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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. The revised plan is to construct a WAP-SNX1 cDNA vector for transgenic mouse production.

A Career Development Award was a second component of the application. Career development activities include: presentation of the inaugural seminar for the Arkansas Cancer Research Center Forum, 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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RCK In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA RCK In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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

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

[signature] 5-12-99

PI - Signature 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.**

In collaboration with H. Steven Wiley, University of Utah School of Medicine, 3 bacterial articicial 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 has been prepared. Preparation of a manuscript for submission to the journal Gene, based on the results outlined and anticipated is in progress.

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 not yet 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 (Sympson, et al., 1994) and IGF (Neuenschwander, et al. 1996). 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 are in the process of using these plasmids to generate a WAP-SNX1 cDNA vector. Our approach is 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 will be 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 will be cleaved with SalI and EcoRI and cloned into the vector pBSIIKS(+) to generate pWAP 5'. Next, a 3' PCR product will be generated using oligonucleotides

5'-GATCGAATTCAATGGCTGTATCATGAGTTG-3'

5'-GATCGCGGCCGCTCATTCTGTCAAGAGCTCAG-3'

as primers and pbTAPW3' as the template. The 3'WAP PCR product will be 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 will be used for all PCR reactions reactions. An EcoRI fragment of SNX1 will be cloned into the EcoRI site in pWAP 5' & 3'. The correct orientation of the SNX1 insert will be determined by restriction enzyme mapping and nucleotide sequencing. The resusltant plasmid, pWAP-SNX1cDNA (Figure 5) will be 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: This task will be initiated when the pWAP-SNX1cDNA vector construction is completed (anticipated time to completion - 2 months).

KEY RESEARCH ACCOMPLISHMENTS

- Chromosomal Mapping of SNX1 and SNX2
- Partial Characterization of the 60kb human SNX1 gene
- Initiation of WAP-SNX1 Minigene Construction

REPORTABLE OUTCOMES

There are no reportable outcomes for this funding period.

CONCLUSIONS

We have cloned and made considerable progress in characterizing the human gene for SNX1. The SNX1 gene is alternatively spliced and characterization of its structure will aid in understanding the mechanisms, regulation, and significance of the alternative splices. In addition, the clone and subclones that we now have in hand will also provide a useful reagent for use in gene knockout studies in the future to better understand the function of SNX1 inside 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. Given the large size of the introns in the SNX1 gene, we will not pursue our first choice of replacing coding exons and intervening sequences of whey acidic protein gene with SNX1 sequences. Instead, we will insert a SNX1 cDNA fragment to generate our minigene for injection into mouse oocytes. With the generation of transgenic animals, we expect to make considerable progress in understanding the role of SNX1 in cell proliferation and EGF receptor trafficking the mammary gland .

REFERENCES

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2. Neuenschwander S, Schwartz A, Wood TL, Roberts CT Jr, Henninghausen L, LeRoith D (1996). Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model.*J Clin Invest* 97(10):2225-32.

APPENDICES

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 will be 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 will be used for insertion of the SNX1 cDNA.

Figure 5. Map of the plasmid from which the linearized SNX1 1 minigene will be derived. Plasmid pWAP-SNX1cDNA will be generated as described. Then indicated SalI-NotI minigene fragment will be gel purified and used for microinijection into mouse oocytes to generate transgenic mice.

