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Chromosome 3p

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13. ABSTRACT (Maximum 200 words) Loss of tumor suppressor genes by genetic mechanisms represent critical molecular events in the development and progression of breast cancer. One or more of these tumor suppressor genes likely resides on the short arm of chromosome 3 (3p) and appears to be involved in nearly 50% of breast cancers. We have identified a region in 3p14 which undergoes recurrent homozygous deletion or rearrangement in breast cancer cell lines. A set of cloned DNA molecules spanning the target region has been established and a gene search is underway. In order to develop a functional tumor suppressor test of the 3p14 and other target regions, we have begun to modify specific YACs with the Neomycin resistance gene that will permit their selective retention in mammalian cells. The identification of this homozygous deletion region is an exciting development that has resulted in a modification of the order for our Specific Aims.				
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INTRODUCTION

A. Purpose of the Present Work

Our project concerns the identification and isolation of a breast cancer tumor suppressor gene from the short arm of chromosome 3 (3p). Importantly, we have recently identified a region of homozygous deletion in a subset of breast cancer cell lines. Our findings strongly suggest that a tumor suppressor gene will be found in the region of loss. The deletion occurs in a segment of DNA within a few hundred kilobases** of a constitutional translocation breakpoint between chromosomes 3 and 8 which is associated with hereditary renal carcinoma (**kb = 1000 base pairs of DNA, a measure of distance along the DNA molecule). This breakpoint at 3p14.2 was one of the possible target loci described in our original application. Our finding of homozygous deletions near this site has caused us to modify the order of our experimental design and has required development of new DNA reagents to study the target area. This Revised Progress Report first deals with the 3p14 homozygous deletion region representing Specific Aim 3, and then lastly covers progress made on Specific Aim 2 (which was unintentionally omitted in the earlier version of this report). We have attempted to explain, in lay terms, technical concepts throughout.

B. Nature of the Problem

The malignant potential of any tumor, including breast cancer, is a consequence of specific alterations (mutations, deletions, amplifications, overexpression, etc.) in target genes that regulate the growth and biologic behavior of those cells. (Genes are segments of DNA which encode proteins; DNA is "transcribed" into RNA and RNA in turn is "translated" into protein.) Whether cells grow slowly and remain localized, or proliferate rapidly and spread to distant sites (metastasize) is a complex process involving a host of regulatory genes. In some instances, specific genes are known. For example, amplification/overexpression of the Her2-neu oncogene (1), a growth factor receptor, is associated with aggressive disease. Similarly, loss or mutation of the p53 tumor suppressor gene, located on chromosome 17, is associated with instability of the genome (entire DNA of the cell) (2). This instability results in an enhanced capacity of the malignant cell to undergo DNA rearrangements leading to alterations in critical regulatory genes. Loss of normal p53 function is also associated with the cell's ability to escape death or cell cycle arrest resulting from therapeutic radiation or chemotherapy (3).

In other instances, the critical regulatory genes have yet to be identified. This is the case for genes located on 3p. Nevertheless, scientific investigations have provided strong evidence pointing to where certain types of critical genes are likely to be located. For example, cytogenetic studies, which examine the content and nature of chromosomes within cells, have identified certain recurrent abnormalities in cancers. Specific chromosomal segments have been found to be increased in number (amplified). This finding is expected to be associated with overexpression of a gene (because of its increased copy number). Such genes, for example, may encode growth factor receptors or may encode proteins that mediate resistance to chemotherapeutic agents. An example is provided by amplification of the MDM2 gene whose protein product inhibits the activity of p53; overexpression of MDM2 is thought to have consequences similar to mutation in p53. In contrast, cytogenetic studies have also pointed to recurrent deletions involving specific chromosomal regions. The critical genes believed to be encoded in these regions are referred to as tumor suppressor genes, the type of gene located on 3p which is the focus of our investigation.

The nature of known tumor suppressor genes is quite varied. Certain tumor suppressor genes, e.g. p16 (an inhibitor of the cyclin dependent kinases or CDKs) and Rb control cell division by regulating the process of DNA replication. Some tumor suppressor genes, such as the chromosome 18 gene DCC (Deleted in Colon Carcinoma), may function in cell-cell adhesion while others such as the neurofibromatosis-2 gene (NF-2 encodes the protein *merlin*) are suspected of affecting the cells internal network of protein fibers (the cytoskeleton). These interactions are

critical for proper signal transduction from the cell's exterior to the nucleus where control of proliferation and differentiation occurs. A common feature in this class of tumor suppressor genes is that their normal function is lost as part of tumor development. As a consequence, a regulatory function generally affecting growth and differentiation is also lost.

