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PRINCIPAL INVESTIGATOR: David A. Talmage, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

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David A. Talmage, Ph.D.

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Columbia University
New York, New York 10032

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Initially, I predicted that the constellation of interesting genes induced by retinoic acid in T47-D cells would include: (1) phosphoprotein phosphatases,,,(2) GTPase activating proteins,,,(3) protein kinases,,,(4) transcriptional repressors." As documented in Cho et al. 1997 and Table 1, each of these classes of targets has been identified by other means, either in T47-D cells (Cho et al., 1997 and Tighe et al., in prep) or in rat cells using the alternative strategy outlined in Figure 3 (Revised Task 7). Therefore we remain confident of the success of this project.

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Table of Contents.

Introduction.

Breast cancer is the leading cause of female cancer deaths in this country. Genetic causes account for some of the early onset (pre-menopausal) cases but the causative agents for post-menopausal tumors remain elusive. Retinoid therapy provides an alternative approach for treating or preventing breast cancer. However, it is still unclear who would benefit most from retinoid based therapies. Resolving this requires an understanding of the molecular basis for retinoid regulation of breast cancer cell proliferation. The identification of retinoic acid regulated genes whose products account for the biological response of breast cancer cells to retinoids should provide mechanistic insights into this question.

The project funded by this award proposed to utilize an "enhancer trap" protocol to target genes whose expression is induced by retinoic acid and whose products mediate the anti-proliferative action of retinoids in human breast cancer cells (Forrester et al., 1996; Li and Cohen, 1996). Progress using the "enhancer trap" approach has bee limited due to unexpected technical issues (discussed below). However, complementary approaches (funded in part by pre and post-doctoral awards from the USARMC BCRP) have identified several candidate genes. Therefore I am revising my statement-of-work to incorporate these findings, while continuing to overcome technical issues related to retroviral infection of the T47-D human breast cancer cell line.

Body of Report.

Background.

Initially I proposed to test the hypothesis that "Biologically active derivatives of vitamin A (retinoids) inhibit mammary carcinoma cell proliferation by disrupting one or more growth factor activates serine/threonine protein kinase signaling cascades". I proposed to do this by "Identify(ing) and isolat(ing) genes whose expression is regulated by retinoic acid in hormone-dependent, but not hormone-independent cells and determining if these genes encode proteins involved in cell cycle progression". The approach originally taken to accomplish this goal consisted of construction of a defective retroviral vector allowing selection of living cells in which the vector was integrated adjacent to actively transcribed genes. The design of the vector allows for a two step method for inactivating the target genes; first by insertional mutagenesis and second by inducible expression of antisense RNA complementary to the 5' end of the disrupted transcription unit (thereby inhibiting expression from the non-targeted allele)(Li and Cohen, 1996).

Three experiments were proposed:

(1) Insertional mutagenesis and molecular tagging of RARα-induced genes using an "enhancer trap" retroviral vector;

(2) Cloning RAR–induced growth suppressing genes,

(3) Functional characterization of RARα-regulated genes.

Results and progress.

These experiments were broken down into 10 specific tasks, four of which I had proposed to have finished by the end of project year 1.

As such, we have made the following progress:

Task 1. Construct pLLGFPSV and pRARE-tk-GFP by standard recombinant DNA protocols.

In order to replace the β-geo cassette in pLLGSV (Li and Cohen, 1996), with the enhanced green fluorescent protein gene (GFP; Cubitt et al., 1995, Prashner, 1995), pLLGSV was engineered to contain a unique Bgl II restriction site at the junction between the β-geo gene and the reverse orientation, SV40 promoter (Figure 1), and a unique Nhe 1 site near the junction of the 3' end of the β-geo gene and the 3' LTR. This allowed straightforward removal and replacement of the β-geo cassette

with a fragment encoding GFP (PCR amplified with appropriate primers introducing unique Bgl II and Nhe 1 sites. Note: the Bgl II primer was designed to destroy the single Nhe 1 site at codons 2/3 without changing the amino acid sequence).

In addition, pRARE-SV-GFP has just been constructed by replacing the pCMV immediate early promoter of pCMV-GFP-myc (purchased from Invitrogen, Inc.) with a enhancer-promoter from pRARE3-SV-CAT (Talmage, unpublished) containing 3 tandem repeats of the RARE from the RARβ2 enhancer and the core early promoter from SV40 virus. Standard recombinant DNA protocols were used for these manipulations (Sambrook et al., 1989).

