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PRINCIPAL INVESTIGATOR: Raymond L. White, Ph.D.

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, Utah 84102

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Raymond L. White, Ph.D.

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During the past year we have made good progress on several aspects of the research outlined in our original proposal. In particular, we have identified the human homologue of the mouse FKBP65 gene and a novel gene with strong protein homology to an uncharacterized gene in C. elegans in the region of LOH surrounding plakoglobin, a region which is lost in human breast tumors. We have also begun to establish a physical map in the telomeric region of the short arm of chromosome 17, as part of an approach to cloning the tumor suppressor gene(s) thought to be present there. In the process, by comparing available LOH mapping information with our genetic maps we have identified a preliminary 15-cM target region for the putative tumor suppressor gene(s). In our other focus of investigation, we are characterizing the biological effects of inhibiting the two DLG genes we have identified as located 1Mb telomeric of BRCA1. We have also tested 9 paired samples of breast tumors and corresponding normal tissues for LOH in the region containing the DLG2 and DLG3 loci. As 3 of the tumors in these sets exhibited LOH, we are determining whether either the DLG2 or DLG3 gene is the target by means of sequencing experiments based on templates prepared from RNA isolated from all the tumors showing LOH. In one sample tested for LOH, micro-satellite instability was observed.

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Ray White 7/29/86
PI - Signature Date

Table of Contents

Introduction

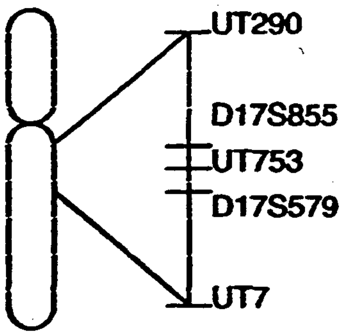
The identification and characterization of genes involved in development and progression of breast cancer is critical to an understanding of the biological mechanisms that regulate growth of cells in breast epithelium. Two genes relevant to breast cancer, BRCA1 and BRCA2, which were identified largely as a consequence of their obvious hereditary patterns of segregation in families with a high incidence of breast and/or ovarian cancer (Miki et al., 1994; Wooster et al., 1995), assert their biological effects at the level of the cell in a recessive fashion according to the well documented paradigm of tumor suppressor genes. However, only a fairly small percentage, less than 10%, of all breast cancer cases analyzed display a familial pattern of segregation; sporadically occurring cases of breast disease constitute the majority of cases detected. Further, molecular analysis of BRCA1 and BRCA2 genes in sporadic breast cancers has revealed that mutations in these genes have contributed only rarely to development of those tumors. One must conclude that defects in other genes must be responsible for the development of most breast cancers. Several independent studies support that conclusion; for example, the p53 locus often displays loss of heterozygosity (LOH) in breast tumors (Cropp et al., 1993; Cropp et al., 1994; Godwin et al., 1994). Also of interest is a recent study of LOH in *prostate* tumors which identified a region of allelic loss immediately distal (1Mb) to BRCA1 (Brothman et al., 1995) which encompasses DLG-2 and DLG-3 (Mazoyer et al., 1995; Smith et al., 1996); the founding member of the DLG gene family, originally identified in Drosophila, has been characterized as a tumor suppressor gene. The DLG2 and DLG3 genes have become even more interesting since the recent discovery that APC protein, the product of the *APC* gene which is responsible for the inherited colon cancer syndrome, familial polyposis, binds the human homolog of the Drosophila discs large tumor suppressor protein (Matsumine et al., 1996).

Body

During the past 12 months we have made progress in several areas relating to our research proposal. In the following sections we will describe in detail the current status of our project and how each line of experiments relates to our Statement of Work (SOW). With respect to Task 1, which focuses on "Isolation and characterization of two potential tumor suppressor genes approximately 1MB proximal and 1Mb distal of BRCA1", I mentioned in my previous report that we had identified and published descriptions of two novel members of the DLG family of genes, DLG2 (Mazoyer et al., 1995), and DLG3 (Smith et al., 1996). The presumed biological properties of these genes as protein components of tight junctions in epithelial cells make them likely candidates for tumor suppressor activity through a mechanism analogous to that of the lethal disc large (DLG) gene found in Drosophila (Woods and Bryant, 1991).

