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13. ABSTRACT *(Maximum 200 words)*

My project, as decribed in the last annual report, was to coexpress E2F wild type and mutant proteins, ras, and GFP from the same viral vector and infuse the viruses into rat mammary glands to see if E2F wild type and mutant proteins could cooperate or interfere with ras mediated mammary carcinogenesis. While cloning of the viral vectors was successful and protein expression of the E2F proteins was evident, there was no ras expression from the IRES (internal ribosome entry site) as monitered by Westerns or NIH 3T3 transformation assays. Therefore, using viral infusion to investigate the interaction between ras and E2F proteins was not possible. As a result, I used tissue culture assays to investigate these two proteins in transformation. While E2F1 was were unable to cause transformation by by itself, a E2F1 del417-437 mutant interfered with ras transformation. Interestingly, this mutant does not affect cell cycle progression and does not appear to be toxic. Therefore, E2F1 del417-437 may be affecting transformation-specific E2F targets. Endogenous E2F targets are being investigated by a chromatin immunoprecipitation assay.

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Peggy Faulkner 8/13/98

PI - Signature Date

Grace Lee 8/12/98

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INTRODUCTION

Tumors arise when cells escape the controls of cell growth and undergo an inappropriate expansion in cell number. The decision for a cell to divide, and thus expand in number, is made in the G1 phase of the cell cycle before cells replicate their DNA in S phase. It is during the G1 to S phase transition that a cell commits to activating genes whose products are required for DNA replication and cell cycle progression. Many factors which control the G1 to S phase transition of the cell cycle are implicated in the formation of cancer. The activation of several G1/S-phase regulated genes is mediated via a family of transcription factors called E2F (26). The abundance and activity of the E2F transcription factors are tightly regulated by positive and negative regulators of the G1 to S phase transition. These regulators are often mutated in breast cancer in such a way that they could upregulate E2F activity (11). The retinoblastoma (Rb) tumor supressor protein, a direct negative regulator of E2F activity, is lost or inactivated in approximately 30% of breast carcinomas (27, 29). In addition, the activity of positive regulators of E2F activity, such as cyclin D1 and cyclin E, is increased in many breast cancers. Cyclin D1 has been found to be increased in 45% of breast tumor biopsies (2) and studies show altered patterns of expression for cyclin E in numerous breast cancer cell lines and primary breast tumors (13, 14, 15, 21).

The mechanism by which Rb and the G1 cyclins (cyclin D1 and cyclin E) are involved in the formation of breast cancer is unknown, but they are all regulators of E2F activity. While E2F family members have been shown to function as oncogenes in standard transformation assays (1, 8, 12, 25, 30), an *in vivo* investigation of the involvement of E2F in breast cancer development has yet to be done. Because rat mammary carcinoma mirrors human breast cancer in many ways, I proposed to overexpress wildtype E2F family members in rat mammary glands using a replication defective retrovirus (9). Last year, I determined that the E2F4 virus did not cause a visible phenotype in the rat mammary gland. This result suggested that E2F4 was not able to cause breast cancer when overexpressed alone. Since activated ras is able to cause tumors in the rat mammary gland when expressed alone in the rat mammary gland, I proposed to overexpress either wildtype or mutant E2F family members in combination with the oncogenic protein ras in rat mammary glands using a replication defective retrovirus. By looking at the effect of E2F proteins on ras mediated mammary carcinogenesis, I could determine if E2F wild type or mutant proteins can cooperate or interfere with activated ras to cause mammary carcinoma.

BODY

Materials and Methods

Cloning and plasmids used

The pJLR, pLCG, and pJLR-HrasA plasmids were obtained from the Gould laboratory (28). The priboEJras plasmid, containing the second and third exons of an activated c-Ha-ras under the expression of the ribosomal L323A promoter, was obtained from Mary Ellen Perry's laboratory (7). The pEMC-F vector which contains an IRES (Internal Ribosomal Entry Site) was obtained from W. French Anderson's laboratory (19). The mE2F1 cDNA was available in our laboraory and the hE2F4 cDNA was obtained from Rene Benards (1, 16). The pCMV E2F1 and pCMV E2F1 E138 vectors were from Joseph Nevins laboratory (5). The pE2F1 del417-437 vector was obtained from Ed Harlow's laboratory (10).