Task 2. Establish conditions for FACS separation of GFP expressing cells.

Initially I proposed to use the RARE-tk-GFP for establishing conditions for isolating live, GFP expressing cells by FACS. This has not been completed because we were unable to get GFP expression in transient assays using the first construct that was tried. Recently we purchased the pCMV-GFP-myc vector DNA from Invitrogen to overcome this problem. These experiments will be completed by mid-October, 1998. We have determined that RA induces CAT expression during transient transfections of T47-D cells by up to 10 fold (Figure 2) without co-transfecting RAR expression vectors. Assuming that endogenous target genes respond similarly, this sets the signal-to-noise ratio with which to work when optimizing the FACS.

Task 3. Generate NIH3T3/GP+E-Amph12 amphotropic retrovirus packaging cells stably producing LLGFPSV;

Task 4. Isolate T47-D cells expressing GFP in an RARα-dependent fashion.

We have not packaged pLLGFPSV. During the past 2 years my lab has isolated stable transfectants of T47-D cells. Different approaches have been used to do this, including standard calcium phosphate co-precipitation, liposome mediated carriers (lipofectamine, Life Technologies Inc., and Superfect for Qiagen, Inc.), and retroviral infection (using either stable producing packaging cells or transient packaging). Only the Superfect reagent resulted in consistent and efficient stable transfections (30-100 drug resistant colonies/microgram of DNA compared to 5-10 colonies/μg with lipofectamine, and <1 colony/μg using CaPO). This was somewhat surprising since we know from transient transfection experiments that there is no block to DNA uptake and initial expression in these cells (e.g., Figure 2).

The inability to obtain more than 1-5 G418 resistant colonies following retroviral infection is particularly distressing. This represents <0.1% of the efficiency obtained on other human breast cancer cells lines and <0.01% of that using rodent fibroblasts. This seriously limits the feasibility of performing saturation mutagenesis with the "enhancer trap" vector. This has caused the most serious delay in progress during project year (and has precluded initiating Task 4).

The basis for the poor stable infection of T47-D cells is not known. It is reminiscent of the low infectability of F9 embryonal carcinoma cells with murine retroviruses, a phenomenon that results from a transcriptional silencing, in part from de novo methylation of the provirus (Gautch and Wilson, 1983; Niwa et al., 1983). This possibility will be examined in project year 3 as a modification of Task 3/4 (detailed below).

Other progress relevant to the overall goals of this project.

A. We have identified two proteins whose expression is increased in retinoic acid treated T47-D cells (Cho et al., 1997; Tighe et al., in preparation). These two are protein kinase Cα (PKCα) and phosphotyrosine phosphatase 1C (a.k.a. SH-PTP-1). Expression of PKCα in T47-D cells results in decreased proliferation, essentially equivalent to that seen following treatment with 10^{-8} M retinoic acid (Cho et al.,

1997). Both the PKCα effect on proliferation, and the retinoic acid affect on proliferation were reversed by the inhibitor of conventional PKCs, Go6976. Since among the conventional PKC isotypes expressed in T47-D cells, only PKCα was detected, we take this as evidence that PKCα mediates at least part of the anti-proliferative effects of retinoic acid. Similar experiments to determine the effect of constitutive (i.e. retinoic acid independent) expression of PTP-1C on T47-D cell proliferation (Task 9) and to determine if antisense expression blocks the retinoic acid induced increase in PTP-1C expression and growth arrest (Task 8) will be completed during the coming year (Project year 2, months 12-24).

B. We have been studying a rodent model in which retinoic acid activation of RARα inhibits de novo transformation by preventing oncogene activation of the c-fos proto-oncogene (Talmage and Lackey, 1992; Talmage and Listerud, 1994). Recently we have shown that the block is in a signaling pathway involving PtdIns 3-kinase, the small G protein Rac1 and Jun N-terminal kinases (Chen et al.1998; see Hill et al., 1995 and Minden et al. 1995 for the initial characterization of this pathway). By using a PCR based subtractive cDNA cloning strategy, we isolated 8 cDNAs whose expression is induced by retinoic acid. Four of these encode proteins known to down-modulate signaling through this, or similar pathways. The others encode novel proteins. We do not know if any of these target genes are induced in T47-D cells during retinoic acid induced growth arrest. Preliminary Southern blot analysis indicates that we will need to isolate human homologues of these cDNAs before northern blot hybridization can be done. Therefore, we are: (1) proceeding with Task 6, construction of a cDNA library from retinoid treated T47-D cells; and (2) obtaining partial human cDNA clones from Expressed Sequence Tag (ESTs) libraries.

