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The key to better understanding the cellular and molecular phenomena associated with the development of breast cancer is to reconstruct the early events in the breast carcinogenesis pathway. Mutations in genes governing cell cycle and growth regulation, such as retinoblastoma (RB) and p53, are expected to have a significant role in breast cancer. Both RB and p53 deficient human mammary epithelial cells (HMEC) were created by infecting retroviruses that express the human papilloma virus genes E6 and E7, previously shown to abrogate p53 and RB protein (pRB) function, respectively. Western blot analysis of these HMEC showed an absence of p53 in cells expressing E6 and a reduction of pRB in cells expressing E7. The functional activity of pRB in E7 cells is currently being examined using phosphorylation studies. E6 and E7 HMEC were investigated for growth response to TGF-B, an indicator of cellular transformation. HMEC containing just E6 or E6 and E7 were only partially growth inhibited. The ability of these cells to still grow in the presence of TGF-B indicates a significant change from the normal cellular response. It would be interesting to conduct future experiments on HMEC grown in an extracellular matrix (ECM), creating a functionally relevant model system.

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INTRODUCTION

Genetic Construction and Molecular Characterization of Breast Cancer Precursor Cells

Approximately 184,300 new diagnoses and 44,300 deaths due to breast cancer are projected to occur in the United States in 1996[1]. The estimated breast cancer incidence for women this year is almost three-fold greater than lung cancer however, unlike lung cancer, an obvious epidemiological risk factor has yet to be identified for breast cancer. Substantial progress has been made in the early detection of breast cancer, but molecular mechanisms involved in the progression of normal cells to carcinoma have not been determined.

Many genetic alterations have been found in breast tumors, including mutations in a class of genes collectively referred to as tumor suppressors[2]. These genes are responsible for controlling normal cellular proliferation and, more specifically, have been shown to play a role in cell cycle control and cellular transcription. The tumor suppressor genes retinoblastoma (*RB*), *p53*, type 1 neurofibromatosis (*NF1*), and BRCA1 are found to be mutated in breast carcinomas and therefore implicated in breast carcinogenesis[3,4]. Mutation in just one of these genes does not lead to a fully transformed phenotype; therefore, it is assumed that mutations in several genes are required for breast carcinogenesis to occur. The number of possible pathways and combinations of mutations is currently unknown.

The consequences of mutations in regulatory genes, such as tumor suppressors, should be associated with observable cellular and molecular changes. These changes would be difficult to detect in primary breast tumors due to the genetically heterogeneous nature of the cell population in a tumor. Additionally, identifying mutations in tumors does not allow one to discern those primary genetic events which are critical and rate-limiting from those which do not contribute to early tumorigenesis. It is my goal to establish a functional and manipulatable system to study the molecular effects of mutating specific genes or perturbing the function of the corresponding protein product.

The initial focus of my experiments was on *RB* but has been expanded to partially include *p53*. Loss of heterozygosity of both the retinoblastoma and *p53* genes has been demonstrated in sporadic breast carcinomas[5,6]. Additionally, families with constitutional mutations in either gene have been found to have an increase in the incidence of breast cancer[7,8]. The *p53* protein product is thought to have a "guardian" relationship over the *RB* protein (pRB). When normal p53 is present in the cell and *RB* is mutated the cell will undergo apoptosis, if, however, p53 is absent, the cell is capable of becoming tumorigenic [9,10,11]. These data suggest that *RB* and *p53* should be examined together in the breast carcinogenesis pathway.

BODY OF REPORT

Task 1 from my original Statement of Work has been completed by an alternative and more effective approach than the two methods described in the proposal. Work on Task 2 has occurred during the last six months and, as hoped, new, promising experiments have developed since the initial grant submission.

Task 1: Construction of human mammary epithelial cell lines (HMEC) deficient in pRB.

I originally proposed two approaches to construct RB deficient cells either by mutating the retinoblastoma gene directly or by abrogating the protein's normal cellular function. Neither of these approaches was successful and the reasoning is outlined below.

Approach #1: Construct HMEC containing homozygous deletions of RB.