C. Methods of Approach

C.1 Methods to Isolate/Identify Tumor Suppressor Genes

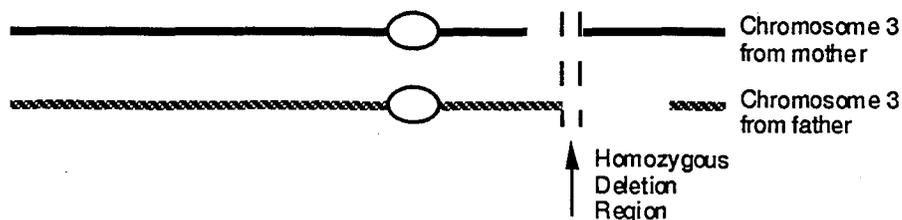
Tumor suppressor genes have been isolated by two approaches. Perhaps the most frequently used method is referred to as "positional cloning". In positional cloning, the region of chromosomal loss is defined by molecular (DNA) probes and cytogenetic analysis. Because a visible chromosome deletion represents a large expanse of actual DNA, it is necessary to narrow the target region as much as possible. In one approach, this is done by using "polymorphic" probes which can distinguish between the two copies of the chromosome in question (each being inherited from one parent). In the tumor DNA, loss of one copy (by a variety of mechanisms) is referred to as "loss of heterozygosity" (abbreviated LOH). To detect LOH, a DNA probe must exist which corresponds to the target DNA in question. Importantly, this bit of DNA must exhibit a fairly frequent naturally occurring variation in the population in order to have a reasonable chance of detecting differences between the two chromosome copies in any individual tumor sample. Naturally occurring differences in the DNA sequence are not uncommon although some types of DNA sequences, referred to as "microsatellites", exhibit much more variation than other types. Microsatellites consist of repeated pairs of nucleotides (usually cytosine followed by adenosine, i.e. CACACACA, etc.) at specific chromosomal sites with the important feature that the number of pairs is variable from person to person. Since the natural variation of markers is often a limiting factor even with microsatellite markers, this explains the general necessity to test fairly large numbers of samples with different DNA probes. The goals of LOH experiments are to identify one or more target regions and to narrow such regions as much as possible prior to performing gene searches.

A second approach in identifying tumor suppressor genes is through the testing of candidate genes. These candidates come in two forms; those in target chromosomal regions (which is essentially the positional cloning approach except that the genes have already been identified), and secondly by testing candidates whose known function suggests they might be targets independent of chromosomal position. Our studies involve the positional cloning approach. As will be discussed below, LOH studies can lead to the identification of homozygous deletions in tumors. These often rare events provide a powerful counterpart to LOH and candidate gene studies.

C.2 Identification of Homozygous Deletions Greatly Facilitates Positional Cloning of Tumor Suppress Genes

An extremely important finding, which can greatly facilitate the precise positioning of tumor suppressor genes, is the identification of homozygous deletions in tumor DNA samples. A homozygous deletion means that both copies of a chromosome have undergone loss for a particular segment of DNA (see diagram below).

Fig. 1 Homozygous Deletion in Tumor



Instead of being restricted to using DNA probes and tumor sample combinations which may or may not detect a naturally occurring variation (polymorphism), homozygous deletions allow direct identification of a potentially critical DNA segment. The DNA of interest is simply missing and therefore the limits of the deletion can be easily defined. Homozygous deletions usually vary in size. Obviously, the smallest deletions define the smallest target area. Alternatively, deletions from different tumors can define the critical target area from their common overlap. In practice, the identification of homozygous deletions greatly expedites the search to identify a segment of DNA expected to contain a tumor suppressor gene. Homozygous deletions may obviate the need to test large numbers of probes against many tumors in order to identify/narrow a region by LOH analysis. However, it is also important to develop polymorphic DNA probes corresponding to the deletion region in order to assay other tumor samples for involvement of the same area.

C. Additional Background Information

In this section, we present a summary of data from the literature regarding genetic deletions of chromosome 3 in breast cancer. These selected studies relate to our identification of a homozygous deletion in 3p14. We have placed these comments here for the convenience of the reader, since the concepts of loss of heterozygosity (LOH) and homozygous deletions have been introduced earlier.

