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13. ABSTRACT *(Maximum 200*

In order to study the role of Raf-1 kinase signaling in the progression of breast cancer from estrogen-dependent to estrogen-independent growth, we had previously established several clones of MCF-7 cells stably expressing a truncated, constitutively active Raf and examined these clones for their ability to grow in the absence of estrogen. Constitutive activation of Raf did result in estrogen-independent growth. In addition, the constitutively active Raf also induced apoptosis in these cells. In this study, we report that these Raf transfected cells growing long-term in the absence of estrogen have lost ER expression. This loss is at both the protein and RNA level. Potential mechanisms underlying this loss are discussed. In addition, we are assessing the role that known modulators of apoptosis may play in the Raf-induced apoptosis. After establishing conditions for Western blot analysis of the expression of these factors, we have examined two potentiators of apoptosis, Bax and Bad, and an inhibitor of cell cycle progression, WAF1/Cip1. There were no significant alterations in the expression levels of these proteins that could be correlated with either the presence of high levels of Raf kinase activity or with apoptosis in our clones.

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Donaya El-Ashry 8/30/96

PI - Signature Date

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INTRODUCTION

Breast cancer growth can be estrogen-dependent or -independent. Estrogen-dependent breast cancer, that is breast cancer that expresses functional estrogen receptors (ER) and requires estrogen for growth, has a better prognosis than estrogen-independent, ER-negative breast cancer (1-4). This better prognosis is reflected in both a longer disease-free survival and overall survival, as well as, response to endocrine therapies such as the anti-estrogen tamoxifen.

It is well established that estrogen treatment of ER+ breast cancer cells results in increased growth and the increased expression of several growth factors. As a result, it has long been hypothesized that estrogen promotes cellular proliferation via the induction of specific growth factors and their cognate receptors thus setting up an autocrine loop. Upon progression to estrogen-independent growth, there is loss of ER expression and overexpression of certain growth factors/receptors, such as TGF-α and EGFR. Given this, it is also possible that signal transduction via growth factor tyrosine kinase receptors is a separate and alternate pathway and thus a mechanism for bypassing estrogen-mediated effects. Upregulation of these pathways may therefore be an early event in progression to ER-, thus resulting in an intermediate ER+/estrogen-independent phenotype. Many studies support this idea. Breast cancer cells overexpressing FGF-4, FGF-1, or heregulin become tumorigenic in nude mice in the absence of estrogen (5,6). Overexpression of EGFR or erbB-2 in breast cancer cells confers a growth advantage in estrogen-depleted media (6-8) and can increase tumorigenesis in nude mice in the absence of estrogen.

One of the major pathways initiated by the EGFR ligands is the mitogen activated protein kinase (MAP kinase or MAPK) phosphorylation cascade [reviewed in (9)]. Upon binding of ligand, the activated receptor interacts with and activates Ras, resulting in the subsequent activation of the Raf protooncogene. Raf-1, one of three Rafs [reviewed in (10,11)], is a serine/threonine kinase that can directly phosphorylate nuclear transcription factors like p53 (12). It also activates MAP kinase kinase (MAPKK or MEK), a dual specificity kinase, which then activates MAPK. The activated MAPK, which is a serine/threonine kinase, is able to phosphorylate several nuclear transcription factors including Myc, Elk, and Rsk (13-18). It is now known that not only can the activated Ras oncogene cause cellular transformation (19,20), but that activated v-Raf or a constitutively activated MAPKK can also result in transformation (21-24). Furthermore, in a recent study of renal cell carcinomas, it was found that about 50% demonstrated upregulation of Raf, MEK, and MAPK (25), implicating this signal transduction pathway in the carcinogenic process.

We were interested in studying the role of Raf in human breast cancer for several reasons. First, overexpression of growth factor receptors such as EGFR and erbB-2 occurs in a significant percentage of breast tumors and is correlated with a poor prognosis. Such overexpression would be expected to have an effect on downstream factors. Second, there are several studies indicating that oncogenic Ras transfected into ER+ MCF-7 cells renders them estrogen-independent (26-30). While activating mutations of Ras are very rare in breast cancer, overexpression does occur in ~70% of breast cancer cases (31,32). This overexpression may have significant impact on the downstream effectors of Ras, such as Raf. A third reason to study Raf is that many other signaling systems such as protein kinase C (PKC) and protein kinase A (PKA) have been shown to activate or inactivate Raf respectively (33-35), suggesting that Raf may play a pivotal role in signal transduction by several different pathways known to be important in breast cancer.

