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PRINCIPAL INVESTIGATOR: Doctor Jeffrey M. Rosen

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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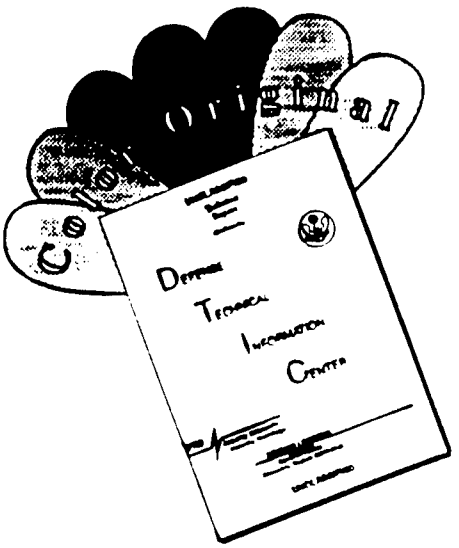

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This proposal is based upon the hypothesis that the protective effects of an early pregnancy and lactation on the incidence of breast cancer result from estrogen (E)- and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and terminal end bud (TEB) growth and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. The TEB is a dynamic structure regulated by a balance of proliferation and apoptosis. The initial objective of this grant is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. Thus far, 14 clones isolated from the TEB DD-PCR fraction have been sequenced. Three clones of unknown identity are preferentially expressed in the TEB fraction. Antibodies to two other clones encoding p190-B and adrenomedullin are being used to localize and study their function. Procedures have been developed to isolate nuclear matrix proteins from the TEB and preliminary 2D PAGE analysis has identified several unique proteins in this fraction.

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INTRODUCTION

A woman's reproductive history is one of the principal determinants of her susceptibility to breast cancer. An early full-term pregnancy is protective and the length of time between menarche and the first full term-pregnancy appears to be critical for the initiation of breast cancer. This proposal is based upon the hypothesis that the protective effects of an early pregnancy and lactation result from estrogen (E)- and progesterone (P)-induced differentiation and the resultant loss of cells susceptible to carcinogenesis. These effects of E and P are mediated by the induction of specific "local mediators", i. e. growth factors that act via autocrine and paracrine mechanisms to influence terminal duct (TD) and terminal end bud (TEB) growth and differentiation. These rapidly proliferating cells are the most susceptible to neoplastic transformation. No molecular markers are available to identify and follow the fate of these susceptible cells, yet this information is required to develop effective diagnostic tools and preventive therapies for breast cancer. Thus, the initial objective of this grant is to identify molecular markers for TEB and TD cells in order to follow their fate during mammary development and carcinogenesis. To do so, genes expressed specifically in the TEBs of the nulliparous rat mammary gland will be isolated, cloned and characterized. These genes will then serve as molecular markers in TEB cell fate studies. Candidates for TEB molecular markers may include cell cycle factors, proteins which interact with the extracellular matrix, cytoskeletal elements and growth factor receptors. In addition, we proposed to define the topology and compare stages of the cell cycle of susceptible and refractory cells, and to identify local mediators of E- and P-treatment in the end buds and surrounding stroma, and characterize the changes in their expression patterns.

The following specific tasks were proposed for the second twelve months of this proposal.

Task 2, Characterization of molecular markers for TEB and TD cells. Months 6-30.

a. Cloning and sequencing of unique DD-PCR products.
b. RT-PCR analysis of RNA isolated from initial fractions.
c. Northern and quantitative RT-PCR analysis to follow the fate of these unique gene transcripts during mammary development and carcinogenesis.
d. *In situ* hybridization analysis.

Task 3, Definition of the topology and cell cycle analysis of susceptible and refractory cells. Months 12-30.

a. Confocal microscopic analysis of BrdU injected animals as described in Methods.

BODY

Experimental Methods

RT-PCR

The reverse-transcription reactions were performed with total RNA from the TEB, mid-gland and stroma using the Superscript II enzyme (Gibco-BRL). Varying amounts of the cDNA (5-100ng) were then added to PCR reactions to determine relative levels of expression in each tissue fraction. PCR reactions were electrophoresed on 10% polyacrylamide gels, exposed overnight in a Molecular Dynamics Phosphoimager cassette and quantitated.

Immunohistochemistry - adrenomedullin

Mammary gland tissue from different stages of development was fixed either 6 hours or overnight in 10% neutral buffered formalin. Antigen unmasking was performed with Target

Unmasking Fluid (Signet Laboratories). Immunohistochemical detection of adrenomedullin was carried out as described by Martinez et al., 1995 (1).

Immunofluorescence - p190B

Mammary tissue from a 45 day-old rat was cryosectioned for immunofluorescence studies. The tissue was immunostained for p190B (1:50) conjugated to FITC, Keratin 14 (1:200) conjugated to Texas Red, and nuclei were detected with DAPI.

Confocal microscopic analysis of DNA synthesis

Having established in the first year of this project that it was possible to preserve cells of the rat mammary gland and observe them in 3-dimensions by confocal microscopy, our effort this year has been to fine-tune the procedure in order to quantitate cell cycle kinetics in control and hormone-treated animals. Much of our effort during this funding period has been aimed at establishing the proper conditions for fixing rat mammary gland cells for BrdU labeling the proliferating cells in terminal end buds, alveolar buds and ducts.

Freezing and cryosectioning

Our initial experiments involved confocal analyses of small segments of intact mammary glands that had been fixed and permeabilized *in situ* and examined by confocal microscopy. Although some of the cells were well preserved, we found this method to be unsatisfactory for large scale quantitative analysis due to incomplete penetration of antibodies. We circumvented this problem by freezing at -80°C and cryosectioning the tissues prior to fixation. Subsequently, sections up to 50 μm thick were mounted on pre-coated slides, permeabilized with 0.5% Triton-X100 for 3 min and fixed in 4% formaldehyde in phosphate buffered saline for 15 min at room temperature. As shown in Figs. 1 and 2 in this report, this method provided complete penetration and quantitative morphological preservation of all cell types.

Three color immunofluorescence

Although our initial efforts gave us striking images of the 3-D structure of mammary glands (see previous progress report), we experienced several technical problems when we attempted to fix and immunostain for quantitative analysis of the proliferating cell populations. In order to determine the growth fraction (GF), labeling index (LI), and S-phase (Ts), we found it necessary to develop conditions for staining that enabled us to detect three emission wavelengths (channels) of illumination. Unfortunately, our Molecular Dynamics confocal microscope was designed to provide only two channels, red and green. We solved this problem by modifying our immunostaining protocols to permit a third color by blending red-emitting immunostains with green to produce yellow. Thus anti-BrdU-FITC was used to identify cells in S-phase (green), Propidium iodide (red emission) was used to identify total nuclei in the preparation. Keratin in the myoepithelium was stained with rabbit anti-keratin 14 and detected with a 1:1 mixture of FITC- and RITC-conjugated goat anti-rabbit antibodies. This color combination allows us to identify and analyze myoepithelial, lumenal epithelium and stromal cells associated with terminal end-buds (TEBs), alveolar buds (AB) and terminal ducts (TD). Examples are shown in Figs. 1 and 2.

Tissue preparation and scoring

For the determination of S-phase and labeling index, BrdU was injected i.p. for periods of either 0.5 or 3.5 hrs. Afterwards the mammary gland was excised and dissected to remove the mid-gland and terminal end bud region. These were cut into 0.5 cm^3 blocks and frozen at -80°C. In order to determine GF, continuous labeling for 24 hrs was accomplished by implanting a small

mini-osmotic Alzet pump under the rat's skin containing BrdU (200 µl at 50mg/kg) prior to excision of glands. Afterwards, cryosections were cut, collected on slides and processed for immunostaining. A data set consisting of up to 10 optical sections (5 µm depths/section) of each sample was obtained in the confocal microscope and analyzed by a cell scoring procedure developed in our laboratory using Optimas software.

Nuclear matrix isolation and analysis

The full set of 2-D patterns of nuclear matrix proteins from TEB, AB, and the stroma adjacent to these regions was obtained for the first time by the following procedure: 45 day-old rats were injected with 0.5% Trypan Blue from the nipple. TEB and AB regions were then separately collected by dissection under the microscope, and were digested with 2 mg/ml collagenase twice at 37°C. The process of digestion was monitored by microscopy assays, until clean ducts and buds were obtained (Fig. 7). The stroma cells in the supernant and the buds/ducts were then separated by centrifugation. The nuclear matrix proteins were extracted from each fragment as described previously. After dissolving in urea containing 2-D sample buffer, approximately 120 µg protein was loaded for each gel. The first dimensional isoelectric focusing (IF) was carried out using ampholites pH 2-11 and 4-6 in a three to one mixture at 18,000 v hrs. The second dimensional IF was in 10%-20% SDS PAGE and run for 5 hrs at a constant temperature of ~12°C. Gels were stained with high resolution color-based silver stain and processed in the 2-D gel electrophoresis core laboratory in the Department of Cell Biology.

Results and Discussion

Task 2a. Characterization of molecular markers for TEB and TD cells

Differential display PCR (DD-PCR) has been used to identify several genes expressed in the virgin mammary gland which may play a role in mammary gland development and the initiation of carcinogenesis. DD-PCR was performed with Terminal End Bud (TEB), Mid-gland (M) and Stroma (S) tissue fractions from nulliparous Wistar-Furth rats at 45 days of age, a time point during which ductal elongation is occurring and TEBs are highly susceptible to chemical carcinogens. Thus far, 14 clones isolated from the TEB DD-PCR fraction have been sequenced and compared to GenBank sequences (Table I). Ten of these clones have identity to GenBank sequences. EDD-C2 (End bud Differential Display - C2) is homologous to human p190-B, a new member of the RhoGAP family. EDD-C12 is 100% identical to adrenomedullin, a secreted peptide factor. EDD-C11 is similar to a calcium binding protein. Two clones are mitochondrial genes: EDD-C16 (mitochondrial rRNA) and EDD-C17 (Cytochrome C oxidase). EDD-G5, EDD-C3, EDD-C6, EDD-C14 and EDD-C15 are similar to Expressed Sequence Tags (EST) cloned from various human tissue libraries. EDD-G6, EDD-G7, EDD-C13 and EDD-C18 have no similarities to GenBank sequences. Two immediate goals of the project are to characterize and localize mRNA expression of the DD-PCR clones in virgin development and to study the protein and mRNA expression of p190-B and adrenomedullin as they relate to mammary gland development.

Experiments are in progress to determine mRNA expression levels of the DD-PCR clones in the virgin mammary gland. To determine the relative levels of transcripts in the TEB, mid-gland and stroma, RT-PCR has been performed. Of nine clones studied three are expressed at greater levels in TEB mRNA; EDD-G5, EDD-G7 and EDD-C18. An example of the RT-PCR analysis is shown in Fig. 3. The data are plotted with respect to cDNA input on a semilog scale to demonstrate the semi-quantitative nature of the assay. The results shown for clone EDD-C3 (Fig. 3A) suggest that this transcript is not actually enriched in the TEB fraction, while clone EDD-G5 appears to be enriched (Fig. 3B). When feasible, depending on the transcript abundance, these results will be confirmed by RNase protection and Northern blot analyses. Messenger RNA expression studies are continuing on the remaining clones.

Clones preferentially expressed in the TEB by RT-PCR will be studied by *in situ* PCR to determine cell type specific mRNA localization. Currently, we are optimizing this protocol for the virgin mammary gland comparing digoxygenin- versus biotin-based secondary detection methods and direct *in situ* PCR using biotinylated- or digoxygenin-substituted nucleotides versus indirect *in situ* PCR using a third biotinylated oligonucleotide to detect the specific amplified cDNA. These experiments are currently being performed using primers specific for adrenomedullin mRNA. Once mRNA localization has been examined, 1 or 2 clones which are expressed in the TEBs will be chosen for further studies. These studies will determine mRNA expression during different stages of mammary gland development and carcinogenesis. In two cases where specific antibodies are available, p190-RhoGAP and adenomedullin, immunohistochemical analysis is being performed to identify the patterns of expression of these proteins (see below).

p190-RhoGAP

p190-B is a new member of the RhoGAP family which functions by catalyzing the hydrolysis of GTP to GDP by Rho GTPases. Rho GTPases can modulate the actin cytoskeleton, induce focal adhesion assembly and participate in intracellular signaling pathways. p190-B and Rho are induced to cluster after integrin cross-linking (2). An affinity purified rabbit polyclonal antibody directed against amino acid residues 1203-1499 of p190-B has been generously donated by Dr. Yoshi Yamada at the National Institute of Dental Research. This antibody was used to characterize expression of the p190-B protein in the virgin mammary gland. Preliminary immunofluorescence experiments to localize p190-B protein expression were performed by Dacheng He in Dr. Brinkley's laboratory (Fig. 4). p190-B was found in the cytoplasm of TEB epithelial cells and in the surrounding stroma, however, p190-B was excluded from the epithelium of the alveolar buds. This suggests that p190-B may be located primarily in less differentiated epithelial cells of the mammary gland and excluded from the more terminally differentiated cells.

In addition p190-B protein expression was characterized in the virgin mammary gland by Western analysis using the p190-B antibody. In Western blots performed with whole cell RIPA extracts from TEB, M and S mammary tissue, the p190-B antibody cross-reacted with proteins of 120 and 75 kDa, but not 190 kDa (data not shown). Similar results were obtained using kidney extracts. It had been reported previously that immunoprecipitation with the p190-B antibody could detect p190-B protein in HT1080 extracts (2). In Westerns with HT1080 extracts, the p190-B antibody detected multiple proteins including the 75 kDa and 120 kDa proteins as well as a 190 kDa protein (data not shown). These results suggest that p190-B may be expressed at extremely low levels in the mammary gland which can not be detected by Western analysis. Thus, immunoprecipitations may be necessary to detect p190-B in the mammary gland. Additionally, Yamada's antibody cross-reacts with several other proteins of lower molecular weights. This lack of specificity makes the interpretation of the immunofluorescence results difficult.

To resolve issues of specificity, two new rabbit polyclonal antibodies raised against unique peptide sequences in p190-B have been generated. Two peptides, p190-B/1 (a.a. 1018-1033) and p190-B/2 (a.a. 1130-1145) were selected because of their high antigenicity and hydrophilicity, as well as low identity to the p190-A and other rhoGAP proteins. Affinity purification of these polyclonal antibodies is currently in progress. Once affinity purified, studies to determine p190-B protein expression and localization will be undertaken, as well as, parallel experiments to determine levels and localization of mRNA expression throughout mammary gland development.