In an experiment designed to directly determine if DLG2 and DLG3 are the targets for the LOH observed in sporadic breast cancers (Task 1a), we obtained paired tumor and normal tissue samples from 10 individuals who underwent surgical removal of malignant carcinomas. We extracted DNA and RNA from these samples using the Trizol reagent, and subsequently tested the paired DNA samples for loss of heterozygosity. As indicated in figure 1, we used five highly polymorphic genetic markers located along the long arm of chromosome 17 (Albertsen et al., 1994a), three of which lie relatively close to the BRCA1 locus (Albertsen et al., 1994b). From among the ten tumors we identified 3 which displayed LOH around BRCA1. According to the established model for LOH involving tumor suppressor genes (Knudson et al., 1975), the allele remaining in the tumor sample would harbor the deleterious mutation. Using RNA extracted from these tumors we prepared first-strand cDNAs specific to each of the two DLG genes and submitted these templates for automated sequencing on an ABI373A sequencer (Applied Biosystems, Foster City, CA). However, none of the samples we have sequenced have yet revealed any mutations, and we are moving on to expression studies. This is an especially interesting line of research now because of the report that the APC protein binds the DLG protein; although the *APC* gene is not yet implicated in human breast carcinogenesis, it is very important to note that mice heterozygous for the mutant APC allele, *min*, have an approximately 10% risk of mammary cancer at 12 months of age.

Figure 1



1C p	2C p	2C t	3C p	3C t	4C p	4C t	5C p	5C t	6C p	6C t	8C p	8C t	9C p	9C t	10C t
0.90	1.23	1.22	0.85	[illegible]	MIN*	MIN*	NI	NI	[illegible]	[illegible]	MIN*	MIN*	1.11	1.24	1.23
1.16	1.11	1.16	1.03	1.01	MIN*	MIN*	1.05	1.06	1.26	[illegible]	0.80	0.98	[illegible]	[illegible]	[illegible]
NI	0.93	0.95	NI	NI	NI	NI	0.79	1.16	1.10	[illegible]	1.29	[illegible]	[illegible]	[illegible]	1.04
[illegible]	1.19	0.83	1.17	0.89	1.00	1.24	NI	NI	0.96	[illegible]	[illegible]	[illegible]	NI	NI	0.99
[illegible]	[illegible]	1.09	1.14	0.82	NI	NI	NI	NI	-	[illegible]	0.79	0.85	[illegible]	[illegible]	-

Thirteen normal and 16 tumor samples were amplified with fluorescently-tagged primers. Product was visualized on an ABI 373 sequencer. An allelic imbalance factor was calculated by dividing the ratio of the allele intensities (area under the peaks) in the normal alleles by the ratio of the allelic intensities in the tumor alleles. A factor less than .77 or greater than 1.3 was considered conclusive of allelic imbalance. (See Gruis, et al, Cancer Res., 68, 308-313)

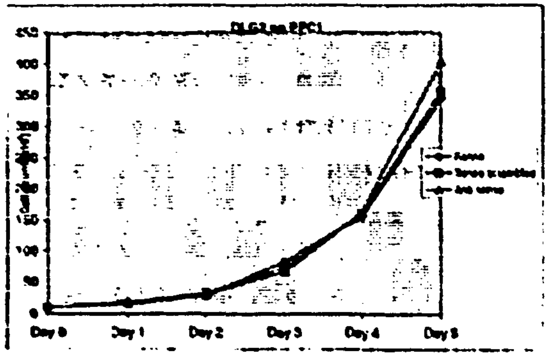
*Microsatellite instability was seen in 4 tumor samples.