We have previously reported on the stable transfection of a constitutively active Raf kinase (referred to as Δ-raf) into estrogen-dependent MCF-7 breast cancer cells. We had studied the growth growth characteristics of 6 Δ-raf clones arising from this transfection in comparison to the vector-transfected control pool of cell (referred to as Hcopool) and found that Δ-raf expression results in estrogen-independent growth, but the cells are responsive to estrogen (36). However, while the high Raf activity seems to allow for growth in the absence of estrogen, this high activity is not compatible with growth in the presence of estrogen and continued growth in estrogen-containing medium results in down-regulated expression of the transfected Raf. A second observation was that high levels of Raf activity in these cells leads to apoptosis as measured by an assay (refer to previous annual report) that specifically measures apoptosis by assessing the presence of histone-bound DNA fragments in the cytoplasm of cells -- a hallmark of apoptosis that distinguishes it from necrosis or other types of cell death. This apoptosis most likely is a result of the extremely high levels of constitutively active kinase in these cells, however we can use this as a model to study apoptotic pathways in breast cancer cells.

The objectives for the past year were to assess the mechanism by which Raf induced estrogen-independent growth, and to begin to assess the role that known effectors and modulators of apoptosis play in the Raf-induced apoptosis in our transfectants.

RESULTS

Estrogen and Anti-estrogen effects on Δ-raf clones growing long-term in the absence of estrogen (CCS). We had previously analyzed the growth of Δ-raf clones in the short-term absence of estrogen, and as shown previously, these clones were capable of estrogen-independent growth but were still responded to estrogen with further increased growth. Since then, we now had each of the clones growing long-term in the absence of estrogen [IMEM without phenol red supplemented with 10% charcoal-stripped serum (CCS)]. In order to begin to dissect the mechanism of Raf-induced estrogen-independent growth in our transfectants, we therefore assessed the growth capabilities and response to estrogen and anti-estrogens of these clones growing long-term in the absence of estrogen. Figure 1A represents results from an anchorage-dependent growth assay of HCopool (the vector-transfected control pool of MCF-7 cells that normally grow in FBS and for this experiment have been quick-stripped of estrogen and assayed for growth in CCS conditions), MCNC4 (a vector-transfected control clone of MCF-7 cells long-term adapted for growth in the absence of estrogen, and Raf 14c (a Δ-raf transfected clone of MCF-7 cells growing long-term in CCS). As shown, HCopool exhibits no significant growth in CCS after the first two days of the assay (since the cells have been quick-stripped and some residual estrogens initially remain, there is a short lag before the cells stop growing). The anti-estrogens tamoxifen and ICI 182 780 have no significant impact, however, the addition of estrogen or growth in FBS restores the growth capacity of these cells. MCNC4 cells are capable of slow growth in CCS, and this growth is slightly reduced in the presence of the anti-estrogens. Both estrogen and FBS are capable of inducing growth of these cells. Interestingly, the Δ-raf transfected clone, Raf 14c, exhibits growth not only in the absence of estrogen (CCS), but also in the presence of either anti-estrogen. Furthermore, the addition of estrogen or growth in FBS does not further increase the growth capacity of these cells. Thus the Δ-raf transfected cells growing long-term in the absence of estrogen no longer seem to respond to

estrogen.

Anchorage-independent growth in soft agar, a more stringent test of growth capacity, was next assessed. As shown in Figure 1B, Raf 14c produces a large number of colonies in CCS, about 650, compared to the ~ 50 colonies that quick-stripped HCopool produces. Again, while HCopool is responsive to estrogen, in that estrogen induces colony formation (~ 3-fold induction), and the anti-estrogens have a slightly inhibitory or no effect, Raf 14c produces the same high number of colonies under all conditions -- that is there is no response to either estrogen or the anti-estrogens. These unexpected results seemed to indicate that these cells were no longer expressing a functional ER.

