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PRINCIPAL INVESTIGATOR: Donna G. Albertson, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Donna G. Albertson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Francisco
San Francisco, California 94143-0962

E-MAIL:
albertson@cc.ucsf.edu

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The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. However, the increasing number of candidate genes whose expression needs to be evaluated for prognostic, diagnostic, therapeutic, or research purposes will require obtaining material from numerous tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens. This will entail adapting/developing procedures to amplify with fidelity the mRNA repertoire expressed in small numbers of normal, pre-cancerous and malignant breast epithelia. To this end, in this project period, we have concentrated effort on establishing and validating microarray-based assays for measuring gene expression levels and have demonstrated the capability to isolate and amplify mRNA from cultured cells. Realization of these objectives will allow, in the future, development of a resource, consisting of amplified mRNA populations from individual cells from normal and tumor material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

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Donna A. Albertson 10/24/99

PI - Signature Date

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INTRODUCTION:

The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. Therefore it is desirable to be able to both characterize and compare the levels of expression of particular genes in normal and tumor cells. Currently, assays of gene expression are carried out on mRNA isolated in bulk from tissue specimens, or at the individual cell level by *in situ* hybridization or immunohistochemistry. However, neither approach will meet all the needs of the research and clinical communities. Conventional mass biochemical extraction procedures are not appropriate in breast cancer, because extraction of mRNA from single cell types is difficult due to the intermingling of epithelial and stromal components, and the fact that the amount of malignant or pre-malignant tissue available in the specimen is small. In addition, very few cells in the specimen make up the normal ductal epithelium. Cell type-specific gene expression can be visualized in tissue sections, but there are now increasing numbers of candidate genes to be evaluated for prognostic, diagnostic, therapeutic, or research purposes, and expression analysis of all these genes will require numerous individual tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens for analysis of gene expression in normal and diseased tissue. This will entail adapting/developing a new approach to archiving the repertoire of genes expressed in normal, pre-cancerous and malignant breast epithelia. Procedures used to obtain gene expression profiles from single neurons (Eberwine *et al.*, 1992) will be adapted for use with clinical breast cancer specimens, allowing amplification of the mRNA repertoire from small numbers of cells from normal ductal epithelium, DCIS and invasive carcinoma. The procedure has been reported to provide 10^6 fold amplification and uses an oligo-dT primer incorporating the promoter binding site for T7 polymerase to prime first strand cDNA synthesis. Subsequently linear amplification of this small quantity of cDNA is accomplished by *in vitro* transcription using T7 RNA polymerase (Figure 1). Thus, this procedure could provide enough material for multiple and diverse assays of gene expression and/or for the generation of cDNA libraries. Therefore the purpose of this proposal is to develop the capability to isolate and amplify with fidelity total mRNA from small numbers of microdissected cells of histologically defined types. We will then apply these procedures to obtain expression profiles for cells representing normal epithelium, DCIS and invasive carcinoma from frozen and paraffin embedded sections of tumors. Our objectives are to: (1) Demonstrate linear amplification of high complexity RNA from a homogeneous population of cells; optimize the techniques to maximize the amount and complexity of amplification that can be obtained while preserving relative copy number of different mRNA species, and (2) apply these techniques to amplify mRNA from microdissected cells from frozen and formalin fixed sections containing normal ductal epithelial cells, DCIS and invasive carcinoma, and use this material to obtain expression profiles for these different cell types. Realization of these objectives will allow development of a resource, consisting of amplified mRNA populations from individual normal and tumor-specific material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

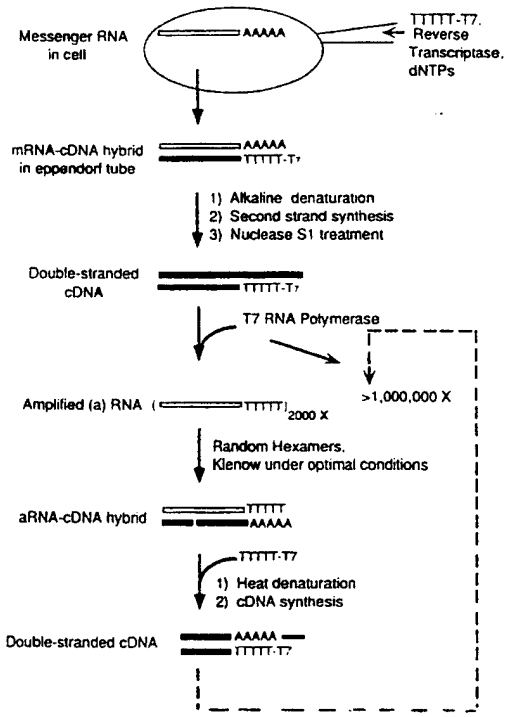

Figure 1. aRNA amplification scheme (from Eberwine *et al.*, 1992)