To assess the biological effects and functions of the DLG2 and DLG3 genes (Task 1b), we are currently using cultured breast cells BE40 (established from a fresh mastectomy in our laboratory) and two cell lines , PPC1 and SF15-2, to test inhibition of normal expression of these genes by supplementing the growth medium with antisense DLG2 or antisense DLG3. PPC1 is a rapidly dividing prostate cancer cell line which has lost the long arm from one of its chromosome 17; SF15-2 is a derivative of PPC1 which by micro cell fusion has been given a normal copy of the long arm of chromosome 17; SF15-2 displays much more moderate growth than PPC1 (Murakami et al., 1995). To differentiate between the specific biological effects of the antisense oligonucleotides and possible nonspecific chemical effects, parallel experiments with either a sense oligonucleotide or a scrambled antisense oligonucleotide are included to serve as controls. There were no observable differences in the growth potential of these cell lines with respect to the given oligonucleotides as shown in Table 1. Experiments are ongoing with an additional twelve oligonucleotides designed with different specificities for the 5' regions of DLG2 and DLG3.

Table 1

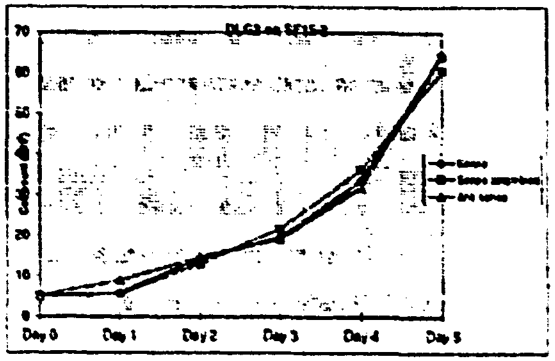
DLG2 oligo's on PPC1 cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	10	16	23.6	83	156.6	345.6
Sense scrambled	10	17.2	33.3	73.6	160	356
Anti sense	10	19.2	30.4	67.8	163	404



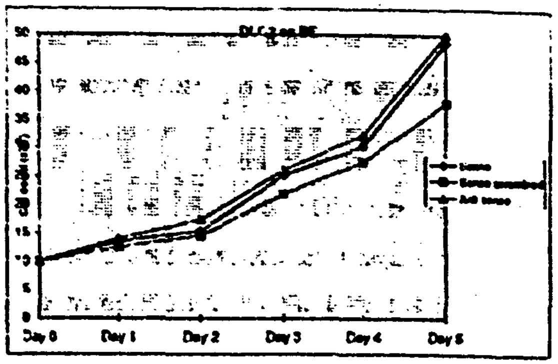
DLG2 oligo's on SF15-2 cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	5	5.3	14.4	19.5	33.6	64.5
Sense scrambled	5	5.6	13.2	21.7	36.2	60.6
Anti sense	5	9	14.7	19.3	31.9	64.3



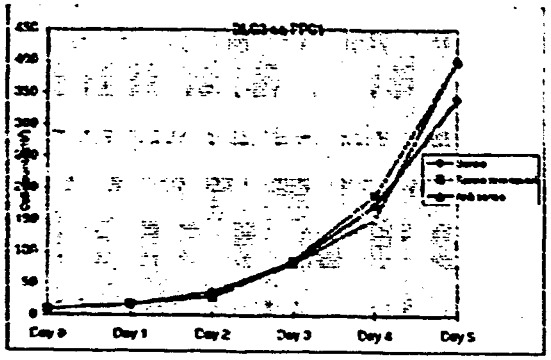
DLG2 oligo's on BE cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	10	13.5	15.4	25.2	30.3	48.6
Sense scrambled	10	12.5	14.6	22	27.6	38
Anti sense	10	14	17.4	26.1	32.3	50



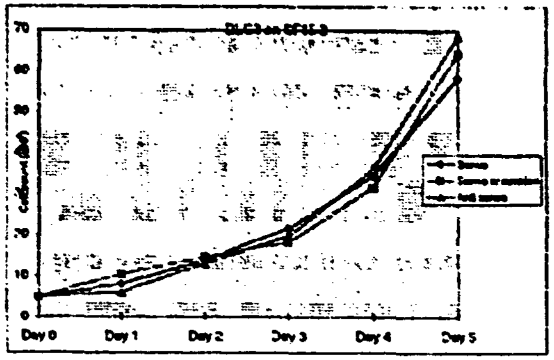
DLG3 oligo's on PPC1 cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	10	17	37.7	83.4	172.8	340
Sense scrambled	10	18.3	31.7	86.7	187.8	398
Anti sense	10	17.7	29.4	82	152.2	402