Plans for Project Year 2 (including revisions to the statement-of-work).
Technical objective 1. Insertional mutagenesis and molecular tagging of RARα-induced genes using an "enhancer trap" retroviral vector.

Task 1. Completed.

Task 2. We will establish conditions for FACS mediated isolation of GFP expressing cells using the newly constructed RARE-GFP vector. This should be completed during months 12-14.

Task 3. All future packaging will be done using the transient packaging system, using the HEK 293T derived, BING cell line (Pear et al., 1993). It has been our experience that this method yields routinely higher titer supernatants and is accomplished in 3 days rather than 2-4 weeks.

Task 4 (revised). Determine if the low yield of stably infected T47-D cells results from a failure of retrovirus to integrate or to de novo methylation of proviral sequences. As noted above before attempting the "enhancer trap" experiment, we will determine if integration or methylation defects account for the low frequency of stable retrovirally infected T47-D isolates. If integration is the problem, then this approach will be abandoned (see below for alternative strategy). If methylation explains the encountered difficulty, we will proceed as described.

To determine the role of de novo methylation in low retroviral infection of T47-D cells we will do the following experiments:

1. T47-D and NIH3T3 fibroblasts will be infected with high titer stocks of MV-7 (a retroviral vector encoding G418 resistance; stocks are available with titer of ~10^5 cfu/ml; Housey et al., 1988). After 72 hrs high molecular weight DNA will be isolated, digested with Eco R1 (which cleaves the proviral DNA at a single site) and the presence of proviral integrants will be confirmed and partially quantified by Southern

blot analysis (Sambrook et al. 1989). If the block to infection occurs at the level of expression of the provirus LTR, similar levels of proviral integration should be seen in both the non-permissive T47-D and the permissive NIH3T3 cells.

2. If T47D cells contain proviruses, proviral methylation will be examined by comparing the restriction pattern following digestion with the isoschizomers Msp 1 (which is methylation insensitive) and Hpa II (which is methylation sensitive). Southern blots will be probed with either the entire retroviral vector, or restriction fragments corresponding to the LTRs. If de novo methylation of regulatory sequences has occurred during or following integration, then Msp 1 will digest the provirus sequences to a greater extent than Hpa II.

3. If methylation, in particular of LTR sequences has occurred we will determine if this results in the lack of proviral gene expression. T47-D and NIH3T3 cells will be infected in the presence of 0 - 20 μg/ml 5-azacytoside (5azaC; which inhibits DNA methyltransferases). Infected cultures will be maintained under G418 selection for 2-3 weeks. Infected cultures will be treated with 5azaC for either 72 hrs or the entire selection period. If methylation is preventing proviral gene expression, than increasing amounts of 5azaC should result in increased numbers of G418 resistant T47-D cells (and is not expected to have much affect on NIH3T3 cells).

If methylation accounts for the poor infection levels we will proceed with Task 4, using optimal levels of 5azaC during the infection and selection process. I expect these additional experiments to be completed during months 12-15. This will delay the proposed timetable for completion of Tasks 4-7 to months 15-36.

Technical Objective 2: Clone RARα-induced growth suppressing cDNAs.

Task 6. Construct cDNA library with RNA from Am580 treated T47-D cells.

Plans for Task 6 are unchanged.

Task 7. Clone RARα-regulated cDNAs.

If further attempts at infection of T47-D cells proves futile, we will alter our cloning strategy. We will (a) generate a cDNA library from Am580 treated T47-D cells (as described in the original Task 6; Am580 is a potent, RARα-selective synthetic retinoid; Cho et al., 1997; Delecluse et al., 1991) and (b) we will perform PCR based subtraction, using the PCR-select system developed by Clonetech, Inc. (Figure 3). cDNAs that will be used in the subtraction will include Am580 treated T47-D cells (10^{-8} M, 18 hrs) as the target, and solvent treated T47-D cells as the driver in hybridization reactions. We used this approach to successfully identify eight retinoic acid induced cDNAs in a different set of experiments. This process took ~3 months. Sequences expressed preferentially in Am580 treated T47-D cells will be PCR amplified in the presence of ^{32}P-dCTP and used to screen the T47-D cDNA library (Task 6). Positive colonies will be analyzed by restriction digestion, used as probes for northern blots to confirm retinoid induction and partially sequenced (Sambrook et al. 1989; sequence analysis will be done by the Herbert Irving Comprehensive Cancer Center's DNA core facility). Sequences will be compared to all known sequences available in data bases and any cDNAs corresponding to known genes involved in regulating signal transduction pathways, cell cycle progression or gene expression will be studied further (Tasks 8-10).

