones has not been practical due to the unexpectedly large size of the SNX1 gene.

Task 3: **Months 5-6: Subclone phage inserts that appear to contain the entire SNX1 coding region; confirm that they do by sequencing using primers complimentary to the ends of the SNX1 coding region and select the largest one for further use.**

Progress: The complete SNX1 coding region lies within 55kb of genomic DNA as defined by sequencing and oligonucleotide hybridization (Figure 3).

Task 4: **Month 8-12: Determine the complete sequence of the SNX1 gene.**

Progress: We have sequenced 6.2kb of the SNX1 gene. This represents 5 of an estimated 9 exons and considerable intronic sequence. We will proceed to sequence the remaining exonic regions in the identified subclones to completely define the intron-exon structure of the SNX1 gene and to describe the intron-exon boundaries (Figure 3). Given the unexpectedly large size of the SNX1 gene, we will not determine the sequence of the remaining intronic DNA.

Task 5: **Months 11-12: Prepare annual project report and a manuscript describing the human SNX1 gene.**

Progress: This annual report was prepared and submitted. Preparation of a manuscript for submission to the journal Gene, based on the results outlined is in progress. This work will be presented in poster format at the department of defense Breast Cancer Research Progam "Era of Hope Meeting" in June 2000.

Technical Objective 2. Prepare a transgenic mouse line with SNX1 expression targeted to the mammary gland using the WAP promoter.

Task 6: **Months 7-9: Prepare the transgenic expression vectors.**

Progress: Transgenic expression vectors have now been prepared. The SNX1 genomic clone was too large for the proposed construction. Therefore we have adopted a cDNA approach that has been use successfully for stromolysin 1 (1) and for IGF (2). We obtained plasmid pbl103 containing bases -949 to +33 of the rat whey acidic protein (WAP) gene and plasmid pbTAPW3' containing 843 base pairs of WAP 3' sequence including the exons 3, 4 and the 3' untranslated region from Jeffrey Rosen, Baylor College of Medicine, Houston, Texas. We used these plasmids to generate a WAP-SNX1 cDNA vector. Our approach wass to ligate the SNX1 cDNA into a WAP vector consisting of 943bp rat WAP 5' sequence and 675 bp WAP 3' sequence (Figure 4). To generate this construction, a 5' WAP PCR product was generated using oligonucleotides

5'-GATCGTCGACAAGGAGTATGGGCTGCACCA-3'

5'-GATCGAATTCGGCGGCGGCAGGCAAGTGAT-3'

as primers and pbl103 as the template. This PCR product contains rat WAP sequences -949 to -7. The 5' WAP PCR product was cleaved with SalI and EcoRI and cloned into the vector pBSIIKS(+) to generate pWAP 5'. Next, a 3' PCR product was generated using oligonucleotides

5'-GATCGAATTCAATGGCTGTATCATGAGTTG-3'

5'-GATCGCGGCCGCTCATTCTGTCAAGAGCTCAG-3'

as primers and pbTAPW3' as the template. The 3'WAP PCR product was cleaved with EcoRI and NotI and cloned into EcoRI/NotI cleaved pWAP 5' to generate pWAP 5' & 3' (Figure 4). The high fidelity polymerase Pfu was used for all PCR reactions reactions. An EcoRI fragment of SNX1 was cloned into the EcoRI site in pWAP 5' & 3'. The correct orientation of the SNX1 insert was determined by restriction enzyme mapping and nucleotide sequencing. The resultant plasmid, pWAP-SNX1cDNA (Figure 5) was cleaved with SalI and NotI and the linearized minigene purified for mouse oocyte microinjection.

Task 7: **Months 10-13: Generate founder mice in collaboration with Jeffrey M. Rosen, Baylor College of Medicine.**

Progress: pWAP-SNX1cDNA vector construction is completed and a linearized DNA fragment has been purified. Next, the DNA will be sent to the NICHD transgenic Mouse Development Facility at the University of Alabama at Birmingham for the production of transgenic mice.

Task 8: **Months 16-18: Identify transgenic lines, confirm SNX1 protein expression.**

Progress: A PCR assay has been developed and validated for detecting the SNX1 transgene in FVB mouse tail blot DNA (figure 6). A graduate student, Anthony Eddington M.S., has been identified who will be responsible for the project. Stocks of an affinity-purified polyclonal antibody (#3904) that was to be used to measure SNX1 protein expression, was depleted in the last year. New antibodies have been generated (figure 7).

