bcl-2 Overexpression Inhibits Cell Death and Promotes the Morphogenesis, but Not Tumorigenesis of Human Mammary Epithelial Cells

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Abstract. Overexpression of the B cell leukemia/lymphoma-2 (*bcl-2*) gene has been shown to confer a survival advantage on cells by inhibiting apoptosis. In epithelia, the *bcl-2* gene is also related to development and differentiation, and the protein is strongly expressed in the embryo in the epithelial cells of the developing mammary gland. To investigate directly the effect of *bcl-2* on human epithelial cells, we used an amphotropic recombinant retrovirus to introduce the gene into nontumorigenic cell lines developed from luminal epithelial cells cultured from milk. Here we demonstrate that while *bcl-2* overexpression does not directly induce the tumorigenic phenotype, it provides a survival advantage to the mammary epithelial cells by inhibiting cell death at confluence or under conditions of serum starvation. *bcl-2* can also affect the phenotype of the original epithelial cells, and promote epithelial–mesenchymal conversion, accompanied by loss of the cell adhesion molecules E-cadherin and $\alpha 2\beta 1$ integrin. The extent of the epithelial–mesenchymal conversion varies with small differences in the phenotype of the parental line and with the level of expression of Bcl-2 and in some cases cell lines emerge with a mixed phenotype. The increased survival of Bcl-2–expressing cells at confluence results in multilayering, and the development of three- dimensional structures. Where a mixed phenotype is observed these structures consist of an outer layer of polarized epithelial cells separated by a basement membrane-like layer from an inner mass of fibroblastoid cells. Branching morphogenesis of *bcl-2* transfectants is also observed in collagen gels (in the absence of fibroblast growth factors). The results strongly indicate that by increasing their survival under restrictive growth conditions, and by modifying the epithelial phenotype, *bcl-2* can influence the specific morphogenetic behavior of mammary epithelial cells.

THE B cell leukemia/lymphoma-2 (*bcl-2*)[1] gene was identified while studying the t(14;18) chromosome translocations which frequently occur in B cell leukemia and follicular lymphoma (Yunis et al., 1982; Tsujimoto et al., 1984; Bakhshi et al., 1985; Clearly et al., 1986). *bcl-2* expression confers a survival advantage to a variety of cell types including lymphoid cells, fibroblasts, and neuronal cells by inhibiting apoptosis induced by growth factor deprivation (Tsujimoto, 1989; Alnemri et al., 1992; Baffy et al., 1993) or applied stress such as heat shock and ethanol treatment (Deng and Podack, 1993). Cooperative expression of *bcl-2* with *c-myc*, *ras*, or viral genes has been suggested to be important in cell immortalization and tumorigenesis (Vaux et al., 1988; Reed et al., 1990; Strasser et al., 1990; Fanidi et al., 1992).

Recently, *bcl-2* expression has been described in epithelial cells and in various types of epithelial malignancies (Lu et al., 1993*a*; Leek et al., 1994). The expression of Bcl-2 in epithelial organs is mainly associated with cell populations capable of proliferation (Hockenbery et al., 1991; Lu et al., 1993*b*) and with tissue development and morphogenesis. In the resting breast, Bcl-2 is expressed in some luminal epithelial cells (Nathan et al., 1994), and the level of expression shows a cyclic variation during the menstrual cycle (Sabourin et al., 1994). In the developing fetal breast, Bcl-2 is expressed in the basal cell layer of the budding mammary gland. Bcl-2 is also expressed at a high level in more than 70% of breast carcinomas and the expression has been found to be inversely related to the apoptotic cell index (Chan et al., 1993; Leek et al., 1994). These observations suggest that Bcl-2 expression may be

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1. *Abbreviations used in this paper*: Ab, antibodies; bcl-2, B cell leukemia/lymphoma-2; CAT, chloramphenicol acetyl transferase; CFU, colony-forming units; DAB, 3,3'-diaminobenzidine tetrahydrochloride; PAP, peroxidase anti- peroxidase; PEM, polymorphic epithelial mucin.

involved in the growth and morphogenesis of the human breast and possibly in the evolution of breast malignancies. However, there has been no direct demonstration of an effect of Bcl-2 overexpression on the behavioral properties of the normal or malignant mammary epithelial cells.

To investigate the effect of overexpression of *bcl-2* on human mammary epithelial cells, we have used an amphotropic recombinant retrovirus to introduce the gene into nontumorigenic cell lines developed from mammary epithelial cells cultured from milk (Bartek et al., 1991). The original cell line MTSV1-7 and a subclone HB2 (Berdechevsky et al., 1994) both retain many features of the luminal epithelial cells from which the majority of breast carcinomas develop. These cell lines also undergo morphogenesis in collagen gels in vitro (Berdechevsky and Taylor-Papadimitriou, 1991; Berdechevsky et al., 1994), thus allowing analysis of the effects of Bcl-2 overexpression on these parameters. Analysis of the transfectants showed that although overexpression of Bcl-2 did not induce the tumorigenic phenotype, the transfectants grew to higher cell densities that resulted in the formation of multilayers and three-dimensional structures at confluence. Epithelial polarity was also affected and an epithelial to mesenchyme transition was seen to varying degrees in the different clones. In some cases this led to a pleiotypic differentiation and formation of organ-like structure in dense culture on plastic. Branching morphogenesis in collagen gels was also seen. The results suggest that overexpression of *bcl-2* can provide survival advantages under normally restricted growth conditions and promote phenotypic and morphological differentiation of mammary epithelial cells.

Materials and Methods

Cell Culture

The cell lines MTSV1-7 and HB2 (Bartek et al., 1991; Berdechevsky et al., 1994) were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10 μg/ml insulin (Sigma Immunochemicals, St. Louis, MO), 5 μg/ml hydrocortisone (Sigma), 0.3 μg/ml glutamine and 10% FCS (GIBCO BRL, Gaithersburg, MD) (referred to as growth medium). The retroviral infectants derived from MTSV1-7 or HB2 were maintained in the same medium with the addition of 0.4 μg/ml of puromycin (Sigma).

Development of Helper-free Amphotropic bcl-2/Retrovirus Producer Cell Lines

Amphotropic *bcl-2*/retrovirus producer cell lines were developed by infection of AM12, an amphotropic packaging cell line, with an ecotropic *bcl-2*/retrovirus based on the pBabe puro vector according to the method described by Morgenstern and Land (1990). Infected cells were selected with 2 μg/ml puromycin. The retroviral titres of the isolated clones ranged from 10^5 to 10^6 colony-forming units (CFU)/ml as detected by titration for puromycin resistance on NIH 3T3 cells. The amphotropic *bcl-2*/retrovirus producer cell lines were grown to full confluence and the spent medium was replaced with half as much fresh medium. The virus-containing medium was harvested following 3-d incubation, filtered, quick-frozen in dry ice, and stored in aliquots at −70°C.

Development of Human Mammary Epithelial Cell Lines Expressing bcl-2 and or the Puromycin-resistance Marker

For infection of the epithelial cell lines, MTSV1-7 and HB2, 2 ml of viral stock (with or without dilution) containing 8 μg/ml polybrene was added to each dish. Infection proceeded for 4 h and cells were cultured for 2 d before being split 1 in 10 in selective medium (0.4 μg/ml puromycin) until drug-resistant cells grew out. Individual colonies could only be identified when the viral stock were used as 1:10 or more dilution and ring cloned using Whatman filter paper soaked in trypsin/versine. Selected clones were referred to as MTSV1-7 B1 or HB2 B1 etc. Where a high concentration of virus was used, most cells survived selection and the cultures from MTSV1-7 were also expanded and the mixed culture studied here is referred to as MTSV1-7Bcl-2. Cells were also infected with an amphotropic retrovirus derived from the pBabe puro vector and the cell lines which were derived from clones are referred to as MTSV1-7Puro1 etc. or HB2Puro1 etc. The cell line derived from the MTSV1-7 mixed culture after using a high multiplicity of infection is referred to as MTSV1-7Puro. The experiments were carried out in accordance with the British Advisory Committee for Genetic Manipulation/Health and Safety Executive Guidelines.

Antibodies

The name, specificity and source of antibodies (Ab) are listed in Table I.

Immunofluorescence and Immunoperoxidase Staining

Cells were grown on glass coverslips, washed with PBS, air-dried and fixed with methanol/acetone (1:1, precooled to 20°C) for 10 min for cytoplasmic protein detection, and with 4% formaldehyde for 10 min for membrane protein detection. After blocking with 20% FCS/PBS for 30 min, cells were incubated with appropriate first Ab and then detected with FITC-conjugated rabbit anti-mouse Ig (1:40; Dako Corp., Carpinteria, CA) or FITC conjugated sheep anti-rabbit Ig (1:40; Dako).

Table I. Antibodies

Antibody	Antigen	Species	Dilution	Source (reference)
bcl-2/124	Bcl-2	Mouse	1:50	Dako (Pezzella et al., 1990)
HECD-1	E-cadherin	Mouse	1:100	Dr. M. Takeichi* (Shimoyama et al., 1989)
HAS4	α2 integrin	Mouse	1:100	Dr. F. Watt‡ (Tenchini et al., 1993)
J143	α3 integrin	Mouse	1:20	Dr. A. Albino§ (Wayner and Carter, 1987)
MAR4	β1 integrin	Mouse	1:100	Dr. M. Colnaghi‖ (Pellegrini et al., 1992)
LLOO2	Keratin 14	Mouse	Undiluted	(Leigh et al., 1988)‡
DA7	Keratin 18	Mouse	Undiluted	Dr. J. Bartek (Lauerova et al., 1988)
BA16	Keratin 19	Mouse	Undiluted	(Bartek et al., 1985)‡
HMFG1	PEM	Mouse	Undiluted	(Burchell et al., 1983)‡
HMFG2	PEM	Mouse	Undiluted	(Burchell et al., 1983)‡
Dako-vimentin	Vimentin	Mouse	1:100	Dako (Osborn et al., 1984)
ZO-1	ZO-1 protein	Rabbit	1:100	ZYMED (Willott et al., 1992)
Collagen Type IV	Collagen Type IV	Rabbit	1:100	Euro-Diagnostics (Visser et al., 1986)

PEM, polymorphic epithelial mucin.
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§ Sloan-Kettering Institute, New York.
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For structural examination, cultured cells were stripped off tissue culture flasks with 20 mg/ml dispase (Boehringer Mannheim Corp., Indianapolis, IN) and cell sheets were fixed with 10% neutral-buffered formalin and wax-embedded for paraffin sections, and then stained by H & E or with antibodies using the three step peroxidase anti-peroxidase (PAP) method. Following the first Ab, rabbit anti–mouse Ig (1:20; Dako) or swine anti–rabbit Ig (1:100; Dako), and then mouse PAP (1:50; Dako) or rabbit PAP (1:50; Dako) were applied. Peroxidase activity was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB, 1 mg/ml in PBS) and H_2O_2 (1 μl/ml) and the cells were counterstained with haemotoxylin.