Technical Objective 3: Characterization of function of RARα-regulated genes.

Task 8. Determine if expressing antisense cDNAs in T47-D cells confers retinoid resistance;

Task 9. Establish effect on cell cycle progression following constitutive expression of full length cDNAs in T47-D and MDA-MB-231 cells;

Task 10. Sequence cDNA inserts.

Tasks 8-10 were to be performed during months 20 -36. In fact, for two candidate genes identified by independent means, these tasks have been (for PKCα;

Cho et al., 1997; Tighe et al., in prep.) or are being (for PTP-1C) done as of month 12. We will complete Tasks 8 and 9 for PTP-1C and if warranted (i.e. if their human homologues are retinoid induced in T47-D cells), begin similar studies on our other 8 candidate retinoic acid target genes.

Conclusions.

Year 1 has uncovered unexpected difficulty in infecting T47-D cells with recombinant retroviruses. Possible explanations are discussed above and will be addressed prior to proceeding, since the "enhancer trap" strategy requires efficient infection. Two additional points will be considered. First, de novo methylation of provirus might not compromise the enhancer trap strategy since it is the flanking cellular genes that provide the transcriptional elements in this system. Second, it is possible to essentially multiply infect entire cell populations by co-culturing with mitomycin C treated producing cell lines for 3-5 days. Therefore I do not feel that this approach need be abandoned yet.

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Tighe, A.P., Cho, Y. and Talmage, D.A. manuscript in preparation.

Appendices.

Figure Legends.

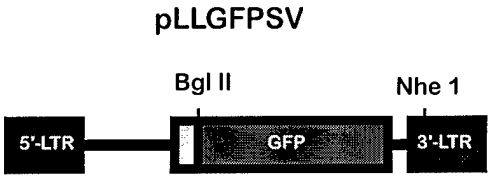
Figure 1. The following plasmid were manipulated for Task 1. A. ppLLGSV, the original enhancer trap vector (Li and Cohen, 1996), showing the gene organization and the restriction sites that were utilized. The first 2 Nhe 1 and the first Bgl II sites were destroyed by sequential partial digestions and fill in reactions. The β-geo gene cassette was then replaced. B. pCMV-GFP-myc, from Invitrogen, Inc. The GFP coding regions was amplified with primers Pr1 and Pr2 by the PCR. Primer 1 contains a Bgl II site and primer 2 a Nhe 1 site allowing direct replacement of the β-geo cassette. The resulting plasmid is referred to as pLLGFPSV. C. pRARE-Sv-CAT contains 3 copies of the DR5 element of the human RARβ2 retinoic acid response element (RARE), the core promoter for the early transcription unit of the SV40 virus and the bacterial chloramphenicol acetyltransferase gene (CAT). The RARE-SV40 enhancer /promoter are flanked by Eco RI and Hind III restriction sites. D. pCMV-GFP-myc, as in B. Note that the CMV immediate early promoter is flanked by unique Eco RI and Pst 1 sites. This regulatory region was removed by digestion with Pst 1, followed by generation of blunt ends with T4 DNA polymerase, and then Eco RI. The RARE-Sv sequences from C were removed by digestion with Hin dIII, filling in the overhand with Klenow, and then Eco RI. Ligation of the RARE-Sv insert with the pCMV-GFP-myc backbone yields pRARE-Sv-GFP.

Figure 2. Transactivation of pRARE-Sv-CAT in T47-D cells treated with retinoids. T47-D cells were transfected with 1 μg of plasmid and treated with the indicated concentrations of either all trans retinoic acid or Am580 for 24 hours. CAT activity in extracts was measured in the linear range of the reaction and background subtracted. Note that at nanomolar concentrations of all trans retinoic acid, RARγ is preferentially activated relative to RARα, and that at $<10^{-8}$ M Am580 is strongly selective for RARα over RARγ. This allows us to approximate the relative contribution each promoter makes to reporter gene expression.