Δ-raf Clones Growing Long-term in CCS Have Lost ER. To determine if this was indeed the case, Δ-raf clones were assessed for ER levels, both at the protein and RNA level. ER protein was analyzed both by a steroid-binding assay, which will measure all ER capable of binding estrogen, and by Western blotting with an anti-ER monoclonal antibody, which will detect all immunogenic ER. Results from these assays are shown in Figure 2 where the steroid-binding assay results are expressed in fmol/mg protein in parenthesis beneath each lane of Western-blotted lysate. Compared to the MCNC4 control cell line, which exhibits ~206 fmol ER/mg protein, each of the Δ-raf clones has significantly reduced levels of ER expression, with Raf 14c, the clone growing in CCS the longest and the least responsive to estrogen and anti-estrogens, having only ~10 fmol/mg protein. Thus, all of the clones had reduced levels of ER protein expression.

RNase protection assays, shown in Figure 3, were performed to determine ER RNA levels in the clones. Compared to either the Hcopool (grow in FBS) or the MCNC4 (grow in CCS) control cells, the Δ-raf clones exhibited drastically reduced RNA levels, and again, Raf 14c was the most dramatic with virtually no detectable expression.

Loss of ER Expression is, in part, at the Level of Transcription. Since we have now recapitulated the clinical progression of breast cancer, that is from ER+ to ER-, for the first time in a laboratory model, an important next step is to determine the mechanism to this Δ-raf induced loss of ER. As a first step to try and determine the mechanism underlying this loss, we were next interested in determining if there was a transcriptional component to the reduction of ER in the clones. A nuclear run-on assay was performed to assess the level of transcription of the ER gene in Raf 14c cells and in the control MCNC4 cell line (Figure 4). Raf 14c cells displayed a 7-fold reduction in transcription of ER as compared to that occurring in MCNC4 cells indicating that decreased transcription of the gene played a role in the significantly reduced levels of ER observed in these cells.

Assessment of the Expression of Known Modulators of Apoptosis. Since Δ-raf also induced apoptosis, our other line of investigation is to dissect the mechanisms underlying this in order to gain an understanding of specific apoptosis pathways in breast cancer cells. As a first step, we have been further characterizing the Raf-induced apoptosis that occurs in our transfected cells. Initially, we wanted to confirm that what we were observing in these cells was truly apoptosis by a second method, the Tunel assay which measures the presence of fragmented DNA in the cytoplasm of cells by flow cytometry (FACS). In the Tunel Assay, both floating and adherent cells were collected and fixed. Following incubation with terminal deoxytransferase and fluorescein-labeled nucleotides, the cells were analyzed by FACS. The positive control, human leukemia cells treated with campothecin to produce ~30% apoptotic cells, show the expected second peak representative

of apoptotic cells (Figure 5A). The negative controls, HCopool, HCopool stripped of estrogen for 5 days, and HCopool adapted long-term to grow in CCS, all look very similar with a single peak of low fluorescence (Figure 5B). Since the Δ-raf transfectants undergo apoptosis at some constant rate, that is they are not treated with an apoptosis inducer, it did not seem likely that two distinct peaks would be seen. Rather, one would expect a single peak that is much broader or with a shoulder as compared to non-apoptotic cells. In fact, the peak obtained with raf14c is much broader and shifted to the right compared with HCopool cells adapted to grow in CCS (Figure 5C). Since we had previously observed that the floating cell population was enriched for apoptotic cells, we also assayed these cells by Tunel. And, although the cell number was low resulting in more scatter, a second distinct peak of higher fluorescence is evident (Figure 5D). Next, we were interested in determining if any alterations in the expression levels of factors known to potentiate or inhibit apoptosis had occurred in the Δ-raf clones. Therefore, we have spent some time working out conditions for Western blot analysis of the expression of Bax, Bcl-2, Bcl-X_L and Bcl-X_S, Bad, Myc, p53, and WAF1/Cip1. At this time, we have determined that there are no significant alterations in the levels of two potentiators of apoptosis, Bax and Bad. Nor is there any change in the expression of WAF1/Cip1, a cell cycle inhibitor that is induced by p53. Further analysis of the other factors is ongoing.