Sato et al. (4) examined 120 breast cancers for loss of heterozygosity (LOH) using a series of 3p polymorphic loci. Nearly 50% of the informative tumors (56/120) demonstrated LOH involving 3p. In this study, the 3p region undergoing the greatest loss was 3p14.2-p13 which should include the homozygous deletion region we have identified. This study has been one of the largest in terms of the number of tumors examined and 3p-derived probes tested. One significant limitation was that the type of probe used did not detect sufficient polymorphisms to allow the limits of the 3p target region to be defined more precisely. Another recent study using LOH analysis demonstrated that 3p loss was most frequently seen in familial breast cancers (5). Excitingly, the highest frequency of loss (68%) was with a probe (D3S1217) also located in the 3p14 region. A cytogenetic study (chromosome analysis by direct microscopic visualization) by Pandis et al. (6) identified 3p deletions in 5 of 41 breast carcinomas. Importantly, the deletions appeared nearly identical and involved the 3p13(p14) region implicated above using LOH analysis. Intriguingly, in the study of Pandis et al., in 3 of the 5 cases the 3p deletion was seen as the only recognizable cytogenetic abnormality. This suggests that 3p deletion may be an early event in a subgroup of breast cancers.

Taken together, there is considerable evidence from multiple investigators using different techniques which supports the existence of a putative tumor suppressor gene located within 3p14, the region containing our homozygous deletion. From a scientific standpoint it is extremely compelling to pursue the modified approach we have taken to identify a 3p tumor suppressor gene operative in breast cancer. This is the purpose of our project.

D. Review of Previous Specific Aims and Modifications to their Experimental Order

D.1. Original Specific Aims.

Our original Specific Aims were written before we discovered a recurrent homozygous deletion involving 3p14 in a subset of breast carcinoma cell lines. The original Aims were as follows:

1. To accurately define, using critically placed highly polymorphic markers, the region (or regions) of 3p that most consistently undergo LOH in breast cancer and to further narrow the critical region in order to facilitate molecular cloning of the disease gene. Fluorescence *in situ*

hybridization will be employed as an adjunct to the analysis of 3p loss. These studies will also provide data on possible microsatellite instability.

2. To test already isolated 3p candidate tumor suppressor genes and flanking DNA sequences for mutations or other alterations occurring in breast cancers or breast cancer cell lines.
3. To isolate additional candidate tumor suppressor genes from involved regions of 3p.
4. Once a breast cancer gene from the 3p region is identified and the DNA sequence analyzed, we will proceed to a more in-depth analysis of mutations in breast cancer specimens and cell lines. We will also correlate the presence or absence of mutations with other prognostic markers and begin studies to characterize the gene product.

D.2. A Modified Approach

From the information presented above, we believe that the most immediate and important challenge is to explore the 3p14 homozygous deletions observed in breast cancer cell lines. If one or more genes can be identified from this region, they will be tested for deletion, mutation and expression in other breast cancer cell lines and direct tumors. In addition, during the next period we will develop polymorphic microsatellite probes (which allow frequent discrimination between the two parental copies of chromosome 3) from the 3p14 homozygous deletion region. These will be used to ascertain LOH of this region in direct tumors (in contrast to cell lines). Therefore, the order of our experimental design is changed with Specific Aim 3 being performed before the survey studies indicated in Aim 1.

BODY

(UNPUBLISHED DATA)

A. Experimental Methods Used and Results Obtained

A.1. Examination of breast cancer cell line DNAs with probes from 3p14.

The following experiments used Southern blot hybridizations with probes from 3p14 and DNAs from breast cancer cell lines. To study the extent of the homozygous deletion region, we developed overlapping cloned DNA molecules using both cosmid and lambda (cloning) vectors. This region, encompassing approximately 180 kb (180,000 bp) of DNA, appears to encompass all or most of the deletions and rearrangements identified to date in breast cancer and other tumor cell lines. Restriction enzyme analysis and Southern blot hybridizations of the cloned molecules allowed us to develop a "restriction map" of the region (see figure below). This enabled us to choose various probes in a directed manner to look for alterations in the target region. The Southern blot analysis is only capable of detecting gross rearrangements of the DNA. Individual base pair changes within a specific gene would not be detected.

Southern blot hybridizations were used to test DNA probes against restriction enzyme digested DNAs from breast cancer cell lines. No deletions or rearrangements were detected using probes from the immediate region of the hereditary renal carcinoma 3;8 chromosomal translocation breakpoint at 3p14.2. This was a possible target region indicated in our original application. Instead we have identified a homozygous deletion region which is located within a few hundred thousand base pairs of the 3;8 translocation. Of 10 breast cancer cell lines, 1/10 demonstrated a homozygous deletion and probable rearrangements have been identified in 3 additional lines. These numbers may represent the "tip of the iceberg" in that many mutations that could negatively affect gene function would be unrecognized by this analysis. We then asked whether we could identify corresponding deletions in other carcinoma cell lines. When tested against a series of