The pIR vector was made by PCR amplifying the activated form of the c-Ha-ras gene from the pJLR-HrasA vector with primers containing the apropriate restriction sites. PCR products were digested with the proper restriction enzymes, isolated, and inserted into the the pEMC-F vector, placing it in frame downstream of the IRES. Then the IRES-activated c-Ha-ras cassette was removed from the pEMC-F-ras vector with the apprpriate restriction enzymes, isolated, and inserted into the pLCG SalI site. The pLCG-E2F and pIR-E2F vectors were cloned by PCR amplification of different E2F cDNA vectors with primers which have BamHI sites. PCR products were digested with BamHI, isolated, and inserted into the BamHI site of the vector. Cloning was done using standard procedures (23). All inserts were sequenced for proper orientation and high fidelity PCR.

Cell Culture

NIH 3T3 cells used in focus forming transformation assays are from ATCC (American Type Culture Collection) and were grown in DMEM with 10% BCS (Bovine Calf Serum) and 1% P/S (Penicillin / Streptomycin) in a 5% CO_2 incubator at 37°C.

NIH 3T3 cells used for serum starvation stimulation assays were grown in DMEM with 5% BCS and 1% P/S in a 5% CO_2 incubator at 37°C. In a starvation-stimulation procedure, Cells at about 50% confluency were placed in starvation media for 48-60 hours. Cells were then stimulated by adding stimulation media for the indicated times. Starvation media used was DMEM with 0.5% BCS and 1% P/S. Stimulation media used was DMEM with 10% BCS and 1% P/S.

Hela cells were grown in suspension in Joklik's media with 10% BCS and 1% P/S, and 1% glutamine using a stirring flask at 37°C without CO2 regulation.

Whole cell protein extraction

Cells were grown in T225 flasks and harvested by trypsinization. Cells were pelleted by centrifugation at 1000 rpm for 5 min. in a tabletop centrifuge and the supernatent was removed. Cell pellets were stored at -20° C. Whole cell protein extractions were done by thawing the cell pellet that was harvested from one T225 flask briefly at 37° C. The cells were then kept on ice and 0.5 ml of lysis buffer [50mM Tris, pH 8.0; 5mM EDTA; 150mM NaCl; 0.5% NP-40; 1mM PMSF] was mixed with the pellet. After leaving the cells on ice for 20 min., the cells were further mixed for 1 min. and then the lysate was placed in a 1.5 ml microcentrifuge tube and spun at 14,000 rpm for 5 min. at 4° C in a microcentrifuge. The supernatent is then assayed for protein content and kept for Western studies.

Western analysis

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 10% gel by standard techniques (23). Proteins were transferred to nitrocellulose electrophoretically and probed with antibodies against E2F proteins (antibodies from Santa Cruz) or with antibodies against TBP as a standardization control (from Richard Burgess's laboratory) (3). Proteins were detected by enhanced chemiluminescence (reagents and protocol from Amersham).

NIH 3T3 focus forming transformation assay

Transfection buffers and procedures used are as described in Chen and Okayama (4) except for the following: 10 ugs of E2F or empty expressor plasmid and indicated amounts of the priboEJras vector were used for each transfection. Sonicated salmon sperm DNA was added to the transfection DNA to make a total DNA content equal 20ugs per transfection. After cells were allowed to grow for 2 weeks, cells were fixed and stained using the following procedure. Media was removed and cells were rinsed with PBS. Cells were fixed in 10% buffered formalin (formaldehyde) for at least 5 min. Formalin was removed and cells were stained with 0.14% methylene blue in H20 for 10 min or longer. Stain was removed and cells were washed extensively with H20 and allowed to dry.

Flow analysis

Cells were harvested by trypsinization and counted with a hemacytometer. Cells were then resuspended 5 X 10^5 cells per 300ul media. An equal volume of 20ug/ml Hoecsht 33342 was added and cells were then incubated for 30 min. at 37° C , filtered through a 30um nylon mesh, and treated with propidium iodide (PI) to a final concentration of 5ug/ml. Cells were then analyzed using a FACSstar machine to detect transfected (using GFP as a marker) and live (by gating out PI stained cells) cells and to determine the