Task 9: **Months 19-22: Determine the effect of overexpressing SNX1 in the mammary gland on the concentration of mammary gland EGF receptors**

Progress: We have begun to assemble the reagents to measure EGF receptor levels in saturation binding experiments. We have purchased the EGF and identified a vendor of the ^{125}I-EGF we will require for the assays. In a separate project, we have generated a HEK 293 cell line overexpressing EGF receptors. We will use this cell line to validate the ligand binding assay. A technician, Ms. Susan Foreman, has been identified in a colleague's laboratory adjacent to my own to perform the assays. Susan has experience in more difficult measurements of β_2-adrenergic receptors in crude membranes and should have no problems performing the more sensitive EGF binding assays to measure EGF receptors in mouse mammary glands.

Task 10: **Months 23-24: Prepare annual project report and a manuscript describing the WAP-SNX1 mice and the consequences of SNX1 overexpression on EGF receptor levels in vivo.**

Progress: This annual report was been prepared. Preparation of a manuscript has not been initiated as that awaits the completion of tasks 7-9.

Technical Objective 3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

Progress: The tasks in technical objective (task 11-14) cannot be begun until task 8 is completed. Thus we are currently 5 months behind schedule. We will apply for additional funding to ensure completion of these tasks.

KEY RESEARCH ACCOMPLISHMENTS

- Chromosomal Mapping of SNX1 and SNX2
- Characterization of the 60kb human SNX1 gene
- Analysis of alternative splicing of SNX1 in cDNA from human tissues
- Functional identification of the SNX1 promoter
- Completion of WAP-SNX1 minigene construction
- Validation of PCR assay to detect SNX1 minigenes in mouse tail DNA
- Generation and characterization of affinity-purified antibodies to detect the SNX1 protein

REPORTABLE OUTCOMES

An abstract outlining the work completed for Technical Objective 1 has been submitted and will be presented in poster format at the department of defense Breast Cancer Research Progam "Era of Hope Meeting" in June 2000.

Work from my laboratory was presented in April 2000 at the American Association of Cancer Research meeting (Appendix 2). Although not directly related to the Statement of Work, the lead author, Cynthia Burroughs, Ph.D., was awarded a HBCU Faculty Scholar Research

Award for this poster. Dr. Burroughs is on the faculty at a neighboring undergraduate institution, The University of Arkansas at Pine Bluff, and has experience with mouse mammary glands. She works in the laboratory full-time during the summer and part-time during the academic year. This collaboration represents a Career Development Effort that is especially relevant to procuring additional research support for the laboratory from the State of Arkansas Breast Cancer research Program.

CONCLUSIONS

We have cloned and characterized the human gene for SNX1. The SNX1 gene is alternatively spliced and characterization of its structure will aid in understanding the regulation, and significance of the alternative splices. We have also identified the functional promoter in transfection experiments and find that it contains features characteristic of a house keeping gene. The SNX1 genomic clone and subclones that we now have in hand have also provided useful reagents for gene knockout studies to better understand the function of SNX1 inside cells. A graduate student, Parag Chowdhurry, has constructed a targeting vector and will attempt to use it to knockout the SNX1 gene in HEK 293 cells. Our determination of the chromosomal localization of SNX1 could facilitate the potential assignment of SNX1 as a disease locus identified in human genetic mapping studies. Currently, no diseases map to the SNX1 locus. Given the large size of the introns in the SNX1 gene, we decided not to pursue our first choice of replacing coding exons and intervening sequences of the whey acidic protein gene with SNX1 genomic sequences. Instead, we have inserted a SNX1 cDNA fragment to generate our minigene for injection into mouse oocytes.

Over the last year, other projects in my laboratory and in other laboratories (3-5) have led me to question the specificity and the utility of SNX1 in down-regulating EGF receptor expression. In part, these reservations may have arisen from our use of overexpression strategies to study SNX1. For this reason, transgenic mouse production was temporarily delayed while we evaluated the problem. We think that our plans for gene knockouts will provide additional support for the functional identification of SNX1 as a down regulator of EGF receptors using an experimental approach distinct from overexpression. We now have evidence that SNX1 exists in a multi-protein complex consisting of oligomers of SNX1, SNX2 and possibly 3 additional proteins. The fact that SNX1 is part of a multi-protein complex may account for variability problems in our cultured cell assays. In any event, the conservation of function and biochemical properties between SNX1 and its yeast homologue, Vps5p (6), make it clear that SNX1 has a role in endosomal membrane trafficking. With our continued studies in cell culture models and transgenic animals, we expect to make considerable progress in understanding the role of SNX1 in cell proliferation and EGF receptor trafficking the mammary gland.

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APPENDICES

Appendix 1 - Figure Legends

Figure 1. Chromosomal localization of SNX1 and SNX2. Fluorescence in situ hybridization of P1 genomic DNA clones isolated using SNX1 and SNX2 cDNA fragments. Biotinylated DNAs corresponding to genomic clones for SNX1 (3E2) and SNX2 (B100-3) hybridized with efficiencies of 87.5% and 85%, respectively. Two additional P1 clones isolated with the SNX1 cDNA (6K1 and 6K7) yielded similar results.