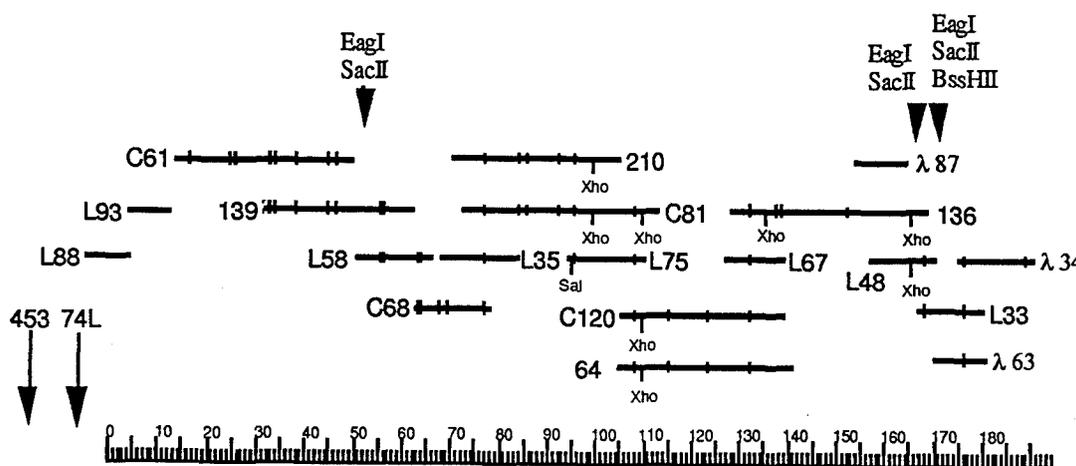
cervical carcinoma lines, homozygous deletions were identified in nearly all. In contrast, no deletions were identified in a series of renal carcinoma cell lines. Thus, the deletion appears to occur in a tissue specific manner suggesting that it does not result from a tissue culture artifact. A table summarizing the results in breast cancer cell lines with various 3p14 probes is provided below. A schematic showing the overlapping DNA clones (contig) is also given below. The accompanying Figure in the Appendix shows a photocopy of an autoradiogram demonstrating the homozygous deletion in cell line #3.

TABLE I. Breast Cancer Cell Lines and Results with 3p14 Probes

cell line	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10
probe:										
453	N	N	N	N	N	N	N	N	N	N
74L	N	N	DEL	N	N	N	N	N	N	N
L88	N	N	DEL	N	N	N				
L93	N	N	DEL	N	N	N				
C61	N	N	N	N	N	N	N	N	N	?
L58	N	N	N	N	Rearr.	N				
C68	N	N	N	N	Rearr.	N	N	N	N	N
L35	N	N	Rearr.	N	N	N				
C81	N	N	Del/ Rearr.	N	N	N				
L75	N	N	N	N	N	N	N	N	N	N
C120	N	N	N	N	N	N				
L67	N	Rearr.	Rearr.		N	N	N	Rearr.	N	N
L48	N	N	N	N	N	N				
L33	N	N	N	N	N	N				

N = normal, DEL = deleted, Rearr. = rearranged, blank = not tested

Figure 2. Overlapping clone set spanning the 3p14 homozygous deletion region. The positions of putative CpG-islands are indicated by arrowheads. These CpG-islands represent clusters of un-methylated CpG rich regions usually associated within genes. Cleavage sites for EcoRI are indicated by short vertical bars, other restriction endonuclease sites are indicated.



A.2. Significance of the homozygous deletion region and the cloned set of DNA molecules spanning the deletion region.

The identification of a DNA segment which undergoes recurrent homozygous deletion and rearrangement, as we have observed for this region of 3p14, strongly suggests that it encodes a tumor suppressor gene. The set of cloned DNA molecules, shown above in Fig. 2, provides the necessary reagents to search for genes which will then be tested for mutation in other breast cancers. Thus, these cloned molecules provide essential materials for subsequent studies. The restriction map we have developed has allowed us to identify the position of potential CpG-islands, marked by the presence of clustered *EagI*, *SacII* and *BssHIII* restriction enzyme sites. These restriction sites are relatively infrequent in the human genome, but are commonly found in the 5'-region of many genes. Our results suggest that 2 genes may lie within this region.

B. Methods Used to Identify Genes

B.1. Gene searches using conserved DNA fragments

We initiated the search for genes in the 180 kb segment using 3 experimental approaches. The first approach involves looking for evolutionarily conserved sequences in the DNA of other species. This approach is based on the fact that the DNA sequence of some genes tends to be very conserved among species and we have had success with this technique in the past (7). To perform these experiments, we isolated selected restriction enzyme cleaved fragments across the 180 kb segment. These were labeled with radioactive phosphorus (^{32}P) and hybridized under reduced stringency conditions to Southern blots containing hamster, mouse, cow, pig and sheep DNAs (referred to as "zoo" blots). Two human DNA fragments detected strong bands in cow, pig and sheep DNAs. The human DNAs were subcloned, their DNA sequences determined and the results were compared to other sequences in GenBank database using the BLAST utility. Unfortunately, the DNA sequence data indicated they were derived from repetitive elements (LINES sequences). Other fragments have detected fairly weak signals in the zoo blots but have not yet been investigated.