Confocal Microscopy

Cells were grown on 9-mm glass coverslips for immunofluorescence staining as described above. Cells were mounted in glycerol/PBS solution and analyzed on a Nikon Optiphot Microscope viewing horizonal (Z-series) and vertical (XZ-series) sections through the cells.

Fluorescence Activated Cell Sorting Analysis

Cells grown to confluence were harvested by trypsinization and washed with 5% FCS/PBS. The suspended cells were incubated with first Ab for 45 min, washed twice, and then incubated with FITC-conjugated rabbit anti–mouse Ig (1:40; Dako) for an additional 45 min. Finally, cells were washed and resuspended in 1 ml PBS. All steps were carried out at 4°C after trypsinization. A minimum of 5,000 cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, Cockeysville, MD).

Growth and Survival Assays

Subconfluent growing cells were suspended by trypsinization and for each cell line, 1×10^5 cells were plated in 6-cm culture dish in triplicate in DME supplemented with 10, 2, or 1% FCS. Medium was changed every 3 d and the number of attached cells counted at day 3, 6, 9, 15, and 18.

To evaluate cell death at confluence, floating cells in culture medium, indicative of epithelial cell death, were collected at time of medium changes (days 9, 15, and 18). Since the number of dead cells was difficult to be determined accurately due to degradation, DNA was instead extracted from the floating cells by phenol–chloroform and measured by spectrophotometry.

Electron Microscopy Examination

Floating cells from supernatant after confluence were pelleted and fixed with glutaraldehyde (2.5% wt/vol in 0.1 M Sorensen's phosphate buffer, pH 7.4) for 2 h at room temperature. The pellets were washed with the buffer and postfixed in 1% osmium tetraoxide for an hour. Samples were dehydrated and embedded in araldite resin (Taab Laboratories Equipment Ltd., Alder Maston, UK). 100-nm sections were mounted on carbon/formvar-coated grids and stained with uranyl acetate for 10 min followed by saturated lead citrate for 4 min before being examined using a Jeol 1200 transmission electron microscope.

Assay for Anchorage-independent Growth and Tumorigenicity

For each cell line, 2×10^4 cells were seeded in 0.3% agar on top of a 0.5% agar base in 6-cm culture dish in duplicate. The cultures were maintained in growth medium changed every 3 d for 3 wk. T47D, a breast carcinoma cell line was used as positive control.

To assess the tumorigenicity, 1×10^7 cells per site were injected subcutaneously in *nu-nu* mice. For each cell line four mice were implanted at two sites per mouse. The mice were monitored for 3 mo. ce1, a *cerbB*-2 transformed tumorigenic cell line originated from MTSV1-7 (D'Souza et al., 1993), was used as tumor control.

Cell Growth in Collagen Gel

2×10^4 cells were suspended in 2 ml of neutralized isotonic bovine dermal collagen (Vitrogen 100; Imperial Laboratories Ltd., Andover, Hants, UK) according to manufacturer's instructions. The mixture was poured into 3-cm tissue culture dishes and incubated at 37°C for 2 h to gel. Medium was then added and the cells allowed to grow for 2 wk.

Probes and Northern Blot Analysis

A 950-bp EcoRI fragment of *bcl-2* cDNA, 4.5-kb XbaI/KpnI fragment of the α2 integrin cDNA, 2.6- and 0.6-kb EcoRI fragments of E-cadherin cDNA and a 330-bp fragment of vimentin cDNA were labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) by random priming and used as probes for appropriate mRNA detection.

Total cellular RNA from the cell lines was isolated according to the method of Chomczynski and Sacchi (1987). 25 μg of RNA from each cell line was denatured in 1 × MOPS buffer, containing 0.66 M formaldehyde and 50% (vol/vol) formamide, run on a 1.2% agarose-formaldehyde gel, and transferred onto membranes (Hybond-N; Amersham). The membrane-bound RNA was hybridized to labeled probe according to the protocol described by Church and Gilbert (1984). Hybridization was performed in 0.2 M sodium phosphate, pH 7.2, 0.1 M EDTA, 7% SDS, 45% formamide and 250 μg/ml denatured salmon sperm DNA at 42°C for 16 h. The filters were sequentially washed twice with 0.5% SSC/0.1% SDS at 65°C for 20 min and 0.1% SSC/0.1% SDS at 65°C for 20 min. Filters were exposed to Fuji film at 70°C. To assess the loading of RNA, the membranes were reprobed for the glyceraldehyde-3-phosphate dehydrogenase gene.

Western Blot Analysis of Protein Expression

The level of expression of Bcl-2 and E-cadherin was assessed by subjecting 100 μg of total cell lysates to immunoblot analysis. Confluent cell cultures were washed with complete PBS and lysed in HNET buffer (50 mM Hepes, pH 7.5, 100 mM sodium chloride, 1 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, and 1 mM PMSF) for Bcl-2 detection, and in HNET buffer containing 1 mM calcium chloride for E-cadherin detection. After clarification of the lysates by centrifugation at 15,000 *g* for 10 min at 4°C, the protein concentration of the lysates was estimated using the Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA). Samples were then electrophoretically separated on a 5% stacking/12.5% running SDS–polyacrylamide gel for Bcl-2 detection and on a 5% stacking/7.5% running SDS–polyacrylamide gel for E-cadherin detection. Immunoblots for Bcl-2 were blocked with 5% BSA and 0.1% Tween 20 in PBS (blocking buffer) for 2 h followed by incubation with Ab bcl-2/124 (1:50) at room temperature for one hour. Immunoblots for E-cadherin were blocked with 5% skimmed milk and 1% BSA in complete PBS (blocking buffer) for 3 h followed by incubation with Ab HECD-1 (1:100) in blocking buffer at 4°C overnight. The first Abs were detected using ^{125}I-labeled sheep anti–mouse Ig (0.5 μci/ml).

Transfections and CAT Activity Assays

The chloramphenicol acetyl transferase (CAT) constructs containing the E-cadherin regulatory sequences (p0.2CAT and p6.5CAT) were kindly provided by Dr. J. Behrens (Delbruck Centre, Berlin, Germany), the control plasmid, pGCAT-A, by Drs. T. Frebourg and O. Brison (Laboratory of Molecular Oncology Institute, Gustave Roussy, France). pJ3 Luc containing the luciferase gene driven from the SV40 promoter was obtained from Dr. S. Goodbourn (St. Georges Medical Hospital, London, UK).

Cells (2×10^5) were plated on 6-cm plates and incubated overnight and then transiently transfected by the calcium phosphate coprecipitation method as previously described (D'Souza and Taylor-Papadimitriou, 1994). 10 μg of the CAT plasmid together with 2 μg of the luciferase expression plasmid, pJ3 Luc, were used for each 6-cm dish in duplicate for each sample in individual experiments. Transfection was carried out for 12 h and the precipitate removed by repeated washing of the cells with serum-free medium before replacing with fresh medium. Cells were grown for 48 h after transfection, and then lysed in 200 μl of NP-40 buffer (0.65% NP-40, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl). 30 μl of lysate was incubated at 68°C for 10 min and the CAT activity was assayed essentially as described previously (Kovarik et al., 1993). CAT activities have been expressed as the ratio of the CAT activity (counts/min/2 h) to luciferase activity (arbitrary units of chemiluminescence).

Results

Isolation of Clones of MTSV1-7 and HB2 Cells Overexpressing the bcl-2 Gene

MTSV1-7 is a cloned cell line originally developed by im-

Table II. Cell Lines Derived from MTSV1-7 and HB2 Cells

Cell lines	Expression of Bcl-2	Cell lines	Expression of Bcl-2
From mixed cultures			
MTSV1-7Bcl-2*	Moderate		
MTSV1-7Puro‡	Negative		
From single clones			
		HB2 B4*	High
		HB2 B6*	Moderate
MTSV1-7 B1*	Low	HB2 B9*	Low
MTSV1-7 B7*	High	HB2 B12*	High
MTSV1-7 B10*	High	HB2 B17*	High
MTSV1-7 Puro 3‡		HB2 Puro2‡	
MTSV1-7 Puro 14‡		HB2 Puro4‡	
		HB2 Puro8‡	

*Cells infected with recombinant amphotropic virus expressing Bcl-2 and the puromycin resistance markers.
‡ Cells infected with recombinant amphotropic virus expressing puromycin resistance marker only.

mortalizing cells cultured from human milk with SV40TAg. The cells are non tumorigenic, do not show anchorage independent growth and reflect the phenotype of the luminal epithelial cell in the profile of keratins they express (7, 8, 18, and 19) and in their expression of an epithelial mucin (polymorphic epithelial mucin [PEM]) (Bartek et al., 1991). In collagen gels, MTSV1-7 cells form ball-like structures, a fraction of which can be induced to branch by hepatocyte growth factor or other fibroblast-derived growth factors (Berdechevsky et al., 1992, 1994). The HB2 cell line is a subline of MTSV1-7 selected in collagen for a more homogeneous branching response to fibroblast-derived

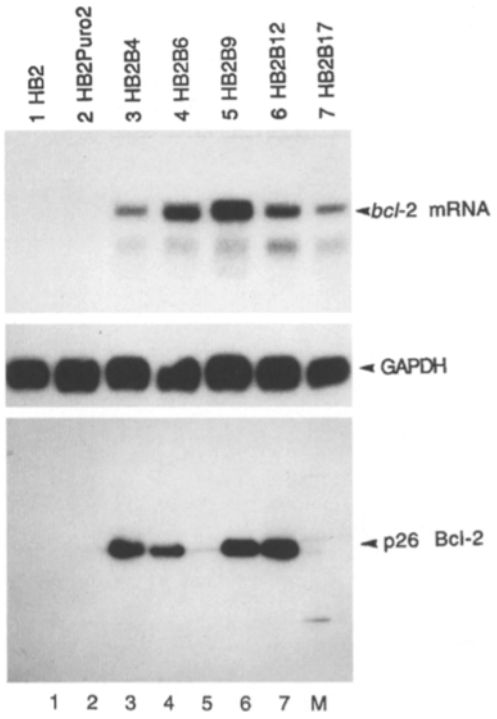

Figure 1. Analysis of the levels of *bcl-2* mRNA and protein in the HB2 clones. Northern blot showing major transcripts of 5.5 kb in the *bcl-2* transfectants, but not in the parental and puro vector control cell lines (*upper panel*). Western blot showing expression of Bcl-2 protein (*lower panel*).