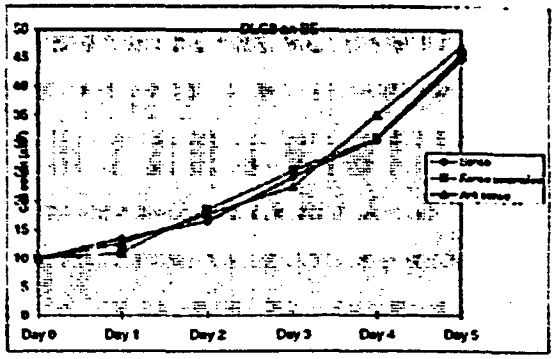
DLG3 oligo's on SF15-2 cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	5	8	13.9	21.6	34.4	58.2
Sense scrambled	5	10.5	14.7	18.2	31.6	64.2
Anti sense	5	6.2	13.1	19.7	36	68.4



DLG3 oligo's on BE cells (x 1,000)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Sense	10	13.4	16.5	24.2	30.8	44.8
Sense scrambled	10	11	18.5	25.3	31	45.8
Anti sense	10	12.6	17.7	22.5	35	47



Additional, and quite compelling, evidence for the biological importance of the DLG family of genes was recently discovered when it was found that APC, the tumor suppressor gene responsible for adenomatous polyposis coli (Groden et al., 1991), interacts on the protein level with the human DLG homolog (Matsumine et al., 1996). To determine whether the DLG2 and DLG3 products can interact with APC protein in a similar fashion , we have initiated a collaboration with Dr. Tetsu Akiyama to test whether the APC constructs he developed for finding the protein interaction between APC and DLG also show affinity for the DLG2 and DLG3 proteins (see Dr. Akiyama's letter of collaboration in the appendix).

Another part of our project that has yielded good progress during the past year is the identification of two novel genes from the region of LOH encompassing the plakoglobin locus. We discovered these genes in collaboration with Dr. Robert Callahan at the National Cancer Institute using the P1 clones 50H1 and 122F4 identified in our laboratory (Albertsen et al., 1994b). The first of these genes is the presumed human homologue of the mouse FKBP65 gene (Coss et al., 1995). Genes of the FKBP family derive their names from the immunosuppressant macrolide antibiotic FK506, because they mediate its activity (in part) by binding to a ubiquitous family of highly conserved intracellular receptors termed immunophilins (Sigal and Dumont, 1992). Although FK506 is known to block various signal transduction pathways in normal T-cells, FKBP genes (including FKBP65) are expressed in most tissues that have been analyzed. The biological relevance of human FKBP65 to cancer development remains unclear, but once its full-length sequence is ascertained we will undertake a detailed analysis of the gene and its functional domains. The second gene we identified in the plakoglobin region was ascertained by coincidence. As part of the process of determining the genomic structure of the FKBP65 gene, we sequenced several genomic subclones derived from P1 phage clones 50H1 and 122F4. While analyzing the sequence of one of these subclones, named 1H2M, we identified a small collection of human ESTs that shared a segment of almost 300 nucleotides of perfect homology to 1H2M. Further analysis and database comparisons extended the DNA sequence to approximately 1300 nucleotides, and revealed that the novel gene shared homology with no other currently known vertebrate gene. However, the protein translation of the nucleotide sequence showed 40%

homology, over a segment of almost 200 amino acids, to an uncharacterized gene from C. elegans. It is impossible at present to predict the biological relevance of the novel gene with respect to tumor formation, but the high degree of protein homology preserved across such distant species suggests a fundamental and probably critical role. Further characterization of this gene will obviously have a high priority during the coming year and we anticipate providing a complete description in our next annual report.