Adrenomedullin

Adrenomedullin is a secreted peptide factor which was originally isolated from a human pheochromocytoma (3). Adrenomedullin is synthesized as a 185 a.a. precursor which is first processed by enzymatic cleavage of the signal sequence. The proprotein is further cleaved into two biologically active peptides; Adrenomedullin (52 a.a.) and PAMP (20 a.a.), which are then

secreted by the cell. Adrenomedullin is expressed in a variety of tissues (stomach, colon and chorid plexus (4)). Polyclonal antiserum and peptide antigen to adrenomedullin have been kindly provided from Dr. Frank Cuttitta at the National Cancer Institute Division of Biomarkers (1). The rabbit polyclonal antiserum was generated against the most C-terminal 30 amino acids of the adrenomedullin active peptide (116-146 a.a.). This antiserum reacts with the 18 kDa proprotein, a 14 kDa intermediate form and the 6 kDa active peptide. The polyclonal antiserum has been used to determine the temporal and spatial expression of adrenomedullin in the mammary gland by immunohistochemistry.

Immunohistochemistry was performed with fixed sections of rat mammary glands from various time points throughout mammary development. Adrenomedullin was localized to the cytoplasm of epithelial and stromal cells of the virgin gland. Staining was most prominent in TEB and ductal epithelium (Fig. 5). However, staining was also detected in the alveolar buds, stroma, blood vessels and lymph node. In the 12 day pregnant gland, adrenomedullin expression was pronounced throughout the cytoplasm of alveoli, ducts and stroma (data not shown). Interestingly, in the 18 day pregnant gland adrenomedullin was localized to the nucleus and cytoplasm of alveoli and cells of the stroma (Fig. 6). Nuclear staining was also observed in lactating (2 days) and involuting (3 days) tissue but to a lesser extent (data not shown). The cytoplasmic staining of the epithelium and stroma also continued through lactation and involution. In all cases, adrenomedullin staining could be competed with the specific peptide (Fig. 5C and 6C).

Since adrenomedullin is a secreted growth factor, experiments are in progress to ascertain the mRNA localization of adrenomedullin in the virgin mammary gland by *in situ* PCR. Comparable studies performed by Gloria Jahnke at NIEHS have localized adrenomedullin expression to the TEBs in the virgin mouse mammary gland (personal communication). Additionally, to examine the physiological effect of adrenomedullin on mammary gland development, elvax pellets with either adrenomedullin or a neutralizing antibody (kindly provided by Dr. Cuttitta) will be implanted into the fat pad of ovariectomized 45 day-old virgin rats. The mammary glands will then be fixed for whole mounts and examined for alterations in TEB development and ductal branching in comparison to untreated animals.

Task 3a. Definition of the topology and cell cycle analysis of susceptible and refractory cells by confocal microscopic analysis of BrdU injected animals

Our initial experiments have focused on determining the growth fraction (GF), labeling index (LI) and fraction of cells in S phase (Ts) in 45 day-old virgin rats in frozen sections using confocal microscopy. The results of these experiments are illustrated in Figs. 1A-F and are summarized in Table II. The values obtained for LI, Ts, and GF are comparable to published reports obtained from conventional paraffin sections (5). In a separate study, we analyzed cell proliferation in two regions of the ductal tree in 96 day-old virgin and parous rats. Although this analysis is still underway at the time of this report, we present preliminary images (Figs. 2A-D) and the labeling index in Table II.

Related studies on TEB proliferation and apoptosis

We have performed an independent study of the role of apoptosis in ductal morphogenesis using several available transgenic and knockout mouse models. The results obtained from these experiments are relevant to the overall objectives of this grant, but were funded by other sources. These studies could not be performed in the rat, because of the lack of knockout and transgenic models, but the information learned from these studies should be directly applicable to studies of the protective effects of pregnancy on mammary carcinogenesis in the rat. The results of these experiments are summarized below in an appended manuscript accepted for publication in

Development. In this manuscript we report that the TEB is a dynamic structure regulated by a balance of proliferation and apoptosis, and that the high level of apoptosis in the TEB could provide another mechanism sensitive to transformation as summarized below.

Ductal morphogenesis in the rodent mammary gland is characterized by the rapid penetration of the stromal fat pad by the highly proliferative terminal end bud and subsequent formation of an arborized pattern of ducts. The role of apoptosis in ductal morphogenesis of the murine mammary gland and its potential regulatory mechanisms was investigated in this study. Significant apoptosis was observed in the body cells of the terminal end bud during the early stage of mammary ductal development. Apoptosis occurred predominately in defined zones of the terminal end bud; 14.5% of the cells within three cell layers of the lumen were undergoing apoptosis compared to 7.9% outside this boundary. Interestingly, DNA synthesis in the terminal end bud demonstrated a reciprocal pattern; 21.1% outside three cell layers and 13.8% within. Apoptosis was very low in the highly proliferative cap cell layer and in regions of active proliferation within the terminal end bud. In comparison to other stages of murine mammary gland development, the terminal end bud possesses the highest level of programmed cell death observed to date. These data suggest that apoptosis is an important mechanism in ductal morphogenesis. In *p53*-deficient mice the level of apoptosis was reduced, but did not manifest a detectable change in ductal morphology, suggesting that p53-dependent apoptosis is not primarily involved in formation of the duct. Immunohistochemical examination of the expression of the apoptotic checkpoint proteins, Bcl-x, Bax, and Bcl-2 demonstrated that they are expressed in the terminal end bud. Bcl-x and Bcl-2 expression is highest in the body cells and lowest in the non-apoptotic cap cells, implying that their expression is associated with increased apoptotic potential. Bax expression was distributed throughout the terminal end bud independent of the observed pattern of apoptosis. A functional role for Bcl-2 family members in regulating end bud apoptosis was demonstrated by the significantly reduced level of apoptosis observed in WAP-Bcl-2 transgenic mice. The pattern of apoptosis and ductal structure of end buds in these mice was also disrupted. These data demonstrate that p53-independent apoptosis may play a critical role in the early development of the mammary gland.

Task 1b and c (continued from year 1). Isolation and analysis of nuclear matrix proteins by 2D PAGE.

One of our major aims has been to analyze the nuclear matrix protein components of terminal end buds (TEB) and alveolar buds (AB) in control rat mammary glands and after the treatments with E and P hormones and MNU. In our previous progress report, we demonstrated differences in the 2-D pattern between total TEB protein and total AB protein, and identified differences between total protein and proteins in the nuclear matrix in TEB. However, these results were inconclusive because of our inability to separate the different cell types in the tissue samples. During the current year we made important progress in the separation of epithelia cells and the stroma fibroblast cells by testing a variety of collagenase types and different combinations of conditions.

A comparative analysis of nuclear matrix proteins from TEB, AB, and the stroma adjacent to these regions is shown in Figs. 8A-D. The proteins marked with numbers 1 to 4 were used as inner markers that demonstrated in every gel and their positions remained constant. Some preliminary but encouraging results were obtained: 1) A few proteins were shown to be specifically localized in either TEB or AB [arrows in Fig. 8. A and B]; 2) Some protein spots (arrowheads), presumably keratins, were missing in the stroma samples indicating that the enzyme-separation procession was successful [Fig. 7, compare C,D to A,B]; and 3) Interestingly, some differences were detected between two stroma samples [Fig. 8. C and D] suggesting differentiation of stroma cells in different areas, therefore, having potential influence in mammary gland development. Although preliminary, this 2-D gel set supports our hypothesis that nuclear matrix protein composition may be a useful index in rat mammary gland development. In our

future work, we will attempt to identify and characterize nuclear matrix proteins in control and hormone-treated animals using Western blots and microsequencing.

CONCLUSIONS

Thus far, 14 clones isolated from the TEB DD-PCR fraction have been sequenced. While some of the clones identified by DD-PCR appear to be false positives, three clones of unknown identity are preferentially expressed in the TEB fraction. Antibodies to two other clones encoding p190-B and adrennomedullin are being used to localize and study their function. Both of these proteins appear to be expressed in the TEB and may play important roles in regulating TEB proliferation and apoptosis. Procedures have been developed to isolate nuclear matrix proteins from the TEB and preliminary 2D PAGE analysis has identified several unique proteins in this fraction. Procedures have also been optimized for measuring cell cycle kinetics in frozen sections using confocal microscopy. Finally, the TEB is a dynamic structure regulated by a balance of proliferation and apoptosis. Based upon parallel studies performed in transgenic and knockout mouse models, a functional role for Bcl-2 family members, but not for p53, in regulating end bud apoptosis was demonstrated. Overall, the progress made during this past year is consistent with the original tasks proposed for the second year in the Statement of Work.

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FIGURE LEGENDS

Figure 1. Confocal microscopy of TEBs and ABs from a 45 day-old virgin rat showing BrdU labeling patterns after 0.5 hrs (A,B) 3.5 hrs (C,D) and 24 hrs (E,F). Red = Propidium iodide (all nuclei), Green = FITC-anti BrdU (nuclei in S-phase), and Yellow = keratin 14 (myoepithelium). Panels A, C and E are single optical sections (5 μm step-size) while Panels B, D, and F are composites representing 10 contiguous sections. Magnifications are shown as μm above each bar.

Figure 2. Confocal microscopy of ABs in 96 day-old virgin (A, B) and parous (C, D) rats showing very low LI after 2 hr exposure to BrdU. Color code same as in Fig. 1. Magnifications are shown inside bars.

Figure 3. RT-PCR experiments with two EDD clones. A. EDD-C3. B. EDD-G5. PCR experiments were carried out using varying cDNA inputs (C3; 5, 10, 25ng and G5; 12.5, 25 and 50ng). PCR products were electrophoresed and quantitated by phosphoimager analysis.

Figure 4. p190-B expression in the 45 day virgin mammary gland by immunofluorescence. A. Immunofluorescence of TEB with p190-B (green). Nuclei are stained with DAPI (blue). B. Immunofluorescence of TEB with DAPI, p190-B and keratin 14 (red). C. p190-B expression in alveolar bud. Nuclei are stained with DAPI. D. Alveolar bud stained for p190-B, keratin 14 and nuclei stained with DAPI.

Figure 5. Adrenomedullin protein expression in the Terminal End Buds (TEB) of a 45 day virgin rat. A. 40x magnification of adrenomedullin staining. B. 100x magnification. C. Antiserum to adrenomedullin pre-absorbed with antigenic peptide (10uM). D. Pre-absorption of antiserum with nonspecific peptide (10uM).

Figure 6. Adrenomedullin protein expression in 18 day pregnant rat. A. 40x magnification of alveoli. B. 100x magnification. C. Pre-absorption of adrenomedullin antiserum with antigenic peptide (10uM). D. Pre-absorption with nonspecific peptide (10uM).

Figure 7. Phase contrast images of TEBs treated to remove stromal cells with collagenase and microdissection. Note absence of stromal cells around TEBs (A) and around ducts and AB (B). These preparations provided the cell fractions used for nuclear matrix preparations as described in text.

Figure 8. Silver-stained 2-D gels from epithelial from TEBs and ABs, as well as of stromal cells around these areas as discussed in the text.

TABLE I

End Bud-Differential Display (EDD) Clones

Clone[1]	Primers[2]	Size (b.p.)[3]	Identity[4]
EDD-G5	AP-7, T_{11}-G	257	yb27d04.s1; cDNA clone 72391; human fetal spleen; 87%
EDD-G6	AP-2, T_{11}-G	183	
EDD-G7	AP-2, T_{11}-G	230	
EDD-C2	AP-7, T_{11}-C	241	p190B Rho GAP; 87%
EDD-C3	AP-7, T_{11}-C	204	yy55h09.s1; cDNA clone 277505; human ovary; 80%
EDD-C6	AP-1, T_{11}-C	122	yf99a06.r1; cDNA clone 30458; human infant brain; 81%
EDD-C11	AP-8, T_{11}-C	92	Calcium binding protein Cab45; 86%
EDD-C12	AP-8, T_{11}-C	108	Adrenomedullin; 100%
EDD-C13	AP-8, T_{11}-C	125	
EDD-C14	AP-8, T_{11}-C	132	yn84b02.s1; cDNA clone 175083; human adult brain; 78%
EDD-C15	AP-8, T_{11}-C	145	yw25b09.s1; cDNA clone 253241; human fetal cochlea; 91%
EDD-C16	AP-8, T_{11}-C	151	rat mitochondrial rRNA; 98%
EDD-C17	AP-8, T_{11}-C	181	rat cytochrome C oxidase; 99%
EDD-C18	AP-8, T_{11}-C	422	

1 EDD clones.

2 Primer sets used to amplify clones. AP denotes 5' anchored primer. T11N represents 3' polyT primer with anchored base.

3 Length in base pairs of each clone.

4 Sequences in Genbank with similarity to EDD clones and the percent similarity.

TABLE II

Calculation of labeling index (LI), duration of S-phase (Ts) and growth fraction (GF) by confocal microscopy

		Cells Scored	TEB	AB	TD	Combined
45 day-old virgin, N=4	LI	15,079	12.80	9.97		
	Ts	3,585	7.74 hrs	8.96 hrs		
	GF	18,079	-------	-------		0.57
96 day-old virgin	LI	4,999	-------	-------	-------	0.0229
96 day-old parous	LI	7,771	-------	-------	-------	0.0299