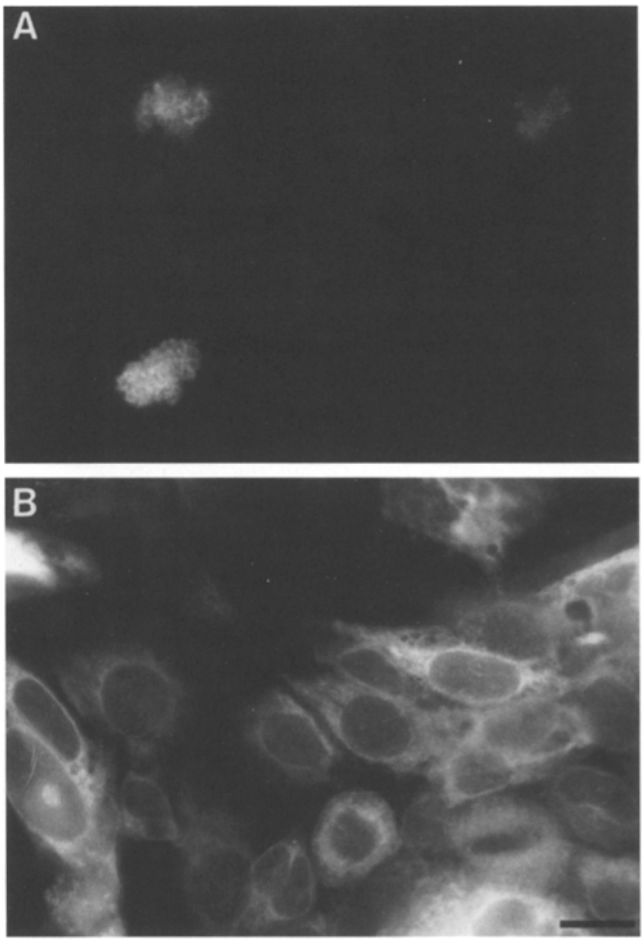

Figure 2. Detection of Bcl-2 protein by immunofluorescent staining with Ab bcl-2/124. (*A*) MTSV1-7Puro cells show staining only in a few cell nuclei. (*B*) A diffuse cytoplasmic staining is observed in nearly all cells of MTSV1-7 B7 clone. Bar, 25 μm.

factors. Since these factors can also affect cell proliferation, HB2 cells grow to a higher cell density than MTSV1-7 cells. Both cell lines exhibit well-defined epithelial polarity showing apical expression of PEM and sub-apical and lateral localization of the ZO-1 and E-cadherin components, respectively (see below).

Because epithelial cells are difficult to transfect, an amphotropic retrovirus was developed expressing the *bcl-2* gene from an LTR promoter and a selectable marker (puromycin resistance) under the control of the SV40 promoter. Cells were also infected with a virus expressing only the selectable puromycin resistance marker for isolation of control cell lines. Infection was extremely efficient and single clones could only be isolated from cultures infected with low concentrations of virus. At higher multiplicities of infection, most cells survived puromycin selection and in the case of the MTSV1-7 cell line, these mixed cultures were also expanded and used in some studies (MTSV1-7Bcl-2 or MTSV1-7Puro). Table II lists the various cell lines which were isolated and characterized.

Analysis of the levels of Bcl-2 protein and mRNA in the clones showed that there was in fact little correlation between the level of mRNA detected by Northern blots and the level of Bcl-2 protein detected by Western blots and

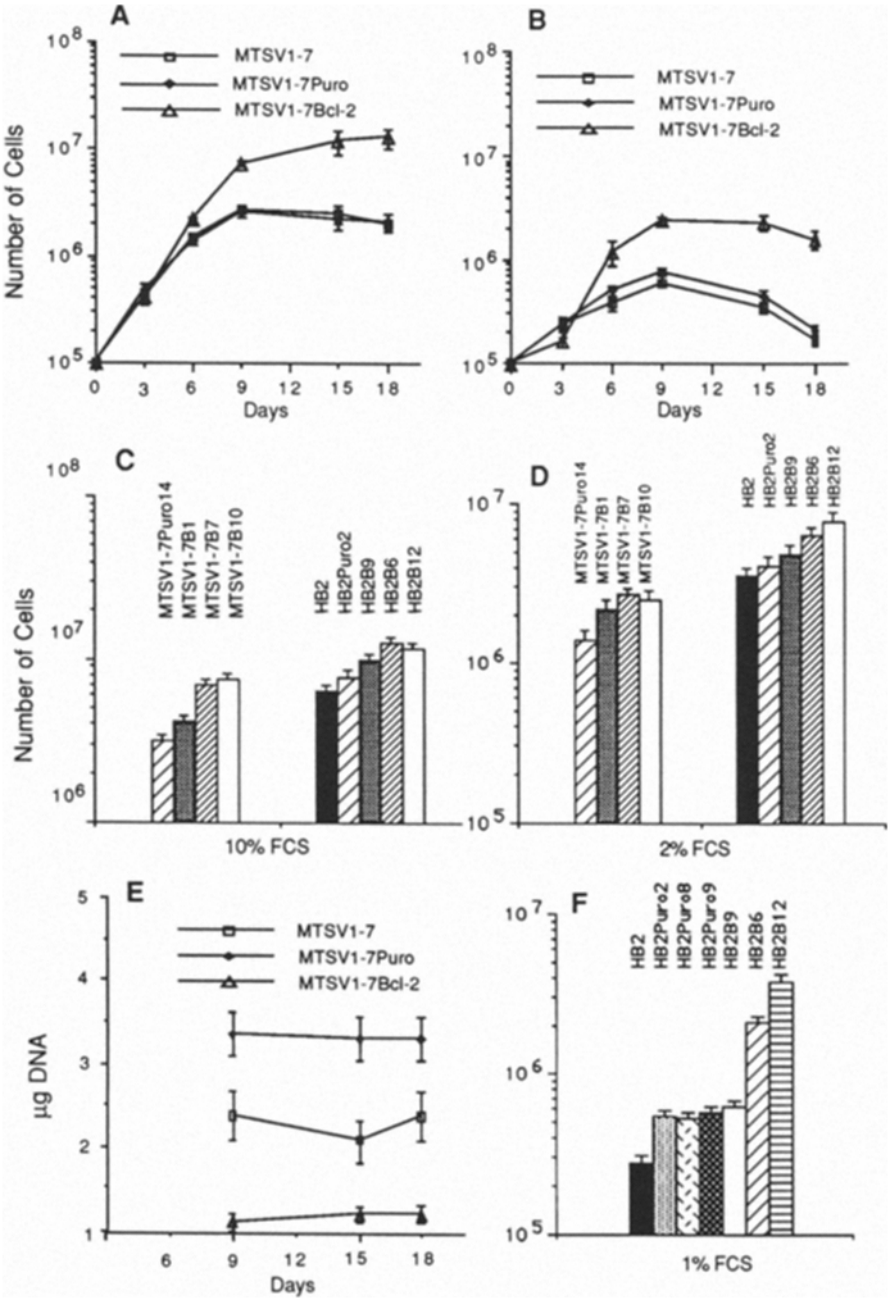

Figure 3. Growth analysis of the MTSV1-7 and HB2. Cells plated at 1×10^5 per 6-cm dish were cultured in growth medium containing 10% FCS (*A*, *C*, and *E*) or medium with 2% FCS (*B* and *D*) and 1% FCS (*F*). *A* and *B* show the growth curves of the mixed culture. *C*, *D*, and *F* shows the cell number of individual clones after 18 d of culture. The number of cells represents means of experiments in triplicate. *E* shows the amount of DNA extracted from floating cells of cultured supernatants at days 9, 15, and 18 when the medium was changed in the experiment shown in *A*. The amount of DNA shown on the ordinate is expressed as µg per 10^6 attached cells.

this is illustrated for the HB2 cloned cell lines in Fig. 1. The discrepancy was particularly evident in HB2 B9 which showed the highest mRNA level, but the lowest expression of the protein. The Bcl-2 protein was detected as a diffuse cytoplasmic stain by indirect immunofluorescence and this is illustrated for the MTSV1-7 B7 clone in Fig. 2 *B*. In the cell lines showing a high protein expression, 90% of the cells showed a high level of staining. In cell lines expressing low levels of the Bcl-2 protein (e.g., MTSV1-7B1 and HB2 B9) staining was more heterogeneous with only ~10% of cells showing strong staining, and the remainder staining positively but weakly. Parental cell lines, and lines expressing only the puromycin selectable marker did not show cytoplasmic staining but, as we have observed for immortalized cell lines (Lu et al., 1994), mitotic cells showed nuclear staining (Fig. 2 *A*).