Figure 2. Restriction map of the 6K1 Clone. Indicated are the locations of sites for some of the restriction enzymes (EcoRI, BamHI, XhoI) used for mapping and subcloning the 6K1 genomic fragment. Also indicated are the locations of defined exons as well as at least 4 undefined exons (Contains Exon) .

Figure 3. Map of the SNX1 Gene as deduced from the 6K1 clone. The cross hatched boxes (Seq) on the map indicate the regions of 6K1 that have been sequenced. Also indicated are exons defined by sequencing and the coordinates in the SNX1 cDNA (U53225) to which they correspond. The hatched boxes indicate subclones containing at least 4 additional exons based on their hybridization with oligonucleotides derived from the SNX1 cDNA.

Figure 4. Map of plasmid pWAP 5'&3'. This plasmid was constructed by cloning PCR fragments corresponding to the 5' (WAP -949 to -7) and the 3'-end (WAP 3' PCR Product) of the rat WAP gene. The unique EcoRI site separating the WAP 5'-end and 3'-end fragments was used for insertion of the SNX1 cDNA.

Figure 5. Map of the plasmid from which the linearized SNX1 minigene is derived. Plasmid pWAP-SNX1cDNA was generated as described. Then indicated SalI-NotI minigene

fragment was gel purified and will be used for microinjection into mouse oocytes to generate transgenic mice.

Figure 6. Specificity and sensitivity of pWAP-SNX1 transgene specific amplimers in the presence of FVB mouse tail genomic DNA. PCR reactions were performed in a volume of 25µl with the indicated amounts of pWAP-SNX1 transgene and/or FVB mouse genomic DNA. One primer was specific for human SNX1 and the other was specific for rat WAP DNA. Assuming that there are $1.6x10^5$ copies of transgene per 100ng genomic DNA, This assay will easily detect the specific transgene in mouse tail DNA assuming that there are $1.6x10^5$ copies of transgene per 1000ng genomic DNA.

Figure 7. Western Blot Characterization of Affinity Purified Anti-SNX1 (Batch 2) using HeLa cytosols containing HA-SNX1. Cytosols were prepared from HeLa cells overexpressing a HA epitope-tagged SNX1. The indicated volumes of the cell extracts were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes and blotted with affinity purified antibodies raised against the peptide CKYLEAFLPEAKAIS. Antibody preparations from two different rabbits (5551/2 and 5552/2) were analyzed either fresh or following aliquoting and a cycle of freezing and thawing. From this analysis, we conclude that 5551/2 is the more sensitive and specific antibody preparation and that both preparations are stable to freeze-thaw.