B.2. Gene searches using exon-trapping

Two other approaches, exon trapping and genomic DNA sequencing, are being used simultaneously. Exon trapping (8) is a method by which genomic DNA fragments can be randomly cloned into an expression vector (e.g., pSPL3) that contains two exons flanking an intron. Gene regions are comprised of coding portions (exons) and non-coding, intervening sequences (introns). Normally, as the messenger RNA is produced from the DNA template the intervening sequences are "spliced" out. There are specific signals in the RNA, located immediately adjacent to the exons, that signal this splicing event. To use the exon-trapping system, genomic DNAs are inserted into a specific cloning site within the pSPL3 intron. The resulting DNA is then introduced (transfected) into a mammalian cell line (e.g., cos cells). A promoter/enhancer element in the pSPL3 vector initiates RNA production across the region. If the inserted DNA (and thus corresponding RNA) contains an exon, and provided it is in the correct 5' to 3' orientation, the RNA will be spliced as shown below in Fig. 3.

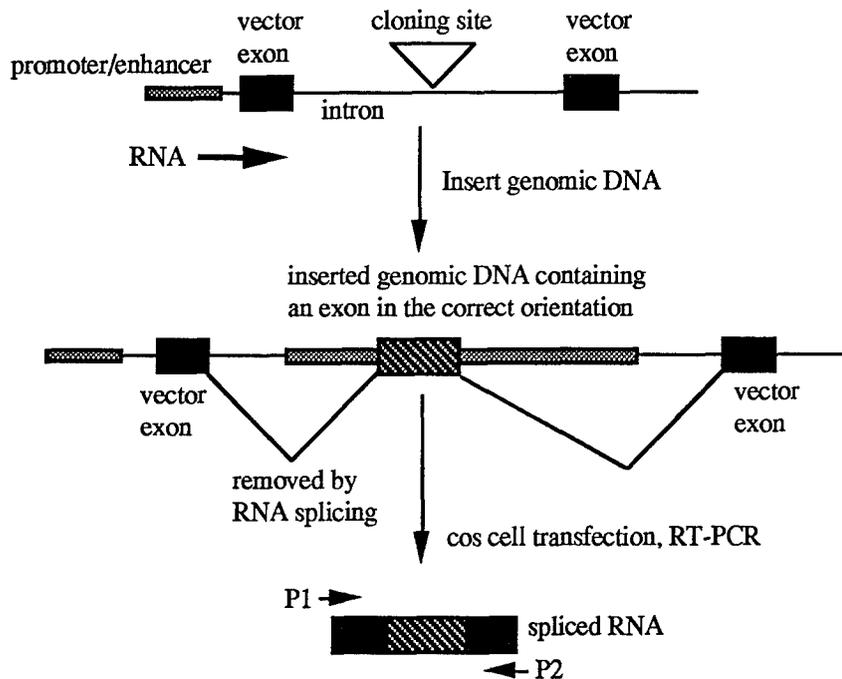


Fig. 3. Diagram of the exon trapping system for identification of coding segments of genes.

RNA is then isolated from the transfected cos cells, corresponding cDNA is prepared using reverse transcriptase (RT), and the polymerase chain reaction (PCR) is used to amplify the specific product. In the above schematic, the PCR primers are indicated as P1 and P2. The product resulting from the incorporation of an additional exon is larger than the product when only the vector exons are spliced together. Larger products are isolated from an agarose gel, subcloned and their DNA sequence determined. The sequences are examined for the presence of an open reading frame (ORF, a DNA segment without translational stop codons) using DNA Strider. Trapped exons are also tested to make sure they are derived from the proper DNA source and have not resulted from contamination or other artifact.

We have taken the group of cosmids spanning the homozygous deletion region, prepared DNA and *Sau3A* partial digests, then inserted these into the cloning site of the pSPL3 exon-trapping vector. This material was transfected into cos cells, RNA was prepared and RT-PCR carried out as outlined above. Larger PCR products were gel isolated, and 200 clones are presently undergoing analysis. So far we have confirmed 2 putative exons of 103 and 154 bp. We know that the 103 bp trapped exon from cosmid 120 (Fig. 2) matches the corresponding genomic DNA sequence from that cosmid. No significant similarities were identified after performing a GenBank search of DNA and protein databases. Trapped exons will also be used to screen cDNA libraries looking for corresponding larger fragments and to identify/confirm which of these may actually represent genes. Similarly, trapped exons will also be used as hybridization probes on Northern blots to look for expression (meaning they represent real genes). We will also carry out RT-PCR experiments using RNAs from a variety of cell lines looking for expression.