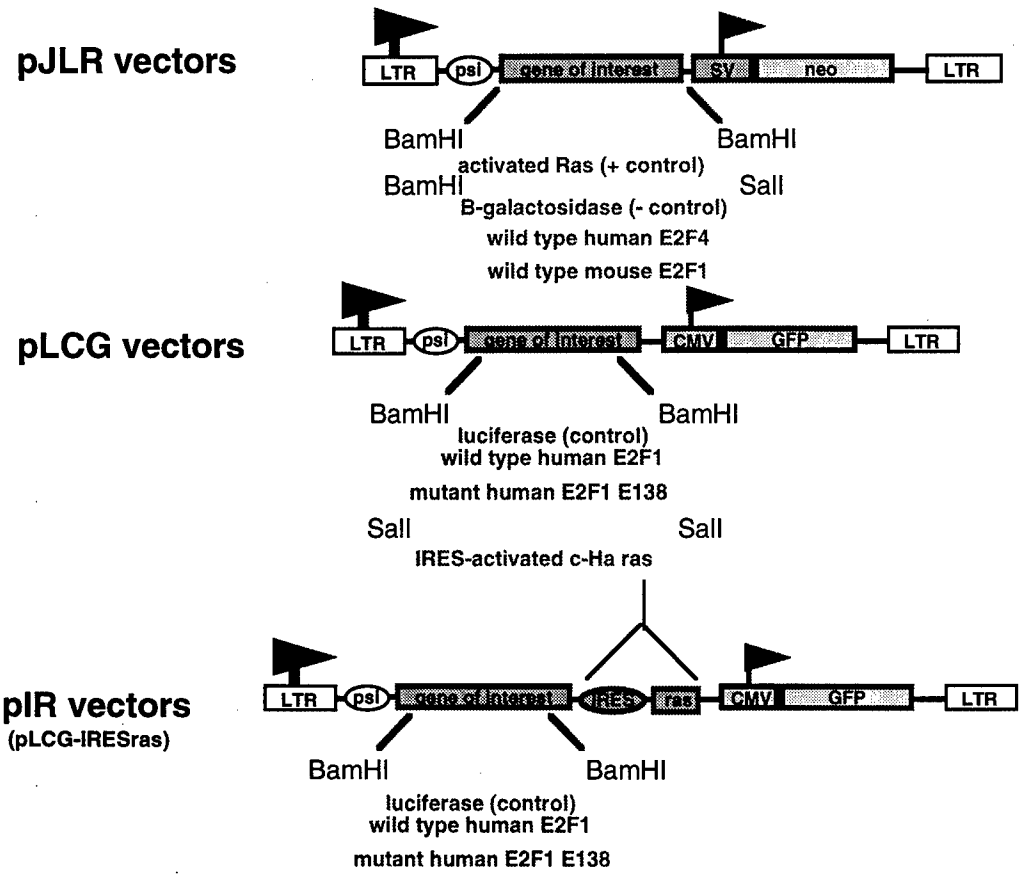
cell cycle stage (using Hoecsht 33342 to stain DNA) of the cells. Analysis was conducted by the staff at the flow cytommetry facility at UW hospital.

Results and Discussion:

Instead of using the pJLR vector as in the studies done last year, I used a different retroviral vector which expresses an enchanced form of the green flourescent protein (eGFP) instead of the neomycin resistance gene as a selectable marker. eGFP is a protein marker that will emit a green light in the presence of blue light. As a result, visualization and selection of infected cell clones and quicker titering of single cell clones *in vitro* and *in vivo* would be possible (20). I received a retroviral vector which contained eGFP, called pLCG, from Phil Watson in the Gould laboratory.

From the last annual report, I had decided to coexpress E2F with ras by adding an IRES to the pLCG vector in order to express the two proteins from the same MMLV LTR promoter. E2F1 would be the the first gene driven by the promoter and the activated form of c-Ha-ras is the second gene (see "pIR" vector). For controls, the parental vector, pLCG, which does not have the IRES-ras cassette, and vectors which expressed luciferase (luc) instead of E2F were used. A schematic of all vectors are shown in Figure 1.