A third gene that occupied a significant amount of our time and effort was DOC-2, whose identification and characterization we published earlier this year (Albertsen et al., 1996). A 788-basepair segment of DOC-2 was originally identified by differential display between ovarian carcinoma and normal ovarian epithelial tissue; its expression was greatly reduced or entirely absent in a panel of 10 ovarian tumors (Mok et al., 1994). Our ascertainment of DOC-2 was based on cDNA screening of a fetal retina library (Stratagene # 937202) with P1 clone 124D3. One of the cDNA clones we identified, 1RA1, harbored a large segment of the genuine DOC-2 gene; however, we did not realize immediately that the 1RA1 cDNA clone was a chimera between DOC-2 and DLG3. Consequently, our original attempt to verify the chromosomal location of the 1RA1 clone clearly indicated that the clone was located in the BRCA1 region. It was not until we were in the process of determining the genomic structure of DOC-2 that we found the correct genomic location of DOC-2 on chromosome 5. Nevertheless, the complete sequence, genomic characterization, and chromosomal location of DOC-2 have been attributed to the present Army grant.

The purpose of Task 2 is "To identify and characterize Breast Cancer Tumor Suppressor Genes on distal 17q and distal 17p using physical reagents identified by the CEPH". We have initiated a physical mapping project to refine the presently rather crude physical map of 17p. We have chosen to focus solely on this region in the initial stages of the physical mapping project for two reasons: a) the 17p region of LOH is better characterized and thereby provides a better possibility for identifying the tumor suppressor gene(s) located there; and b) with only limited manpower available to satisfy all aspects of our research proposal it would be unwise to further dilute our efforts by simultaneously attempting to refine the physical map of the 17q region.

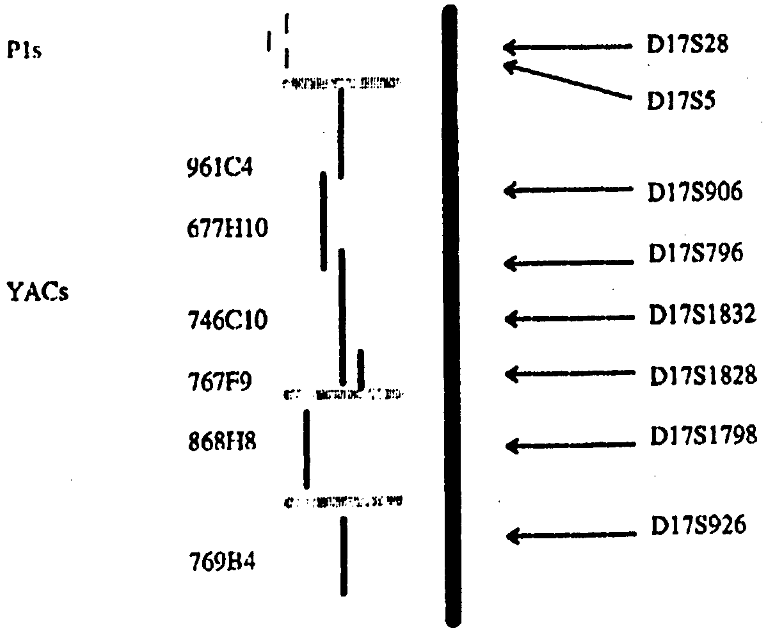
During the past year two developments relating to the chromosome 17p region led us to initiate a refined physical mapping of 17pter. Of greatest importance was the publication of two novel candidate tumor suppressor genes in a quite narrowly delimited region of 17p13.3 (Schultz et al., 1996). While it is not clear whether either of these genes is the target for LOH near the telomere of the short arm of chromosome 17, an important conclusion can be drawn: the very distal location of the presumed tumor suppressor locus at the extremity of the chromosome means that the general numeric relation between genetic distance measured in cM to the physical length measured in Mb, which exists in the central parts of chromosomes, can not be applied in this case because the frequency of genetic recombinations is elevated in telomeric regions. This phenomenon dramatically skews the relationship between genetic distance and physical distance, such that a large genetic distance actually is contained within a relatively short physical segment of DNA. (Adamson et al., 1995; Gerken et al., 1995; Murray et al., 1994) showed that the 3.5 cM of genetic distance between D17S5 and D17S28, which normally would be expected to represent a 3.5-Mb genomic region, in reality is contained within a single cosmid (30kb). If the genetic compression observed in this short telomeric region of the short arm of chromosome 17 extends beyond the confined segment delimited by the markers D17S5 and D17S28, the task of refining the physical map in the approximately 15-cM region which harbors the presumed tumor suppressor gene(s) should be relatively simple. To this end we have obtained yeast artificial chromosomes (YACs) that have been localized to telomeric 17p through the genome mapping efforts at the CEPH and the Whitehead Institute at MIT. By cross referencing these YACs with the genetic markers we and others have developed and mapped to this region (Adamson *et al.*, 1995; Gerken *et al.*, 1995; Murray *et al.*, 1994), we can identify the exact extent of the existing physical coverage of the region. The information obtained in this manner will allow us to localize potential gaps in the physical maps and will provide guidance as to where the genomic coverage must be expanded to complete the physical map of the region. The data shown in Table 2 represent the results of cross-referencing the YACs and genetic markers currently under investigation in our laboratory Figure 2 graphically represents the data from Table 2.