B.3. Gene Search by DNA Sequencing

Genomic DNA sequencing provides a powerful approach to look for genes, provided that the region in question can be efficiently examined. The 180 kb homozygous deletion region can be handled since we have two ABI automated DNA sequencers (models 373 and 377) in our laboratory and we have a complete set of overlapping clones. We have gained considerable experience with large scale DNA sequencing through our efforts in the Human Genome Project.

We have automated the process of transferring information from the ABI generated sequence files, collected on a MacIntosh computer, to a SUN workstation where the information is processed and analyzed. This results in the assembly of data derived from many individual sequencing reactions. Using randomly generated clone libraries (the DNA templates for sequencing), 6-7 fold redundancy of sequence data are required before the actual sequence can be efficiently assembled. Thus, 240-280 kb of raw sequence information is obtained to assemble a 40 kb cosmid (which we have done). From the assembled DNA sequence, we wish to determine if any of its portions contain either known genes, or expressed sequences identified from cDNA libraries. Due to the effort supported by the Genome Project, a considerable portion of all the human gene sequences have been determined. These sequences are stored in a computer database (dbEST) as "expressed sequence tagged sites" or ESTs. Since ESTs have been derived from various cDNA libraries, a match between our genomic DNA sequence and sequences in the dbEST database would indicate the presence of a gene. To avoid artifacts, our DNA sequences are first screened and filtered (using various software packages) to remove all identifiable repetitive DNA sequences.

A second method of analyzing the DNA sequence is through a program called GRAIL (9). This utility searches the genomic DNA sequence for various features including open reading frames, codon usage (which is non-random) and predicted splice sites. This allows the detection of putative exons (coding regions). This approach is entirely analytical whereas the exon-trapping methodology looks for exons biologically on the basis of RNA splicing. Thus, the processes are very complementary.

We have begun the sequencing process using two cosmids (#120 and 136) indicated in Fig. 2. Thirty-seven kb of finished sequence from cosmid 120 has been assembled from 240 kb of raw sequence (6.5 X redundancy). Cosmid 136 is approximately one-half completed with over 3X coverage to date. Cosmid 120 was found to be very rich in repetitive sequences although 4 potential exons have been identified using GRAIL. GRAIL predicted exons are reported in terms of being excellent, good and marginal. "Excellent" exons have an approximately 90% probability of being real, while "good" and "marginal" exons are correct 60% and 20% of the time, respectively. In cosmid 120, GRAIL predicted 1 excellent, 1 good and 2 marginal exons. Independent exon-trapping experiments so far have identified an additional putative exon not detected by GRAIL. Thus, we have evidence for possible gene sequences within this cosmid. Cosmid 136 contains a CpG-island and on that basis is likely to contain a gene. The sequence data from cosmid 136 has been partially assembled into 9 overlapping groups (contigs). While this level of coverage is sufficient to search for database similarities, we have had more success, less artifacts and better understanding of the results when the sequence was more completely assembled. We expect to have the cosmid assembled in the next few weeks. We have also used a PCR-based approach to clone and sequence the region of the EagI site located in lambda 58 (Fig. 2). We have tentatively identified a 470 bp open reading frame which may represent an exon.

.B.4. Significance of the exon-trapping and DNA sequencing studies

These studies represent the basic approach we are using to search for genes in this homozygous deletion region. They are proceeding at a very good and steady rate. We believe we can obtain the complete assembled sequences for the remaining cosmids within approximately 4 months. Our past experience has already helped us streamline this process so that DNA sequences for the remaining cosmids will be obtained more rapidly and with lowered redundancy. Our immediate goal will be to identify as many potential coding segments within the target region, since DNA sequence similarity searches may suggest the most likely candidate(s) for a tumor suppressor gene.

C. Experimental Studies Addressing Specific Aim 2

The stated aim of Specific Aim 2 is "to test already isolated 3p candidate tumor suppressor genes and flanking DNA sequences for mutations or other alterations occurring in breast cancers or breast cancer cell lines." Our original proposed methods to address Aim 2 involved looking for genomic rearrangements using candidate cDNA clones and Southern blots, as well as expression alterations using Northern blot analysis and RT-PCR. We also described experiments to replace a candidate tumor suppressor gene using either specific expression plasmids or, as an alternative, to introduce YACs into recipient cells. YACs (yeast artificial chromosomes) contain large stretches of cloned human DNA in a vector containing elements which allow its propagation in yeast cells. We noted that "YACs provide the opportunity to introduce genes containing all their normal regulatory sequences into recipient cells and provide an alternative if a full-length cDNA is not available. Candidate YACs could be introduced to initially assess the possibility that a TSG was contained in a particular stretch of genomic DNA." To perform these experiments using YACs, a selectable marker active in mammalian cells must be incorporated directly into the YAC DNA. The selectable marker encodes Neomycin resistance (NeoR) and other elements in the retrofitting vector, pRV-1, mediate direct insertion (via homologous recombination) into the right arm of the YAC cloning vector.