Figure 1

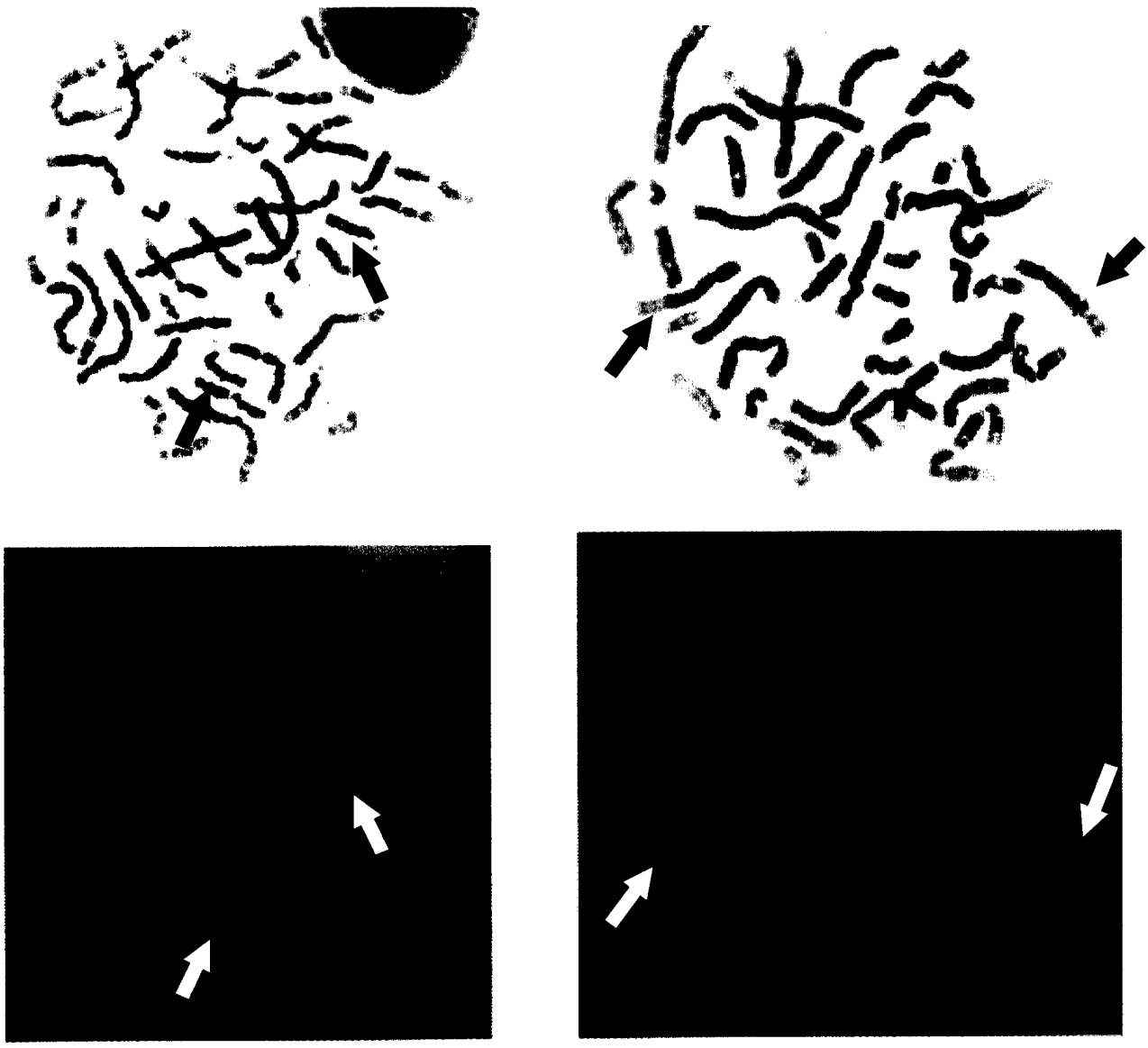
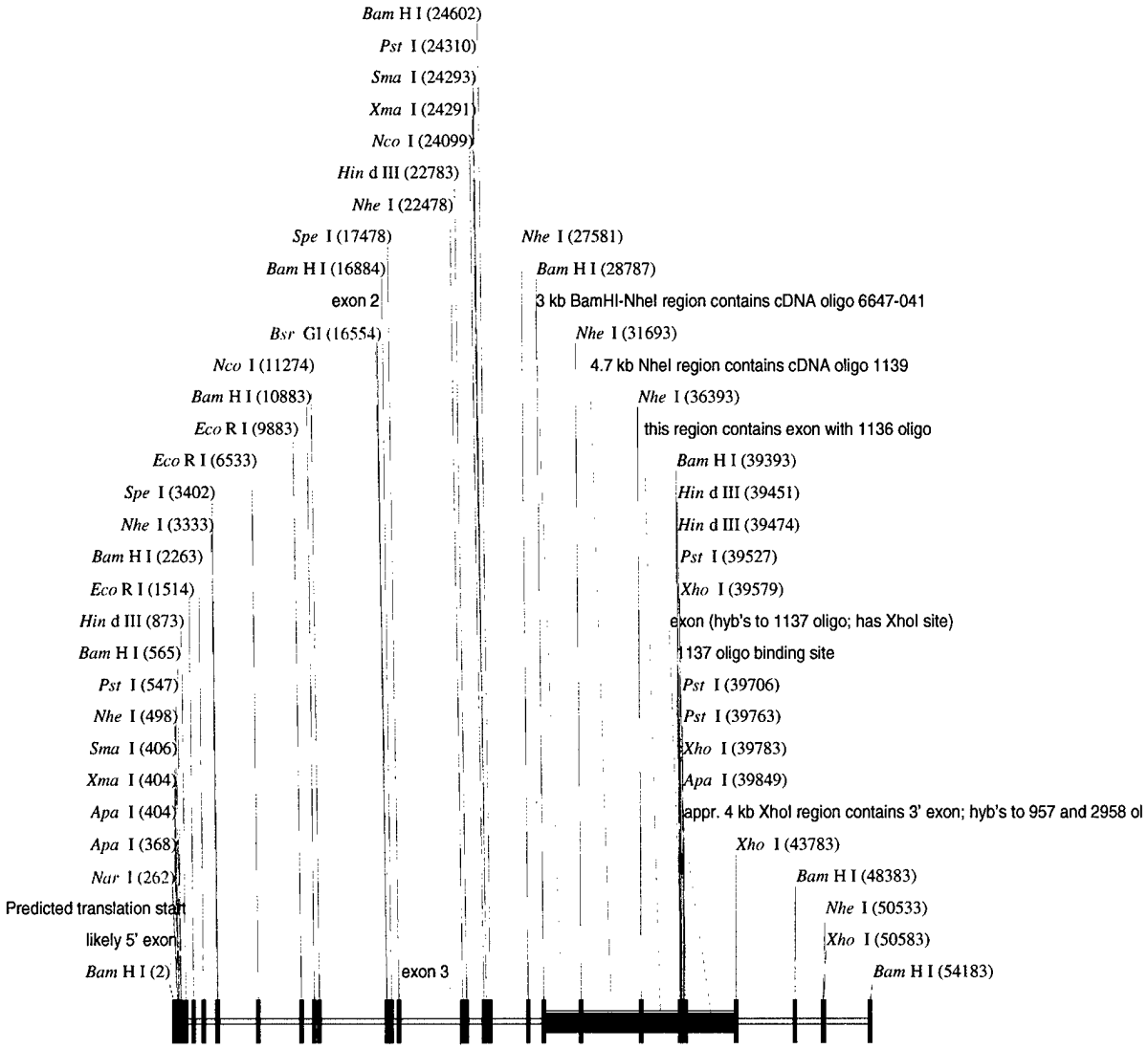