Table 2

	D17 S18 66	D17 S92 6	D17 S18 40	D17 S15 29	D17 S17 98	D17 S18 45	D17 S18 28	D17 S18 76	D17 S18 10	D17 S18 32	D17 S79 6	D17 S67 8	D17 S59 5	D17 S67 5	D17 S90 6	D17 S69 5	D17 S5	D17 S28
626B10																		
629B10																		
650G5																		
677H10											+				+			
680C8																		
688C8																		
746C10										+	+							
767F9							+											
769B4		+																
770F3																		
825H6										(+)								
825H10																		
868H8					+								(+)					
892A1																		
898A10																		
908G7																		
921H5											+		(+)					
927D6																		
931D8																		
961C4															+			

+ Unambiguous placement
(+) Ambiguous placement

Figure 2



Chromosome 17 (pter)

Graphic representation of data from table 2. YACs (▬), P1s (—), gaps in coverage (┅).

Conclusions

Our research during the funding period just past has produced an extensive and intriguing list of results which include the identification or detailed characterization of three new genes, two of which (FKBP65 and the C. elegans homologue) are closely linked to plakoglobin. Another gene, DOC-2, which we also identified through this grant and initially thought to be located immediately distal to BRCA1, was subsequently mapped to chromosome 5. Our investigation of the biological functions of our two DLG genes continues, and it will be interesting to see if either of their products displays affinity for APC protein. We published two papers, describing DLG3 and DOC-2 respectively, earlier this year in Genomics. Our search for the 17p tumor suppressor gene was initiated early in 1996 with a series of STS-content mapping experiments aimed at refining the physical map of the 17p telomeric region.

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Appendix

RESEARCH
INSTITUTE
FOR
MICROBIAL
DISEASES

OSAKA UNIVERSITY
3-1 Yamadaoka, Suita-City OSAKA 565, JAPAN

Telephone 06-877-5121
Facsimile. (06 876 2678

Department of Oncogene Research

Dr. Raymond L. White
Huntsman Cancer Institute
Department of Oncological Sciences
The University of Utah
Building 533, Room 7410
Salt Lake City
Utah 84112

June 19, 1996

Dear Dr. White,

Thank you for your letter of June 13. 1996.
I would be very happy to collaborate with you on the project which you proposed in your letter.
I am sending herewith the APC constructs you requested.

pbluescript-APC-C369 (for in vitro translation with T7 polymerase)

pGEX-APC-C369
pGEX-APC-C369Δ72
pGEX-APC-C72

Please transform these plasmids into E.coli and generate GST-fusion proteins.

Sincerely yours.

Tetsu Akiyama.
E-MAIL: akiyama@biken.osaka-u.ac.jp
FAX: 81 6 879 8305

Publications Resulting from the Present Grant

S.A. Smith, P.R. Holik, J. Stevens, S. Mazoyer, R. Melis, B. Williams, R. White, and H. Albertsen. "Isolation of a gene encoding a second member of the disc-large family on chromosome 17q12-21." Genomics 31: 145-150 (1996).

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List of Salaried Personnel

Name:	Position:	Percent Contribution to salary:
Hans M. Albertsen,	Research Instructor	67%
Jeff Stevens,	Research Associate	100%
Ray White,	P.I., Professor	10%