BODY:

A. Reporting Period.

As of June 1, 1998, I took up my current position at the UCSF Cancer Research Institute and a revised Statement of Work and budget at UCSF were accepted with a start date of 11/16/98. Therefore the work described herein was performed over the period from 11/16/98 to 9/29/99.

B. Progress

Work in this period has focused on Technical Objective 1, Tasks 1-3 and 7.

Statement of Work

1. Technical Objective 1. Demonstrate linear amplification of high complexity aRNA (amplified antisense RNA) from a homogeneous population of cells, the breast tumor cell line BT474.

Task 1	Months 1-3	Validate assay for expression levels. Make test RNA population by transcribing test genes *in vitro*, label and hybridize to array of test clones. Demonstrate linearity of the assay.
Task 2	Months 4-9	Grow BT474 cell cultures, isolate mRNA, measure expression levels of test genes in mRNA isolated from BT474 and estimate complexity by hybridization to IMAGE cDNA array.
Task 3	Month 4-9	Carry out amplification on various amounts of bulk BT474 mRNA down to 0.1 pg, measure expression levels of test genes in aRNA and hybridize to IMAGE cDNA array.
Task 4	Months 10-13	Prepare frozen and fixed samples of BT474 cells and cut sections. Carry out mRNA amplification on sections of BT474 cells, measure expression levels of test genes in aRNA and estimate complexity by hybridization to IMAGE cDNA array.
Task 5	Months 10-13	Prepare fluorescently labeled probes for test genes and carry out *in situ* hybridization to sections of BT474 cells. Measure intensity of fluorescent hybridization signals and determine relative levels of expression of test genes in the cells in the sections.
Task 6	Months 10-13	Compare relative levels of expression of test genes in aRNA and *in situ* as determined by FISH in Task 7. Compare results of hybridization to IMAGE cDNA array with aRNA made to whole sections and microdissected cells.
Task 7	Months 10-13	Optimize protocols (Tasks 4-6).

2. Technical Objective 2. Apply the techniques from Objective 1 to amplify mRNA from microdissected cells from frozen and formalin fixed sections containing normal ductal epithelial cells, DCIS and invasive carcinoma. Use this material to obtain expression profiles for these different cell types using SAGE and hybridization to an array of clones from the IMAGE cDNA library.

Task 8	Months 14-24	Prepare fluorescently labeled probes for test genes and carry out *in situ* hybridization to breast tumor sections. Measure intensity of fluorescent hybridization signals and determine relative levels of expression of test genes in different cell types in the tumor section.

Task 9	Months 14-24	Carry out mRNA amplification on tumor sections.
Task 10	Months 14-24	Measure expression levels of test genes in aRNA from tumors.
Task 11	Months 14-24	Compare relative levels of expression of test genes in aRNA and *in situ* as determined by FISH in Task 8.
Task 12	Months 14-24	Carry out expression analysis on aRNA from tissue sections by hybridizing the aRNA to an array from the IMAGE cDNA library.

Task 1. Validate assay for expression levels.

We are using cDNA microarrays to measure and evaluate the linearity and uniformity of the aRNA amplification procedure. In this assay, differentially fluorescently labeled probes are made from two nucleic acid populations. For example, the cellular mRNA is labelled with one fluorochrome (e.g. Cy3, red) and the aRNA made from it with another fluorochrome (e.g. fluorescein, green). These probes are then hybridized to the microarray and the fluorescence intensity of each probe is determined on each array spot. If all genes in the array are represented at equal levels in the two RNA populations, then the red:green intensity ratios should be constant across all spots. Deviant ratios indicate non-uniform amplification of those particular genes. As an initial validation of the array measurements, hybridization of an aRNA population to itself was carried out and the results are shown in Figure 2. The aRNA was synthesized and divided. One portion was labeled with fluorescein and the other with Texas Red and then hybridized to an array of 10 genes (see Table 1). The intensity measurements fell on a single line as expected, indicating in this case uniformity in the labelling of this single RNA population. In addition, these data demonstrate that our microarray capability (see Pinkel *et al.*, 1998) provides measurement sensitivity over more than two orders of magnitude. Recently, the UCSF Cancer Center purchased an initial set of 200 sequence verified cDNAs and have now ordered the set of 5000 cDNAs to be used for cDNA array production. The 200 gene set has already been processed for microarray production, and the larger set will soon be available to this project for validation of the aRNA procedure.