Figure 2



SNX1genmap

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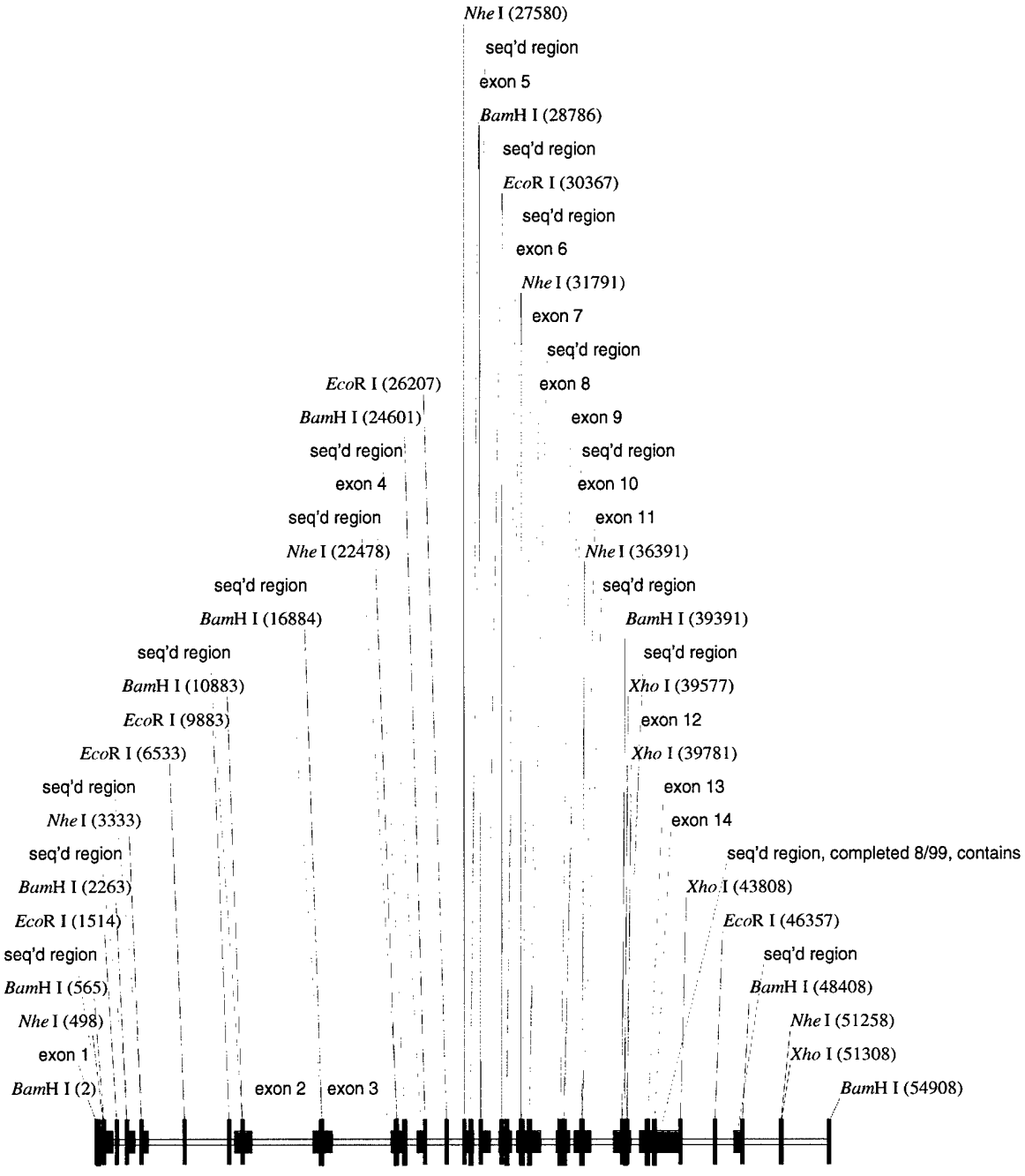
Figure 3