Figure 1. Schematics of all viral vectors

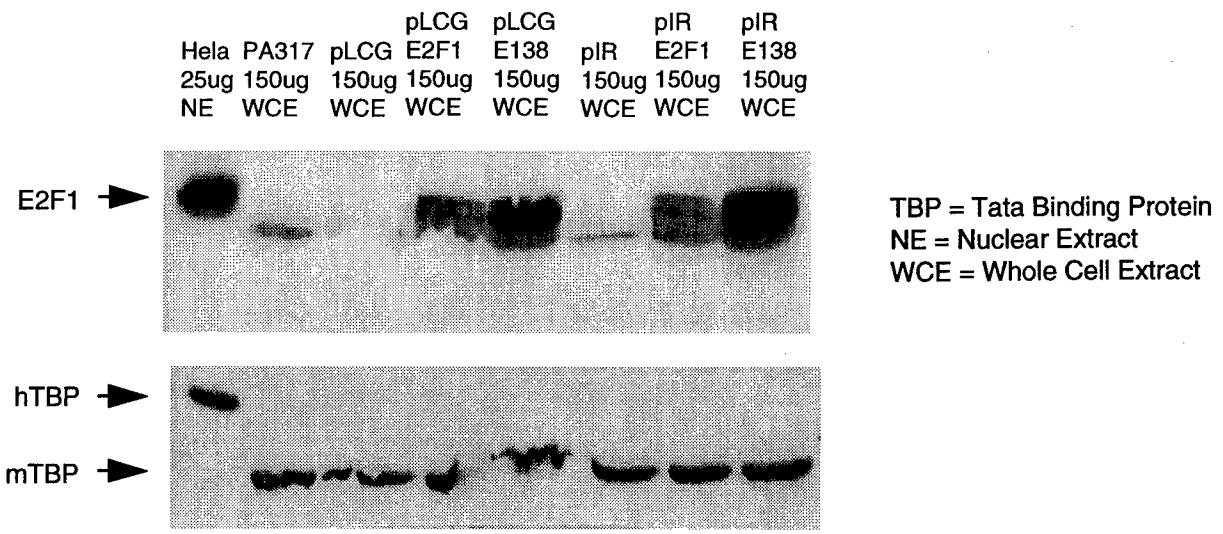


Studies in tissue culture indicate that ras and E2F cooperate to transform rat embryo fibroblasts even though ras or E2F is unable to transform the cells when expressed alone (1, 12). A recent study has shown that transgenic coexpression of E2F1 and ras in mouse skin leads to a severe cancer phenotype which is not seen when ras or E2F1 is expressed alone (22). While cooperation between ras and E2F1 has been demonstrated in vivo, results seen in mouse skin can be very different than what is seen in the rat mammary gland. Since the rat mammary gland is very similar to human mammary tissue, these studies should give insight on the role of E2F1 in the tissue specific environment of the mammary gland for rats and possibly, humans. pIR-wild type E2F1, pIR-luc, pLCG-wild type E2F1, and pLCG-luc would be used to test this issue.

I also coexpressed ras with the E2F1 mutant E138 which contains two amino acid mutations within its DNA binding domain. This mutation interferes with the ability of E2F1 and other E2F proteins to bind to DNA. Ras has been shown to activate many different pathways (18). One of the proteins that ras activates is cyclin D1 which can increase E2F activity (6, 17). If ras causes tumors using a E2F dependent pathway in the mammary gland, expression of a dominant negative E2F should cause a decrease in the number and/or size of ras initiated tumors. pIR-E2F1 E138, pIR-luc, pLCG-E2F1 E138, and pLCG-luc would be used to test this issue.

I first determined if cells which contained the viral vectors were producing the vector proteins properly. I was able to detect human E2F1 by Western analysis (Figure 2) and luciferase by a luciferase assay (data not shown), but I could not detect the ras protein in Westerns (data not shown). I was also unable to detect GFP (by fluorescence) in the pIR vectors but the could be detected in the pLCG vectors (data not shown).

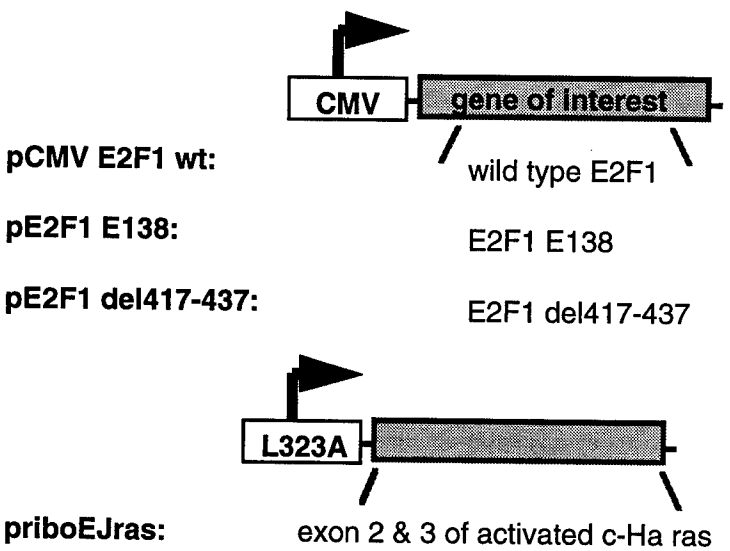
Figure 2. E2F1 Western of PA317 cells lipofected with E2F1 and E2F1 E138 viral vectors. TBP Western was used as a standardization control. Hela nuclear extract used as a positive control for E2F1 detection.