Figure 3. This flow diagram illustrates the strategy that will be used to isolate subtracted cDNA probes as an alternative approach to isolating RA-induced cDNAs in T47-D cells, should the difficulty associated with efficient retroviral infection of this cell line not be overcome. The accompanying table, Table 1, summarizes the success that we have had with this strategy at isolating RA-induced cDNAs that are candidates for disrupting signaling between the middle T:pp60$^{c\text{-}src}$ complex and the c-fos promoter (Chen et al., 1998; Talmage and Lackey, 1992; Talmage and Listerud, 1994). These cDNAs (which are from rat) will be used to first isolate human homologues, and then will be tested to see if they also are induced by RA in T47-D cells, but not in MDA-MB-231 cells (which are insensitive to the anti-proliferative effects of retinoids).

Figure 1.


A. pLLGSV
Nhe 1
Bgl II
Nhe 1
5'-LTR
β-geo
3'-LTR
B. pCMV-GFP-myc
Eco RI
Pst 1
Pr 1
CMV
GFP
myc
Pr 2


pLLGFPSV
Bgl II
Nhe 1
5'-LTR
GFP
3'-LTR


C. pRARE-Sv-CAT
Eco RI
Hin dIII
CAT
D. pCMV-GFP-myc
Eco RI
Pst 1
CMV
GFP
myc

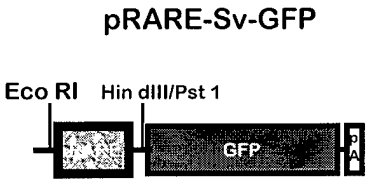
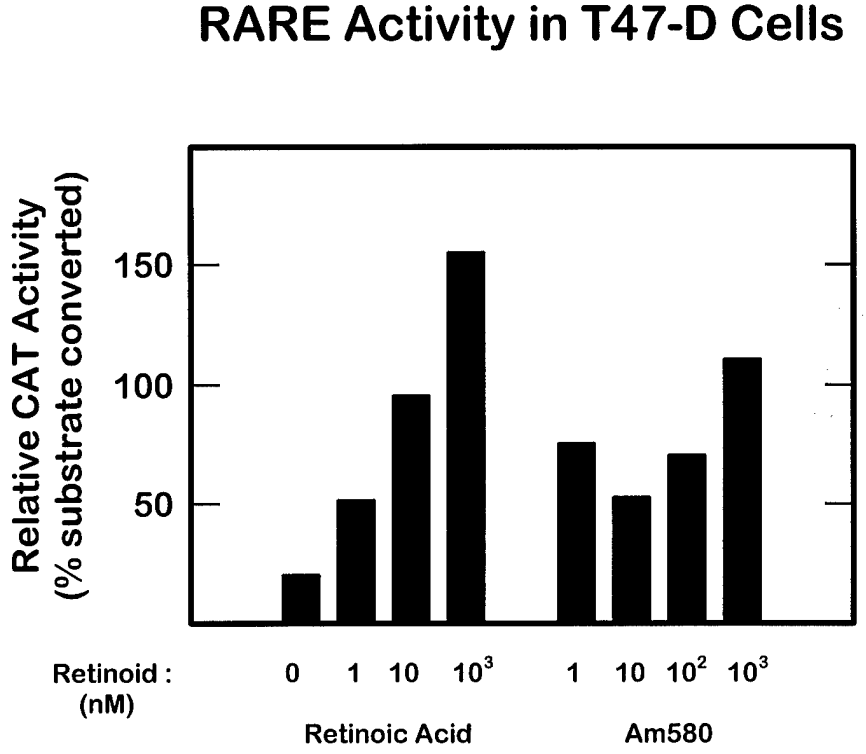

pRARE-Sv-GFP
Eco RI
Hin dIII/Pst 1
GFP

Figure 2.



Relative contribution by:

Basal	13%
RARα	52%
RARγ	23%
Both	12%

Figure 3. Flow chart outline PCR based substraction of differentially expressed cDNAs.