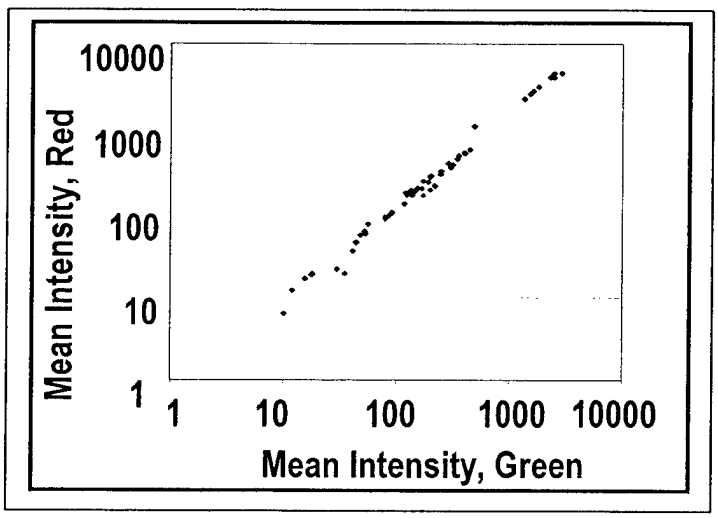

Figure 2. Sensitivity of gene expression detection over more than two orders of magnitude. An aRNA population form MCF7 was divided, labelled with fluorescein or Texas Red and hybridized to an array of 10 genes. The cDNAs were spotted in quadruplicate and ranged in abundance from the lowest based on signal intensity, PI3CA to the most abundantly expressed, actin. The red and green intensity measurements fall on a single line, indicating uniform labelling and detection as expected from a single nucleic acid population.

Table 1. cDNA clones included on microarray

Gene	ATCC Number
Beta actin	37997
ERBB2	57584
TP53	57254
GSK3	369482
FK506BP	381432
P70	389964
PI3CA (bases 2972 to 3' end)	530707
PI3CA (bases 2850 to 3' end)	802544
RACB	597768
P85	703326

Tasks 2. Isolate RNA from BT474 cells.

Since cDNA microarrays are being used to measure the linearity of the aRNA procedure, we evaluated the quality and labelling of the RNA prepared using several commercially available kits for total RNA isolation and/or mRNA isolation. The RNA isolation appears to affect the amount of background fluorescent hybridization signal on the microarray substrate. Thus, both signal intensity and background were assessed. In these experiments, MCF7 cells were used, rather than the proposed BT474 cells, because they grow more rapidly. The RNA was extracted from the cells according to the manufacturer's directions and labelled using superscript reverse transcriptase, incorporating fluorescein or Texas Red UTP. Extraction of total RNA using Trizol (BRL) followed by isolation of mRNA using either the Promega or Invitrogen systems yielded good signal and low background. However, isolation of mRNA directly from cells using the Invitrogen kit resulted in labelled probe that hybridized with high background, possibly due to inadequate removal of contaminants such as carbohydrates.

Task 3. Carry out amplification on various amounts of bulk BT474 mRNA.

The aRNA amplification procedure shown in Figure 1 was carried out on MCF7 cells. First strand cDNA synthesis was performed using Superscript II (Gibco) and essentially followed the manufacturer's recommendations. Synthesis was primed from the poly A tail of the mRNA using 25 ng of an oligo dT primer that included the promoter sequence for the T7 polymerase on the 5' end. Second strand synthesis was also performed according to the manufacturer's directions in a reaction mixture containing 0.2 mM deoxynucleotide mixture, 100 units E. coli DNA polymerase I, 3.5 units RNase H and 12.5 units E. coli DNA ligase. To amplify the cDNA that was generated, *in vitro* transcription was carried out using the Ambion T7 megascript kit. This procedure yielded aRNA with a similar size distribution as the starting mRNA. By gel electrophoresis, the size ranged from 0.5-9 kb, with the majority of the material at ~ 2 kb.