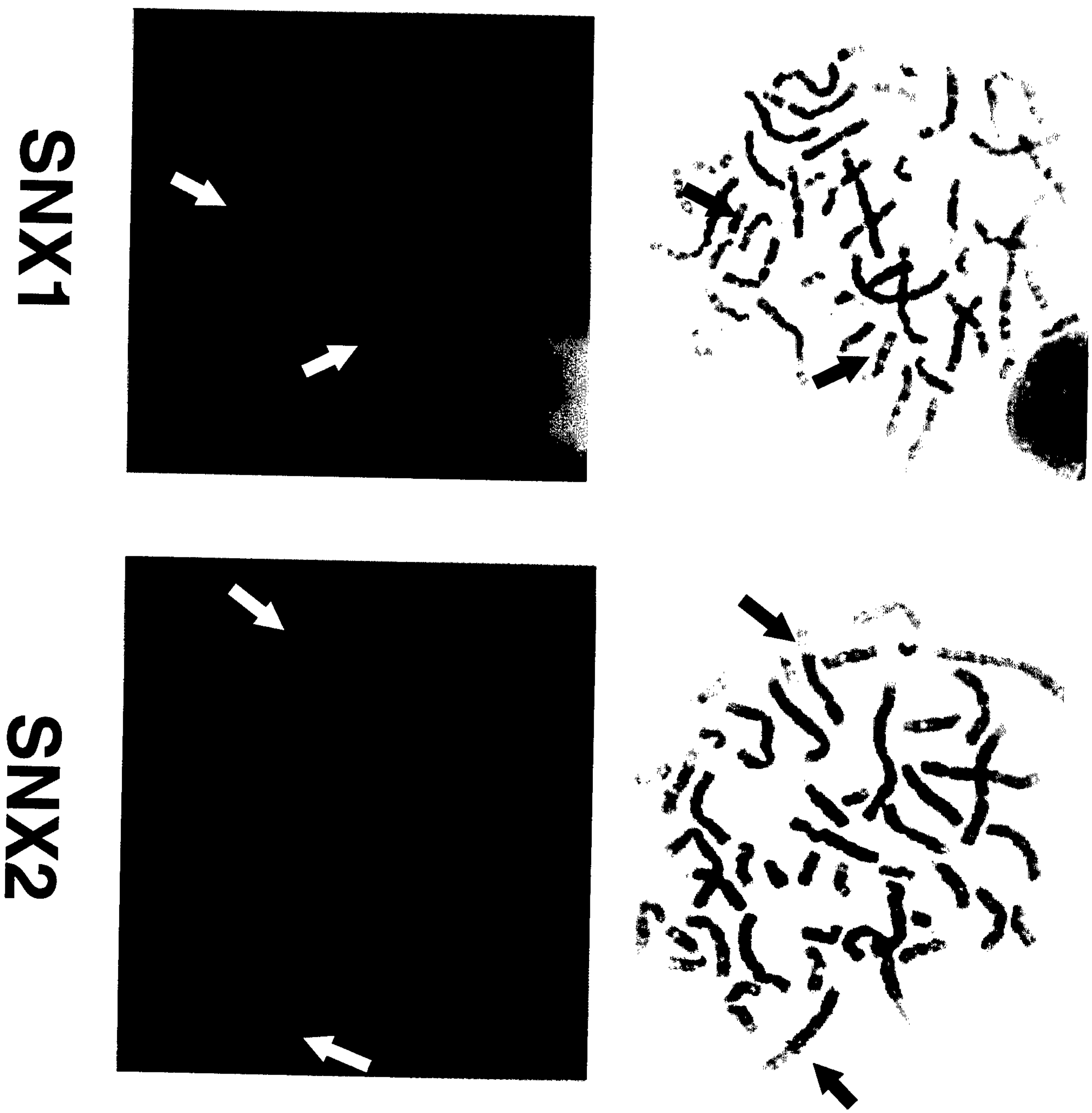
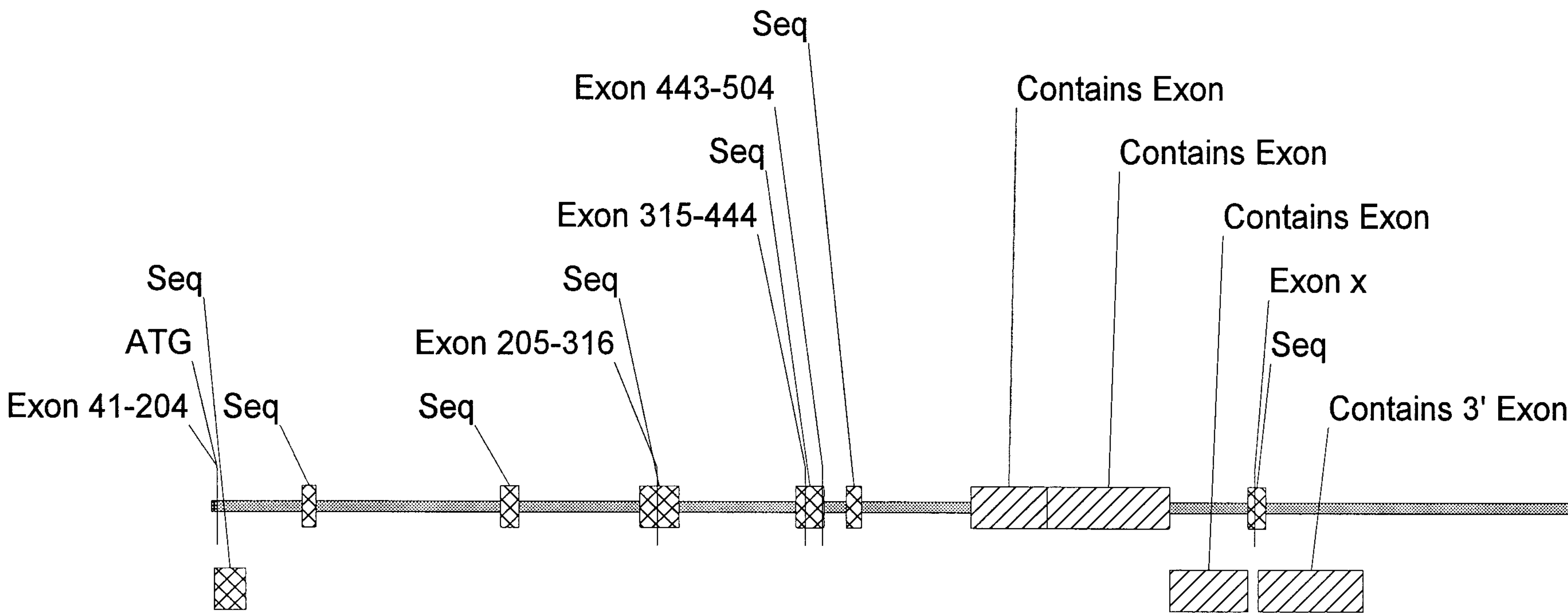