Concordant with my constructing the *RB* targeting vector to be used for knocking out endogenous *RB* in HMEC, two post doctoral fellows in the lab were attempting to create *p53* and *APC* (adenomatous polyposis coli) knockouts in 184A1 cells (HMEC immortalized with benzo(a)pyrene). Neither were able to identify cells that had lost one of the endogenous alleles via homologous recombination of the targeting vector. For the *p53* knockouts, multiple targeting vectors with different positive/negative selectable markers were constructed and transfected into HMEC in an attempt to increase the efficiency of homologous recombination (Lih-Ching Hsu, unpublished work)[12]. Over 300 clones were picked and screened by Southern hybridization and no positive cells were found. The failure of this technique is predominately attributed to the exceptionally low transfection efficiency of the 184A1 cells. Additionally, sequence divergence can effect the frequency of homologous recombination and could be responsible for decreasing the transfection efficiency even further. None of the cells that survived the positive/negative selection were actually knockouts. These findings illustrate the importance of designing a good screening protocol (either by Southern hybridization or polymerase chain reaction) and the magnitude of labor involved in this project. Several years were spent by both fellows and no successful knockouts were obtained. My advisor and I therefore concluded that my time would be better served if the RB deficient cells were constructed by another method.

Approach #2: Construct HMEC with conditionally-deficient pRB.

Last year's annual report included my preliminary findings on the tetracycline-regulated system[13]. These results from the transient transfection of the tetracycline transactivator (tTA) plasmid indicated a 20-30 fold control of this system. The next step was to create a 184A1 derivative cell line that stably expressed the tTA. Eight separate attempts were made to transfect cells and select for positive controls. 184A1 cells were grown to ~80% confluency in 100 mm

dishes and transfected using lipofection (DOTAP, Boehringer Mannheim; previously shown to yield the highest transfection efficiency in 184A1). Several different concentrations of both DOTAP and DNA were tested. Also, different tTA plasmid preparations and transfection incubation times were tested and none of the conditions produced stable transfectants. It is possible that over expression of the tTA is toxic for the 184A1 cells and therefore cells containing the tTA would not survive selection. Since 184A1 is unable to stably express the tTA, the tetracycline-regulatable system can not be used in these cells.

Approach #3: Construct RB deficient HMEC.

Interactions between the tumor suppressor proteins RB and p53 and transforming proteins of various DNA tumor viruses are thought to be essential components of the transformation process. Two Human papilloma virus (HPV) early genes, E6 and E7, bind p53 and RB, respectively[14,15]. Binding of HPV type-16 (HPV-16) E6 to p53 enhances degradation of the p53 protein[16]. Binding of HPV-16 E7 to pRb inactivates pRb by mimicking the hyperphosphorylated state, disrupting pRb interactions with other proteins, and thus effectively deleting pRb without actually knocking out the *RB* gene. Expression of E7 in HMEC should cause perturbation of pRb function, creating an RB deficient cell line.

The retrovirus vector pLXSN containing E7 was obtained from the Denise A. Galloway lab (Figure 1)[17]. This construct contains a neomycin resistance gene allowing for the selection of cells with stably integrated retrovirus. Transcription of the E7 gene is controlled by Moloney murine leukemia virus promoter-enhancer sequences. The retrovirus vector was provided in a packaging cell line previously used to successfully transfect human keratinocytes. Packaging cells were grown for 48 hours, the media was harvested, and primary breast epithelial cultures were infected with 10^6 viral particles per milliliter growth media (work done by Nori Matsunami). As mentioned in the original grant, 184 and 184A1 cells were to be used as a starting point for initial experiments with the intent of switching to primary breast epithelial cultures. Material from reduction mammoplasties and fine needle biopsies has been used to establish numerous primary breast epithelial cultures in our laboratory (work done by Christine Anderson and Leslie Jerominski). Many of these primary cultures have been successfully transfected with the E7 retroviral vector as well as retrovirus constructs containing both E6 and E7 or E6 alone. Cells used in the subsequent experiments are BE46 (reduction mammoplasty from a normal patient) containing E7, E6, or E6 and E7 and BE20 (fine needle biopsy from an *NF1* patient) containing E7, E6, or E6 and E7. Preliminary experiments began with cells expressing E7 but, as noted above, were extended to include cells expressing both E6 and E7 or just E6.