DISCUSSION

Given the importance of growth factor signaling systems in breast cancer progression and reports on the existence of cross-talk between these signaling systems and the estrogen receptor signaling system, we have been using constitutively active Raf as a model of upregulated growth factor signaling, whether this be by overexpressed EGFR or erbB-2, etc. to study the progression of breast cancer from estrogen-dependent growth to estrogen-independent growth. We have previously found that the expression of a constitutively active form of the Raf kinase in MCF-7 ER+ human breast cancer cells results in two phenomena. First, as we hypothesized, it induced estrogen independent growth of these cells, both anchorage-dependent and anchorage-independent. Second, and unexpectedly, it induced apoptosis in these cells. Our focus over the last year has been to begin to dissect the mechanisms underlying both of these phenomena. First, we are interested in determining whether Raf induction of estrogen-independent growth is via an ER-mediated mechanism or if it is via a mechanism that is separate from and bypasses ER. Second, we are interested in examining the potential role that several known modulators of apoptosis in other cell systems play in the Raf-induced apoptosis.

To accomplish the first goal, we wanted to use Δ-raf transfected clones that had been growing long-term in the absence of estrogen (i.e. complete estrogen-independent growth). Before using these cells in experiments, we characterized their growth capabilities in the presence and absence of estrogen or anti-estrogens. Interestingly, in addition to the fact that these cells grew in the absence of estrogen, they no longer responded positively to the addition of estrogen nor did they respond to anti-estrogens as the control cells do. These data suggested that the cells no longer expressed a functional estrogen receptor. Analysis of the cells for ER expression at the levels of steroid-binding, overall protein, and RNA all confirmed the significant reduction in ER expression suggested above. These data are quite exciting because in all other transfections of growth factor signaling

components, i.e. EGFR, erbB-2, or Ras, the cells retained ER expression in spite of estrogen-independent growth. Thus, this is the first system described where the chronic activation of growth signaling pathways has resulted in both estrogen-independent growth and loss of ER. This ER loss could be by one of two mechanisms. First, if Raf activation of the MAPK kinase cascade activates ER in the absence of estrogen (ligand-independent activation), then it is likely that the constitutive activation that occurs in our cells would result in the constitutive activation of ER. And since an end result of ER activation by estrogen is its eventual down-regulation, a constitutive and unending activation of ER would ultimately result in the chronic and total down-regulation of ER. Alternatively, Raf induction of estrogen-independent growth could be via an ER-independent mechanism and the progressive loss of ER in our cells could merely be due to the fact that since the cells no longer need ER for growth, they have down-regulated its expression. We have preliminary data suggesting that the high levels of apoptosis which we originally observed in these cells has decreased along with the decrease in ER expression. We are currently analyzing whether these are linked phenomena or unrelated events. Additionally, we are focusing on determining which of the two above possibilities are responsible for the ER loss. We are currently analyzing the expression of estrogen-induced genes and determining if the ER loss is reversible or if its permanent. And finally, since the expression of the constitutively active Raf in these cells has such strong effects that the cells are continuously going through adaptive processes and changing (i.e. the loss of Raf expression when the transfectants are grown in FBS in our original report, or the decrease of ER expression and apoptosis as described here), our immediate focus is to re-transfect MCF-7 cells with an inducible and regulatable Raf kinase construct. This system, the tetracycline-repressible system, will allow us to specifically turn on Raf kinase expression and immediately assess the effects of its activity.

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Figure Legends

Figure 1. Growth response of Raf14c to estrogen and anti-estrogens. A. HCopool cells were quick-stripped to remove estrogens. 10,000 cells were plated into triplicate 24-well plates in CCS, treated with various hormones, and counted every 2 days. Values represent the average of the triplicates with error bars indicating the standard error. The X-axis represents time in days, and the Y-axis represents cell number. **B.** 20,000 cells (HCopool was quick-stripped) were plated in triplicate in CCS + 0.36% agar onto a bottom layer consisting of CCS + 0.6% agar. Plates were counted at 9 days after plating, and values represent the average of the triplicates with the standard error indicated by error bars.