Figure 1

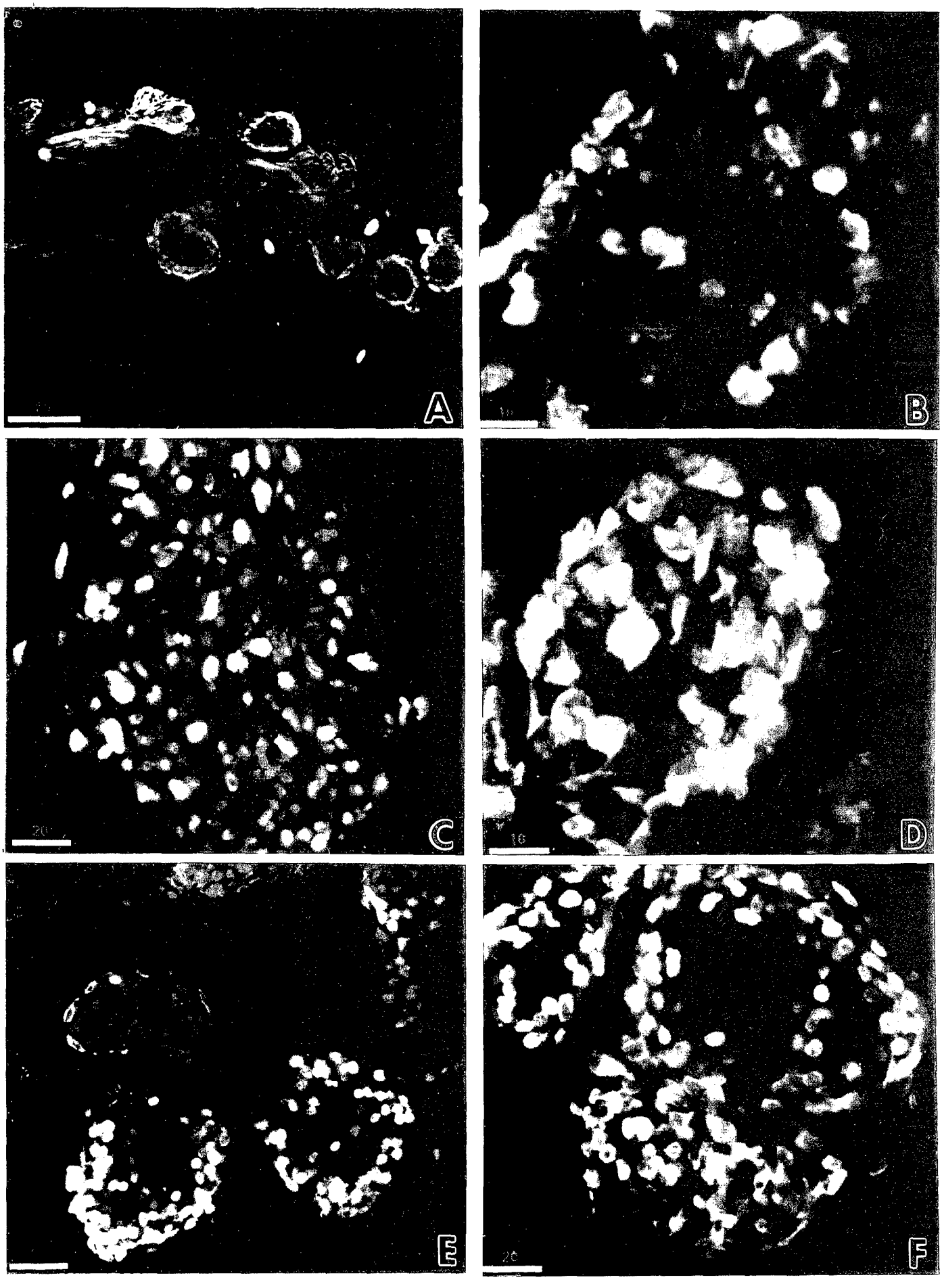


Figure 2

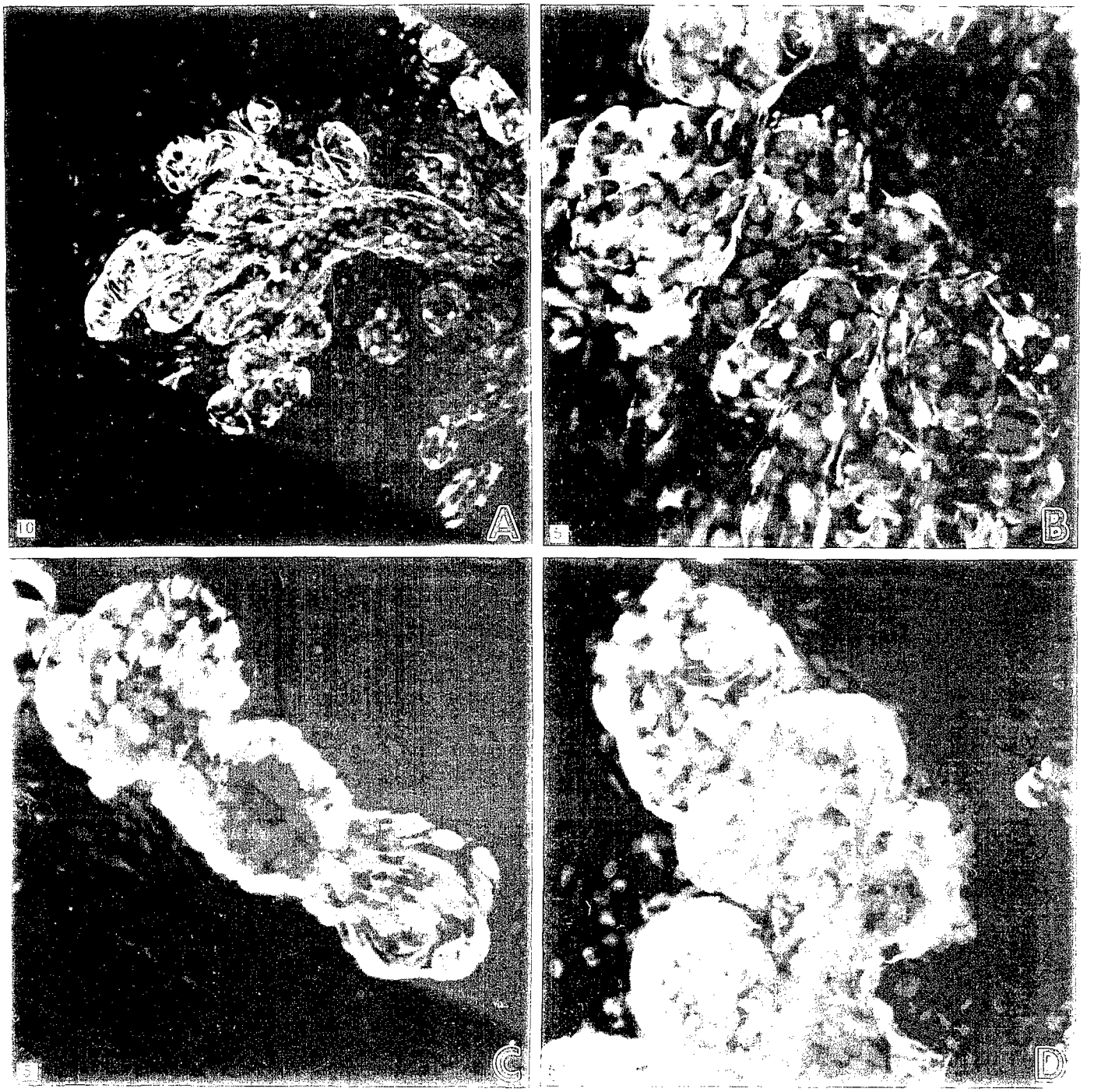


Figure 3A

A

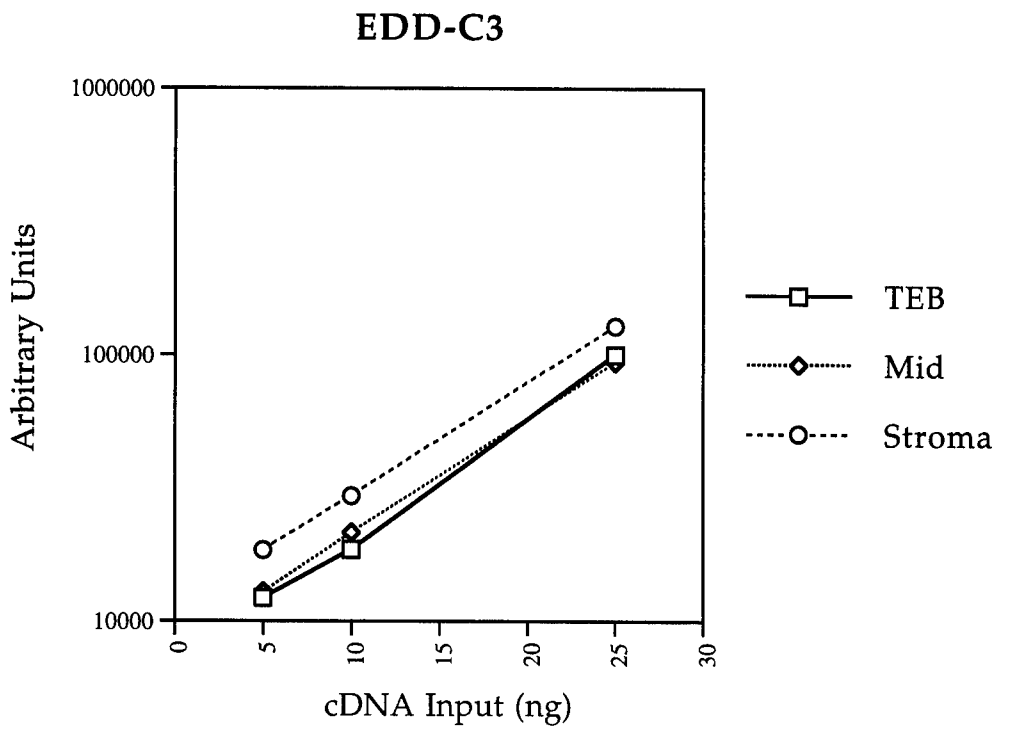

EDD-C3
1000000
100000
10000
Arbitrary Units
0
5
10
15
20
25
30
cDNA Input (ng)
TEB
Mid
Stroma

Figure 3B

B

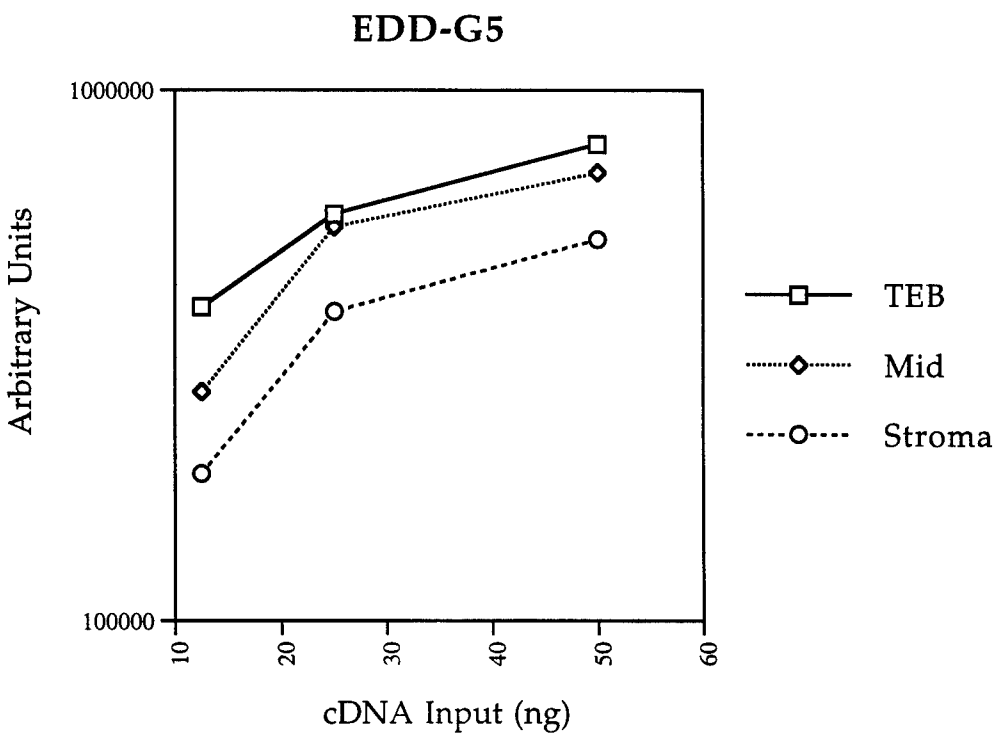

EDD-G5
1000000
100000
Arbitrary Units
10
20
30
40
50
60
cDNA Input (ng)
TEB
Mid
Stroma