Task 2: Characterization of RB-deficient derivatives developed in Task 1.

Assay levels of pRb and p53 in HMEC expressing E7, E6, or E6 and E7.

Since E7 and E6 are reported to effect pRB and p53, respectively, levels of these proteins were assayed in BE46 cells containing E7, E6, or both E6 and E7 by Western blot analysis. Cells were grown to ~75% confluency in 100 mm dishes, rinsed in PBS, scraped in PBS, and pelleted. Cells were resuspended, lysed, and sonicated in lysis buffer (0.1% NP40, 0.2% BSA, and 5 mM EDTA) containing 0.5 μg/ml leupeptin, 1.0 mg/ml pefabloc, 0.7 μg/ml pepstatin, and 2 μg/ml aprotinin. Total protein lysate was quantitated by protein absorbance assays (Bio-Rad) and 100 μg protein sample was loaded per lane on an 8% Tris-glycine SDS-polyacrylamide resolving gel with a 5% stacking gel. Gels were transferred to nitrocellulose membranes and blocked in TBST (Tris-buffered saline + 0.1% Tween-20) and 5% BSA. For pRB Westerns, a monoclonal pRB primary antibody (PharMingen, 1:400 dilution) and a horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody (Zymed, 1:30,000 dilution) were used for probing and blots were developed using the ECL system (Amersham Life Science). RB protein was detected in BE46+E6, BE46+E7, BE46+E6/E7, and parental BE46 cells (Figure 2A). Less pRB was present in cells expressing E7, which has been previously observed in other types of mammary epithelial cells, even though E7 is not believed to directly effect endogenous levels of pRB[18]. In BE46+E6 and BE46+E6/E7 cells, two pRB bands were present presumably representing the hypo and hyperphosphorylated forms of the protein. For p53 Westerns, a monoclonal p53 primary antibody (Oncogene Science, 1:250 dilution) and the above mentioned secondary antibody were used. The p53 protein is present in both normal BE46 and BE46+E7 cells and, as expected, is absent in BE46+E6 and E6/E7 cells due to the targeted degradation conferred by the E6 protein (Figure 2B). This data confirmed that cells containing E6 are deficient in p53 but pRB is still present in all the cell lines.

Assay activity of pRB in HMEC expressing E7, E6, or E6 and E7.

As mentioned previously, E7 is believed to bind pRB and inactivate it by interfering with its normal cellular functions. Hypophosphorylated pRB binds to and inactivates the transcription factor E2F[19]. After pRB is phosphorylated, it can no longer interact with E2F. If E7 is binding to pRB and inactivating it, pRB should no longer be found in association with E2F. To address the amount of pRB activity in the BE46 derivative cells, I initially attempted co-immunoprecipitation assays, precipitating cell lysates with an E2F antibody and then probing with a pRB antibody or vice versa. Co-immunoprecipitations using various conditions yielded inconclusive results (data not shown). Another indicator of pRB activity is phosphorylation state. E7 should bind pRB and prevent its phosphorylation. Cell lysates were incubated with 1 unit potato acid phosphatase

(Boehringer Mannheim) and then compared to untreated lysates by Western blot analysis. These experiments are currently in progress. At this time, I do not know the extent of pRB inactivation in the BE46+E7, E6, or E6/E7 cell lines.

Assay effects of TGF-ß on growth of HMEC expressing E7, E6, or E6 and E7.