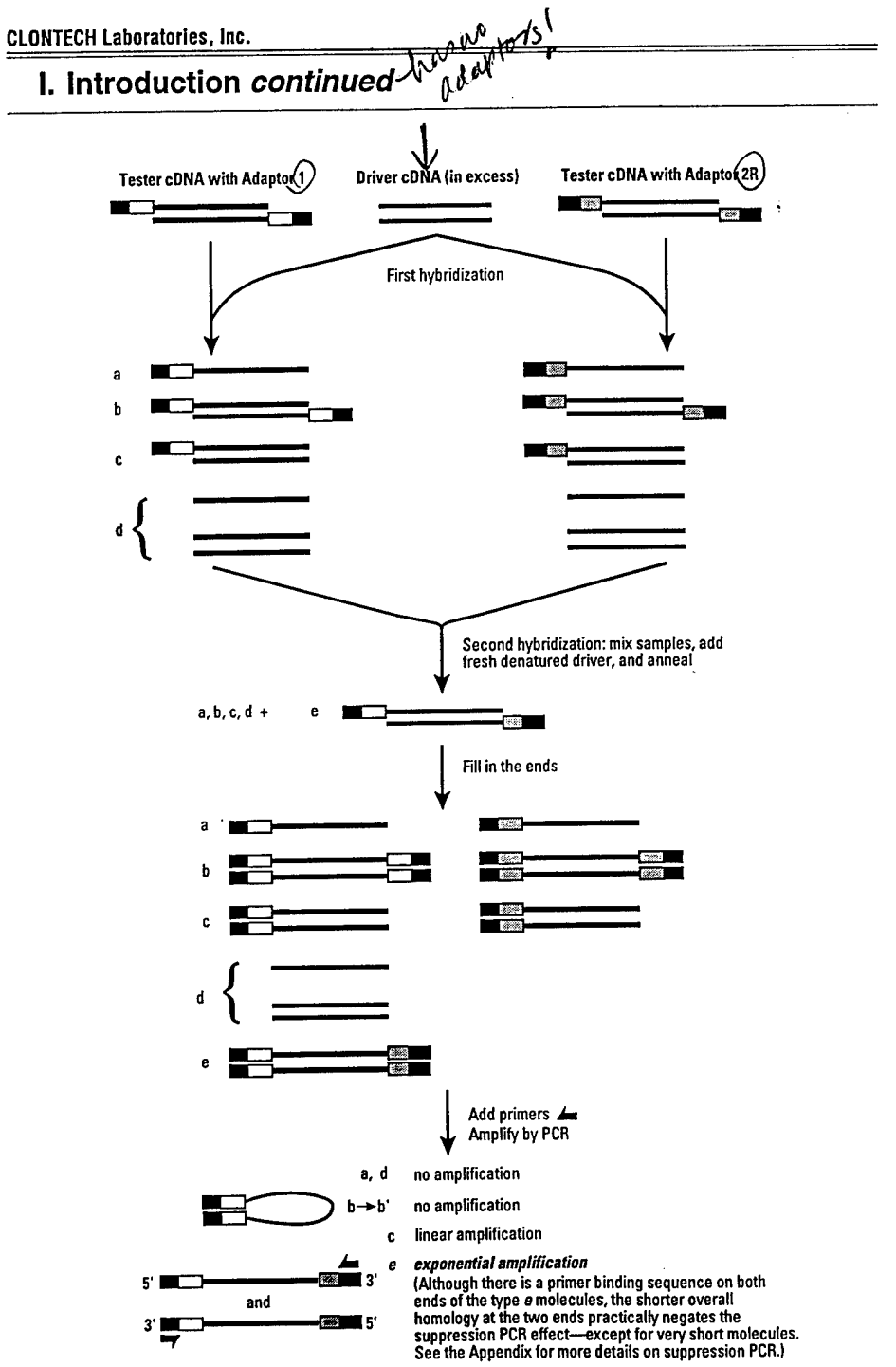


Table I. Summary of retinoic acid induced cDNAs isolated from rat fibroblasts.

cDNA	Description of encoded gene
M33	Novel*
M61	Novel*
M90	Novel*
pRbAp46	interacts with Rb, inhibits Ras signaling interacts with histone deacetylases
p190GAP-AP	Rac1 GAP, has tumor suppressing activity overexpression of GAP domain inhibits c-fos expression
PP1β	serine/threonine protein phosphatase, found bound to chromatin in interphase cells
JIP-1rp	splice variant of JIP-1, and Jun N-terminal kinase interacting protein, can interfere with or regulate signaling via JNK
Sar1a	GTP-binding protein involved in vesicular trafficking

* Novel sequences indicate that either no matching sequence could be found in exisiting data bases, or that if they exist, no known protein function has been attributed to the gene product encoded.

Table 1. Summary of cDNAs that are positively regulated in rat fibroblasts by retinoic acid. With the exception of JIP-1rp, these were isolated in an initial screen using the PCR based method outlined in Figure 3 and proposed as an alternative strategy in Task 7. Human homologues of these sequences will be used to determine if they also are induced by retinioc acid in T47-D breast cancer cells.