Growth Properties of bcl-2 Transfectants

Since *bcl-2* has been found to extend the life span of cells by inhibiting apoptosis, the growth properties of the *bcl-2* transfectants were examined. Overexpression of the gene in MTSV1-7 or HB2 cells did not induce the tumorigenic phenotype as defined by either anchorage independent growth in vitro or formation of tumors in the nude mouse. However, the *bcl-2* transfectants did exhibit different properties when grown on plastic. The mixed culture of Bcl-2–expressing MTSV1-7 cells was found to grow to a higher cell density in growth medium containing 10% serum than that seen with either the parental cell line or with the cell line MTSV1-7Puro (from a mixed culture) (Fig. 3 *A*). Also the MTSV1-7Bcl-2 cells were able to proliferate and maintain cell numbers at higher levels in only 2% serum (Fig. 3

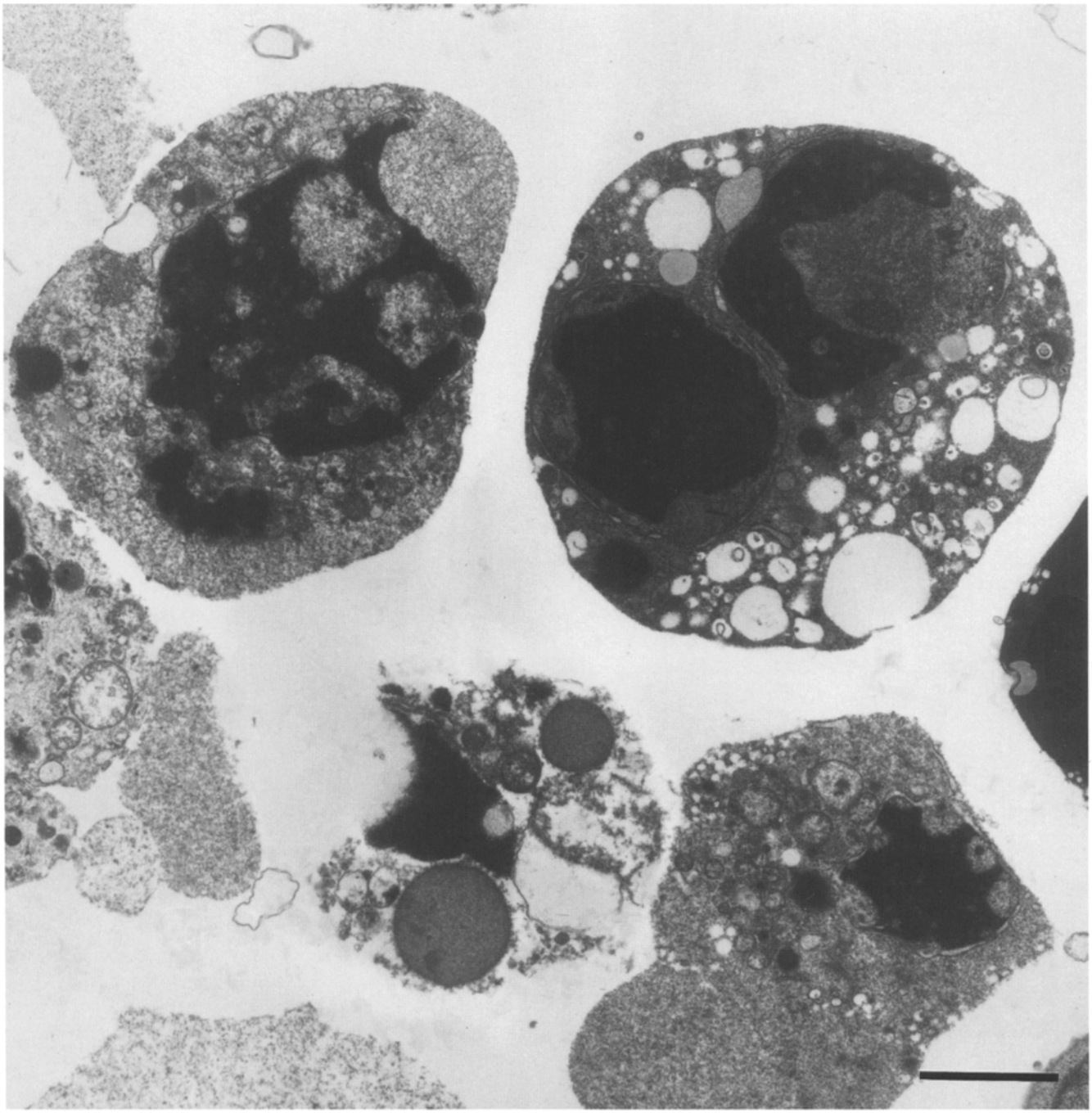

Figure 4. Electron microscopy examination of MTSV1-7. Cells shed into the supernatant from confluent cultures show large apoptotic bodies. Bar, 5 μm.

B). The effect of *bcl-2* expression on the growth of individual clones in medium containing 10 and 2% serum was also examined (Fig. 3, *C* and *D*). Small but significant increases in cell number at confluence were observed, particularly with the clones expressing high levels of Bcl-2 (MTSV1-7 B7, B10, HB2 B6, and B12; Table II and Fig. 1). All of the puromycin resistant lines listed in Table II were examined and the data shown in Fig. 3, *C* and *D* are for one example each of the MTSV1-7− and HB2-derived lines. The puromycin resistant clones showed a smaller increase in cell numbers at confluence in low and high serum as compared to the *bcl-2* transfectants.

The HB2 transfectants could survive in 1% serum (although MTSV1-7 cells and its transfectants could not). A more dramatic effect of overexpressing Bcl-2 in HB2 cells was seen on the survival of two high expressing clones (HB2 B6 and B12) grown in this low serum concentration. Fig. 3 *F* illustrates this point and also shows that none of the three puromycin transfectants of HB2 cells showed such an effect. The HB2 B9 clone which expressed low levels of Bcl-2 (see Fig. 1) did not show increased survival compared to the puromycin clones.

The MTSV1-7 and HB2 are immortal cell lines and when proliferating in sub-confluent cultures in normal

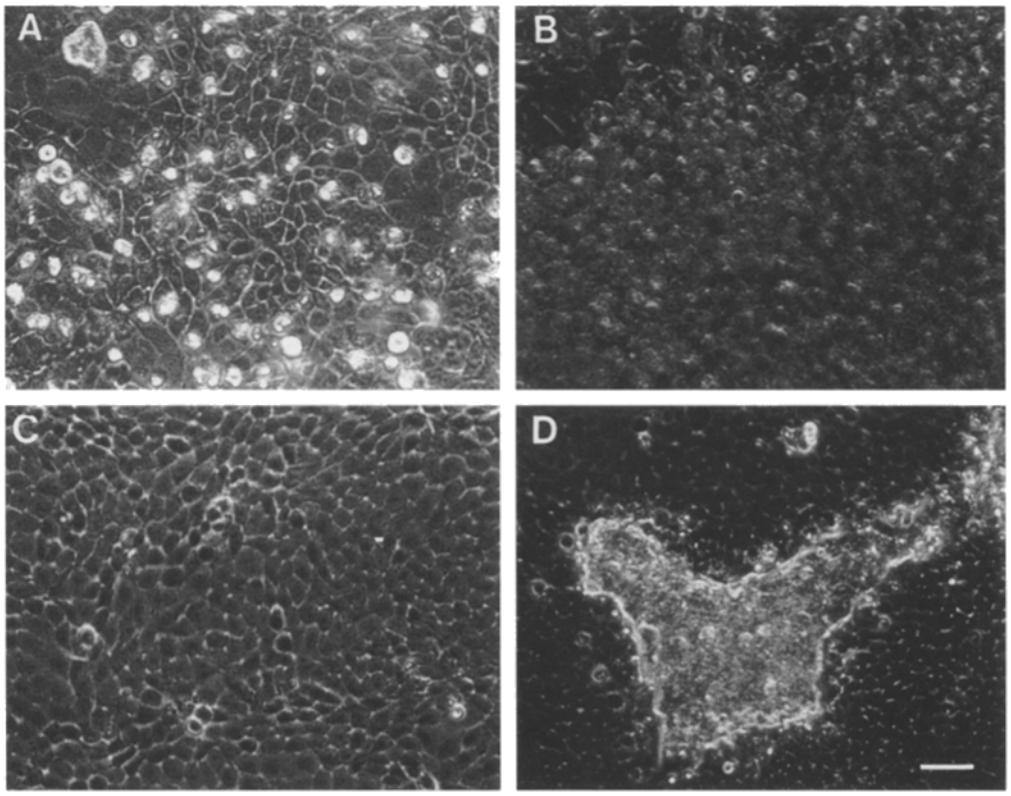

Figure 5. The pattern of growth of cells on plastic after confluence. (*A*) MTSV1-7; (*B*) MTSV1-7 B7; (*C*) HB2; (*D*) HB2 B12. Both parental lines MTSV1-7 and HB2 grow as monolayers. The *bcl-2* transfectant MTSV1-7 B7 forms multilayers (*B*) and HB2 B12 cells pile up to form islands (*D*). Cells were cultured in growth medium for two weeks. Bar, 60 μm.

growth medium do not undergo significant cell death. However after confluence or in low serum concentration, floating cells were detected in the culture medium. Morphological examination by electron microscopy of cells in the supernatant from the MTSV1-7 parental cell showed nuclear fragmentation and the presence of apoptotic bodies (Fig. 4). A possible explanation for the increase in cell numbers seen at confluence in transfectants overexpressing Bcl-2 is that fewer cells were detaching from the monolayer. Cell numbers in the supernatants from MTSV1-7 *bcl-2* transfectants were therefore compared with those found in the parental MTSV1-7 cells or in MTSV1-7Puro cultures by measuring DNA in cells pelleted from the supernatants, and the data corresponding to the experiment shown in Fig. 3 *A* are shown in Fig. 3 *E*. The data clearly show that more cells are shed into the medium in the MTSV1-7 or the MTSV1-7Puro cultures than in the MTSV1-7Bcl-2 cultures. The increased numbers of cells in the supernatants from the puromycin resistant line as compared with the parental line may be due to a slightly increased proliferation rate at confluence, resulting in increased shedding and cell death. Although the cells in the supernatants from MTSV1-7 and MSTV1-7Puro cells show nuclear fragmentation and the apoptotic bodies seen in cells dying by apoptosis, we could not detect the characteristic ladder of fragmented DNA by electrophoresis (data not shown). Whatever the mechanism involved in the cell death occurring at confluence or under serum deprivation, overexpression of Bcl-2 appears to protect the epithelial cells and allows them to grow and maintain a higher density after confluence.

In the *bcl-2* transfectants the higher cell density after confluence was accompanied by the formation of multilayers in contrast to the parental cell line which grew as monolayers (Fig. 5). Moreover, in some cases, cells in the multilayers could develop into ball-like structures which showed morphological differentiation (see below). All the puromycin transfectants listed in Table II behaved like the parental cell lines in forming only monolayers at confluence indicating that the changes in growth characteristics are likely to be due to overexpression of the *bcl-2* gene.