Transforming growth factor ß (TGF-ß) inhibits growth of normal breast epithelial cells but does not effect the proliferation of some breast cancer cells[20]. The loss of TGF-ß response is thought to be an indicator of transformation and could potentially be a marker in the carcinogenesis pathway. Earlier studies with 184 and 184A1 and TGF-ß showed full growth inhibition of 184 cells at 3.0 ng/ml TGF-ß while 184A1 cells had varying levels of incomplete inhibition (10-30% of normal growth)[20]. These results suggested that immortalized cells, such as those containing the different HPV retrovirus constructs, would have a different TGF-ß growth response than the normal parental cells. TGF-ß growth response experiments with BE20+E6 and E6/E7 were performed as previously described[20] (at the time of these experiments these two cell lines were the only ones being cultured in the laboratory). Briefly, two 24 well plates per cell line were seeded with different concentrations of TGF-ß: 0 ng/ml (control), 0.03 ng/ml, 0.1 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 3.0 ng/ml, 10.0 ng/ml,or 20.0 ng/ml TGF-ß (three wells per concentration of TGF-ß). One plate of cells was trypsinized and counted 18 hours after seeding (attached cell number) and the second plate trypsinized and counted when one of the wells is almost confluent (final cell count). A value for cell growth was derived by subtracting the attached cell number from the final cell count. Three wells per concentration of TGF-ß were counted, the values averaged, and the data was expressed as a percentage of the control. This experiment was repeated twice for both BE20+E6 and BE20+E6/E7 (Figure 3A and 3B). BE20+E6 cells were maximally inhibited by 3.0 ng/ml TGF-ß to approximately 20% of normal growth in both experiments. BE20+E6/E7 cells were also maximally inhibited by 3.0 ng/ml TGF-ß but to varying degrees in the two experiments (20% versus 65% of normal growth). These data show the E6 and E6/E7 expressing cells are not completely growth inhibited by TGF-ß, a characteristic not attributed to normal cells, suggesting that these cells may represent a stage along the carcinogenesis pathway. Other researchers in the TGF-ß field use ^{3}H-thymidine or BrdU incorporation assays as a more accurate measure of growth response and these techniques are currently being explored[21,22]. Preliminary data indicate BrdU incorporation to be the superior assay for these breast epithelial cell lines.

Morphologically, BE20+E6 and BE20+E6/E7 cells treated with TGF-ß appear very different from the normal cells (Figure 4). Cells grown with TGF-ß become very large and vacuolated. These observations are characteristics of cells undergoing apoptosis or programmed cell death. Currently, experiments are in progress using an apoptosis detection system (R&D Systems) to examine cells for apoptosis.

The role of cellular environment on molecular mechanisms

Recent data has indicated the need to study cellular processes in the correct cellular microenvironment[23]. It is now possible to grow human mammary epithelial cells in an extracellular matrix (ECM) which allows cells to form ductal-like structures[23]. For future experiments, I am interested in establishing the pRB and p53 deficient cells in an ECM culture to better study the cellular and molecular alterations associated with these tumor suppressor protein deficiencies.

CONCLUSIONS

1. Primary breast epithelial cultures with altered pRB and p53 activity were created using a retrovirus-based approach.

2. Cells deficient for pRB and p53 were analyzed by Western blot analysis. Cells expressing E6 have no detectable p53 protein while cells expressing E7 still have pRB. These data indicate that breast epithelial cells containing no discernable p53 protein have been created and can be treated as p53 negative cells. The activity of the pRB present in these derivative cell lines is currently being examined by determining the phosphorylation state of the RB protein.

3. Cells deficient for pRB and p53 were examined for growth response to TGF-ß. BE20+E6 cells were inhibited to approximately 20% of normal growth while BE20+E6/E7 cells showed a range of inhibition approximately 20% versus 65%. This incomplete growth inhibition response is thought to be indicative of a cell that is progressing through the carcinogenesis pathway.