Figure 4

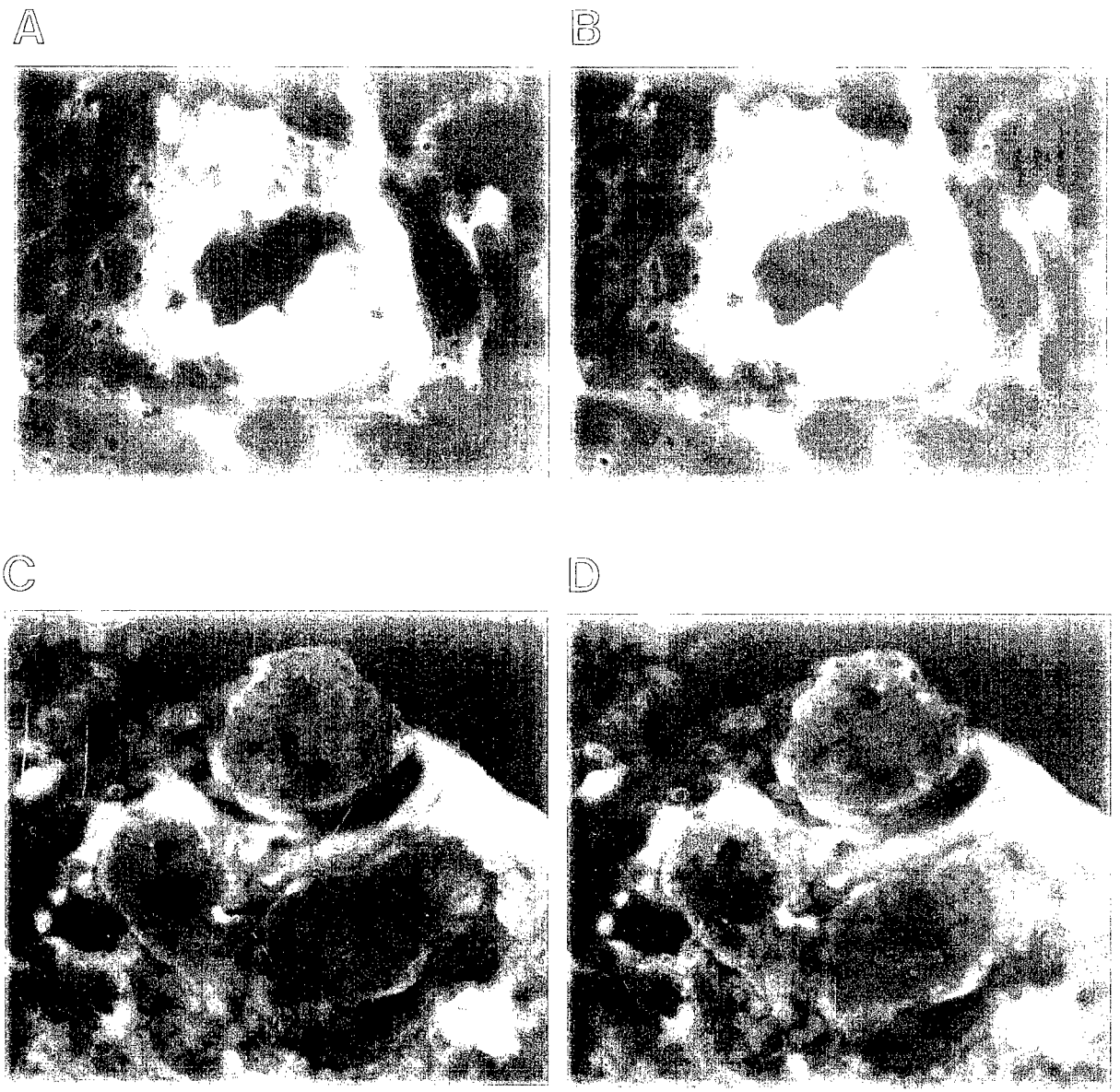


Figure 5

Figure 6

Figure 7

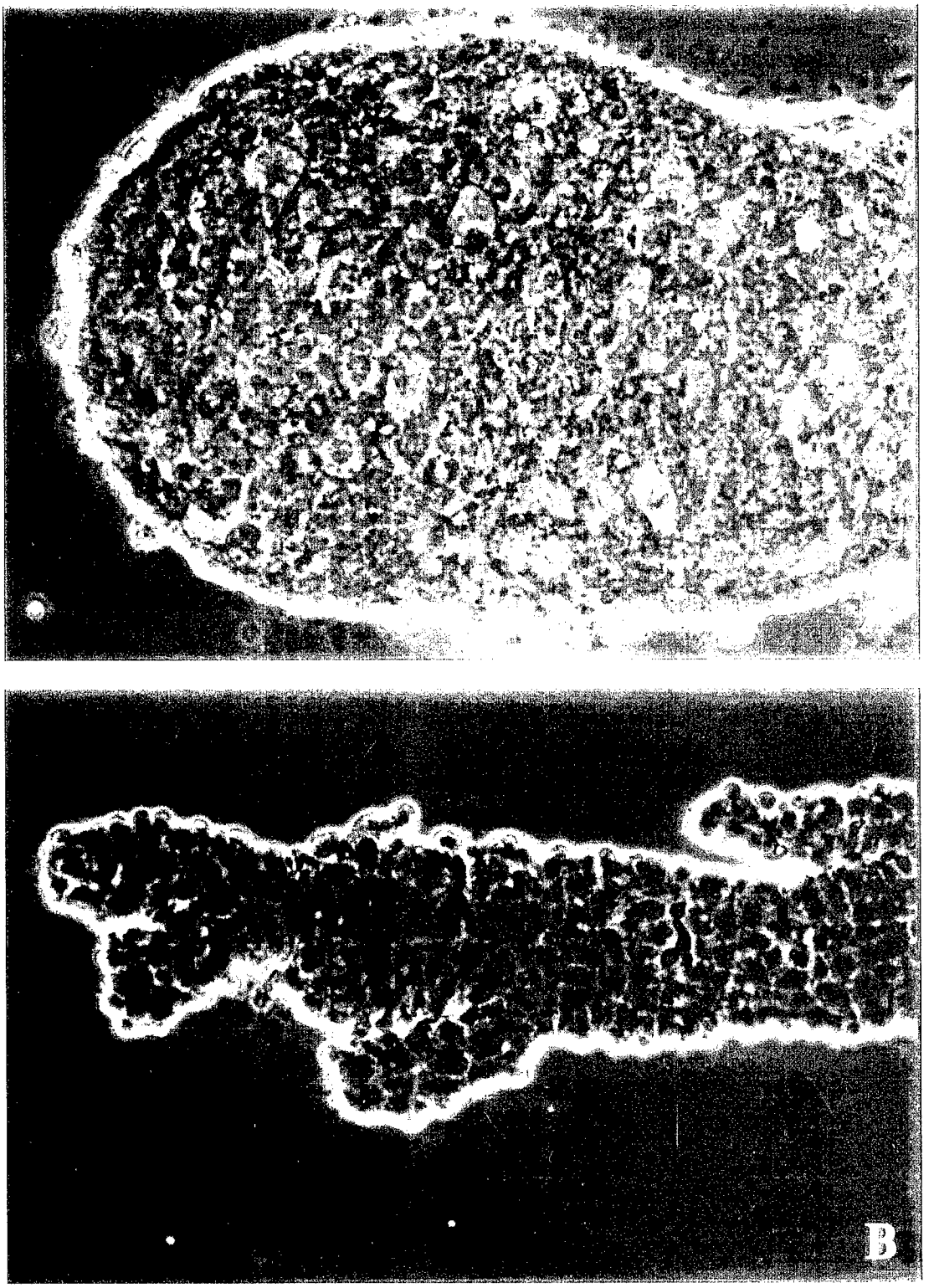
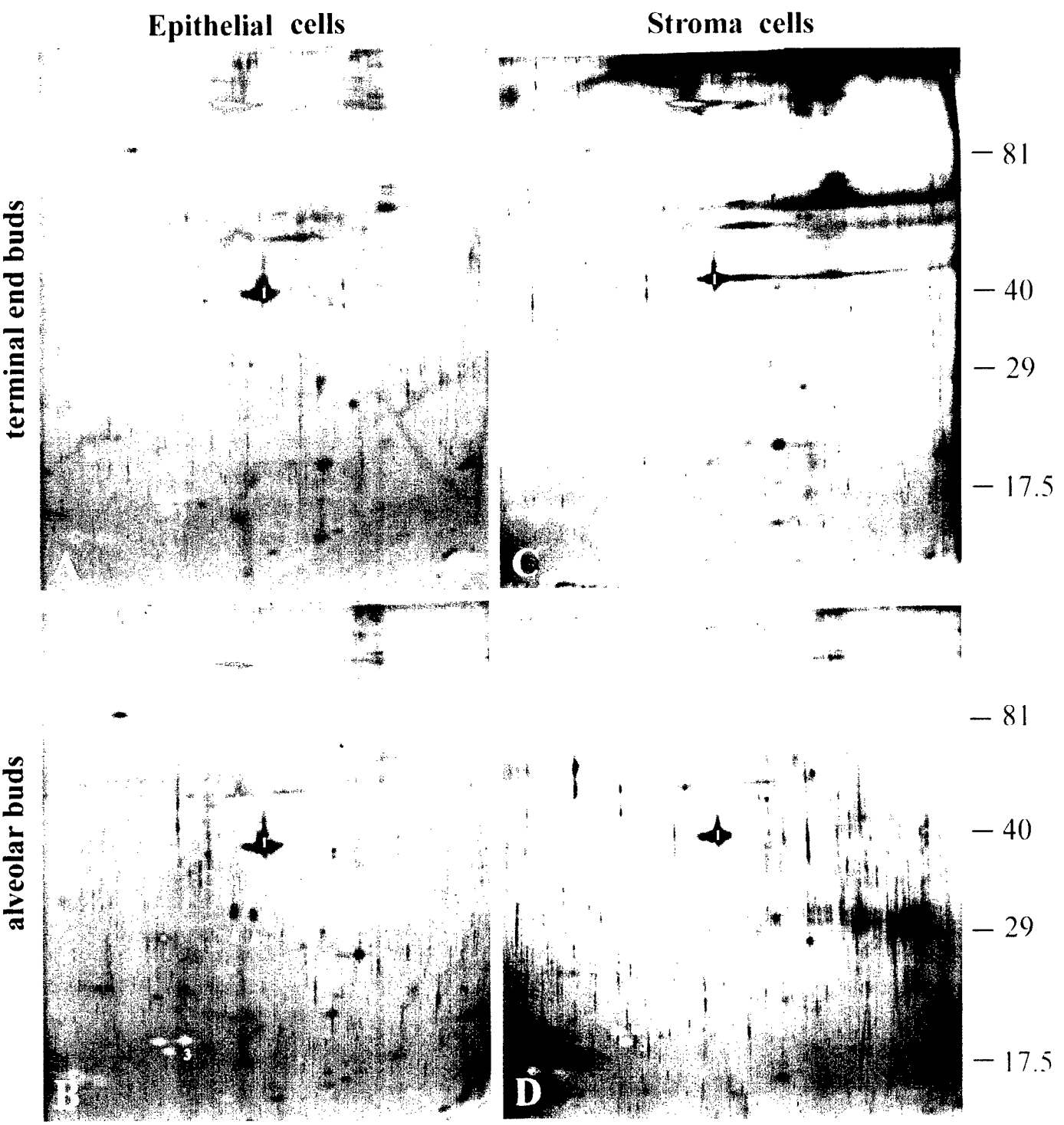


Figure 8


Epithelial cells
Stroma cells
terminal end buds
alveolar buds
81
40
29
17.5
81
40
29
17.5
A
B
C
D

APPENDIX

Humphreys, R.C., Krajewska, M., Krnacik, S., Krajewski, S., Reed, J.C. and Rosen, J.M. (1996) Apoptosis in the terminal end bud of the murine mammary gland: A mechanism of ductal morphogenesis. Development, in press.

Apoptosis in the Terminal Endbud of the Murine Mammary Gland: A Mechanism of Ductal Morphogenesis

Robin C. Humphreys[1], Maryla Krajewska[2], Susanne Krnacik[1], Richard Jæger[3], Hans Weiher[3], Stanislaw Krajewski[2], John C. Reed[2], and Jeffrey M. Rosen[1,4]

[1]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

[2]The Burnham Institute, Cancer Research Center, La Jolla, California 92037

[3]Forschungszentrum Karlsruhe, Instituit für Genetik, Postfach 3640, D-76021 Karlsruhe, Germany.

[4]Author for correspondence

Short Title: TEB Apoptosis and Ductal Morphogenesis

SUMMARY

Ductal morphogenesis in the rodent mammary gland is characterized by the rapid penetration of the stromal fat pad by the highly proliferative terminal endbud and subsequent formation of an arborized pattern of ducts. The role of apoptosis in ductal morphogenesis of the murine mammary gland and its potential regulatory mechanisms was investigated in this study. Significant apoptosis was observed in the body cells of the terminal endbud during the early stage of mammary ductal development. Apoptosis occurred predominately in defined zones of the terminal endbud; 14.5% of the cells within three cell layers of the lumen were undergoing apoptosis compared to 7.9% outside this boundary. Interestingly, DNA synthesis in the terminal endbud demonstrated a reciprocal pattern; 21.1% outside three cell layers and 13.8% within. Apoptosis was very low in the highly proliferative cap cell layer and in regions of active proliferation within the terminal endbud. In comparison to other stages of murine mammary gland development, the terminal endbud possesses the highest level of programmed cell death observed to date. These data suggest that apoptosis is an important mechanism in ductal morphogenesis. In *p53*-deficient mice the level of apoptosis was reduced, but did not manifest a detectable change in ductal morphology, suggesting that p53-dependent apoptosis is not primarily involved in formation of the duct. Immunohistochemical examination of the expression of the apoptotic checkpoint proteins, Bcl-x, Bax, and Bcl-2 demonstrated that they are expressed in the terminal endbud. Bcl-x and Bcl-2 expression is highest in the body cells and lowest in the non-apoptotic cap cells, implying that their expression is associated with increased apoptotic potential. Bax expression was distributed throughout the terminal endbud independent of the observed pattern of apoptosis. A functional role for Bcl-2 family members in regulating endbud apoptosis was demonstrated by the significantly reduced level of apoptosis observed in WAP-Bcl-2 transgenic mice. The pattern of apoptosis and ductal structure of endbuds in these mice was also disrupted. These data demonstrate that

p53-independent apoptosis may play a critical role in the early development of the mammary gland.

Key words: apoptosis, p53, mammary gland, DNA synthesis, Bcl-2, Bcl-x, Bax, terminal endbud, WAP-Bcl-2

INTRODUCTION

Dramatic changes in the morphological organization and molecular expression patterns of the epithelial and stromal cells characterize the ductal and lobular-alveolar stages of postnatal development in the murine mammary gland (MMG). Ductal development is initiated in the virgin animal with the onset of puberty and decreases with the attainment of sexual maturity (Daniel and Silberstein, 1985; Knight and Peaker, 1982). The ductal pattern is created by the penetration of a highly proliferative and dynamic structure, the terminal endbud (TEB), through the stromal fat pad. The pattern of ducts generated during this stage of MMG development is a consequence of the restriction and organization of the proliferation of the TEB by systemic steroid hormones, locally acting growth factors and interaction with the stroma (Coleman et al., 1988; Topper and Freeman, 1980; Vonderhaar, 1987). Studies of the morphological characteristics and the dynamics of DNA synthesis of this early stage of ductal development have revealed little information about the molecular mechanisms that regulate the proliferation of the TEB epithelial cells and the formation of the ductal lumen.

The developing virgin rodent mammary gland contains several cell types that have been characterized by their morphology, differentiated function, ultrastructure (Williams and Daniel, 1983) and expression of cell surface markers such as cadherins (Daniel et al., 1995) and cytokeratins (Dulbecco et al., 1983). The TEB contains two histologically distinct cell types: the body cells and the cap cells. The cap cells are the outermost layer of the TEB and interact through a thin basal lamina with the surrounding stroma. Cap cells are thought to be the progenitors for the myoepithelial cells, which are characterized by their expression of myosin (Dulbecco et al., 1982; Dulbecco et al., 1983; Williams and Daniel, 1983). The interior of the teardrop shaped TEB is filled with 6-10 layers of body cells. Body cells are characterized by the expression of specific cytokeratins (Dulbecco et al., 1986) and E-cadherins (Daniel et al.,

1995). Proximal to the TEB is the neck region which contains several layers of body cells and acts as the transition zone between the TEB and the subtending duct. Adjacent to the neck region is a simple duct with one to three layers of ductal epithelial cells surrounded by a single layer of attenuated myoepithelial cells. How the solid primordium of body cells in the TEB are organized to form the subtending duct and lumen has yet to be determined.

Cell death has been postulated to be an active participant in development for many years (Glucksmann, 1951; Saunders, 1966), but has only recently been demonstrated to be a common event in the development of several organs (Barres et al., 1992; Coles et al., 1993; Coucouvanis and Martin, 1995). Apoptosis is an active process of programmed cell death (PCD) with specific regulators and triggers. One of these regulatory molecules is p53. p53 functions as a guardian of the genome to prevent propagation of mutations to daughter cells. DNA damage induces p53 expression causing cell cycle arrest to effect either DNA repair or induce apoptosis. p53 expression is also associated with a change in the temporal appearance of apoptosis (Colombel et al., 1995). In addition, the members of the Bcl-2 family of proteins; Bcl-2, Bax, Bcl-x_S, Bcl-x_L act together as a rheostat to regulate a cells' apoptotic potential. Bcl-x_S and Bcl-x_L are mRNA splice variants of *Bcl-x* correlated with induction or inhibition of apoptosis, respectively. Importantly, the relative levels of each protein isoform in a particular cell may dictate the progression to apoptosis (Chao et al., 1995; Middleton et al., 1996). However, previous analysis of a wide variety of tissues indicates that Bcl-x_S is only rarely present or present at relatively low levels compared to Bcl-x_L.. The heterodimeric complexes that Bax forms with Bcl-2 regulate the apoptotic potential of various cells (Sedlak et al., 1995). It has been demonstrated that the absolute level of Bax is not the critical factor in the determination of apoptotic potential, but rather the ratio between Bax and Bcl-2 and the cell type in which they are expressed (Knudson et al., 1995).