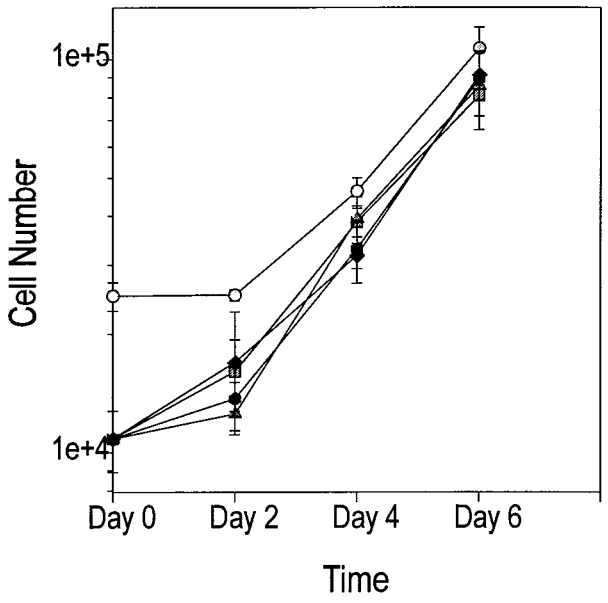
Figure 2. Long-term growth in CCS results in loss of ER protein. Whole cell extracts were prepared from cells and analyzed by Western blotting and by steroid-binding assay. For Western blots, extracts (150 μg) were electrophoresed through 10% polyacrylamide gels, transferred, and probed with an anti-ER monoclonal antibody prepared against the hinge region of ER. For steroid binding assay, extracts were incubated with 10^{-8} M ^{3}H-estradiol plus or minus a 100-fold excess of cold, unlabeled estradiol as competitor. Unbound ligand was removed by incubating with dextran-coated charcoal, and bound receptors were quantitated by counting samples in a Beckman Scintillation Counter. Receptor number is expressed as fmol/mg protein.

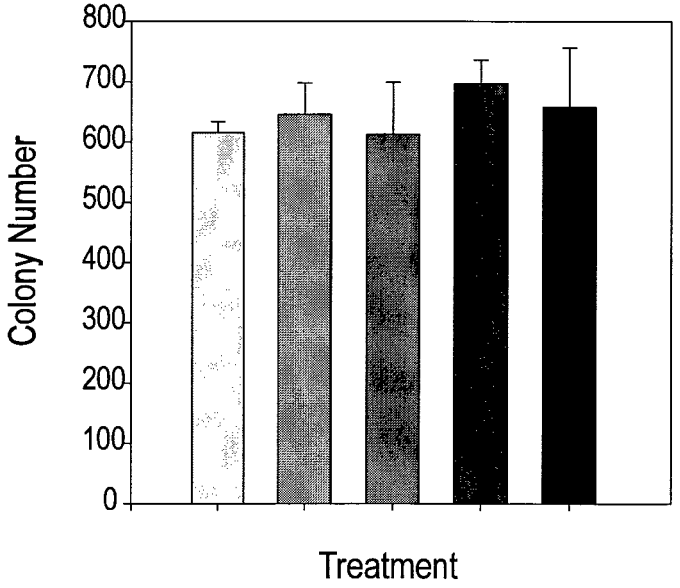
Figure 3. Long-term growth in CCS results in loss of ER mRNA. RNA from Hcopool, MCNC4, MDA-231 (an ER negative control), and the Δ-raf clones was analyzed by RNase protection for expression of ER (top panel) and GAPDH loading control (bottom panel). Migration of molecular weight markers is shown in the first and last lanes, and undigested antisense riboprobes is shown in the second lane. For each Δ-raf clone, FBS refers to cells growing long-term in FBS, CCSe and CCSl refer to long-term growth in CCS for a shorter time (e) or longer time (l).

Figure 4. Reduced transcription of ER in raf14c cells. Nuclei were isolated from the MCNC4 and raf14c cells and nuclear run-on assays were performed. Labeled RNA was hybridized to the immobilized DNA probes (genomic ER and 36B4 as a normalization control). The blot was analyzed and quantitated with a phosphoimager (Molecular Dynamics), and the values for ER were normalized to 36B4.

Figure 5. Confirmation of Apoptosis by Tunel Assay. Cells were fixed in 1% paraformaldehyde, incubated with Tdt and fluorescein-labeled nucleotides, and analyzed by FACS as described. **A.** Positive control cells consisting of human leukemia cells treated for 24 hrs with campothecin to induce ~30% apoptotic cells (left). **B.** Negative control cells consisting of HCopool, HCopool stripped of estrogen for 5 days, and HCopool adapted to grow long-term in CCS (right). **C.** HCopool cells adapted for growth in CCS and total raf14c cell population (floaters and adherents). **D.** Raf14c floater population only.