Changes in Epithelial Phenotype Induced by Overexpression of bcl-2

Several studies have shown that loss of the epithelial phenotype can be induced by overexpression of certain oncogenes (Reichman et al., 1992; D'Souza and Taylor-Papadimitriou, 1994). We therefore examined the epithelial polarity of the *bcl-2* transfectants as well as the level of expression of molecules relating to the epithelial phenotype. Immunofluorescent staining of subconfluent cells with specific antibodies showed that not only was there a reduction in the expression of epithelial-related molecules but this was accompanied by the appearance of vimentin, an intermediate filament protein of mesenchymal cells. The most dramatic changes were seen in the MTSV1-7 B7 and B10 clones and the MTSV1-7Bcl-2 line, where most cells were found to express high levels of vimentin, and only a few cells showed expression of keratin 19 or E-cadherin. These cell lines showed a fibroblastic morphology. In the HB2 clones and the MTSV1-7 B1 clone, most cells retained the epithelial phenotype, and only a few cells could be seen to express vimentin, the fraction being greatest (~10%) in the HB2 B9 clone. All of the puromycin transfectants listed in Table II (and a further eight clones exam-

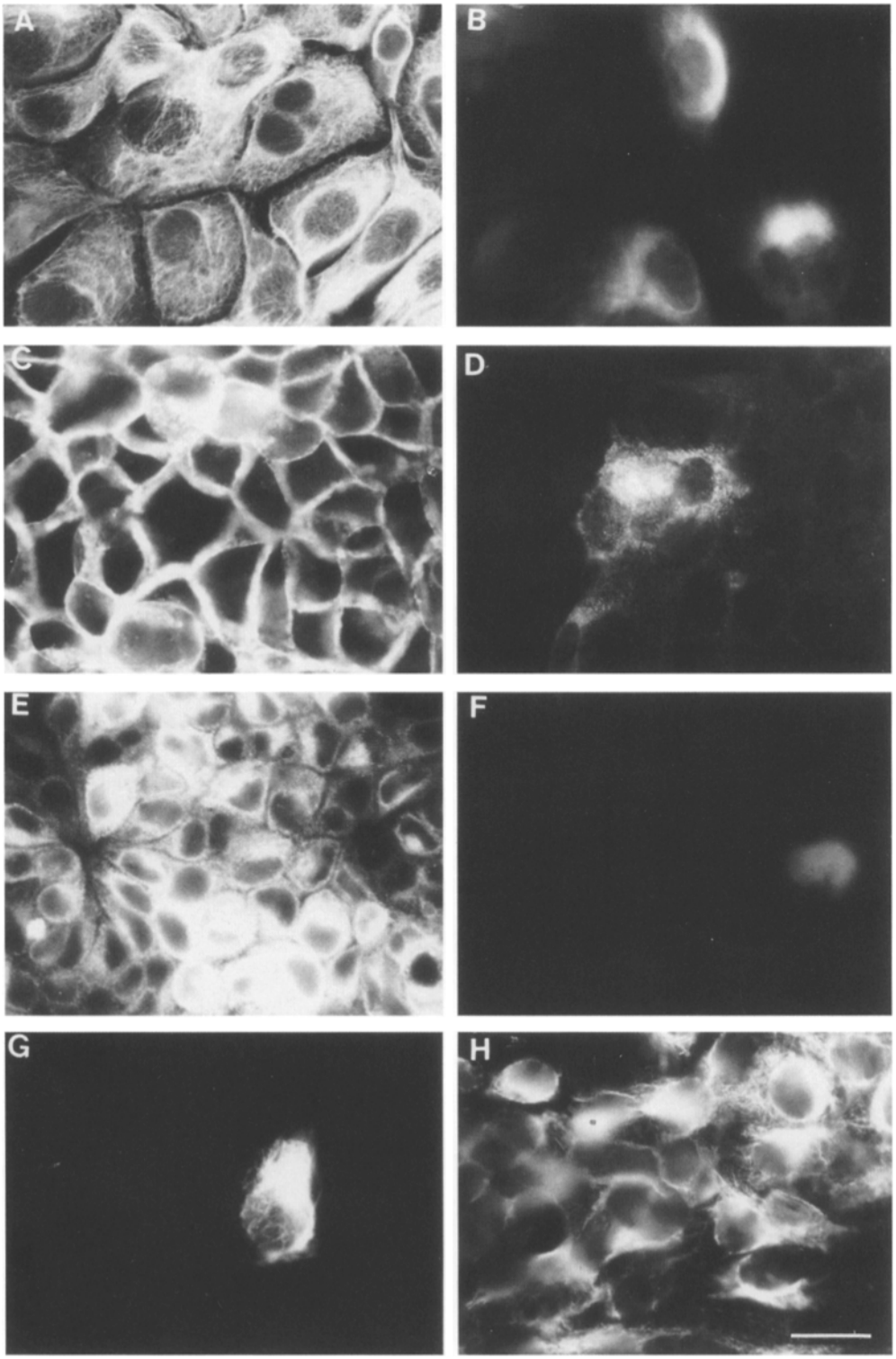

Figure 6. Immunofluorescent staining of MTSV1-7Puro14 (*A*, *C*, *E*, and *G*) and MTSV1-7 B7 (*B*, *D*, *F*, and *H*). Cells were stained with Ab BA16 to CK19 (*A* and *B*), HECD-1 to E-cadherin (*C* and *D*), HMFG2 to PEM (*E* and *F*), and with the Ab Dako-vimentin to vimentin (*G* and *H*). Keratin 19 is strongly expressed in the control cells (*A*), whereas the majority of the B7 cells show loss of expression (*B*). Similarly, peripheral localized E-cadherin is demonstrated in the control cells (*C*). In the B7 cells, the expression of the protein can still be seen in a few cells but with distorted distribution (*D*). PEM is expressed strongly in the control purotransfectant (*E*), but not in the B7 cells (*F*). In contrast, only single cells expressing vimentin are seen in the control cell line (*G*), while the B7 cells become strongly positive for vimentin (*H*). Bar, 20 μm.

ined but not listed) retained the epithelial phenotype exhibited by the parental cell lines, and did not express vimentin.

The differences between the B7 clone and the puro vector transfectant clone, MTSV1-7Puro14, as seen by indirect immunofluorescent staining are illustrated in Fig. 6. In the case of the B7 clone the rare examples of expression of keratin 19 or E-cadherin have been selected to illustrate that even when expression can be detected, its distribution is abnormal. Similarly the single cell expressing vimentin rarely seen in the parental line and in the MTSV1-7Puro clone has been selected for illustration. In the MTSV1-7 B7 and B10 clones the changes in expression of E-cadherin and vimentin could also be seen at the level of mRNA and protein by Northern and Western blot analysis. Fig. 7 illustrates this and also the lack of vimentin expression in the parental cell lines, and in the puro transfectant which express high levels of E-cadherin and keratin

Table III. Activity of E-cadherin Promoter

Constructs	Transfected cells	CAT/Luc	Fold/increase
pGCAT-A	MTSV1-7 neo	45	1.0
	MTSV1-7 B7	52	1.0
0.2 E-cadherin	MTSV1-7 neo	408	9.1
	MTSV1-7 B7	248	4.7
6.5 E-cadherin	MTSV1-7 neo	121	2.7
	MTSV1-7 B7	48	0.9

Cells were transfected with pJ3 Luc and one of the CAT reporter constructs: pGCAT-A (promoterless CAT construct), p6.5 CAT (containing 6.5 kb of mouse E-cadherin 5′ upstream sequence) or p0.2 E-cadherin (containing the −178/+17 E-cadherin promoter fragment). CAT activities are expressed as the ratio of CAT activity (counts/min/2 h). Luciferase activity (arbitrary units of chemiluminescence). The ratios obtained are expressed as an increase over that obtained with pGCAT-A.

19. Expression of keratin 18 was maintained in all the cells in the transfectants of both HB2 and MTSV1-7 although the level was lower in the vimentin-expressing cells (data not shown).

The effect on polarity of the B7 cells is illustrated in Fig. 8 where it can be seen that although some expression of the tight junction-associated protein ZO-1 is seen, the topographical distribution is disorganized and the cells form multilayers (Fig. 8, *E* and *F*). Moreover, even with the MTSV1-7 B1 clone that expressed reasonable levels of the epithelial markers including ZO-1, the distribution of the marker is already changing. Fig. 8, *C* and *D* show that the distribution of ZO-1 in the B1 clone is not as restricted as it is in the Puro14 cell line (Fig. 8, *A* and *B*).

We have recently found that overexpression of the *c-erbB2* proto-oncogene in MTSV1-7 cells inhibited expression of the α2 integrin subunit in association with reduced expression of E-cadherin (D'Souza et al., 1993; D'Souza and Taylor-Papadimitriou, 1994). This is an interesting observation since the α2β1 integrin function is involved in the in vitro morphogenesis of MTSV1-7 cells (Berdechevsky et al., 1992, 1994). We now find that expression of the α2 integrin subunit is also reduced in the Bcl-2 overexpressing MTSV1-7 cells (Fig. 9). This reduction is correlated to the proportion of fibroblastoid cells, and as with the *c-erbB2* transfectants, the effect was specific for the α2 integrin subunit, and was not seen with the α3 or β1 subunits. In the two MTSV1-7 clones, B7 and B10 where the effect on the epithelial phenotype is most marked, the changes in expression of the α2 integrin subunit could be seen at the level of mRNA (illustrated for B7 in Fig. 7). In the B1 clone where only a small proportion of cells showed the change to the mesenchymal phenotype, the reduction in expression was only seen in a subpopulation of cells (Fig. 9). The levels of the α2 integrin subunit in the Puro control lines was the same as that seen in the parental cell lines (illustrated for MTSV1-7Puro14 in Fig. 9).