To determine if functional ras was being made from the pIR vectors as well as test my vectors in a tissue culture assay before using them in the rat mammary gland, I used a NIH 3T3 transformation assay. pIR based vectors did not cause transformation of the NIH 3T3 cells while the positive control for transformation, the priboEJras formed several foci (data not shown). In conclusion, the pIR vectors were not making functional activated ras. The IRES may have not been working and could also have interfered with the expression of GFP driven by the downstream CMV promoter.

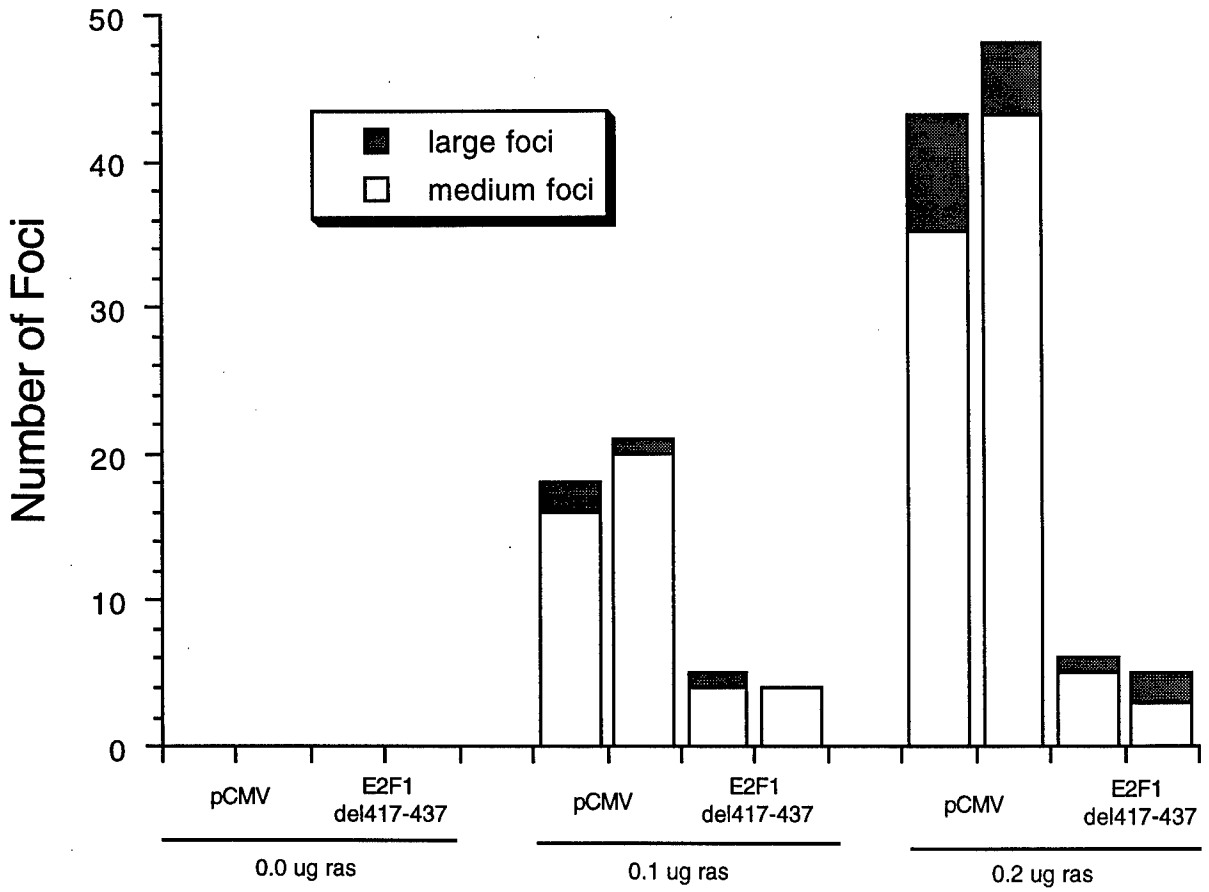
Since the pIR vectors did not produce functional ras or GFP proteins, I could not pursue making viruses for infusion into rat mammary glands. In order to ask the same question, "Can wild type or mutant forms of E2F transcription factors cooperate or interfere with ras mediated transformation?", I used seperate expression constructs for E2F1 and activated ras (see Figure 3) in the NIH 3T3 transformation assay. Transformation assays with the E2F expression vectors alone, produced no foci above background vector alone levels (data not shown). At the recommended level of ras vector, 20ug per transfection, E2F expression constucts seemed to have no effect in tissue culture dishes almost confluent with transformed foci. I proceeded to titrate down the amount of ras vector. While 10ug and 2ug of ras still contained too many foci to count, countable foci were detected at 0.5, 0.2, and 0.1 ug of ras vector (data not shown).