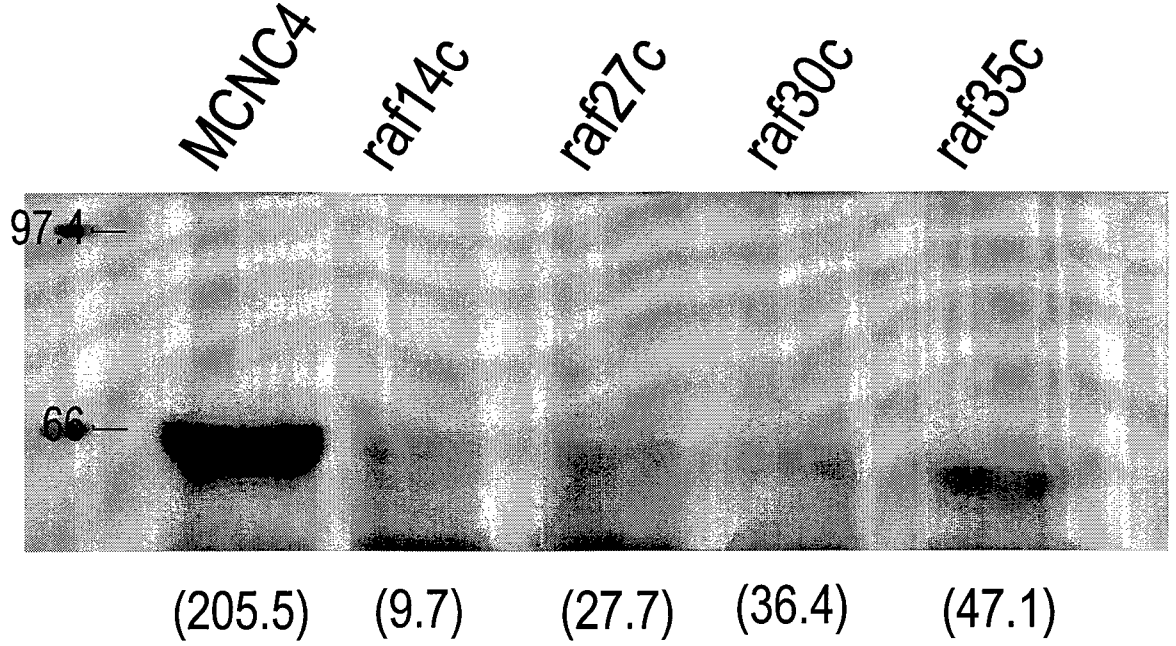
Loss of Estrogen Response after Long-term Growth in CCS