The template mRNA concentration in the reaction affected the amplification. Approximately 100 fold amplification of the mRNA was achieved with a starting concentration of 200 ng (yield 20 μg). At higher concentrations of template, e.g. 1000 ng, the procedure yielded only 40 μg of aRNA, or a 40 fold amplification. Further experiments to investigate the lower limits and optimal template concentration are underway.

As shown in Figure 1, further amplification of the aRNA is possible if the aRNA is used as template for cDNA synthesis, followed by another round of *in vitro* transcription using T7 polymerase. Our current experience with a second round of synthesis resulted in similar yields

of cDNA as in the first round, but the cDNA was significantly decreased in size. Gel electrophoretic analysis of this cDNA indicated a size distribution of 0.1-5 kb, with the majority migrating at ~ 1 kb. Therefore it appears that the second round of synthesis of failed to preserve the representation of the various RNA species in the population.

Task 7. Optimize protocols.

To assess the aRNA amplification procedure on the microarrays, it is possible to incorporate the label into the aRNA, itself or to generate a labelled cDNA. Work in the previous period had indicated problems with cross hybridization when using labelled RNAs as probes on the microarrays. Therefore, since we have most experience using DNA probes, we decided to use reverse transcription to make labelled cDNA from the aRNA for hybridization. We have evaluated and optimized several parameters with respect to signal intensity of the probe.

a. Reverse transcriptase. Superscript and Thermoscript reverse transcriptases were tested. Thermoscript offers a potential advantage, because the reaction can be carried out at 65°C, allowing melting of RNA secondary structure that can interfere with elongation. Superscript can also be stabilized at higher temperatures by the addition of trehalose. However, we did not find any particular advantage when it was added. The additon of 0.1% bovine serum albumen to the superscript reaction, on the other hand, did result in enhanced signal intensity.
b. Primers. The reverse transcription reaction can be primed from the poly A end of the message using an oligo dT primer or randomly using random hexamers. Greater hybridization signal intensity was observed using random priming and signal increased when the concentration of random primers was increased from 500 ng to 1000 ng in a 30 µl reaction.
c. Template RNA. Successful hybridization was achieved using either total RNA or mRNA as the template for labelling. Currently, good hybridization data have been obtained when 1-2 µg of mRNA are used in the hybridization reaction or 25-100 µg of total RNA. Experiments testing the lower limits of mRNA or total RNA required are still underway.

KEY RESEARCH ACCOMPLISHMENTS:

- ~100 fold amplification of starting mRNA in a single round of *in vitro* transcription.
- Demonstration of detection sensitivity in microarray expression measurements over a range greater than two orders of magnitude.

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

Work in this project period was focused on establishing the capability to use cDNA microarrays to evaluate the linearity and fidelity of the aRNA amplification procedure and to begin evaluation and optimization of the aRNA amplification. The results described above indicate that our microarray assay system can be used to measure gene expression levels over more than two orders of magnitude. In the near future, we expect to have available arrays with a larger number of target clones that will provide better assay capability for representation in the

aRNA. Thus, future work will measure linearity and representation of the aRNA relative to the starting mRNA on these larger arrays. Amplification of mRNA samples up to 100 fold has been achieved. Even though, this level is ten fold lower than reported values (Eberwine et al., 1992), it provides a useful enhancement of the starting amount of material and is suitable for measurements of gene expression by techniques such as microarrays or quantitative PCR.

REFERENCES:

Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettel, M. and Coleman, P. 1992. Analysis of gene expression in single live neurons. Proc. Natl. Acad. Sci. U. S. A. 89, 3010-3114.

Pinkel, D., Segraves, R., Sudar, D., Clark, S., Poole, I., Kowbel, D., Collins, C., Kuo, W.-L., Chen, C., Zhai, Y., Dairkee, S. H., Ljung, B.-M., Gray, J. W., and Albertson, D. G. (1998). Quantitative high resolution analysis of DNA copy number variation in breast cancer using comparative genomic hybridization to DNA microarrays. Nature Genet., 20, 207-211.

APPENDIX:

None