Reduction in Transcription of E-cadherin in the MTSV1-7 B7 Clone

Studies with the *c-erbB2* transfectant of MTSV1-7 cells have shown that the reduction in expression of E-cadherin was due to inhibition of transcription of the E-cadherin

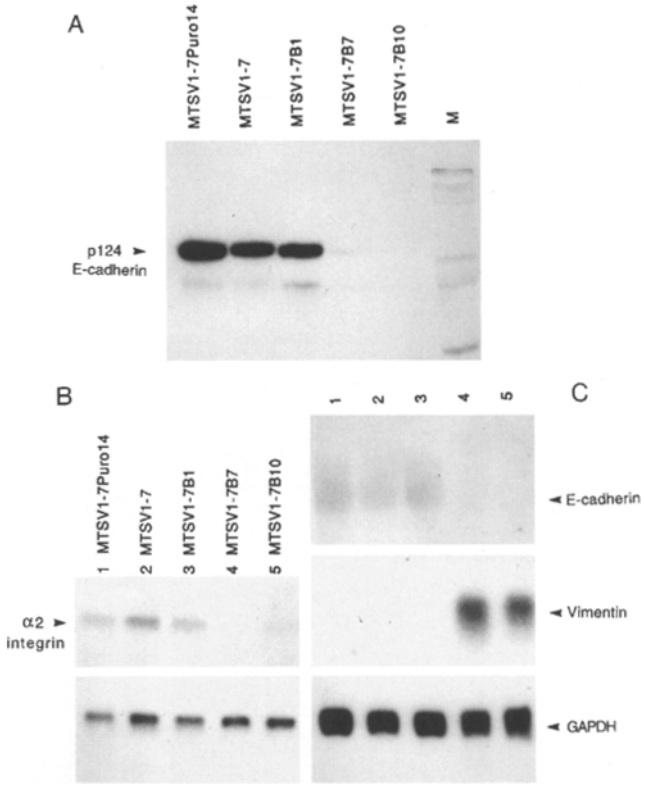

Figure 7. Western blot for the detection of E-cadherin (*A*) and Northern blots for detection of mRNA for α2 integrin (*B*), E-cadherin and vimentin (*C*). E-cadherin protein cannot be detected by MTSV1-7 B7 and B10 clones. The level of the protein in the B1 cell line is comparable to the parental and the vector control cell lines. The mRNA of E-cadherin and α2 integrin subunit is detected in the parental, the puro vector control lines and the B1 clone, but not in B7 and B10 clones. In contrast, strong vimentin expression is only seen in B7 and B10 clones.

gene. To see whether this was also true for the *bcl-2*–expressing clones, the E-cadherin promoter linked to a reporter gene (chloramphenicol transferase, CAT) were transfected into the B7 clone and CAT expression compared to the expression seen in an MTSV1-7 neo clone also transfected with the CAT construct (The neo transfectant was used since it proved to be impossible to transfect the MTSV1-7 puro clones). Table III shows the results of such an experiment that indicates that the E-cadherin promoter is less effective in driving CAT expression in the B7 clone. The decrease in transcription efficiency is ~50%.

Pleiotypic Differentiation in Confluent Cultures of bcl-2 Transfectants

As indicated above, morphologic and phenotypic analysis of the *bcl-2* transfectants showed that the cultures contained two cell types with epithelial or fibroblastic characteristics, the proportion of each varying with the cell line, particularly in the case of the MTSV1-7 transfectants. Moreover, accumulation of cells at confluence resulted in the formation of multilayers and ball-like structures. To examine the morphology and phenotype of the cells within these structures and the relation between the two types of cell, the cell layers were dislodged from the plastic (after

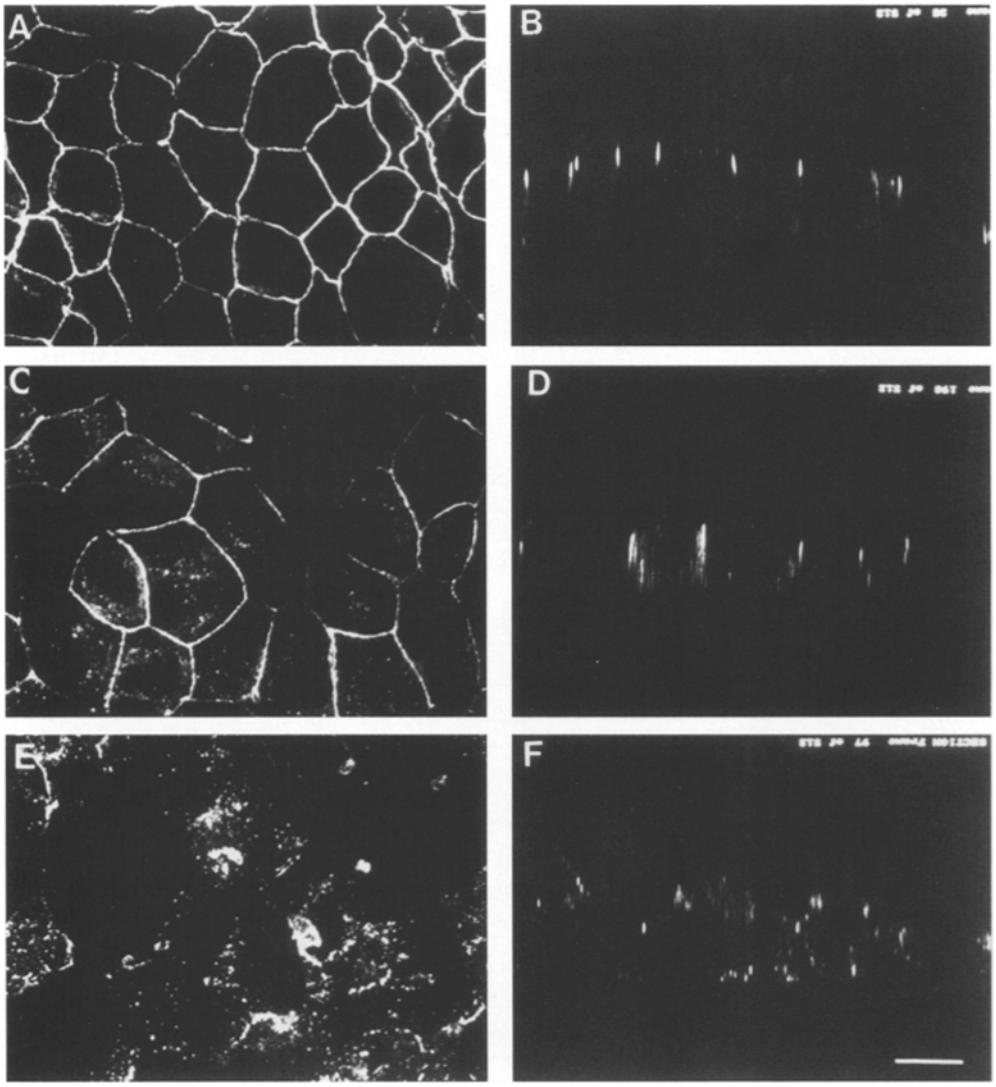

Figure 8. Effect of Bcl-2 expression on cell polarity as demonstrated by immunocytofluorescent staining for ZO-1 protein and examined by confocal microscopy. (*A*, *C* and *E*) horizontal sections; (*B*, *D*, and *F*) vertical sections. (*A* and *B*) MTSV1-7Puro14; (*C* and *D*) MTSV1-7 B1; and (*E* and *F*) MTSV1-7 B7. ZO-1 expression is present around the cell boundary (*A*) and limited to the upper part of the cell junctions (*B*) in the MTSV1-7Puro14. Reduced expression with irregularity in distribution is seen in the B1 clone (*C* and *D*). Expression of the protein is disorganized in the multilayered B7 cells (*E* and *F*). Bar, 20 μm.

being in culture for 3 wk) rolled up and processed for paraffin sections. These sections showed that the parental cell lines and the puro vector control transfectants remained as monolayers with almost all the cells showing an epithelial phenotype. The *bcl-2* transfectants however were either predominantly epithelial as with the MTSV1-7 B1 clone and most of the HB2 clones, or predominantly fibroblastic as with the MTSV1-7 B7 or B10 clones. Both single layer and multilayers were seen with the B1 clone where the majority of cells retained the epithelial phenotype, expressing mucin, keratin 19, E-cadherin, but no vimentin. However the small ball-like structures which developed showed a mixed population with fibroblast-like cells in the middle of the ball surrounded by epithelial cells. As illustrated in Fig. 10, the outer layer of cells expressed E-cadherin, keratin 19, and mucin, but no vimentin, while the inner cells showed reduced expression of E-cadherin but expressed vimentin. Mucin was expressed by both cell types in this case.

While the MTSV1-7 B7 only formed multilayers of cells with fibroblastic features, the bidirectional differentiation indicated in the B1 clone was most dramatically demonstrated in the mixed MTSV1-7Bcl-2 cell line. In the ball-like structures, the outer layer was composed of highly polarized epithelial cells with E-cadherin localized between junctions and the mucin showing a marked apical location, while the inner mass of cells showed high expression of vimentin and a loss of expression of E-cadherin and mucin (Fig. 10). Moreover the outer cell layer was separated from the inner cell mass by a membrane-like structure which could be visualised by staining with an antibody to type IV collagen (Fig. 11).

The above results suggest that while Bcl-2 overexpression protects the epithelial cells from cell death, it can also affect their phenotype and differentiation. The pathway of cell differentiation appears to be affected by both levels of Bcl-2 expression, and the differentiation potential of the individual cells under a given set of conditions. This suggestion is supported by the results from the Bcl-2–expressing HB2 lines. Although MTSV1-7 and HB2 are both cloned cell lines, the HB2 cells are more homogeneous than MTSV1-7 in their morphogenetic phenotype and Bcl-2 did not change this phenotype as significantly as it did the MTSV1-7 lines.

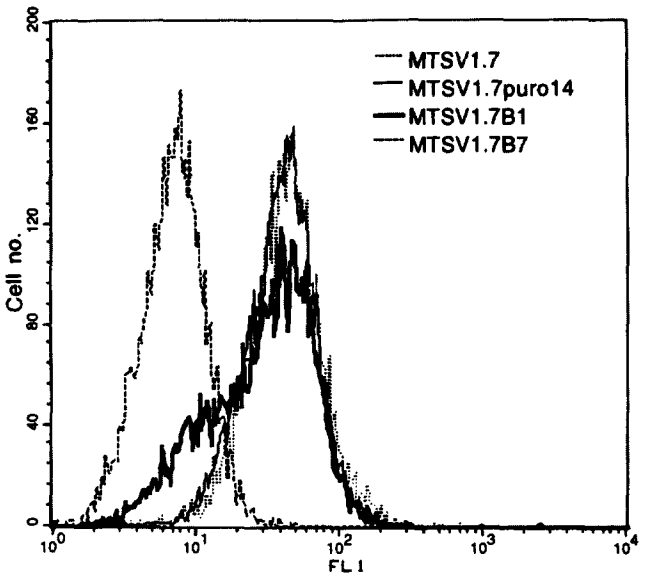

Figure 9. FACS analysis of α2 integrin expression using Ab HSA4. A marked reduction of membrane α2 expression is seen in the B7 clone while only a proportion of B1 cells show a reduced expression of α2 protein compared to the parental and the puro vector control cell lines.