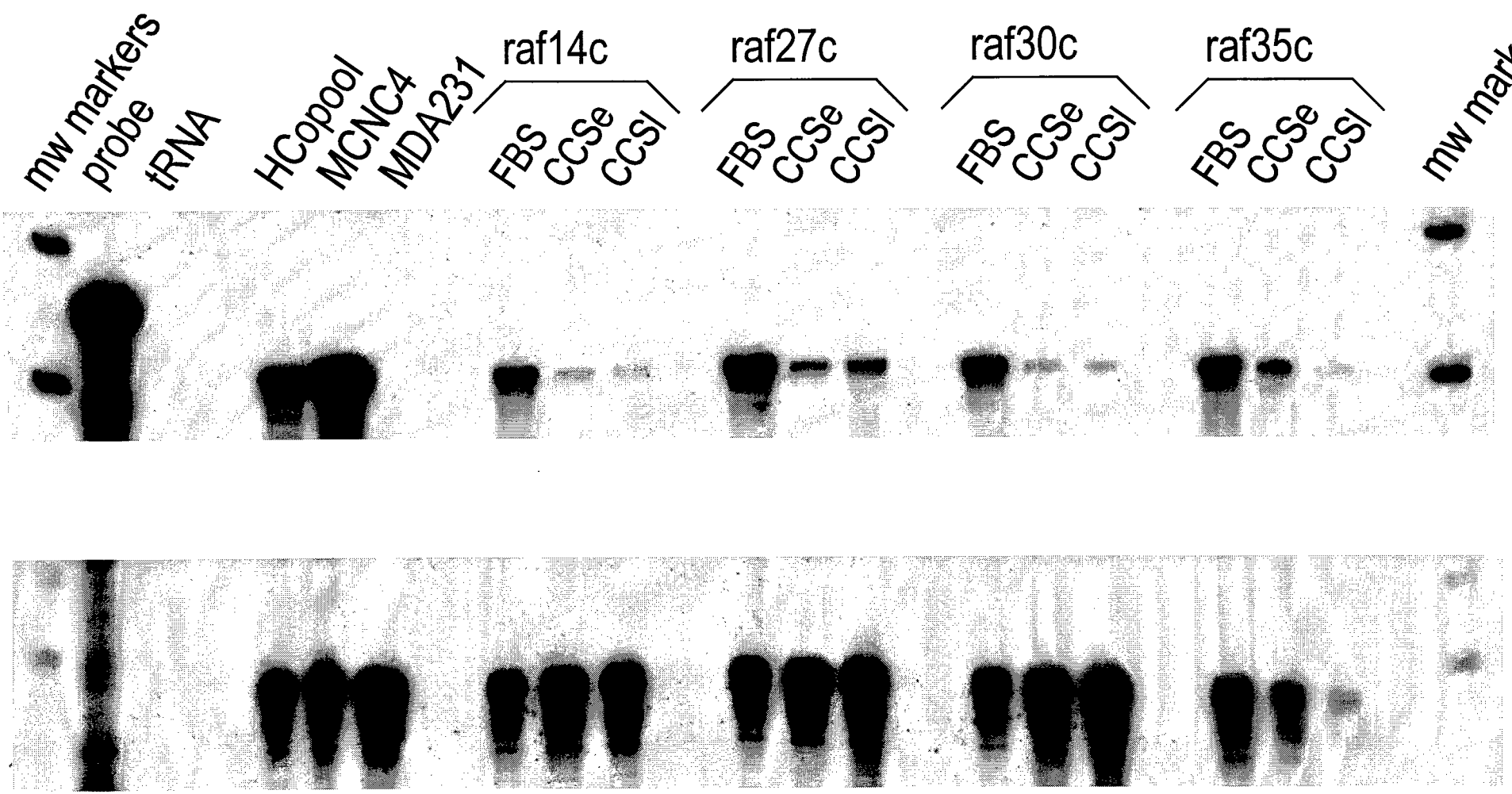


FBS
CCS
CCS+E
CCS+ICI
CCS+Tam

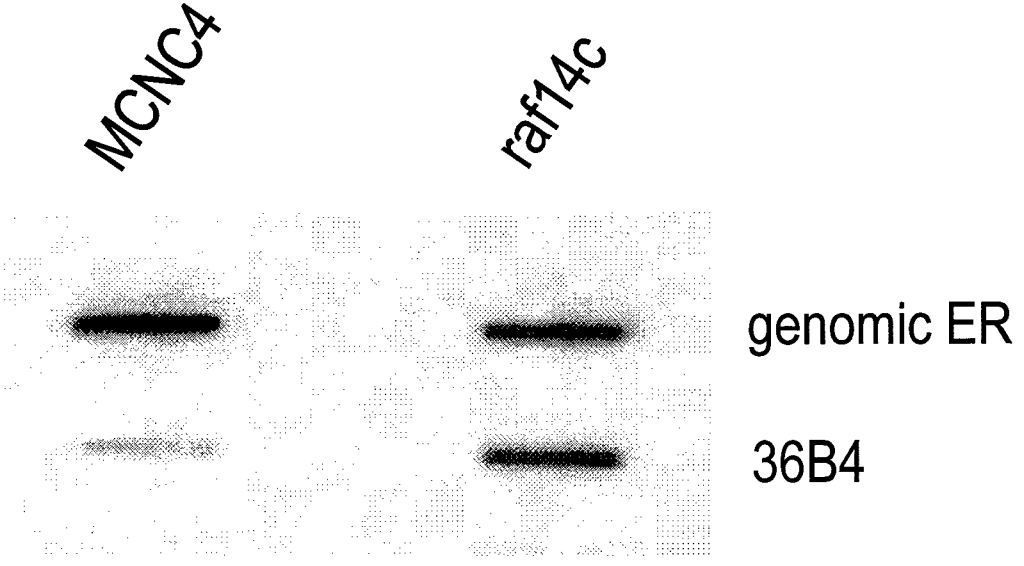
Long-term Growth in CCS Results in Loss of ER Protein