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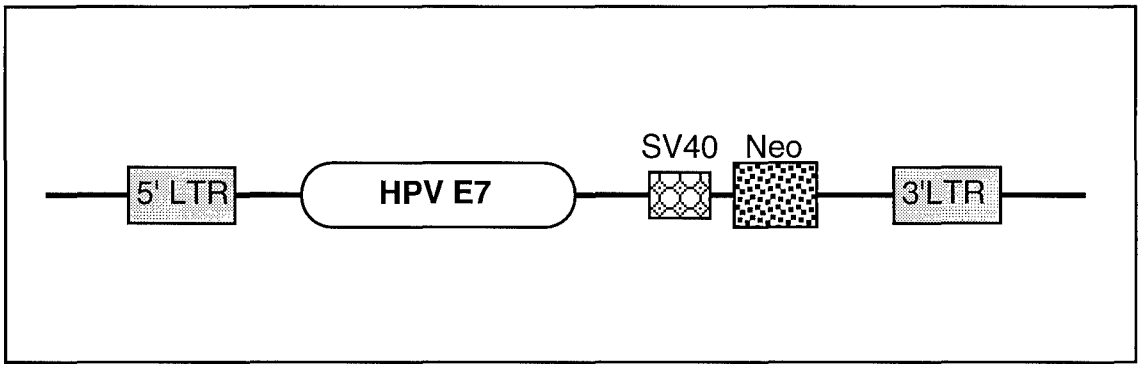

Figure 1. Schematic representation of retrovirus vector pLXSN containing the human papilloma virus (HPV) E7 protein. This construct was used to transfect primary human breast epithelial cells. Abbreviations: LTR, Moloney murine promoter-enhancer; SV40, simian virus 40 promoter; Neo, neomycin resistance gene.

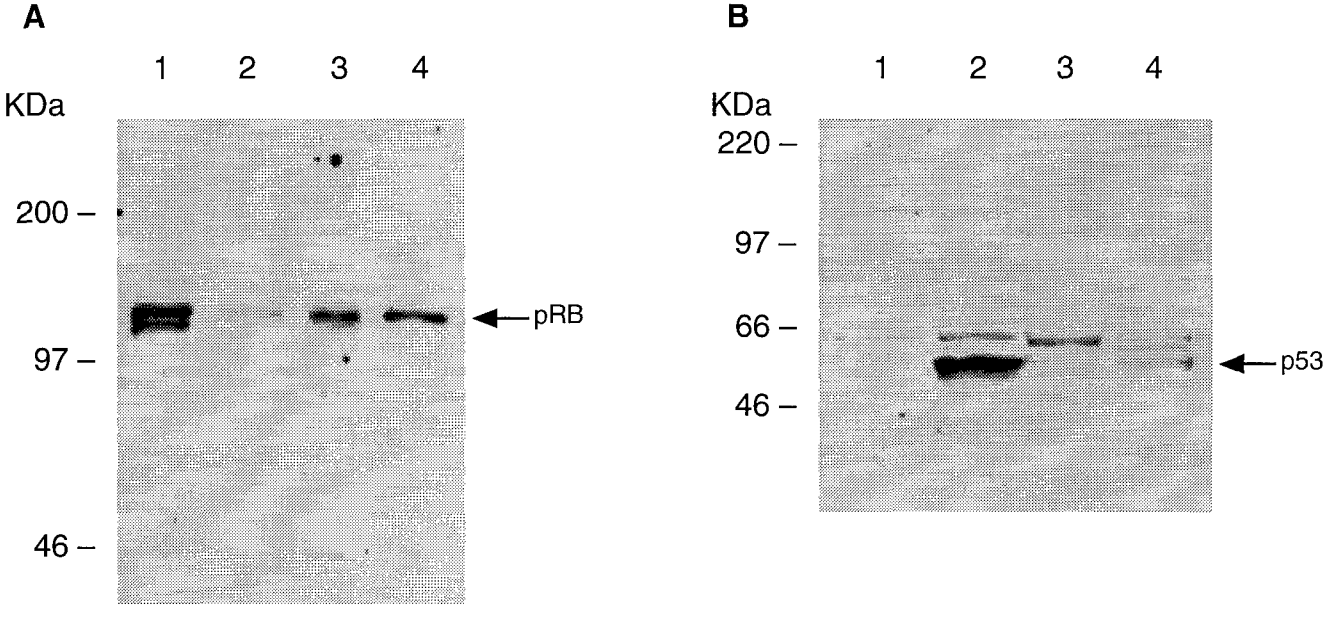

Figure 2. Western blot analysis of BE46 cells. pRB (A) and p53 (B) antibodies were used to probe BE46 derivative cell lines. Lane1, BE46+E6; Lane 2, BE46+E7; Lane 3, BE46+E6/E7; Lane 4, normal BE46. 100 µg protein lysate loaded per lane.