Pursuant to this goal, we have initially chosen 3 YACs spanning the homozygous deletion region for retrofitting and transfer studies. One of these YACs represents a naturally occurring deletion variant in a segment close to the homozygous deletion region. The retrofitting vector, pRV-1, carrying the NeoR gene under control of the mouse metallothionein promoter has now been introduced into each of the 3 YACs. This NeoR marker will be subsequently used to select for the presence of the YAC in mammalian tumor cells. In these types of experiments, it is critical to demonstrate that the NeoR gene be integrated in the YAC and not in other yeast DNA sequences. We have verified that the integrations involved homologous recombination within the actual YAC sequences by the following criteria: 1) the clones became dependent on exogenous uracil for growth (the strain becomes auxotrophic). This occurs since the homologous recombination directs the retrofitting vector to the URA gene on the YAC vector right arm and the AB1380 yeast host strain is *ura-*; 2) By Southern blot analysis using a probe from the right arm of the YAC vector and EcoRI digested DNA, an expected 8 kb larger fragment was detected. Using a NeoR probe, the same fragment was detected indicating that the retrofitting vector had integrated properly; 3) Using pulsed-field gel Southern blots, we demonstrated that the right arm vector and NeoR probes detected bands corresponding to the human insert containing YAC. Thus, by all criteria the proper integrations with the retrofitting vector occurred. Fusions between the yeast cells and rodent cells are underway to test the system and determine if the Neomycin resistance gene is functioning properly. We also described an alternative method, microinjection, for introducing YACs into mammalian cells which will be utilized if necessary. Microinjection facilities are available locally for our use.

C.1. Significance of the YAC Introduction Experiments

This approach provides a direct test of the effects of introducing 3p material into a breast cancer cell line which has deleted the material. Potential consequences that we might observe include altered growth *in vitro* and/or reduction of tumorigenicity of the breast cancer cells in nude mice. This would provide very strong confirmatory evidence, at the functional level, for the importance of this region in breast cancer development/progression. The re-introduction of deleted DNA also provides an alternative approach to gene identification. Hubank and Schatz (10) have described a variation of the representational difference analysis (RDA) method in order to clone the differences between two populations of mRNAs. We have obtained detailed protocols from Dr. Schatz and

will explore this technology once we have the appropriate YAC introductions. Genes which are encoded in the 3p14 deleted material can be directly isolated without having to utilize the gene searching procedures discussed above.

D. Other Target Loci

In our original application, we described a homozygous deletion region in small cell lung cancers located within 3p21.3. We have recently characterized this deletion as well as isolated and characterized a new member of the Semaphorin gene family (11). So far, we have only observed this deletion in SCLC cell lines. In contrast, we also reported the presence of a separate homozygous deletion located approximately 15 cM more telomeric in 3p21.3 which was adjacent to the DNA mismatch repair gene, hMLH1 (11). Interestingly, using probes from this region we identified homozygous deletions in direct lung tumors of both small cell and non-small cell histologies. We have not yet tested probes from this region (or the more proximal 3p21.3 deletion) in breast cancers and breast cancer cell lines, but these studies will commence in the near future.

CONCLUSIONS

The stated goal of our proposal is to identify a tumor suppressor gene located on the short arm of chromosome 3 that is operative in breast cancer. From our studies, as well as those from other investigators discussed above, there are compelling data indicating that 3p14 is a frequent target for deletion in breast cancer. It seems likely that the 180 kb homozygous deletion region we have identified, and are exploring, represents the target suggested by the various loss of heterozygosity and cytogenetic reports described above. Because we have narrowed the target region to a relatively small size, gene searches within this area are now practical. An immediate implication of our results is that the previously proposed order of experimental studies should be modified. We believe that a very focused effort should be placed at identifying genes within this homozygous deletion region. We will then determine if any of these are tumor suppressor candidates by analysis of their DNA sequence and suggested function and by mutational analysis in other breast cancers. We will develop new polymorphic probes from this target region to assess its involvement in tumors where homozygous deletions or rearrangements have not been detected. We will also pursue the introduction of YACs containing the deleted material back into tumor cell lines to examine any possible effects on growth and tumorigenesis. In the identification and molecular cloning of tumor suppressor genes, homozygous deletions have played a very major role in directing gene cloning efforts. The most recent example of the use and importance of homozygous deletions is in the identification of the pancreatic cancer tumor suppressor, DPC4 (12).