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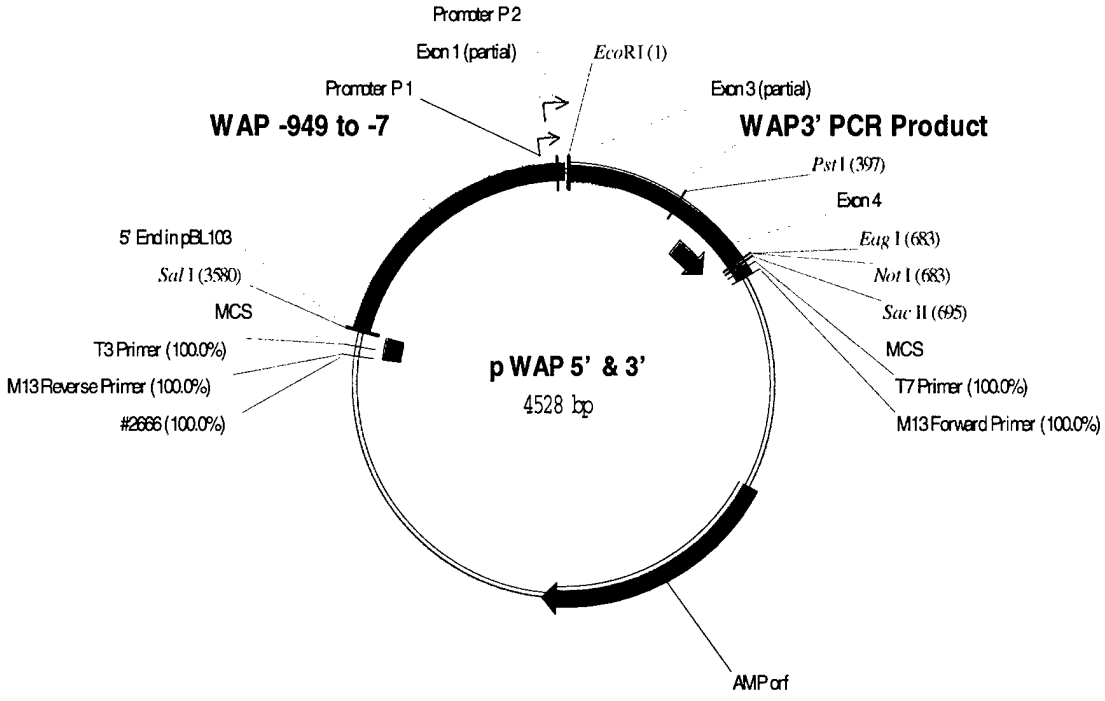
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Sal I (3580)
Not I (683)
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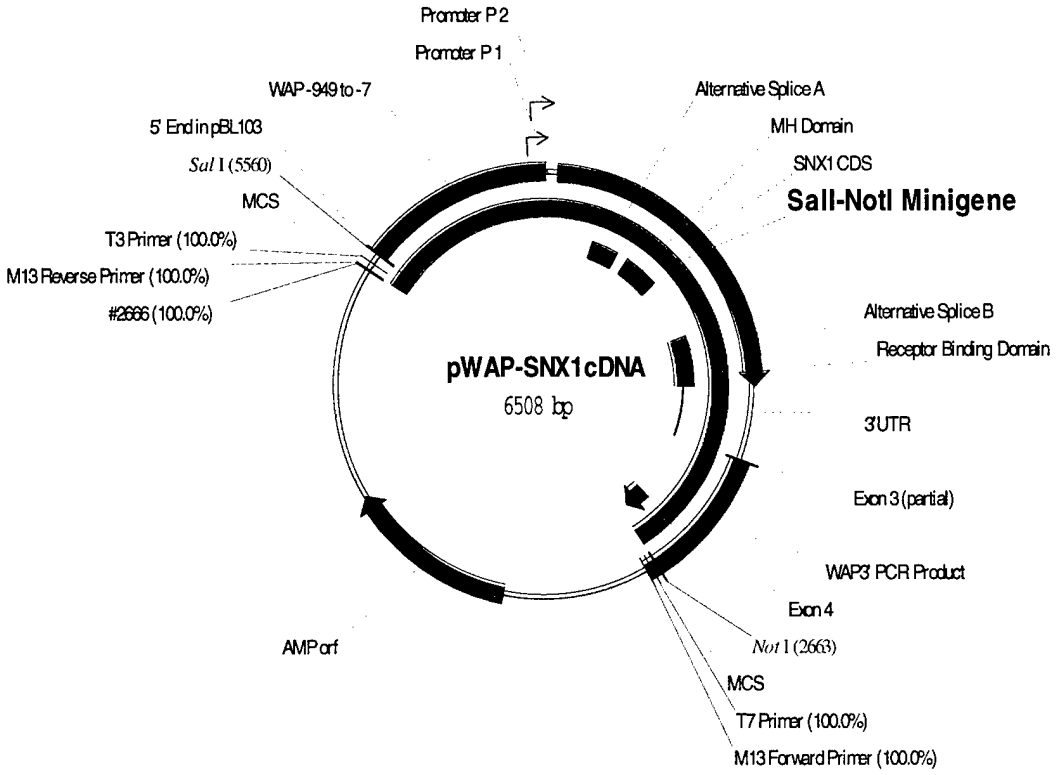
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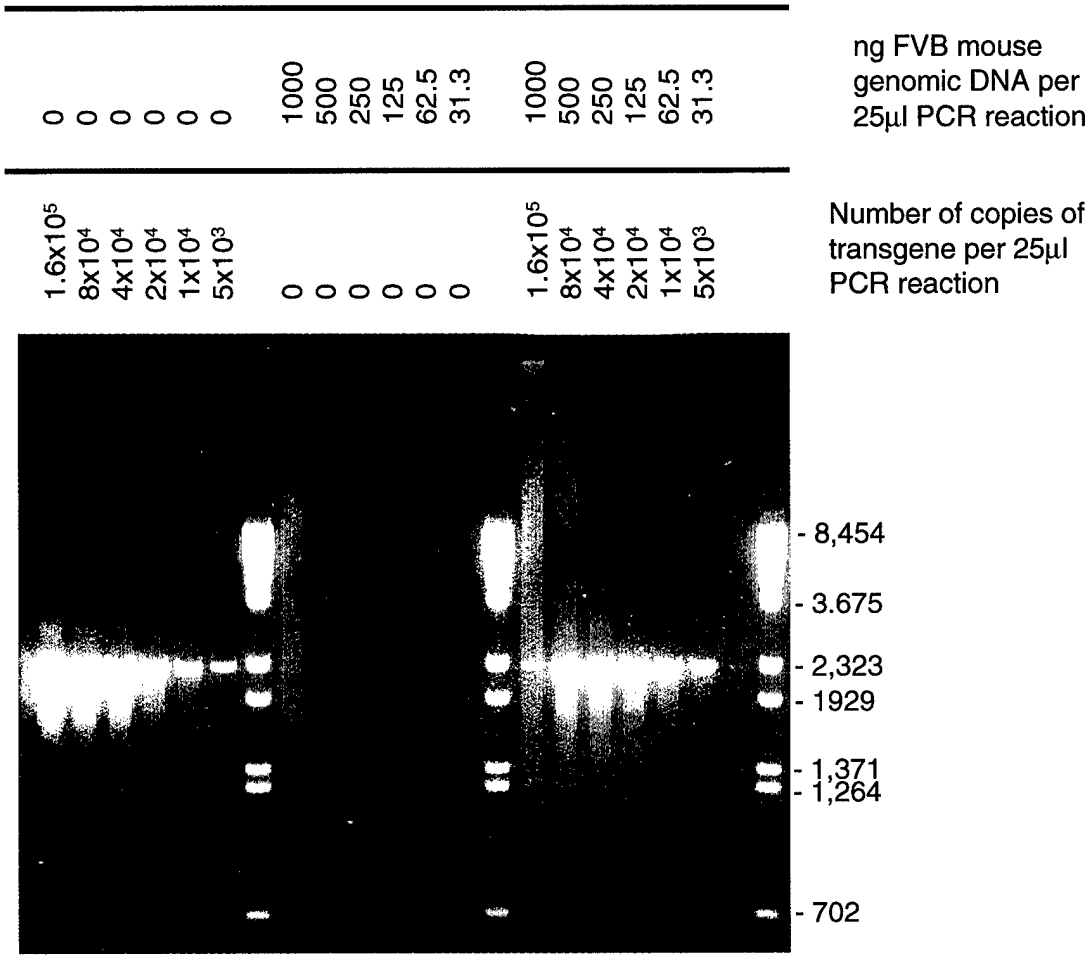