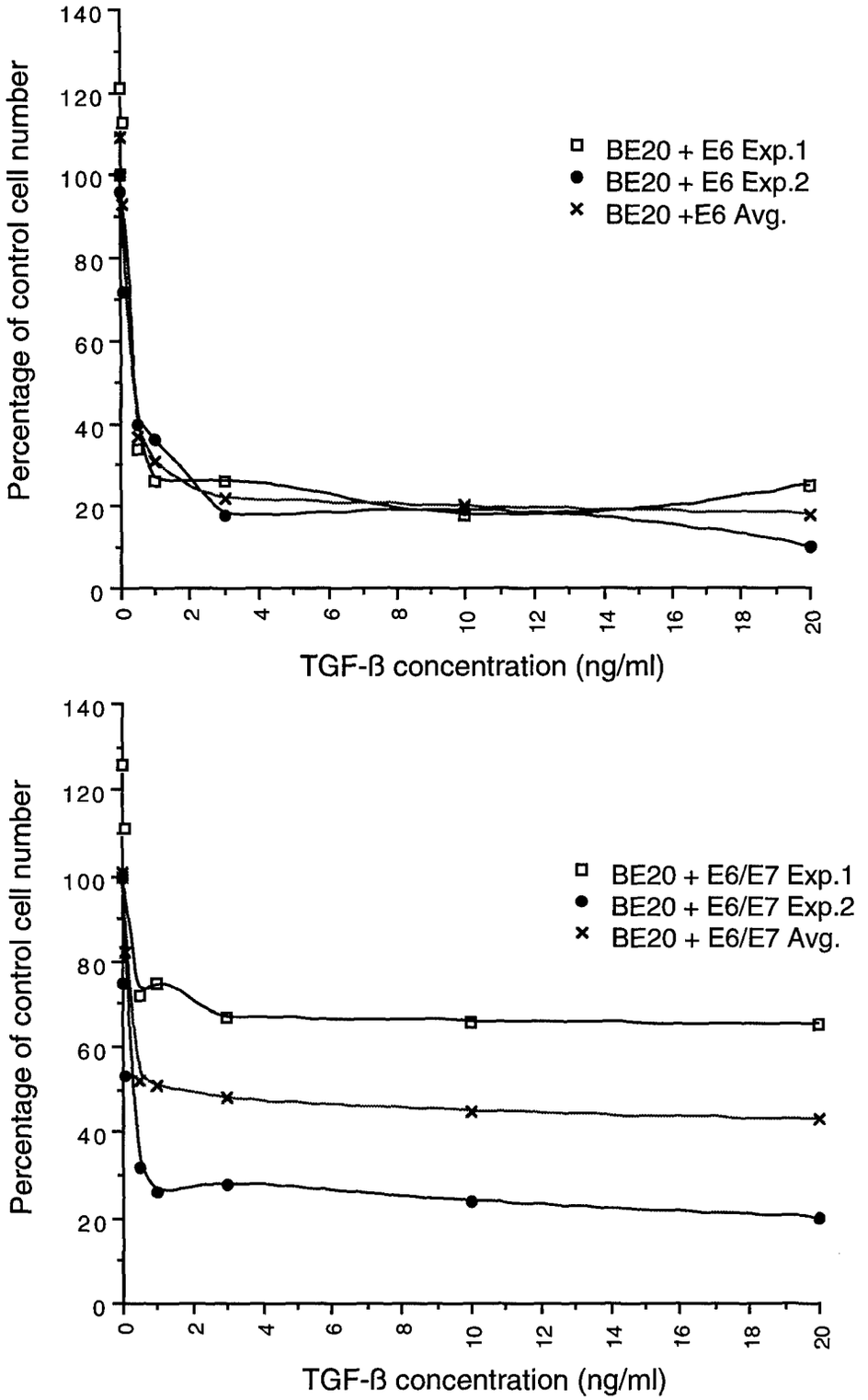

Figure3. TGF-ß effects on growth of (A) BE20+E6 and (B) BE20+E6/E7 cells. Cultures were seeded in the indicated concentration of TGF-ß. Eighteen hours later the number of attached cells was determined. All wells were counted once one well became confluent. The attached cell number was subtracted from the final cell count and data are presented as a percentage of the control (no TGF-ß). Each point represents the average of three wells.