Figure 3: Schematics of nonviral E2F1 and ras vectors used in NIH 3T3 transformation assays



At the levels of 0.1, 0.2, and 0.5ugs of priboEJras, I discovered that the mutant E2F1 protein, del417-437, could consistently inhibit ras mediated transformation. Figure 4 shows a representive graph of transformation results with the del417-437 mutant. Studies of the wild type and the E138 mutant E2F1 are still inprogress.

Figure 4: E2F1 del417-437 inhibited ras mediated transformation in a NIH 3T3 focus forming assay at the ras vector concentrations of 0.1 ug and 0.2 ug per transfection.



To investigate how the E2F1 del417-437 mutant could inhibit ras mediated transformation, I looked at the effect of the mutant on cell cycle kinetics. DNA profiles for NIH 3T3 cells transfected with the del417-437 mutant looked normal when compared to vector alone in 60 hr. serum starved and stimulated cells (see Figure 5). To rule out the idea that E2F1 del417-437 is affecting a cell cycle gene that is very stable, resulting in no effect in a 60hr starvation stimulation assay because the critical target protein is still in sufficient amounts, I also analyzed cell cycle kinetics of 7 day starved and stimulated cells and 3 day log growing cells. Again, I saw no change in cell cycle kinetics between E2F1 del417-437 and vector alone transfected cells (see Figure 6 and 7). In addition, I see the same percentage of cells transfected with del417-437 and vector alone suggesting that apoptosis is not playing a role in inhibiting ras transformation. In support of this, other studies show that E2F1 del417-437 does not cause apoptosis (24). We suggest that E2F1 del417-437 is affecting transformation specific E2F targets. We are currently investigating E2F target genes using a chromatin immunoprecipitation assay.

Figure 5: Comparison of pCMV and E2F1 del417-437 transfected NIH 3T3 60 hr. serum starved and stimulated cells using flow cytommetry.