In the mammary gland, apoptosis has been primarily examined during involution after lactation (Li et al., 1996b; Quarrie et al., 1995; Strange et al., 1992). A basic problem in understanding the development of branching organs like the mammary gland is dissecting the mechanisms that regulate the formation of the lumen of tubes and ducts from a solid primordium. Recently apoptosis has been implicated in the formation of the proamniotic cavity during rodent gastrulation (Coucouvanis and Martin, 1995). During the process of cavitation, apoptosis converts the solid primordium of the embryonic ectoderm into a hollow egg cylinder. While examining the effects of estrogen and progesterone on proliferation and apoptosis in the MMG a significant apoptotic pattern was detected in the TEB of the MMG, prompting a more detailed study of the role of apoptosis in ductal development in the MMG.

This study demonstrates that significant apoptosis occurs in the body cells of the TEB. Apoptosis is most prevalent proximal to the lumen of the TEB and occurs in apoptosis-dense zones. This spatial restriction of apoptosis suggests a possible role for apoptosis in the maintenance of TEB structure and formation of the duct in the developing MMG. Examination of the apoptotic pattern of mammary glands from p53 knockout mice (*p53* $^{-/-}$) revealed that the level of apoptosis is reduced but, that normal development of the TEBs and ducts, in these animals, is not detectably disrupted. Members of the apoptotic checkpoint pathway, Bax, Bcl-2 and Bcl-x are distributed throughout the developing TEB and duct. The exclusion of Bcl-x and Bcl-2 from the cap cell layer and localization of Bcl-2 expression proximal to the lumen of the TEB implies Bcl-x and Bcl-2 may be associated with regulation of apoptotic potential in the body cells of the TEB. In support of this hypothesis, whey acidic protein (WAP)-Bcl-2 transgenic mice had reduced apoptosis and an altered ductal structure in the body cells, and inappropriately increased apoptosis in the neck of the TEB.

MATERIALS AND METHODS

Materials

Terminal deoxytidyl transferase (TdT) was obtained from Pharmacia (Milwaukee, WI). 16-dUTP-biotin and proteinase K were obtained from Boehringer Mannheim (Germany). Probe On Plus slides and Omniset tissue cassettes were from Fisher Scientific (Pittsburgh, PA). ABC reagent, goat serum and diaminobenzidine (DAB) were obtained from Vector Labs (Burlingame, CA). The cell proliferation kit for analysis of bromodeoxyuridine (BrdU) labeling was obtained from Amersham (Buckinghamshire, England). Mice were acquired from Charles River Labs (Wilmington, MA) or from a breeding colony at Baylor College of Medicine courtesy of Dr. Daniel Medina. Aqueous mounting media and hematoxylin were obtained from Biomeda (Foster City, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

WAP- Bcl-2 mice

The WAP-Bcl-2 mice carry a transgene which consists of the human Bcl-2 gene cDNA under the control of a 1.6kb Bgl II/ Kpn I fragment of the mouse WAP promoter. The mice have been bred into DBA/2 background. Details on the construction of the transgene and complete phenotype of these mice is being published elsewhere (Jæger et al., manuscript in preparation). Mice used for this study were backcrossed twice into an FVB background.

Isolation of mammary glands

Age-matched 5-6 week old virgin Balb/c mice (n=6) were injected with 20 µl of BrdU/gm body weight two hours before sacrifice. The 4th (inguinal) and 3rd (thoracic) mammary glands were surgically removed, spread in Omniset tissue cassettes and immediately placed in Tellyesniczky's fixative for 5 hours. Glands were stored in 70%

ethanol then paraffin embedded and sectioned or stained with hematoxylin for whole mount analysis. Sections (5 μm) were mounted on Probe On Plus slides.

Whole mount staining

The whole gland staining was carried out essentially as described (Williams and Daniel, 1983) except that glands were stained for only 2 hours in hematoxylin.

TdT dUTP nick end labeling (TUNEL) analysis

TUNEL was performed as described (Gavrieli et al., 1992) with the following modifications: proteinase K digestion was carried out at 2 μg/ml for 10 minutes at 24°C. Tissue sections were labeled with 1 unit of TdT and 1 nmol of 16-dUTP-biotin in 150 μl of TdT buffer. Labeled sections were incubated with ABC reagent following a standard manufacturer's protocol and then incubated with diaminobenzidine-nickel substrate for 7-8 minutes. Tissue sections were counterstained with 0.1% (w/v) methyl green for one minute. Sections were then rinsed in distilled water, dehydrated through graded alcohols and xylene and mounted with Permount according to standard protocols.

Quantitation of apoptosis and cell proliferation

Images from TUNEL and BrdU analyzed TEBs were captured with a Sony 3CCD color video camera attached to a BX-50 Olympus microscope with Adobe Photoshop software. A manually drawn line on captured images defined a boundary 3 cell layers from the lumen. The percentage of apoptotic and BrdU labeled cells were quantitated both inside (proximal) and outside (distal) this boundary. Thirty TEBs from six mice were counted for apoptosis analysis representing a total of 10983 cells. Eight TEBs from three mice were counted for BrdU quantitation representing 3612 cells. Sixty-two TEBs from eight mice were counted in the Bcl-2 analysis representing 18681 cells. Twenty five TEBs from 8 mice were counted in the p53 analysis representing 4548 cells.

Clusters of TUNEL-labeled cells that occurred within a single cell diameter were considered to be fragments of a central cell and counted as a single apoptotic cell.

Antibodies

The preparation and characterization of anti-peptide rabbit polyclonal antisera specific for the mouse Bcl-2, Bax and Bcl-x proteins have been described in detail previously (Heermeier et al., 1996; Knudson et al., 1995; Middleton et al., 1996).

Analysis of p53 $^{-/-}$ and WAP-Bcl-2 mice

The fourth (inguinal) and third (thoracic) mammary glands from p53$^{-/-}$ (n=5), p53$^{+/+}$ (n=3) (Donehower et al., 1992) and WAP-Bcl-2 (n=3), WAP-Bcl-2 $^{+/+}$ mice (n=5) were collected and stained as whole mounts, as described, and then sectioned. Twelve glands from p53 genotypes and three glands from WAP-Bcl-2 genotypes were used to characterize the changes in ductal morphogenesis. The tissue sections were examined visually for changes in the number and size of ducts, the number of secondary branches, the extent of fat pad penetration and the size and number of TEBs. Eight glands from four mice of each p53 genotype and twelve glands from four mice of each WAP-Bcl-2 genotype were used for TUNEL analysis.

Immunohistochemical analysis

Tissue sections collected and fixed as described, were deparaffinized and rehydrated according to standard protocols. The sections were heated in 0.01 M citrate pH 6.0 or 0.05 M acetate pH 5.5 for Bcl-2 detection, for 10 minutes at 90°C and allowed to cool for 15 minutes at 24°C. The sections were then rinsed in distilled water and endogenous peroxidases were quenched with 3% (v/v) H_2O_2/ 10% (v/v) methanol for 7 minutes in phosphate buffered saline (PBS). After a 30 minute block with 10% (w/v) goat serum in 1xPBS, polyclonal rabbit primary antibody for Bax, Bcl-2 and Bcl-x was

added at 1:500, 1:500 and 1:1000 (v/v) dilution, respectively, diluted in 10% goat serum/PBS. Slides were coverslipped and incubated in a humidified chamber for 60 minutes at 24°C. A PBS wash of 10 minutes preceded application of biotin-conjugated goat anti-rabbit secondary antibody at 1:100 dilution in 10% goat serum/PBS for 45 minutes at 24°C. Biotin-avidin binding and detection was carried out according to manufacturer's protocol (Vector Labs). A 0.05% (v/v) Triton-X 100 wash for 1 minute was followed by detection with DAB for 7 minutes. Sections were counterstained with aqueous hematoxylin diluted 1:10, for 1 minute, mounted with aqueous mounting media and coverslipped. All immunohistochemical analyses included control sections without added primary or secondary antibodies.

RESULTS

Significant levels of apoptosis are present in the body cells of the terminal endbud

TEBs are most prominent and dynamic in the MMG during early ductal development (4-7 weeks of age). Using the TUNEL technique on sections of mammary glands isolated from 5 and 6 week old virgin Balb/c mice revealed significant levels of apoptosis in the body cells of the TEB (Figs 1A, C and 2A, C). Every TEB examined contained apoptotic cells. The majority of the apoptosis is conspicuously localized around the lumen of the newly forming duct. These highly apoptotic regions are primarily restricted to body cells that protrude into, or are proximal to, the ductal lumen (Figs 1A, C and 2A, C). In the densest apoptotic regions up to 60% of the body cells were undergoing apoptosis within a given section. This consistent and striking pattern suggested that apoptosis may be involved in the formation lumen of the duct formed by apoptosis of body cells most proximal to the lumen.

To test this hypothesis the number of apoptotic cells within an arbitrary three cell layers of the lumen were quantitated (Fig. 2C, Table 1). A significantly greater percentage (14.5%) of the cells proximal to the lumen were undergoing apoptosis

compared to those cells distal (7.9%) to this arbitrary division ($p<0.001$). Within an individual TEB the level of apoptosis was as high as 26%. Only 0.5% of the cap cells in the TEB and 1.0% of the ductal epithelial cells and less than 0.01% of the myoepithelial cells in the subtending ducts were undergoing apoptosis (data not shown). The percentage of apoptotic cells detected in the ductal epithelial cells of the subtending ducts of the immature virgin is similar to the value observed in the mature virgin and proliferating pregnant gland (Table 2). These results were consistent between animals, among glands from the same animal, and within endbuds from the same gland. No difference was observed in the apoptotic pattern between TEBs from the third and fourth mammary glands. DNA synthesis can precede a cell's entrance into apoptosis (Barres et al., 1992). This phenomena can be detected in the TEB as fragmented BrdU-labeled nuclei (Fig. 2D arrow) suggesting that some apoptotic cells were very recently generated. The significant level of spatially restricted apoptosis that occurs in the body cells of the TEB suggests that this apoptosis may be involved in organizing TEB structure and possibly contribute to the formation of the lumen of the MMG duct.

Overall, 11.3% of the cells in the TEB were undergoing apoptosis. This represents a significant number of apoptotic cells within a single developing structure. To date, the TEB possesses the highest level of apoptosis detected during any stage of mammary gland development (Table 2). This observation reinforces the dynamic nature of the TEB and suggests that the mechanisms that regulate this apoptosis may be intimately involved in the development of this organ.

DNA synthesis has a reciprocal pattern to apoptosis in the TEB

Studies of DNA synthesis in the rodent mammary gland identify the cap cell layer and the neck region as the primary zones of proliferation in the TEB during ductal development. (Daniel et al., 1987; Dulbecco et al., 1982; Sapino et al., 1990). To understand the relationship between cell proliferation and apoptosis, the pattern of

cellular proliferation in the TEB was examined by immunohistochemical analysis of mammary glands which had been injected with BrdU i.p., 2 hours prior to sacrifice of the mice. DNA synthesis occurred at various locations throughout the TEB, but most significantly in the cap cells and body cells of the neck region (Fig. 1B, D).

Comparing the pattern of apoptosis and DNA synthesis in serial sections of TEBs, revealed reciprocal zones of proliferation and apoptosis occurring within the same endbud and that they can exist in proximity within the TEB (Figs. 1, 2). Cells undergoing DNA synthesis can occur in zones where there is little apoptosis, or adjacent to cells undergoing apoptosis (Fig. 1D arrow). Even though the majority of the apoptosis was around the lumen of the duct, BrdU labeling was not excluded from this region. 13.8% of the cells proximal to the lumen were labeled with BrdU. Distal to the arbitrary boundary 21.1% of the cells were labeled with BrdU. This result represents a significant difference in the level of DNA synthesis depending on a cells proximity to the lumen of the TEB ($p<0.02$) (Table 1).

In the TEB, 17.9% of the cells were undergoing DNA synthesis. This level of BrdU labeling correlates with the previously reported levels of proliferation observed in the TEB of the mouse (Sapino et al., 1990). Compared to other organs, undergoing extensive developmentally associated growth, the TEB of the mammary gland exhibits a high level of DNA synthesis (Barres et al., 1992; Coles et al., 1993).

Apoptosis in the TEB is not dependent on p53

To determine if the pattern of apoptosis observed in the TEB was dependent on the expression of p53, the TEBs of *p53* $^{-/-}$ mice were analyzed by TUNEL. If there was any p53 dependence then the pattern of apoptosis in the TEB and possibly ductal development in *p53* $^{-/-}$ mice should be disrupted. Comparison of whole mount stained mammary glands from *p53* $^{-/-}$ and wildtype mice revealed no difference in the ductal development pattern (Fig. 3). Several morphological characteristics were examined

empirically including the overall ductal organization, number of TEBs, the length of ducts and number of secondary branches. These data support the observation that there are no gross developmental deficiencies in the mammary glands of *p53* $^{-/-}$ mice (Donehower et al., 1992). Microscopic examination of serial sectioned *p53* $^{-/-}$ TEBs showed no consistent changes in TEB morphology. A decrease in the level of apoptosis in *p53* $^{-/-}$ mice (8.3%) is detected when compared to syngeneic, age-matched control mice (11.5%, $p<0.001$)(Table 3) The majority of this decrease in apoptosis occurs in the cells that are distal to the lumen. However, the lack of a detectable effect on the development of ductal structures or TEBs in *p53* $^{-/-}$ mice suggests that p53 does not play an essential role in the development of the ductal structures or the formation of the lumen through apoptotic mechanisms.

Bcl-x, Bax and Bcl-2 expression are non-uniformly distributed in the TEB and reflects a combinatorial regulation of apoptosis

Since apoptosis in TEBs did not appear to be dependent on p53, the expression of other potential apoptosis-regulating molecules was examined. Using an antibody that recognizes both forms of the protein, Bcl-x expression was detected throughout the TEB in the body cells (Fig. 4A, C). Bcl-x is expressed in the ductal epithelial cells and weakly in the myoepithelial cells (Fig. 4B). In the TEB, expression was lowest in the cap cell layer (Fig. 4C arrow) and higher in the body cells. Body cells with the highest expression were located proximal to the lumen (Fig. 4A, C). The pattern of Bcl-x expression in specific body cells doesn't overlap with TUNEL labeling in serial sections. This lack of correlation is not surprising, since Bcl-x regulates the apoptotic checkpoint earlier than the moment when active DNA degradation occurs and can be detected by the TUNEL technique. Thus the pattern of expression of Bcl-x in the body cells and the low level expressed in the cap cell layer implies that Bcl-x may play a role in regulating the apoptotic pattern in the TEB.