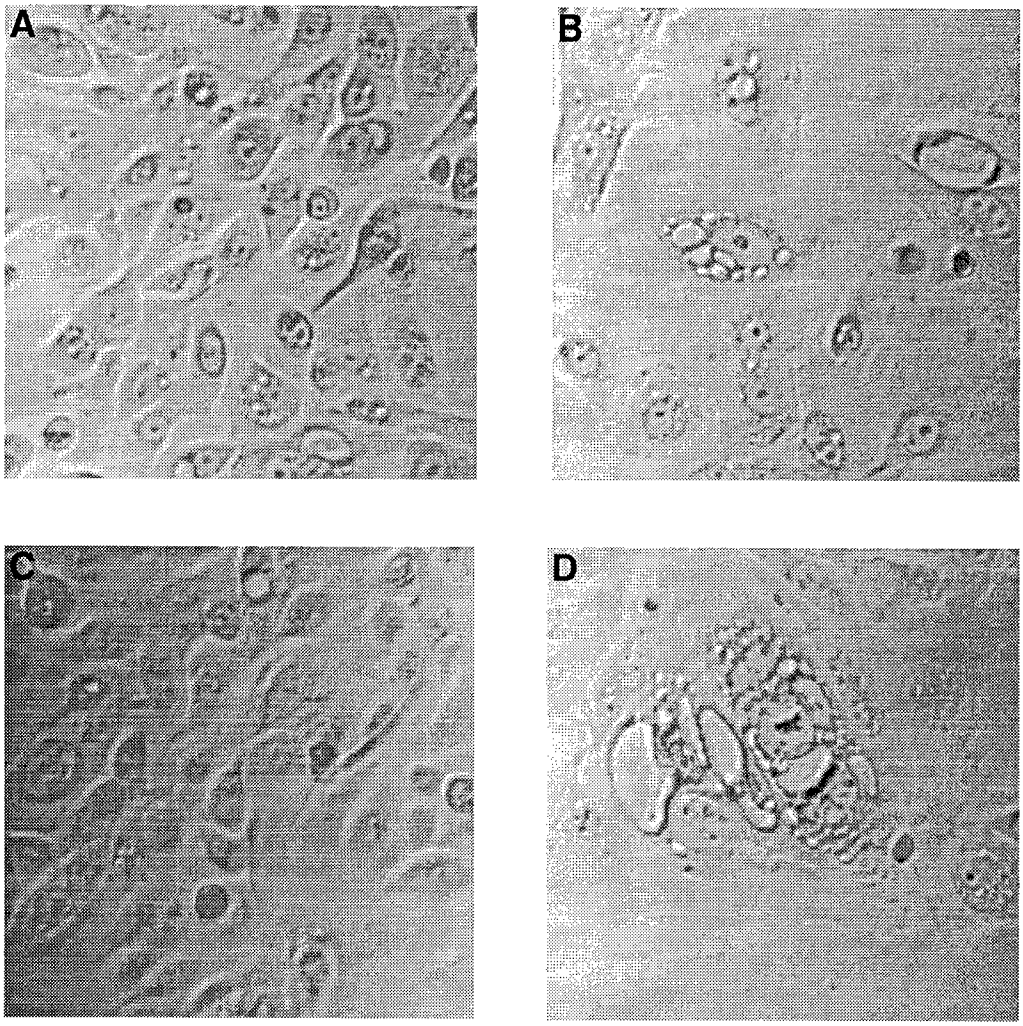

Figure 4. TGF-ß effects on the morphology of BE20 cells infected with retrovirus constructs. Phase-contrast photographs of (A) BE20+E6, (B) BE20+E6 in 20.0 ng/ml TGF-ß, (C) BE20+E6/E7, (D) BE20+E6/E7 in 20.0 ng/ml TGF-ß. Cells grown for one week in TGF-ß containing media became vacuolated and larger in size.