Figure 6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- 1,371
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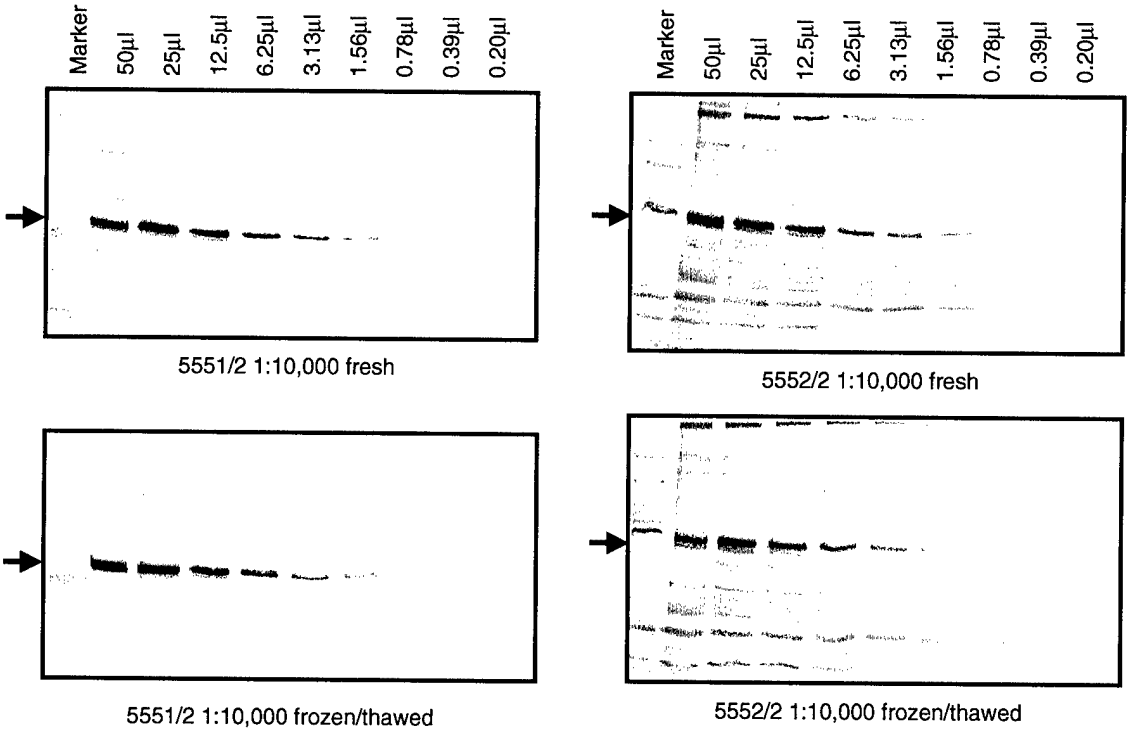
Figure 7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5551/2 1:10,000 fresh
5552/2 1:10,000 fresh
5551/2 1:10,000 frozen/thawed
5552/2 1:10,000 frozen/thawed