Immunohistochemical examination of Bax expression in the TEB of 6 week old Balb/c mice revealed expression of Bax throughout the TEB in both body and cap cells (Fig. 5A, C). High levels of Bax expression was detectable in both body and cap cells (Fig. 5A arrows). Immunoreactive-Bax signal was apparent in the nucleus and cytoplasm. No overlap between the expression of Bax and the apoptotic pattern in serial sections was observed. Bax expression was also detected in the ductal epithelial cells (Fig. 5B). Interestingly, there was significant expression in the non-apoptotic myoepithelial cells (Fig. 5B arrow). This demonstrates that high levels of Bax expression do not necessarily correlate with induction of apoptosis in this cell type.

Bcl-2 is thought to repress apoptosis in most cells (Reed, 1994). Body cells in the TEB displayed significant Bcl-2 expression. Paradoxically, body cells most proximal to the lumen had high levels of Bcl-2 expression (Fig. 6A, B). There was reduced Bcl-2 expression in the cap cell layer. (Fig. 6B). Examining serial sections of TUNEL-labeled TEBs no direct correlation could made between cells non-apoptotic and the expression of Bcl-2. This pattern of Bcl-2 expression suggests that another Bcl-2 family member may be involved in repressing Bcl-2's anti-apoptotic effects. Bcl-2 is expressed in the ductal epithelial cells, but in contrast to Bax expression in myoepithelial cells, Bcl-2 expression was very low (Fig. 6C, arrow).

WAP-Bcl-2 mice have a reduced level and altered distribution of apoptosis in the TEB and disruption in the formation of ductal structures

To demonstrate a functional role for Bcl-2 family members in TEB-apoptosis, the level of apoptosis was examined in transgenic mice where Bcl-2 expression was targeted to the mammary gland using the regulatory sequences from the mouse WAP gene. Overexpression of Bcl-2 has been demonstrated to block p53-mediated apoptosis in vitro (Garcia et al., 1992; Strasser et al., 1991) and in vivo (Vaux et al., 1988). Apoptosis was significantly lowered in WAP-Bcl-2 TEBs (6.3%) when compared to wildtype

controls (10.7%, $p<0.001$, Fig. 8). The persistent pattern of apoptosis observed in the TEBs of wildtype mammary glands was noticeably absent in transgenic glands (Compare Fig. 1A to Fig. 7C). In some large transgenic TEBs there were no apoptotic cells present (data not shown). Additionally, the localization of apoptosis in the TEB was disrupted. A dramatic increase in the level apoptosis was observed in the neck of some TEBs (Fig. 7D). There was also an effect on the structural organization of the TEB. In some TEBs the body cells were present as highly convoluted layers of epithelial cells. These layers of cells were found in the central cavity of the TEB and in the neck region (Fig. 7A,B). Unattached cells could be found in the newly-formed duct (Fig. 7A, arrow). Cells were observed some distance from the TEB into areas that were clearly ductal (data not shown). In addition the cellular organization of the cap cells was disrupted and were often difficult to identify. These data demonstrate that WAP-driven Bcl-2 expression has disruptive effects on the pattern and level of apoptosis and cellular organization in the TEB. These results support the hypothesis apoptosis is involved in ductal morphogenesis, and that Bcl-2 family members play a role in regulating this mechanism.

DISCUSSION

Apoptosis in the TEB is a critical mechanism of fuctal morphogenesis

This study reveals that the TEBs of the MMG exhibit a dramatic pattern and level of apoptosis in the body cells. These apoptotic body cells are often restricted to an area projecting into the presumptive lumenal space. This reproducible pattern of apoptosis suggests that programmed cell death deletes specific body cells and may be a critical mechanism in the ductal morphogenesis of the early mammary gland.

The very high levels of apoptosis (11.3%) detected in the TEB during normal ductal development of the MMG are higher than the published levels for any other stage of mammary gland development. During involution in the mammary gland,

when there is dramatic tissue reorganization, the maximal level of apoptosis observed is approximately 4% (Quarrie et al., 1995) In SV40 T-antigen transgenic pregnant mammary glands approximately 5% apoptosis was detected (Li et al., 1996a). Only in the mammary glands of transgenic mice overexpressing p53 have levels of apoptosis (20%) been detected that exceed those observed in the TEB (Li et al., 1995) (Table 2). The level of apoptosis in most quiescent tissues is usually 0.1-1%. Apoptosis in the TEB exceeds that detected during development in other organs like the kidney, 3% (Coles et al., 1993) and optic nerve, 0.25% (Barres et al., 1992). The clearance rate of apoptotic cells in these structures is rapid and consequently it is estimated that 50% of the cells undergo apoptosis. Thus, there are examples of other organs that utilize large scale apoptosis as a mechanism of development.

How is this pattern of apoptosis in the TEB generated? Given the striking pattern observed in some TEBs, apoptosis is clearly influenced by a cell's position within the TEB. This suggests that there is an apoptotic signal that is regulated by positional cues from the TEB and its environment. Interestingly, for TEBs with large, well-defined lumens, apoptotic body cells are distributed evenly around the lumen (data not shown). Only in TEBs with large numbers of body cells protruding into the presumptive lumenal space is the apoptosis narrowly restricted. Examination of serial sections of TEBs indicate that the zones of apoptosis penetrate several cell layers (data not shown). These observations imply it is not just the distance from the external cap cell layer that induces apoptosis in a particular cell, but that there is another presently undefined mechanism influenced by a cell's position within the overall structure of the TEB.

A precedent for the derivation of an apoptotic signal originating from outside the zone of apoptosis has been provided by studies from Coucouvanis and Martin (1995). These authors demonstrated that the external group of endodermal cells sends an apoptotic signal causing apoptosis in the centrally located ectodermal cells during rodent gastrulation. Attachment to the basal lamina protects the internal ectodermal

cells from this apoptotic signal. There is striking similarity between this system and the TEB, and it is tempting to speculate that this same mechanism of apoptosis induction may be occurring in the MMG. Attachment to the basal lamina by the cap cells may provide a protective effect as indicated by their relatively low apoptotic rate. It is unknown whether the cap cells or the stromal cells are performing the same role as the endodermal cells in the rodent embryo, and are, in fact the source of the apoptotic signal.

This model of developmentally-associated apoptosis implies that structural organization; i.e., attachment to the basal lamina, can protect cells from apoptosis. Attachment to the basal lamina has been demonstrated to regulate the induction of apoptosis and integrins may play a role in the regulation of this response (Coucouvanis and Martin, 1995; Frisch and Francis, 1994; Ruoslahti and Reed, 1994; Zhang et al., 1995). Interaction between mammary epithelial cells, in vitro and in vivo, and the extracellular matrix can regulate their apoptotic potential (Boudreau et al., 1995; Pullan et al., 1996). Specifically, antibodies against β-1 integrin or overexpression of the matrix metalloproteinase, stromelysin-1, can induce apoptosis of mammary epithelial cells. Overexpression of stromelysin-1 can also alter the developmental pattern of transgenic MMGs (Sympson et al., 1994; Witty et al., 1995). Increased expression of several proteinases correlates with an increase in apoptosis during mammary gland involution (Lund et al., 1996). Thus, the signal for apoptosis may originate from an interaction between the cap cells and the extracellular matrix surrounding the TEB. The characteristics of the stroma immediately surrounding the TEB are different from the stroma not involved with the TEB and the thickness and molecular components of the basal lamina change with respect to position on the TEB (Daniel and Silberstein, 1985; Williams and Daniel, 1983). In this way positional cues could be transmitted to the cap cells. Thus, it is conceivable that the interaction between the ECM and the cap cells may

be involved in the generation of the apoptotic pattern in the course of regulating the development of the TEB.

Apoptosis in the TEB is independent of p53 and may depend on combinatorial regulation by several Bcl-2 family members.

Given the presence of apoptosis during ductal morphogenesis, the expression of several proteins that have demonstrated roles in regulating the apoptotic pathway; p53, Bax, Bcl-x_{S+L} and Bcl-2 was examined immunohistochemically. p53 can cause both cell cycle arrest and apoptosis in response to DNA damage (Shaw et al., 1992; Yonish et al., 1993; Zhan et al., 1993) , but is apparently not involved in apoptosis induced by growth factor withdrawal and cell density in mammary epithelial cells in vitro (Merlo et al., 1995). Whether p53 is involved in developmentally-associated apoptosis in vivo is controversial. *p53* gene expression increases with the onset of involution (Strange et al., 1992). However, *p53* $^{-/-}$ mice showed no overt differences in mammary gland ductal or lobular-alveolar development when compared to syngeneic wildtype mice (Donehower et al., 1992; Li et al., 1996b) and this study. The level of apoptosis in SV40 T-antigen transgenic mice is increased abnormally during pregnancy preventing attainment of a complete lactogenic phenotype (Li et al., 1996a). Since p53 is sequestered by T-antigen in these mice, the increased apoptosis probably occurs by a p53-independent mechanism. There is some evidence to suggest that p53 may delay the onset of apoptosis. In *p53*$^{-/-}$ mice prostate involution occurred normally (Berges et al., 1993), but Colombel et al. (1995) demonstrated that prostate gland involution was delayed in the absence of p53. These data suggest the role of p53 in the mammary gland may be of secondary importance in defining the developmental pattern of apoptosis as suggested by Li et al. (1996b). This study supports the conclusion that p53 is not primarily involved in the regulation of the developmentally-associated apoptotic pattern in the TEB.

Bcl-2 is a proto-oncogene that has demonstrated anti-apoptotic effects (Garcia et al., 1992; Reed et al., 1990; Strasser et al., 1991; Vaux et al., 1988). Bax, Bcl-x_L, Bcl-x_S, Bad and Bag can form heterodimeric protein complexes with Bcl-2 and act as apoptotic checkpoint regulators (Boise et al., 1993; Oltvai et al., 1993; Takayama et al., 1995; Yang et al., 1995). Expression of these proteins has been correlated with induction and repression of apoptosis, dependent upon the cell type and expression levels of the competing heterodimer partners. The expression of these proteins during the initial phase of ductal development in the mammary gland has not been characterized to date.

The pattern of Bcl-x expression in the TEB suggested that it may be involved in the regulation of the apoptotic pattern. This is based on the two observations; the cap cell layer has very low Bcl-x staining and Bcl-x staining is correlated with proximity to the ductal lumen. Although the antibody employed in this study recognizes the Bcl-x_S and Bcl-x_L forms of the Bcl-x protein, there may be a role for Bcl-x in the appearance of the apoptotic pattern. Li et al. (1996b) have reported that an increase in the level of both forms of Bcl-x mRNA occurs at the onset of involution when a majority of the apoptosis occurs in MMG development. However, the relative increase in the level of Bcl-x_S mRNA may be responsible for the increased apoptosis. The ratio between the two splice forms regulate the apoptotic potential of the particular cell, and the largest increase in Bcl-x_S expression occurs during mammary gland involution when there is significant apoptosis. In addition, Bcl-x_S overexpression in transfected mammary epithelial cells resulted in an increase in apoptosis(Heermeier et al., 1996). Thus, Bcl-x expression detected in the body cells may be due to an increase in the amount of Bcl-x_S which may in turn, cause increased apoptosis of the body cells. In the absence of antibody reagents that are capable of unambiguously distinguishing between the Bcl-x_S and Bcl-x_L proteins, however, this idea remains speculative.

The presence of Bax in the TEB, and the lack of correlation between its expression and the apoptotic pattern in the TEB, do not exclude Bax from being involved in the

induction of apoptosis. It does suggest, however, that other members of the Bcl-2 family may be required to induce programmed cell death in this structure. Interestingly the high level of Bax in the myoepithelial cells and their characteristically low level of apoptosis shows that expression of Bax does not induce apoptosis in these cells. This may be another example where apoptosis induction is dependent on the ratio of Bcl-2 family members within a cell. Alternatively, studies of Bax knockout mice have suggested that Bax may paradoxically promote cell survival in some cellular contexts (Knudson et al., 1995).

The expression of Bcl-2 in the TEB is similar to that of Bcl-x; i.e. low expression in the cap cells with increased expression in the body cells proximal to the lumen. Bcl-2 was originally characterized as capable of preventing apoptosis and its expression pattern is the inverse of what is expected given the apoptotic pattern in the TEB. This suggests that Bcl-2 expression alone does not regulate TEB-apoptosis. A recent study of the role of matrix attachment and Bax expression in promoting apoptosis in alveolar cells demonstrated that Bcl-2 is expressed in ductal, but not in alveolar cells. These authors suggested that as survival factors, Bcl-2 is dominant in the ductal cell while matrix attachment is dominant in the alveolar cell (Pullan et al., 1996). This supports the theory that Bcl-2 may be a dominant molecule in regulating apoptosis in the ductal epithelial cells and their progenitors in the TEB. The patterns of expression of these three apoptosis-regulating proteins in the TEB imply that no single protein is responsible for regulating apoptosis in the TEB. Thus, although there may be a correlation between the expression of Bcl-2, and possibly Bcl-x, and the apoptotic pattern in the TEB, it is most probably the ratios of these and other Bcl-2 family proteins that decides a cell's fate, as has been suggested previously (Sedlak et al., 1995).

The pattern of apoptosis and ductal development of WAP-Bcl-2 mammary glands is disrupted

In order to establish a functional correlation between the expression of these apoptosis-regulating molecules, the pattern of apoptosis and ductal development was studied in WAP-Bcl-2 transgenic mice. In TEBs of WAP-Bcl-2 mice there was a significant decrease in the level of apoptosis even though there is presumably an effect of incomplete penetrance and estrous cycle-dependent expression on the WAP transgene. Some transgenic TEBs lacked apoptotic cells altogether. In addition to the decrease in the body cells, an increase in apoptosis was observed in the neck region where there is normally little apoptosis. This new localization of apoptosis suggests that there is a possible compensatory mechanism for the lack of normally occuring apoptosis in the TEB. In some transgenic TEBs there was a disruption in the cellular organization of the TEB which was manifested as convoluted layers of body cells in the TEB and neck region. This morphological disruption may have resulted from an increased number of cells present in the TEB forcing a loss of structural integrity. Despite the observed increase in apoptosis in the neck of transgenic TEBs there were still unattached cells present in ductal regions well past the neck. Often these unattached cells were located quite far from the TEB suggesting that compensatory mechanisms could not completely account for the lack of apoptosis in the TEB. These data demonstrate that disruption of the pattern and level of apoptosis in the TEB by altering the balance of apoptosis-regulating molecules can lead to alterations in the ductal development of the mammary gland. Presumably, this occurred by alteration of the balance between Bcl-2 and its co-regulatory molecules.