Formation of Structures in Collagen Gels

The differences in the potential for morphological differentiation are also seen when the cell lines are grown in collagen gels. The parental cell lines form ball-like structures in collagen gels which can be induced to branch by fibroblast-derived factors and the HB2 cells show a more uniform smooth tubular branching response than the MTSV1-7 cells. While the transfectants expressing the puromycin resistance gene also only formed ball-like structures (Fig. 12, *A* and *D*), the cloned *bcl-2* transfectants showed some degree of branching or spiking even in the absence of added factors. The most dramatic effect was seen with MTSV1-7 B7 (Fig. 12 *B*) where most cells exhibit mesenchymal features with reduced expression of epithelial markers and increased expression of vimentin. Although the form of branching was different, it was also seen with the HB2 B9 clone where ~10% of the cells exhibited mesenchymal features (Fig. 12 *E*). Sections of the structure in the gels showed the thinner branches to be made up of fibroblastoid elongated cells, while the thicker branches (Fig. 12 *E*) were made up of epithelial like cells, in some cases surrounding a lumen (data not shown). The different form of branching seen with Bcl-2–expressing MTSV1-7 clones and Bcl-2–expressing HB2 clones, reflects the difference seen in the parental cell lines when they are grown in collagen gels in the presence of fibroblast-derived growth factors (Berdechevsky et al., 1994).

In correlating the changes in levels of the α2β1 integrin with the patterns of morphogenesis seen in the *bcl-2* transfectants, it becomes apparent that the function of the α2β1 integrin in morphogenesis in collagen is complex. Thus we have observed that the complete absence of function (induced by treatment with blocking antibodies) impairs cell–cell interactions and interferes with the formation of compact structures by HB2 cells. On the other hand the level of expression of the integrin is decreased in response to the collagen interaction (Berdechewsky et al., 1994). These observations suggest that while α2β1 integrin function is required for the formation of compact three-dimensional structures, a reduction in the level of the integrin may normally accompany their formation possibly allowing some flexibility of movement of cells relative to each other. This is in keeping with the results reported here where the decrease in α2β1 expression is seen most dramatically with the MTSV1-7 B7 transfectant, which gives very spiky branching structures that are not compact (Fig. 12 *B*). In contrast, the structures formed by the MTSV1-7 B1 transfectant, where only a fraction of the cells show reduced expression of the α2β1 integrin, are largely compact with some peripheral spiky branching (Fig. 12 *C*).

It is possible that the ability and indeed the form of branching in collagen may be related to the profile of expression of mesenchymal characteristics, particularly of the intermediate filaments. This conclusion is supported by the results of an earlier study showing that hybrids of MTSV1-7 cells made with a vimentin-expressing cell line demonstrated elongated branching structures when vimentin was the dominant intermediate filament of the hybrid, while purely keratin-expressing hybrids developed ball-like structures (Berdechevsky and Taylor-Papadimitriou, 1991).

Since fibroblast derived factors are able to induce branching in the parental cell lines, we tested whether the branching of the *bcl-2* transfectants could be attributed to the production of soluble factors by the cells themselves. However, branching of the parental cell lines could not be induced by medium conditioned by the *bcl-2* transfectants, MTSV1-7 B7 and B1, thus making this explanation unlikely. We have also found that a reduction in the level or function of the α3β1 integrin in HB2 cells resulted in branching of structures in collagen gels (Berdechevsky et al., 1994). Again however, no decrease in the level of the α3 integrin subunit was seen in the *bcl-2* transfectants (data not shown). It seems therefore that the molecular mechanisms involved in Bcl-2–induced branching of MTSV1-7 or HB2 cells are different from those previously reported.

Discussion

In the present study, we demonstrate that although *bcl-2* overexpression does not induce the tumorigenic phenotype, it provides a survival advantage to mammary epithelial cells under both standard culture conditions when cells are grown to confluence, and under conditions of serum starvation. This effect appears to be correlated with the level of Bcl-2 protein although other factor(s) may modify the effect. *bcl-2* can also affect the phenotype of the original epithelial cells, and depending on the extent of the conversion to a mesenchymal type cell, this can lead to the emergence of a mixed phenotype. The accumulation of cells at confluence leads to multilayering and to the development of structures where pleiotypic differentiation can occur. The *bcl-2* transfectants also formed branching structures in collagen gels which correlated with the changes in epithelial phenotype. The results suggest that while inhib-

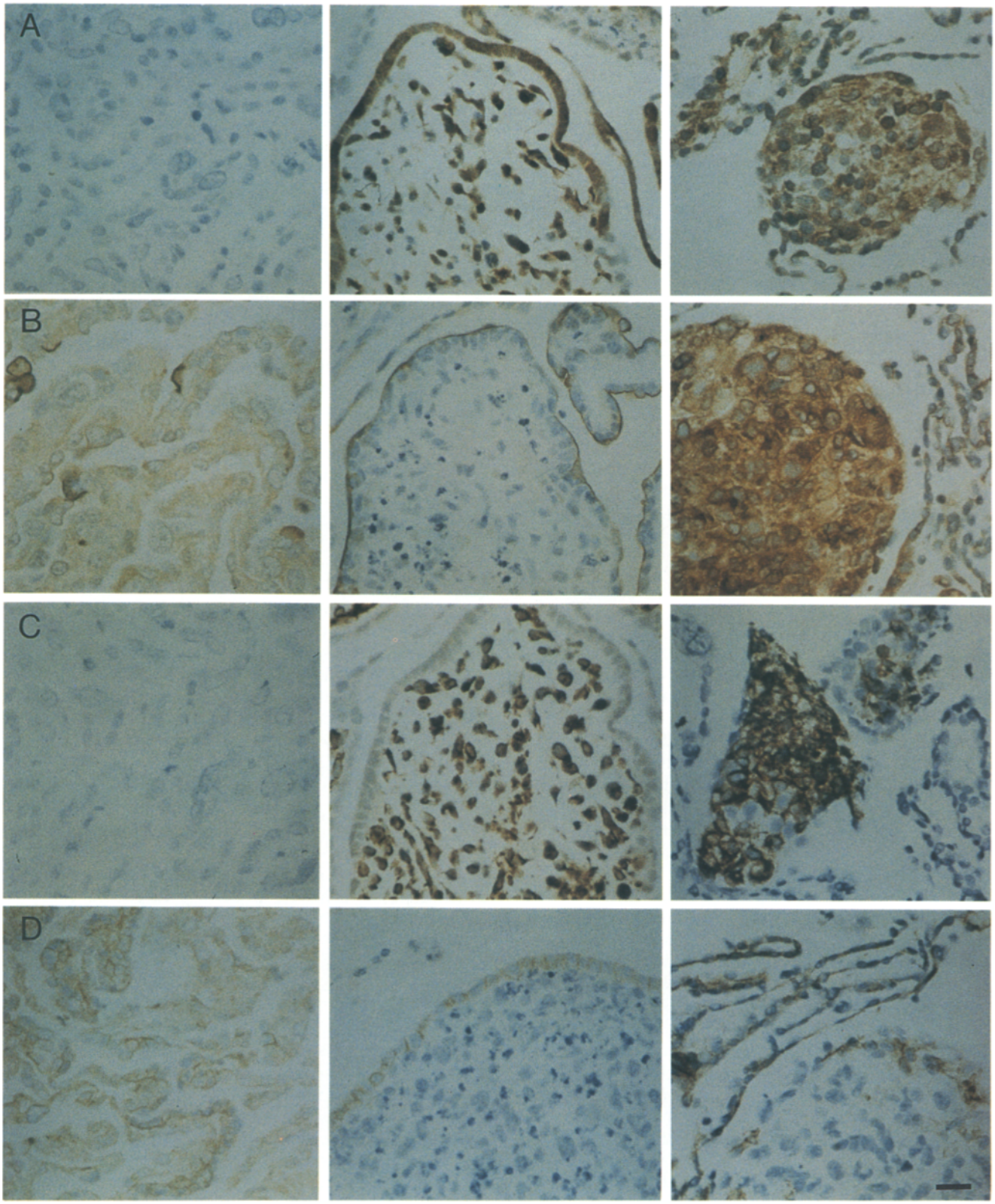

Figure 10. Immunohistochemical analysis of structures formed after confluence. MTSV1-7Puro, *left panels*; MTSV1-7Bcl-2, *middle panels*; MTSV1-7 B1, *right panels*. Immunostaining for Bcl-2 (row *A*), PEM (row *B*), vimentin (row *C*), and E-cadherin (row *D*). The ball-like structures in MTSV1-7Bcl-2 (*middle panels*) and B1 (*right panels*) consist of epithelial cells covering the surface and fibroblastoid cells in the inner ball. Both types of cells are Bcl-2 positive. PEM detected with Ab HMFG1 is apically expressed in the epithelial cells of the ball-like structure in MTSV1-7Bcl-2, but not in the inner fibroblastoid cells. However both epithelial and inner fibroblastoid cells express PEM in the B1 clone. Vimentin expression is restricted to the inner fibroblastoid cells in MTSV1-7Bcl-2 and B1 clones. E-cadherin expression is clearly demonstrated between cell junctions of the surface epithelial cells in MTSV1-7Bcl-2, but is distorted in B1 epithelial cells. Cells were cultured for 3 wk. Bar, 25 μm.

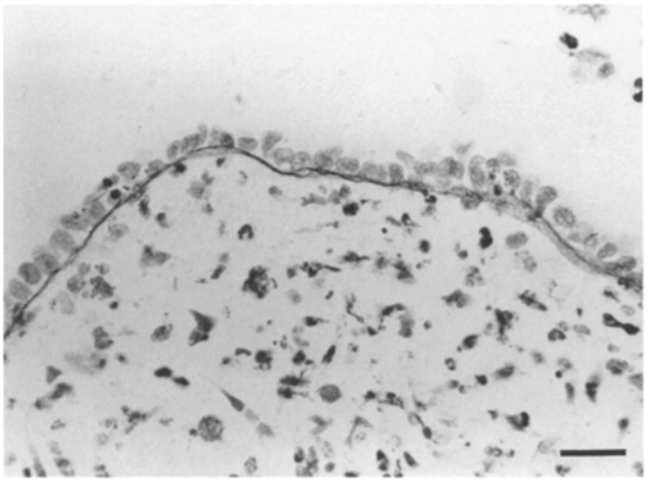

Figure 11. Immunoperoxidase staining of a section from MTSV1-7Bcl-2 (from the same block as those in Fig. 10) for type IV collagen. In the ball-like structure, a clear line of positive staining is seen between the surface epithelial cells and the inner ball of fibroblastoid cells. Bar, 50 μm.

iting cell death and allowing proliferation to proceed in dense cell populations, *bcl-2* may also facilitate differentiation in the mammary gland.