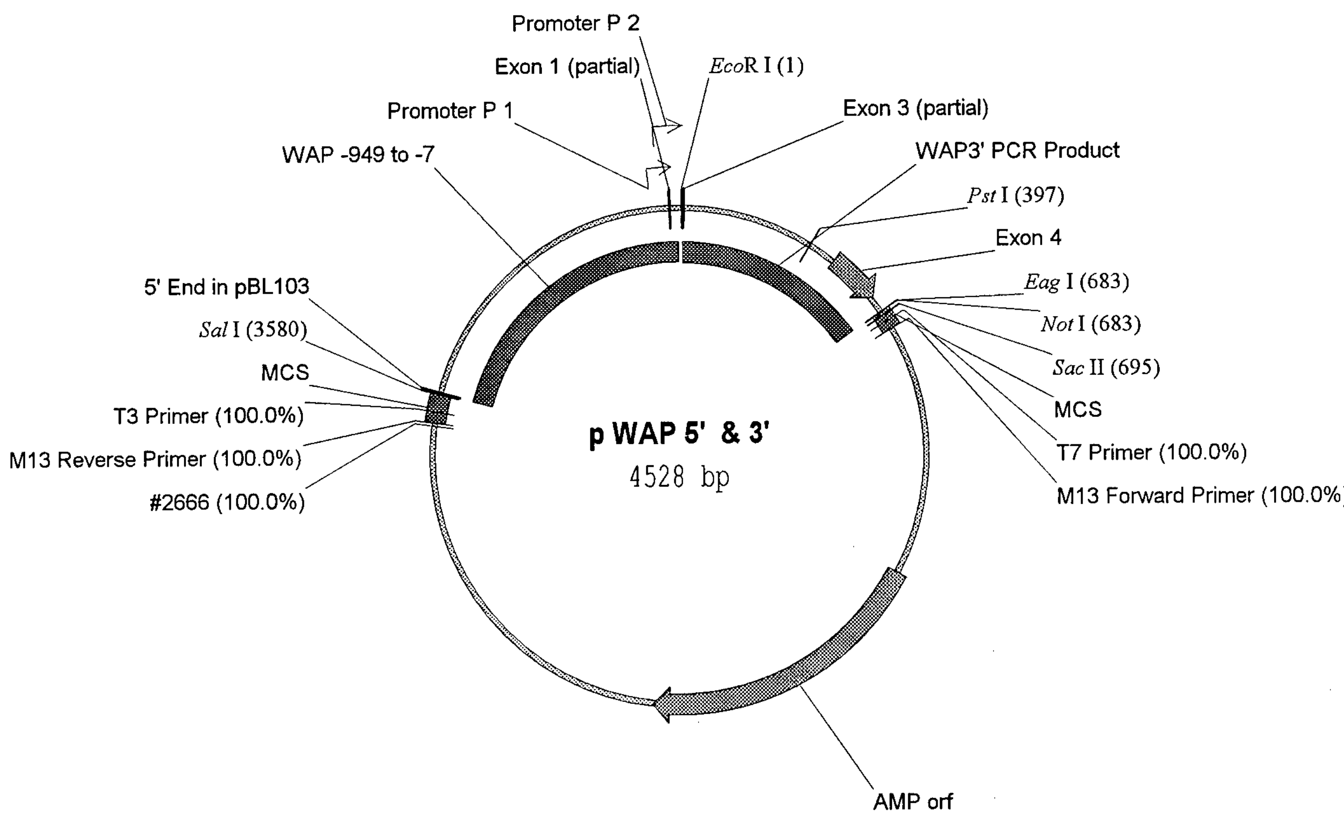