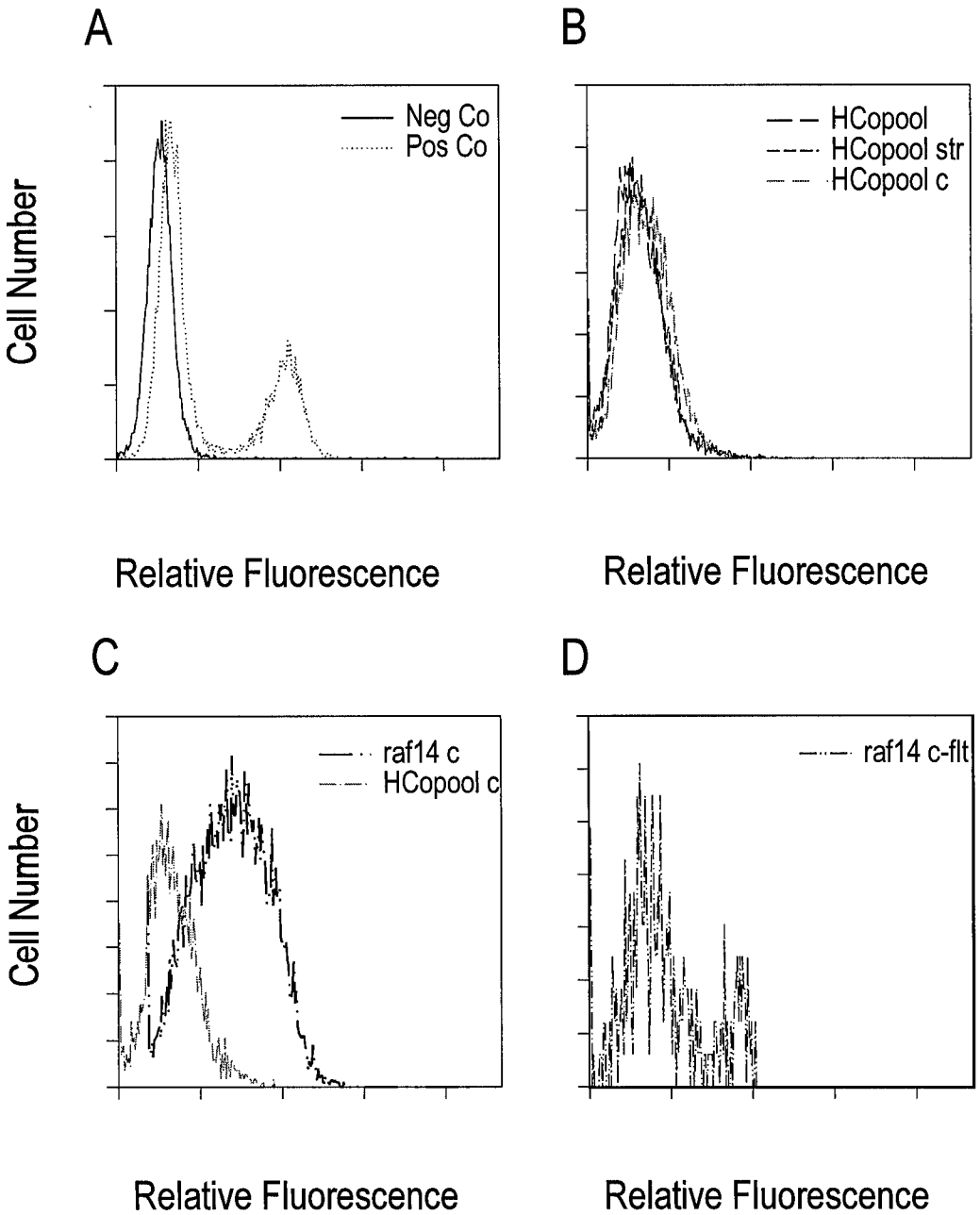
Long-term Growth in CCS Results in Loss of ER mRNA



Reduced Transcription of ER in raf14c Cells



Confirmation of Apoptosis by Tunel Assay



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-94-J-4030	ADB215484
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management