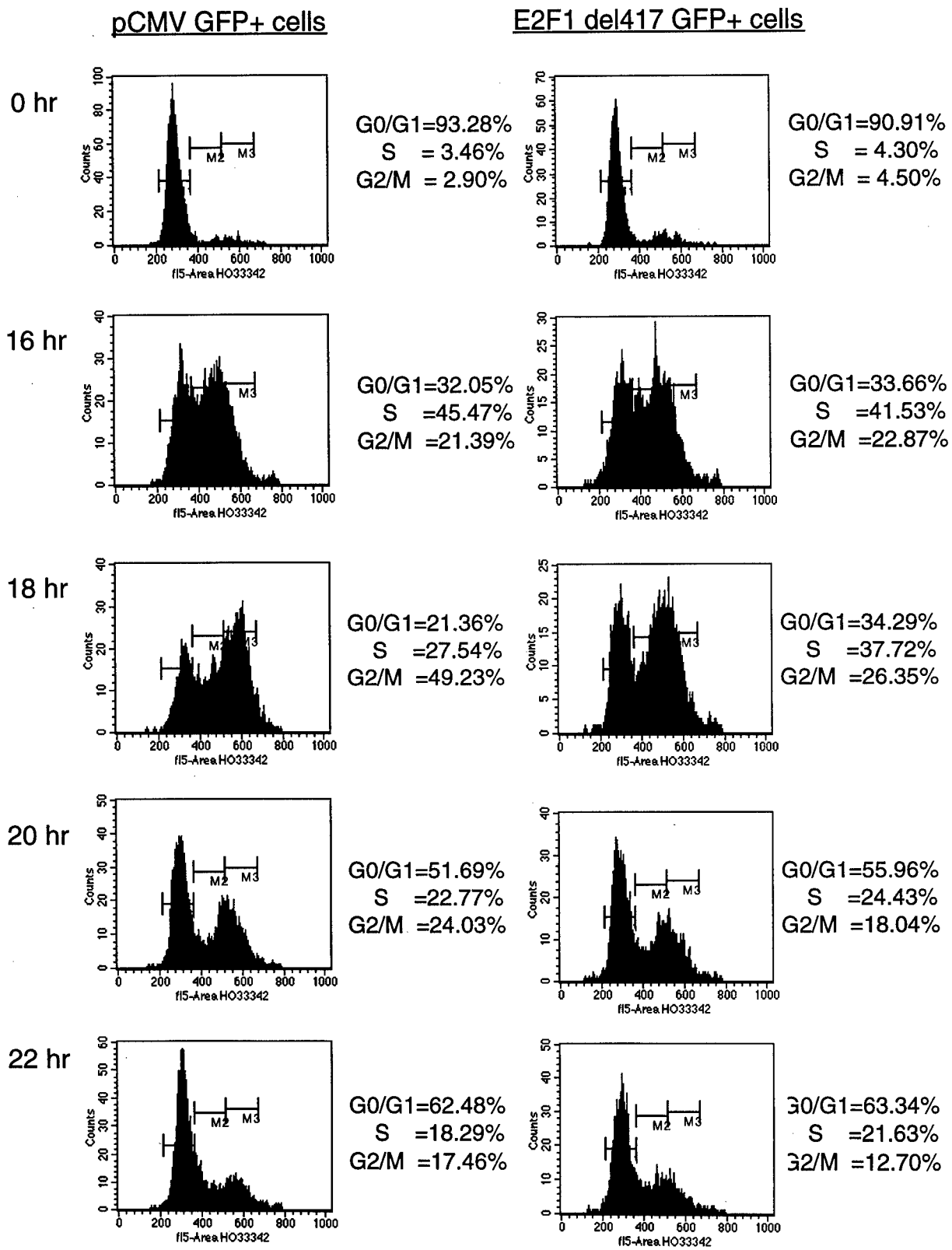


Figure 6: Comparison of pCMV and E2F1 del417-437 transfected NIH 3T3 seven day serum starved and stimulated cells using flow cytommetry.