The identification of a breast cancer 3p tumor suppressor gene, or genes, should enhance our knowledge of the disease process and lead to more effective and specific therapeutic interventions. Similarly, knowledge of gene mutations carries with it the possibility of identifying causes of those mutations and the ability to intervene to prevent their occurrence.

A. Modified Order of Specific Aims for this Project.

1. To isolate additional candidate tumor suppressor genes from involved regions of 3p. In this regard, we will continue our focus on the 3p14 homozygous deletion region. We will search for genes using primarily exon-trapping and genomic DNA sequencing approaches.
2. To test already isolated 3p candidate tumor suppressor genes and flanking DNA sequences for mutations or other alterations occurring in breast cancers or breast cancer cell lines. As discussed, we are proceeding with the introduction of YACs into recipient cells lines. A selectable marker has

been successfully incorporated into YAC DNA sequences and we are now in the process of attempting to introduce these YACs into recipient cells. Once the technology has been satisfactorily implemented, we will perform functional tests on the 3p14 as well as other target regions outlined in the original proposal.

3. To accurately define, using critically placed highly polymorphic markers, the region (or regions) of 3p that most consistently undergo loss of heterozygosity in breast cancer and to further narrow the critical region in order to facilitate molecular cloning of the disease gene. Fluorescence *in situ* hybridization will be employed as an adjunct to the analysis of 3p loss. These studies will also provide data on possible microsatellite instability. Originally, this was Specific Aim 1. Since we have now identified a target region, this survey approach can be delayed until we have more information on genes within the 3p14 deletion region. As noted above, we have identified a second deletion region in 3p21.3 which is deleted in lung cancer direct tumors as well as in cell lines. This deletion is immediately adjacent to the DNA mismatch repair gene, hMLH1, whose alteration is known to be associated with microsatellite instability. It will be important to examine this region in breast cancers and cell lines and to correlate loss with any observed microsatellite instability. Thus, it is noteworthy that we have identified this deletion region in close proximity to the hMLH1 gene. As discussed above, these loss of heterozygosity and fluorescent *in situ* studies will commence after we have gained more information on the 3p14 deletion region and have developed the appropriate markers/probes to effectively assess loss in this area.

4. Once a breast cancer gene from the 3p region is identified and the DNA sequence analyzed, we will proceed to a more in-depth analysis of mutations in breast cancer specimens and cell lines. We will also correlate the presence or absence of mutations with other prognostic markers and begin studies to characterize the gene product.

References.

A. Manuscript in preparation describing the 3p14 homozygous deletion.

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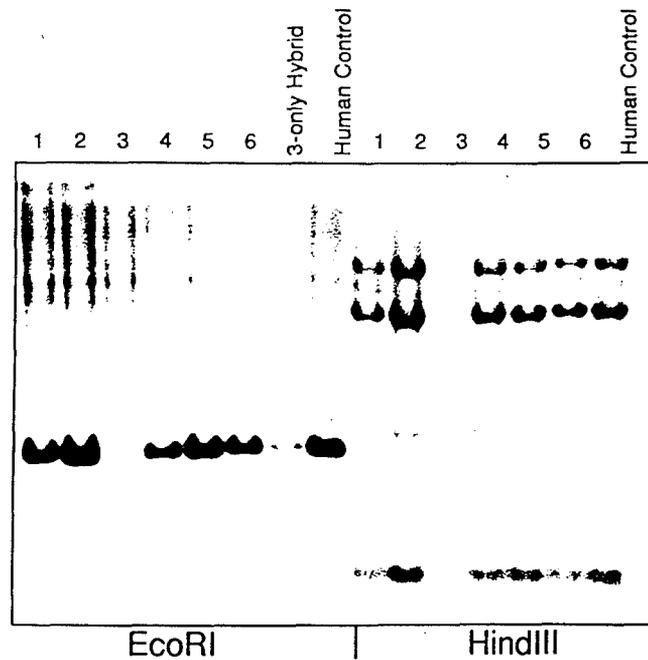
B. Other references:

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APPENDIX

Autoradiogram showing hybridization results from 6 breast cancer cell lines (numbers 1-6 corresponding to the above Table) with a 1.5 kb fragment from clone C81. Note the homozygous deletion seen in cell line #3 observed in both EcoRI and HindIII digests. An adjacent probe demonstrated a rearranged fragment in the same cell line (data not shown).





DEPARTMENT OF THE ARMY

U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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25 Feb 97

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FOR THE COMMANDER:

Gary R. Gilbert
for GARY R. GILBERT
Colonel, MS
Deputy Chief of Staff for
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