Figure 1



SNX1 Gene Map

54187 bp


Promoter P 2
Exon 1 (partial)
EcoR I (1)
Promoter P 1
Exon 3 (partial)
WAP -949 to -7
WAP3' PCR Product
Pst I (397)
Exon 4
Eag I (683)
Not I (683)
Sac II (695)
MCS
T7 Primer (100.0%)
M13 Forward Primer (100.0%)
5' End in pBL103
Sal I (3580)
MCS
T3 Primer (100.0%)
M13 Reverse Primer (100.0%)
#2666 (100.0%)
p WAP 5' & 3'
4528 bp
AMP orf

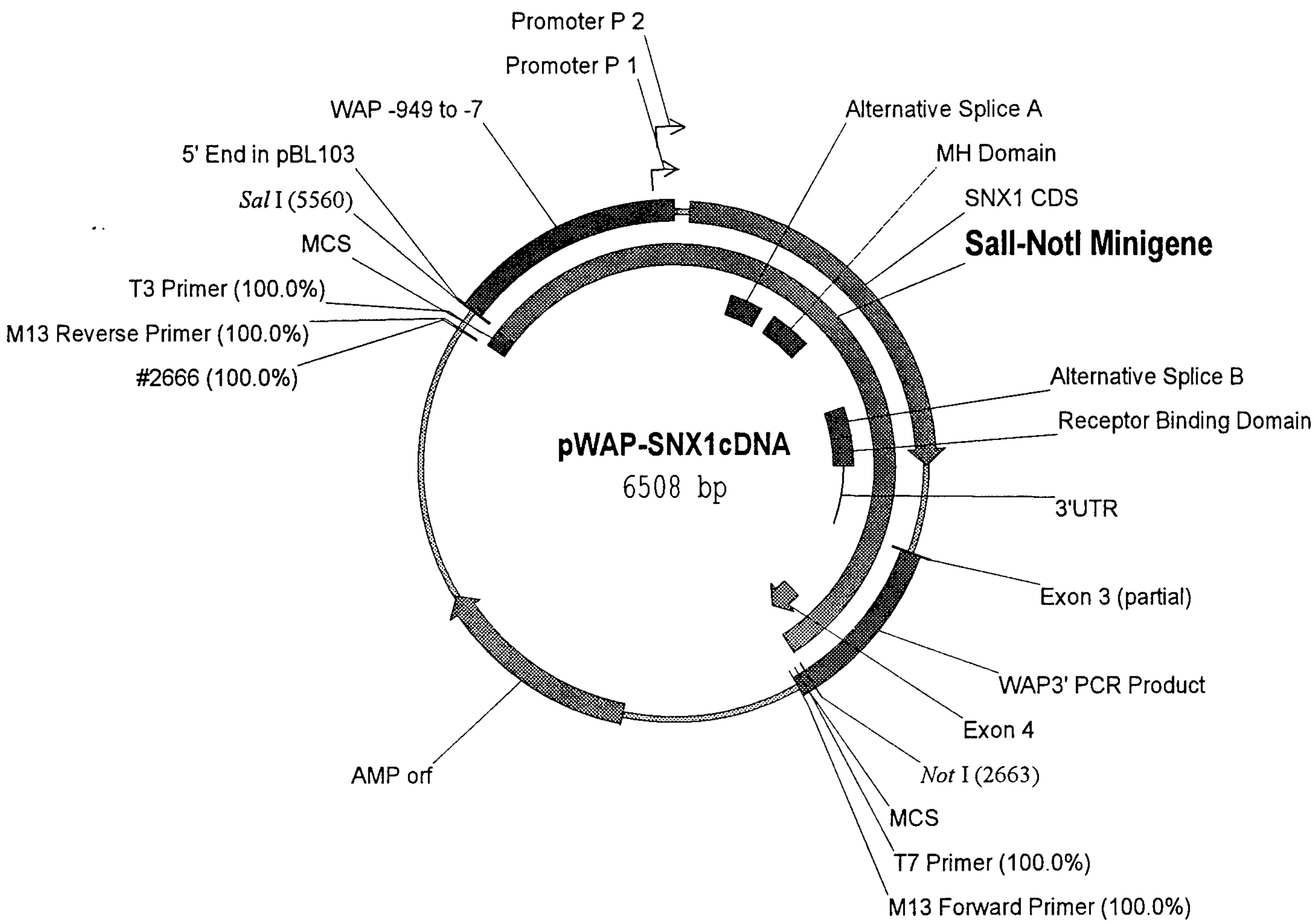

Promoter P 2
Promoter P 1
WAP -949 to -7
5' End in pBL103
Sal I (5560)
MCS
T3 Primer (100.0%)
M13 Reverse Primer (100.0%)
#2666 (100.0%)
Alternative Splice A
MH Domain
SNX1 CDS
SalI-NotI Minigene
Alternative Splice B
Receptor Binding Domain
pWAP-SNX1cDNA
6508 bp
3'UTR
Exon 3 (partial)
WAP3' PCR Product
Exon 4
Not I (2663)
MCS
T7 Primer (100.0%)
M13 Forward Primer (100.0%)
AMP orf

Figure 5