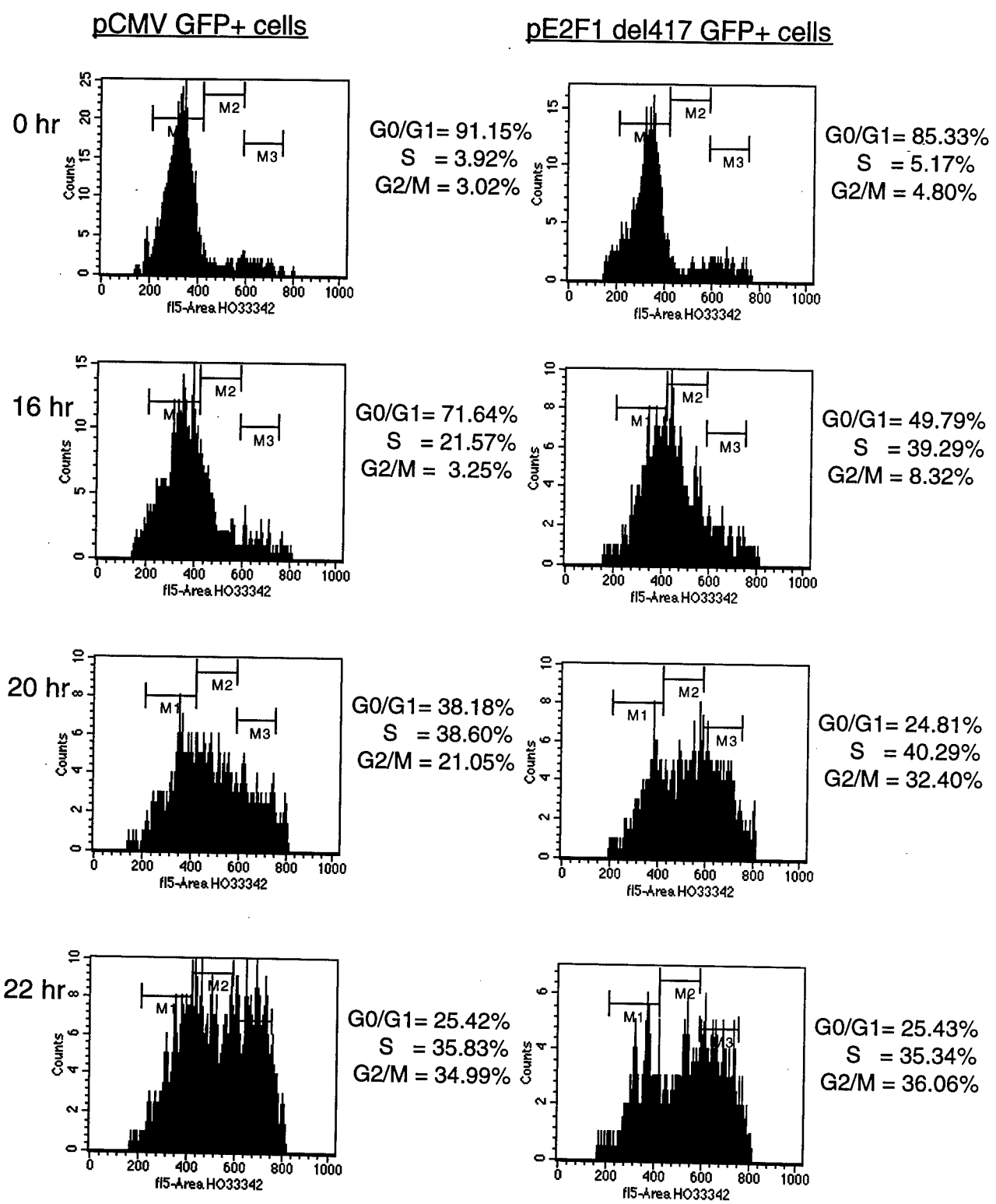
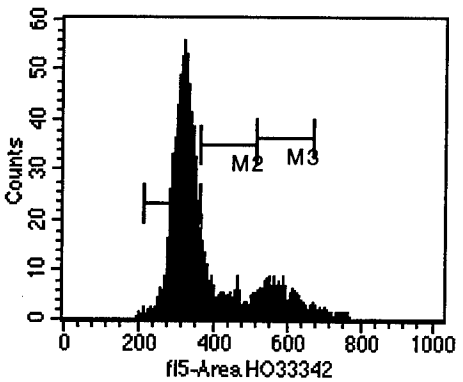


Figure 7: Comparison of pCMV and E2F1 del417-437 transfected NIH 3T3 log growing cells using flow cytommetry.

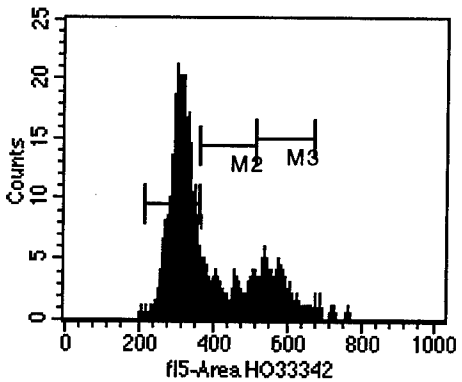
pCMV GFP+ cells

G0/G1 = 76.87%
S = 10.67%
G2/M = 11.21%



E2F1 del417 GFP+ cells

G0/G1 = 72.37%
S = 13.46%
G2/M = 13.22%



CONCLUSIONS

E2F1 mutant del417-437 inhibits ras-mediated transformation apparently without causing a cell cycle arrest or cell death. We suggest that E2F1 del417-437 is affecting transformation specific E2F targets. Comparison of the del417-437 mutant with the wild type and other E2F1 mutant proteins should give more insight into the mechanism of inhibition of ras mediated transformation. These studies are currently being done. Also, we are currently investigating E2F target genes using a chromatin immunoprecipitation assay.

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