The proximity and reciprocity of pattern and levels of DNA synthesis and PCD in the TEB reflect a potential coordination between mechanisms of cell proliferation and cell death during mammary gland morphogenesis. TEBs, the most proliferative structure in the mammary gland, are at their highest density in early ductal

development (Russo and Russo, 1978b). This maximal proliferation and presence of large numbers of TEBs in the young virgin is correlated with an increased sensitivity to carcinogens (Anderson and Beattie, 1992; Russo and Russo, 1978a). Thus, the high level of apoptosis in the TEB could provide another mechanism sensitive to transformation. It will be interesting to test whether disruptions in the apoptotic mechanism will increase the TEBs' sensitivity to transformation.

In conclusion, these studies suggest that the TEB is a dynamic structure regulated by a balance of proliferation and apoptosis. Significant apoptosis occurring in the primordium of body cells may be a critical mechanism of ductal morphogenesis in the MMG.

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FIGURE LEGENDS

Fig. 1 Programmed cell death and DNA synthesis in the TEBs of 5 week old virgin Balb/c mice. A and C are TEBs analyzed by TUNEL. B and D are serial sections analyzed by BrdU immunohistochemistry. CC defines the cap cell layer. BC defines body cells. The open arrow in 1D indicates an apoptotic cell adjacent to a cell undergoing DNA synthesis. All sections are counterstained with methyl green. Bar 15μm.

Fig. 2 Programmed cell death and DNA synthesis in the TEBs of 5 week old virgin Balb/c mice. A and C are TEBs analyzed by TUNEL. B and D are serial sections analyzed by BrdU immunohistochemistry. The dashed line in 2C defines the boundary used for quantitation of apoptosis. The arrow in 2D designates fragmented nuclei labeled with BrdU. CC defines the cap cell layer. BC defines body cells. All sections are counterstained with methyl green. Bar 15μm.

Fig. 3 The effect of *p53* deletion on the ductal development of the virgin mammary gland. Whole mount hematoxylin-stained $p53^{-/-}$ (A) and wild type (B) mammary glands. Bar 0.5mm.

Fig. 4 Immunohistochemical detection of Bcl-x expression in the mammary gland of Balb/c mice. Bcl-x expression in the terminal endbud (A and C) and the duct (B). Arrow in C designates low Bcl-x expression the cap cell layer. The lymph node in D is a positive control for Bcl-x staining. Bar 15μm.

Fig. 5 Immunohistochemical detection of Bax expression in the mammary gland of Balb/c mice. Bax expression in the terminal endbud (A and C) duct (B). Arrow in B

designates Bax expression in the myoepithelial cells. D is control terminal endbud with primary antibody excluded. Bar 15μm.

Fig. 6 Immunohistochemical detection of Bcl-2 expression in the mammary gland of Balb/c mice. Bcl-2 expression in the terminal endbud (A and B) and duct (C). Arrow in C indicates myoepithelial cells with no Bcl-2 staining. D shows staining in the TEB after competing with Bcl-2 control peptide. Bar 15μm.

Fig. 7 Hematoxylin and eosin staining of TEBs from WAP-Bcl-2 mice showing a disruption in the organization of the body cells (A). Arrow in A shows cells in the duct. B shows convoluted layers of epithelial cells in the TEB Bar in A 100μm. TUNEL analysis of WAP-Bcl-2 mammary glands revealing lowered levels of apoptosis in the TEB (C) and increased levels in the neck region (D). Arrow in D indicates the location of the lumen of the TEB. Bar in B, C and D 15μm.

Fig. 8 Quantitation of the reduction of apoptosis levels in WAP-Bcl-2 TEBs by TUNEL analysis. A significant difference is observed between WAP-Bcl-2 (n=3) and wildtype control (n=5) mice ($p<0.001$). Values represent percentage of total cells labeled positive for apoptosis in transgenic and wildtype TEBs.