Many types of epithelial cells, for example, Madin-Darby canine kidney cells, thyroid epithelium, and bladder carcinoma cells, can be converted into mesenchymal cells both in vivo and in vitro (Boyer et al., 1989; Greenburg and Hay, 1988; Zuk et al., 1989). Recent studies have demonstrated that oncogene expression can induce the conversion of mammary epithelial cells to cells with a mesenchymal phenotype in vitro. Thus Reichman et al. (1992) reported that activation of the immediate-early gene *c-fos* causes epithelial–fibroblastoid cell conversion and irreversible loss of epithelial polarity. We have also found that overexpression of the *c-erbB2* oncogene can induce conversion of luminal mammary epithelial cells, (MTSV1-7) to fibroblastoid cells (D'Souza et al., 1993; D'Souza and Taylor-Papadimitriou, 1994). We now show that overexpression of *bcl-2* proto-oncogene can also promote the epithelial–fibroblastoid conversion of the same cell line. However, the biological changes associated with the conversion are different between the two oncogene transfectants in that overexpression of *c-erbB2* results in an anchorage-independent growth and tumorigenicity and loss of morphogenetic ability, whereas cells overexpressing *bcl-2* remain nontumorigenic, do not show anchorage-independent growth and are able to undergo morphogenesis in vitro. These differences are perhaps not surprising since *bcl-2* is involved in the regulation of cell death rather than cell proliferation and its overexpression has rarely led to cells becoming tumorigenic in vitro (Reed et al., 1988). On the other hand, it is surprising that some of the *bcl-2* overexpressing clones show a phenotype strikingly similar to the tumorigenic *c-erbB2* transfectants. Both proto-oncogenes can induce loss of or reduction in the expression of cell–cell adhesion molecules, namely E-cadherin and the tight junction-associated ZO-1 protein. Strikingly, both *c-erbB2* and *bcl-2* transfectants of MTSV1-7 show a reduced expression of the α2β1 integrin which is the major integrin expressed by luminal epithelial cells in vivo and which is crucial for morphogenesis in collagen in vitro (Berdechevsky et al., 1994).

The reduced expression of E-cadherin is of particular interest, since this component plays a central role in maintaining epithelial morphology, and reduced expression of E-cadherin has been shown to be associated with epithelial malignancies. E-cadherin can act as an invasion-suppressor molecule in vitro and cells acquire invasive capability when treated with anti–E-cadherin antibody or transfected with anti-sense mRNA (Takeichi, 1990; Ulminckx et al., 1991). A reduction in E-cadherin expression is also found in a proportion of breast carcinomas, particularly in early stage lesions, and is correlated with invasiveness (Behrens et al., 1989; Oka et al., 1993). The results from this study suggest however that although malignant behavior is often associated with the reduction or loss of E-cadherin expression, down-regulation of this molecule does not necessarily lead to a tumorigenic phenotype even when, as with the cell line MTSV1-7, the SV40TAg is also expressed.

The change to a mesenchymal phenotype was more evident with the transfectants derived from MTSV1-7, which, although a cloned cell line, shows microheterogeneity of morphology and appears to show more plasticity of phenotype. Of the HB2 clones isolated, the B9 contained the highest proportion (10%) of vimentin-expressing cells. The HB2 cell line was selected on matrigel, and in collagen, for a uniform branching response to fibroblast-derived growth factors. HB2 cells are more homogeneous morphologically and the epithelial phenotype appears to be less plastic. Thus the result of overexpressing the *bcl-2* gene on phenotype and morphogenetic behavior appears to be strongly dependent on the phenotype of the individual cell, which in turn reflects a specific stage of differentiation in a particular cell lineage.

The effect of overexpression of Bcl-2 on survival of the mammary epithelial cell lines under restrictive growth conditions appears to be due to a decrease in the proportion of cells shed into the medium. Whether the shed cells are dying by apoptosis is not clear since although they have fragmented nuclei, a typical ladder of fragmented DNA was not seen. If apoptosis is occurring it is unlikely to be mediated by p53 which, in these cells, is wild type but bound by SV40 TAg. An important consequence of the increased cell survival at confluence is the formation of multilayers which can form three-dimensional structures. Where the differentiation is pleiotypic, organized structures are formed where a layer of epithelial cells surrounds a central body of mesenchymal cells, the two cell types being separated by a basement membrane like structure. Since the pleiotypic differentiation is more evident with the MTSV1-7 cells, the organized structures were more readily formed by the MTSV1-7 transfectants. However, in collagen gels, the HB2 clones showed a more physiological type of branching structure with lumen formation.

In considering whether the morphologic and phenotypic conversion seen in the Bcl-2 overexpressing MTSV1-7 cells reflects an in vivo phenomenon it is relevant to note that Bcl-2 has been found to be strongly expressed in the epithelial cells of the mammary bud at the early stage of morphogenesis involving gland branching (Lu et al.,

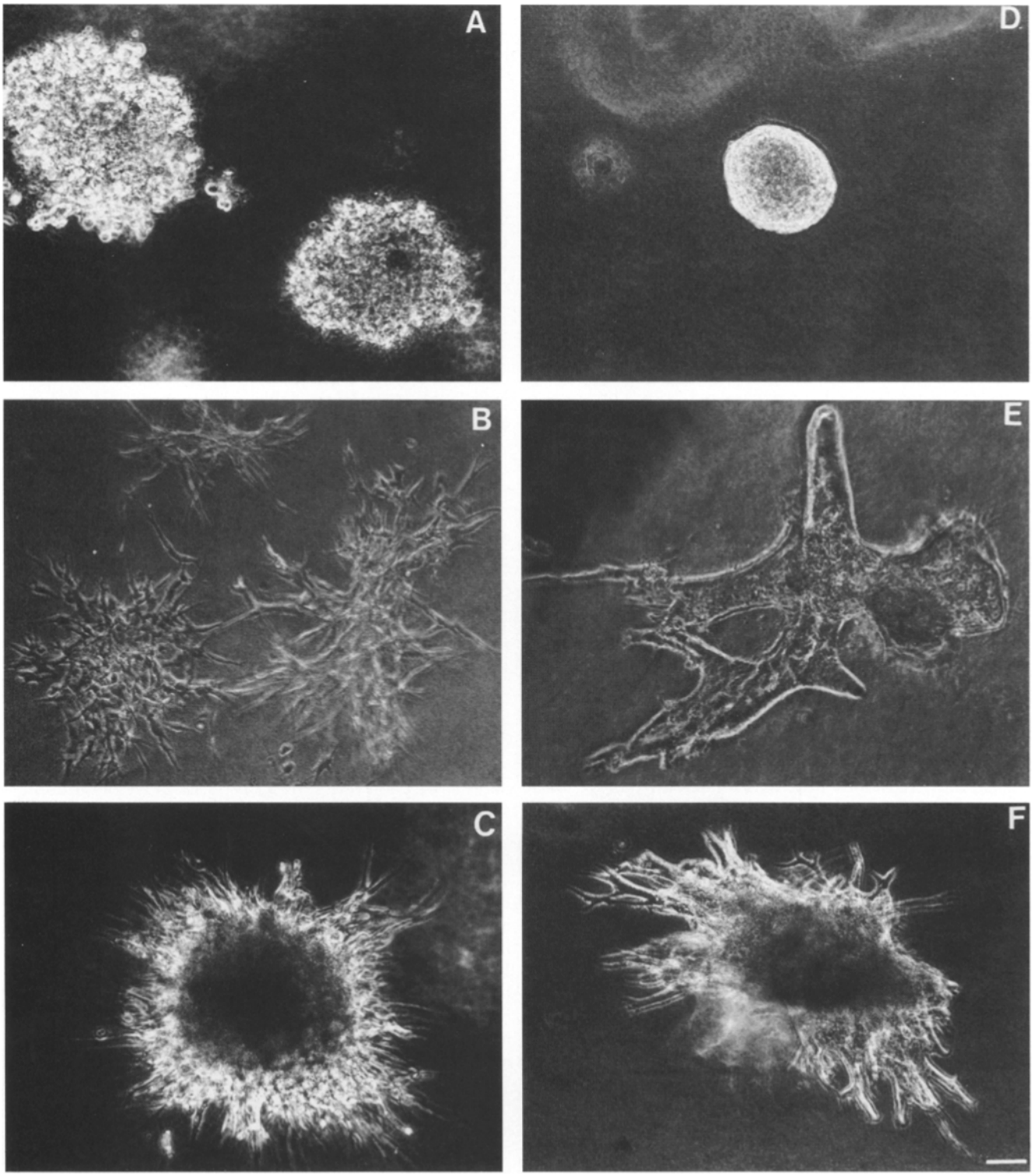

Figure 12. Morphogenesis in collagen gel. (*A*) MTSV1-7Puro; (*B*) MTSV1-7 B7: (*C*) MTSV1-7 B1; (*D*) HB2Puro; (*E*) HB2 B9, and (*F*) HB2 B6. The puro vector controls grow as ball-like structures. The MTSV1-7 B1 and B7 show narrow branches, whereas the HB2 B9 and B6 have thicker and smoother branches. Cells were cultured for 14 d in the absence of fibroblast derived factors. Bar, 100 μm.

1993*a*; Nathan et al., 1994). Also, in the adult gland, the maximum level of Bcl-2 expression is seen during the follicular phase of the menstrual cycle when proliferation (accompanied by morphological differentiation) is occurring. (Sabourin et al., 1994). Certainly branching might be expected to be accompanied by a reduction in the strength of the forces holding the cell to the extracellular matrix and to adjacent cells, and reduced expression of adhesion molecules such as the α2β1 integrin and E-cadherin would achieve this. Furthermore, the changes in cell shape that accompany organ morphogenesis may also require alterations in the cytoskeleton of the cell such as those seen in

the intermediate filaments of the *bcl-2* transfectants. It is therefore quite possible that the effects of Bcl-2 on the growth, differentiation phenotype, and morphogenetic behavior of mammary epithelial cells, which we have observed in vitro, reflect a physiological role of the protein in the regulation of growth and morphogenesis of the mammary gland.

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