Appendix 2 - Abstract resulting in award for the first author

Poster presented at the 91st Annual Meeting of the American Association of Cancer Research April 1-5, 2000 San Francisco, CA.

INTRACELLULAR POOLS OF RECEPTORS MODULATE THE KINETICS OF RECEPTOR DOWNREGULATION. C. Burroughs, R. Smith, M. McIntire and R. Kurten. University of Arkansas at Pine Bluff, Pine Bluff, AR 70611; University of Arkansas School for Medical Sciences, Little Rock, AR 72205

Different distributions of erbB2 receptors have been documented in different cell lines. In some cell lines, most receptors are inside the cell whereas in others most of the receptor is external. We have examine the distribution of epidermal growth factor receptor (EGFR) and the related receptor erbB2 in a virus transformed cell line derived from normal breast tissue (HB2), in an MCF7 cell line, and in a variant cell line derived from MCF7. Each breast cell line was grown to 70% confluence in DMEM/F12 with 10% bovine calf serum and 1% antibiotics. Cells where washed with PBS and serum starved for 24hr in DMEM/F12, 0.01% BSA and 1% antibiotics. Cells were exposed to 100 nM EGF in DMEM/F12, 0.01% BSA and 1% antibiotics and terminated at 2,4,6,12 and 24 hours after treatment. EGF receptor and erbB2 expression was examined in detergent extracts by western blotting. HB2 and MCF-7 cells expressed modest amounts of both EGF and erbB2 receptors. Treatment with EGF for 2 hours efficiently down-regulated both EGF and erbB2 receptors. By contrast, the variant MCF7 cells expressed high levels of receptors and the total mass of receptors was not affected by treatment with EGF for 2 hours. However, when variant MCF7cells were treated for 12 or 24 hours, there was efficient down-regulation of both EGF receptor and erbB2.