Fig. 1

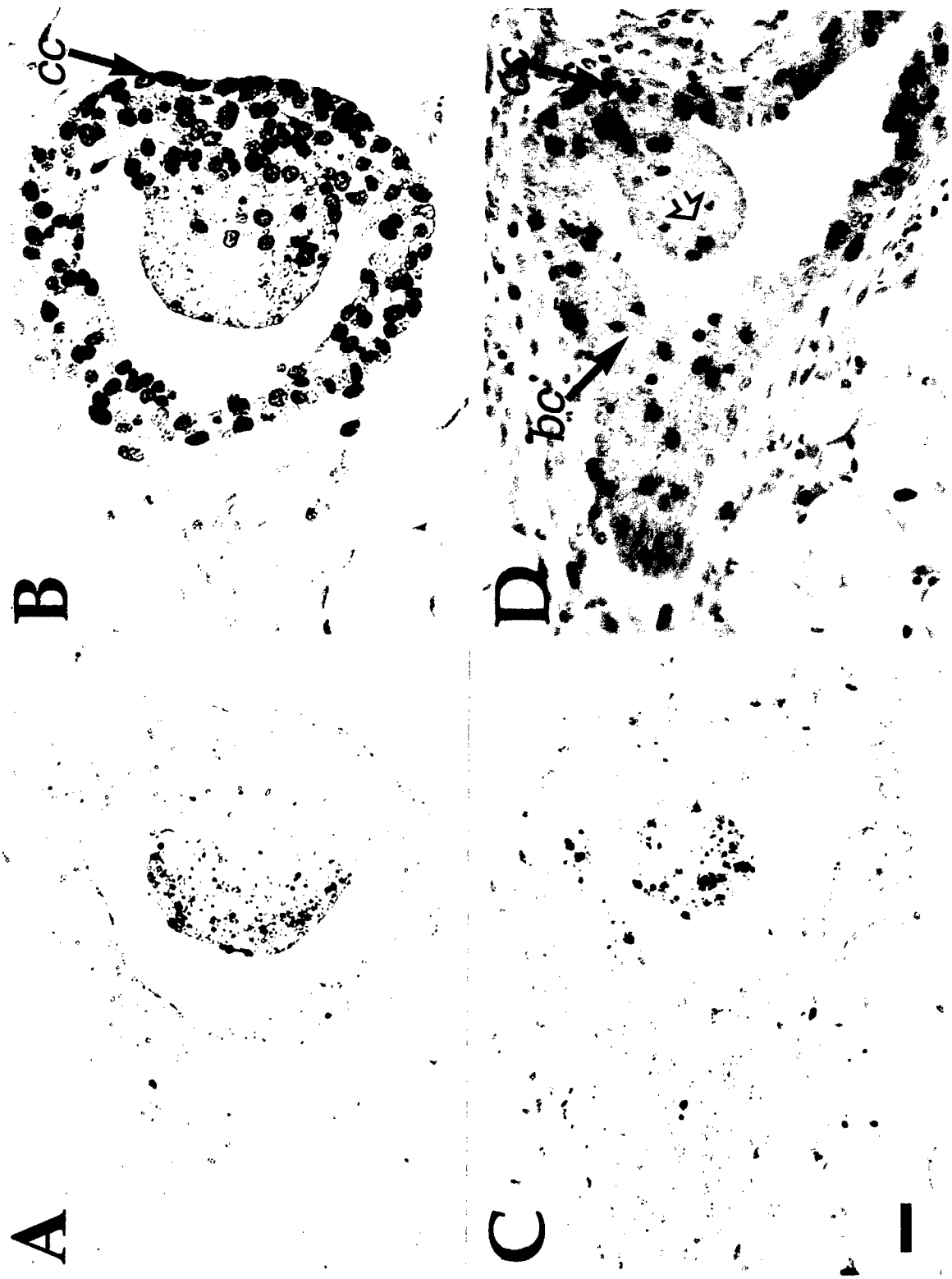


Fig. 2

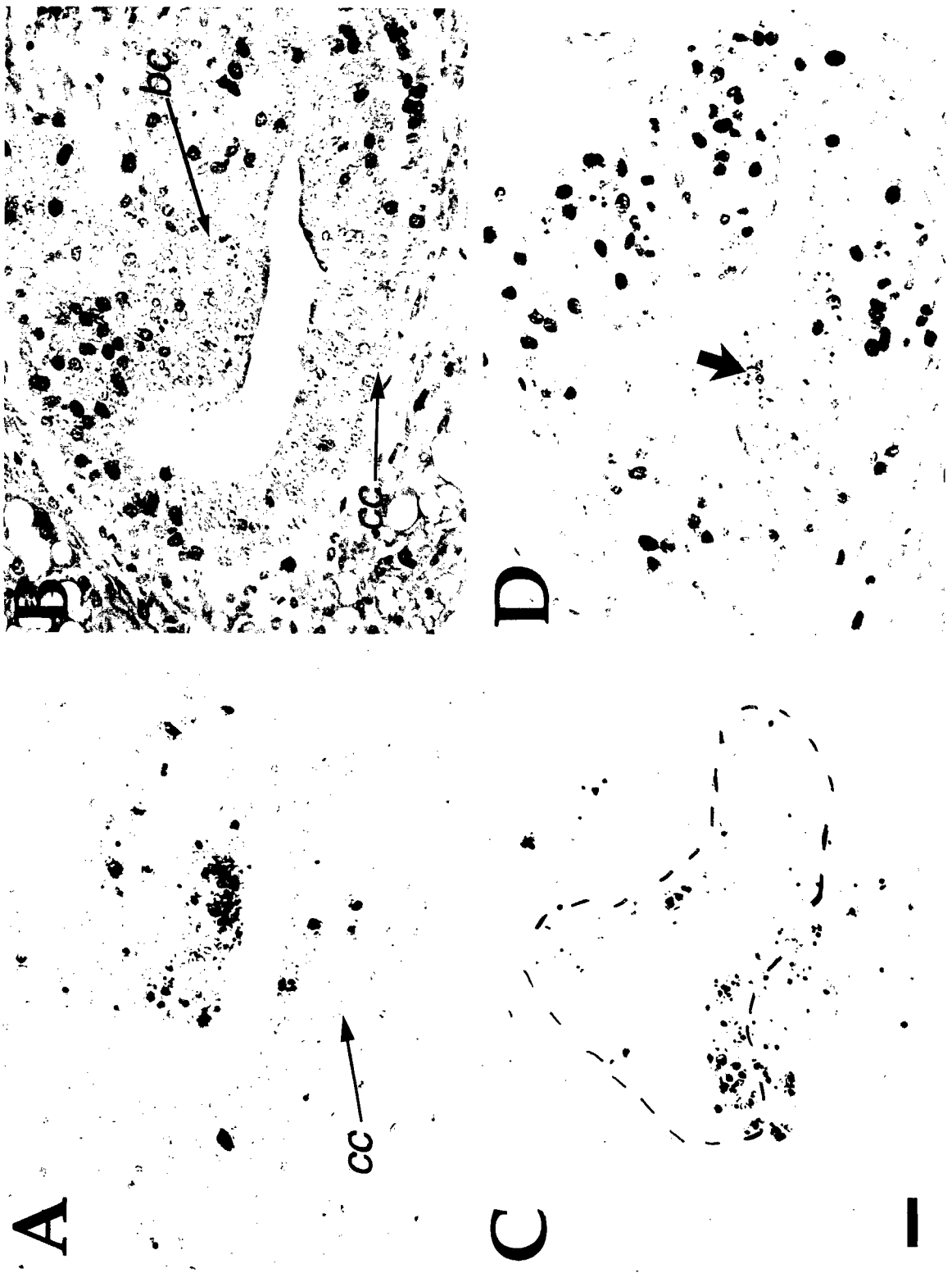


Fig. 3

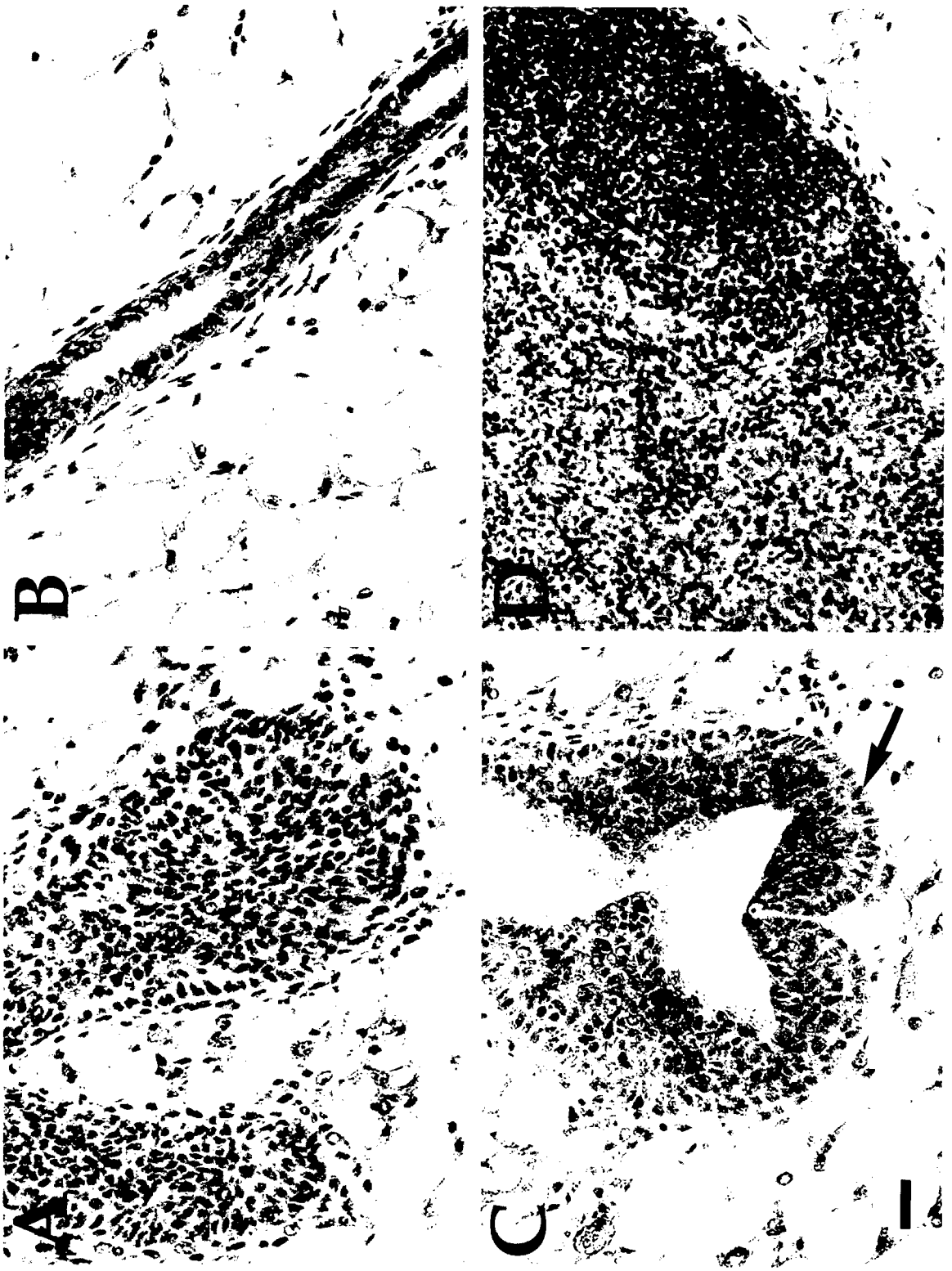

B
D
A
C

Fig. 5

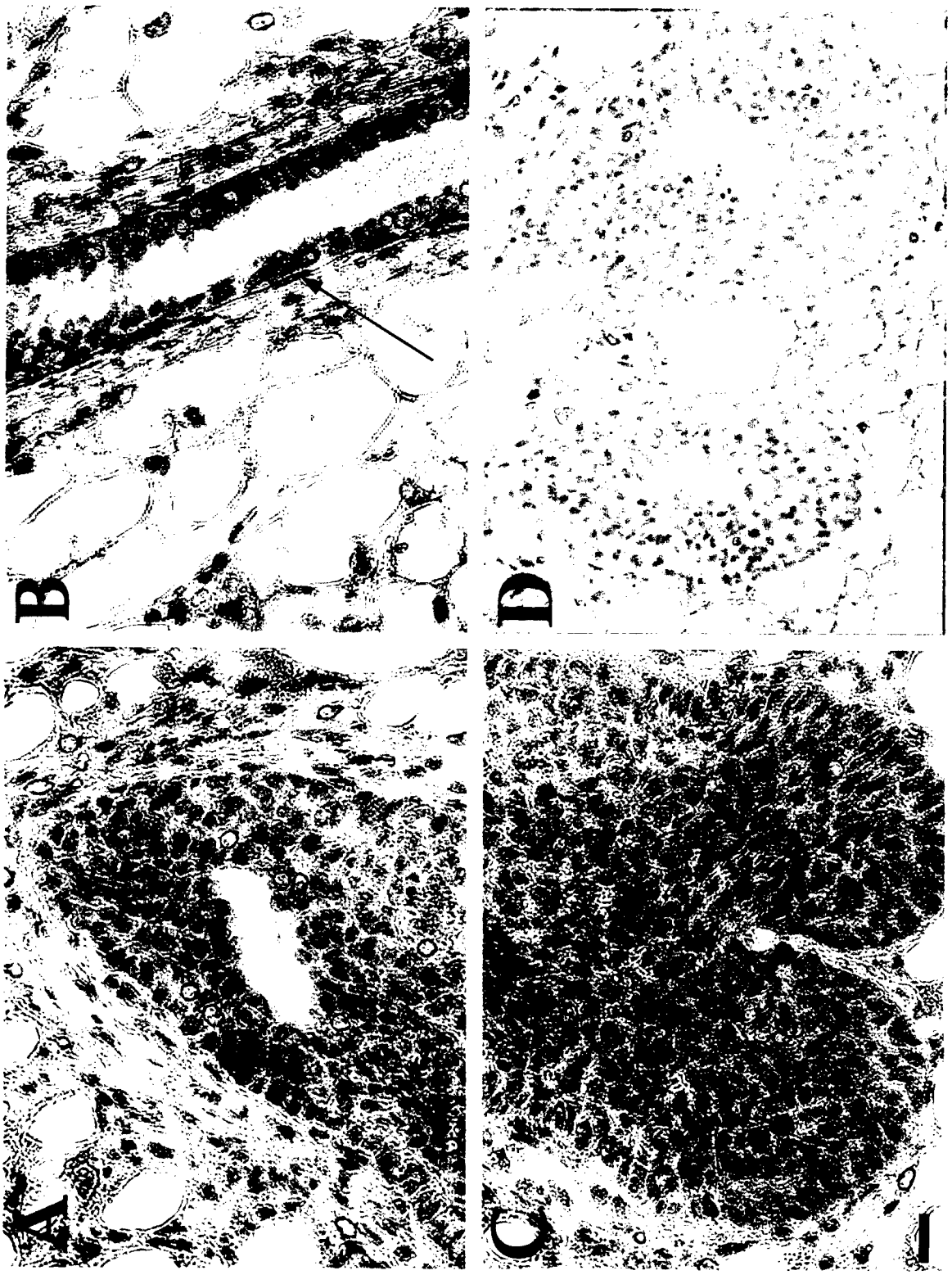


Fig. 6

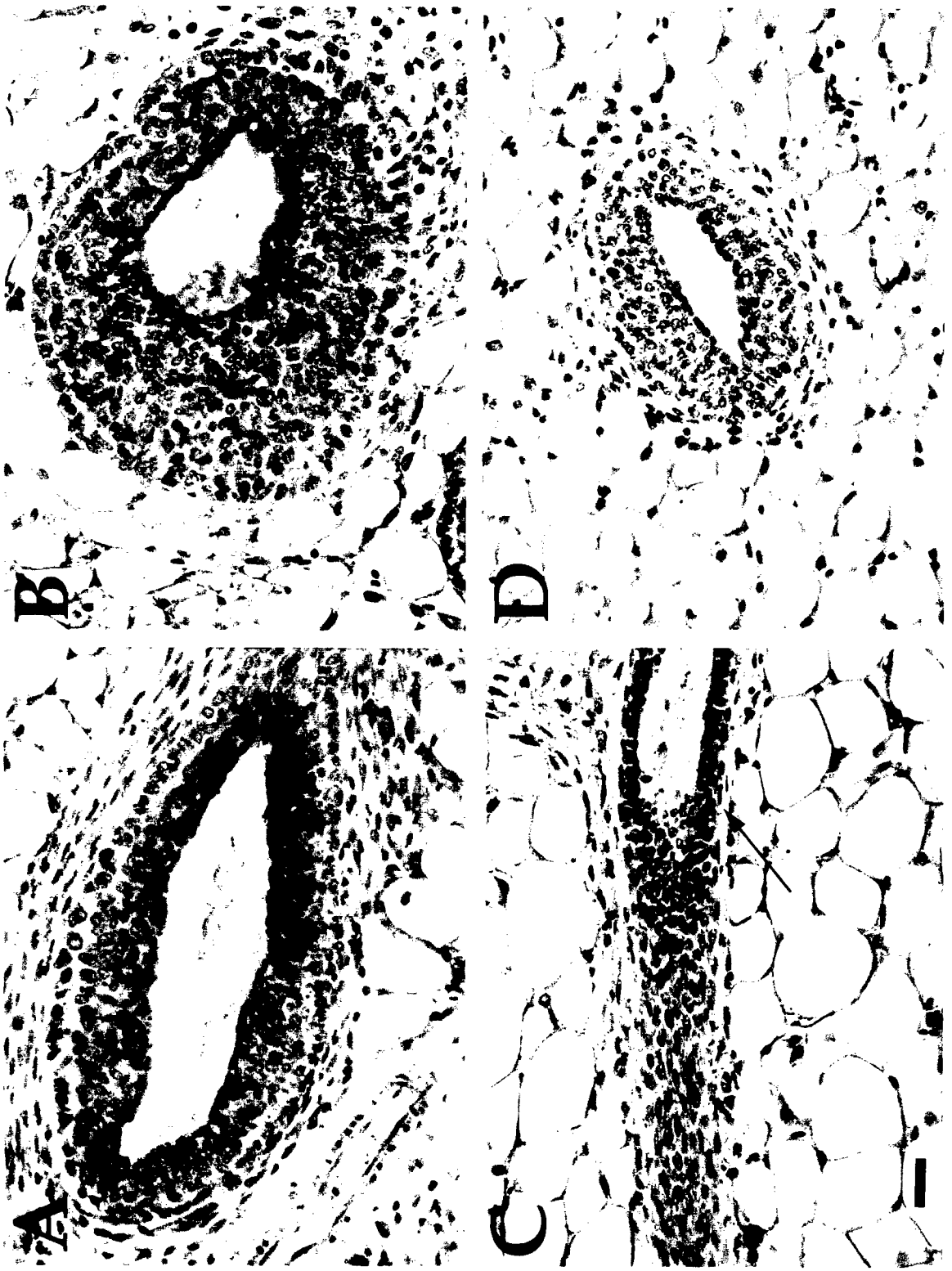
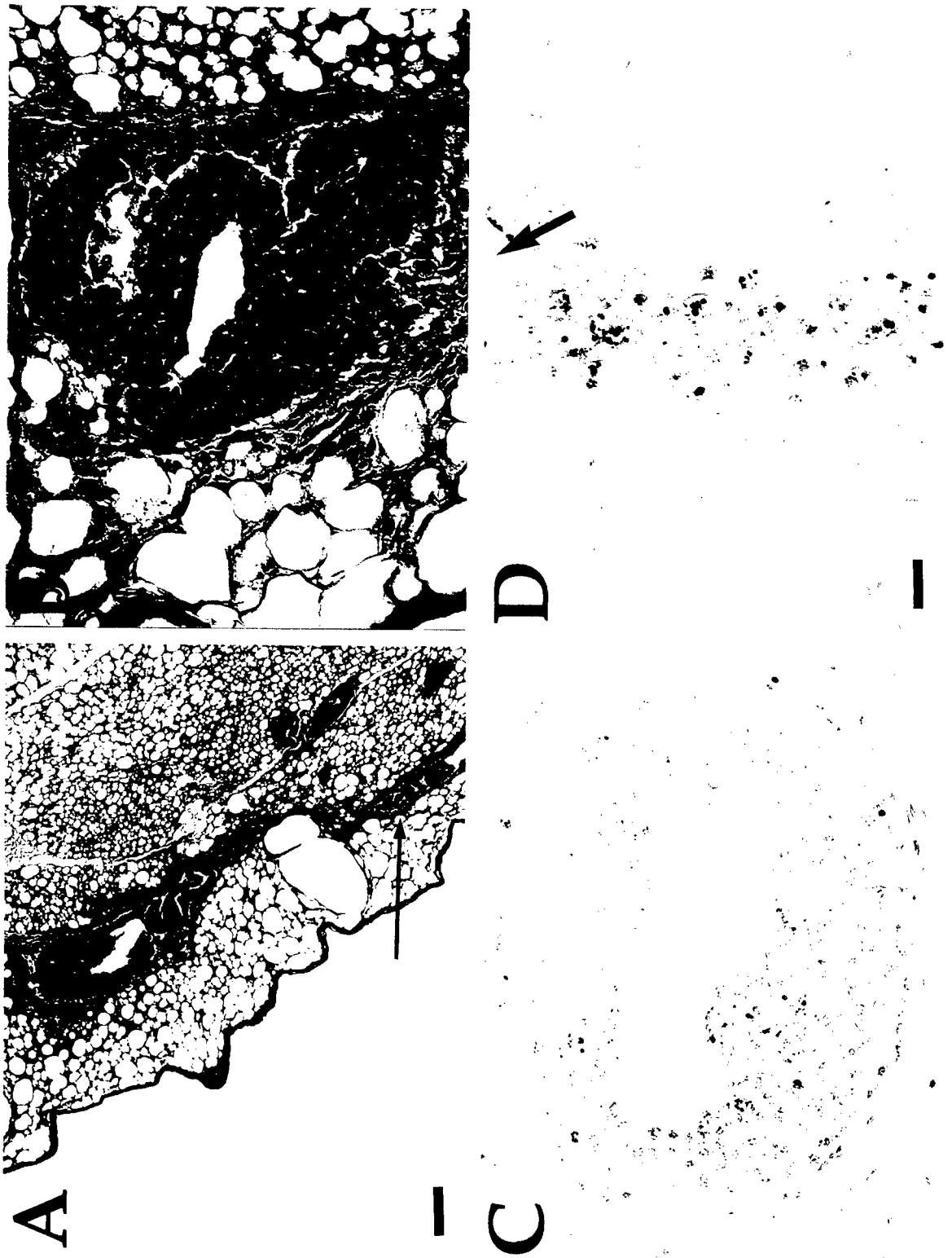
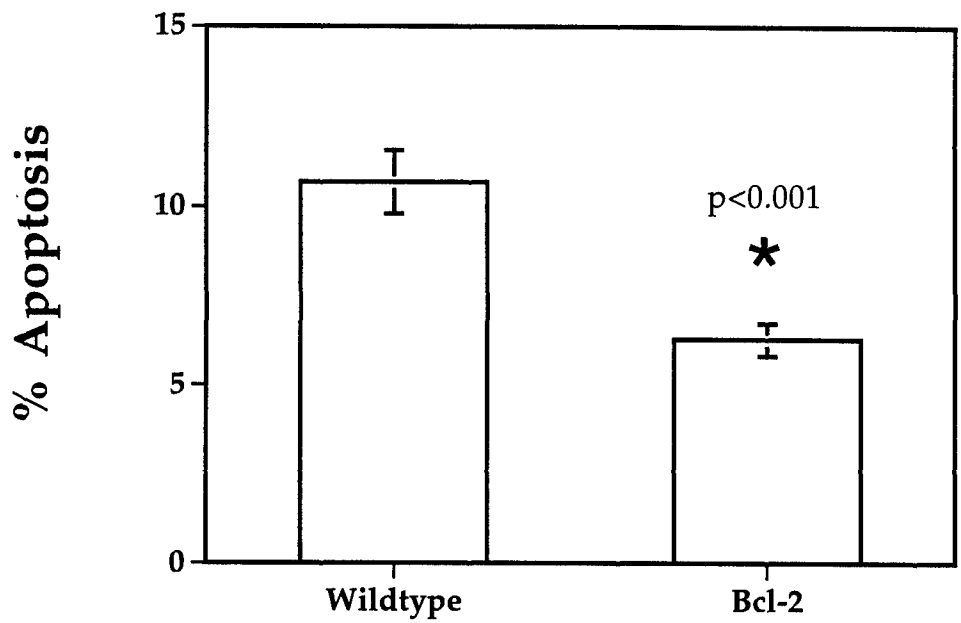


Fig. 7




% Apoptosis
15
10
5
0
p<0.001
*
Wildtype
Bcl-2