

# Regulation of Connexin 43-Mediated Gap Junctional Intercellular Communication by $Ca^{2+}$ in Mouse Epidermal Cells Is Controlled by E-Cadherin

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**Abstract.** Gap junctional intercellular communication (GJIC) of cultured mouse epidermal cells is mediated by a gap junction protein, connexin 43, and is dependent on the calcium concentration in the medium, with higher GJIC in a high-calcium (1.2 mM) medium. In several mouse epidermal cell lines, we found a good correlation between the level of GJIC and that of immunohistochemical staining of E-cadherin, a calcium-dependent cell adhesion molecule, at cell-cell contact areas. The variant cell line P3/22 showed both low GJIC and E-cadherin protein expression in low- and high- $Ca^{2+}$  media. P3/22 cells showed very low E-cadherin mRNA expression. To test directly whether E-cadherin is involved in the  $Ca^{2+}$ -dependent regulation

of GJIC, we transfected the E-cadherin expression vector into P3/22 cells and obtained several stable clones which expressed high levels of E-cadherin mRNA. All transfectants expressed E-cadherin molecules at cell-cell contact areas in a calcium-dependent manner. GJIC was also observed in these transfectants and was calcium dependent. These results suggest that  $Ca^{2+}$ -dependent regulation of GJIC in mouse epidermal cells is directly controlled by a calcium-dependent cell adhesion molecule, E-cadherin. Furthermore, several lines of evidence suggest that GJIC control by E-cadherin involves posttranslational regulation (assembly and/or function) of the gap junction protein connexin 43.

**G**AP junctions are specialized membrane components that permit the direct exchange of ions and small metabolites between cells. Intercellular communication through gap junctions is believed to be intimately involved in the processes of tissue differentiation and homeostasis (Loewenstein, 1979; Pitts and Finbow, 1986). Block of gap junctional intercellular communication (GJIC)<sup>1</sup> during liver regeneration after partial hepatectomy (Finbow et al., 1980; Meyer et al., 1981; Traub et al., 1989) and aberrant GJIC capacity observed during carcinogenesis and in various transformed or cancerous cells (Loewenstein, 1987; Yamasaki, 1990a) support the idea that GJIC plays an important role in the control of cell proliferation and differentiation.

The gap junction channels are made up of oligomeric pro-

teins consisting of six subunits called connexins (Unwin and Zamphigi, 1980; Beyer et al., 1988). The cDNAs encoding several connexins have been sequenced (Kumar and Gilula, 1986; Paul, 1986; Beyer et al., 1987; Zhang and Nicholson, 1989). There are strong overall sequence homologies and similar membrane topology for at least three connexins (connexins 32, 43, and 26) has been proposed (Goodenough et al., 1988; Milks et al., 1988; Beyer et al., 1989; Yancey et al., 1989; Zhang and Nicholson, 1989). Despite these similarities, expression and function of gap junctions appear to be different in various tissues, suggesting complicated functional regulation of connexins. Differences of phosphorylation sites among connexins may represent one such regulatory mechanism of GJIC through changing connexin functions per se (Zhang and Nicholson, 1989).

Another level of regulation proposed for GJIC is cell-cell recognition. Candidate molecules include extracellular matrix and specific cell adhesion molecules (Edelman, 1986; Spray et al., 1987; Mege et al., 1988). GJIC often occurs selectively. For example, selective GJIC patterns are considered to be essential for early development (Lo and Gilula, 1979a, b). Recently, we reported that BALB/c 3T3 cells transformed by a variety of carcinogens show a normal level of GJIC among themselves, but do not communicate with surround-

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1. *Abbreviations used in this paper:* DMBA, 7,12-dimethylbenz[a]anthracene; FAV, fibronectin plus albumin plus vitrogen; GJIC, gap junctional intercellular communication; L-CAM, cell adhesion molecule; N-CAM, neural cell adhesion molecule.

ing nontransformed cells (Enomoto and Yamasaki, 1984; Yamasaki et al., 1987). These results suggest the importance of selective cell-cell recognition mechanisms in the regulation of GJIC. Indeed, cell adhesion molecules are known to play an important role in selective cell recognition and adhesion (Edelman, 1986; Takeichi, 1987).

We have recently characterized the GJIC capacity of a panel of mouse epidermal cell lines derived from different stages of mouse skin carcinogenesis. These cell lines displayed a progressive loss in GJIC with increasing tumorigenicity (Klann et al., 1989). Cell differentiation and proliferation of mouse epidermal cells are calcium dependent, and we have found that GJIC is similarly calcium dependent. We have also studied the relationship between the calcium-dependent cell adhesion molecule, E-cadherin, and GJIC. We report here that gap junction protein (connexin 43) function is regulated by calcium in mouse epidermal cells and that the calcium-dependent cell adhesion molecule, E-cadherin, is involved in this regulation of connexin function.

A possible role of cell adhesion molecules in the regulation of GJIC has been studied by Mege et al. (1988). They transfected liver cell adhesion molecule (L-CAM) cDNA into otherwise L-CAM-deficient and GJIC-incompetent mouse sarcoma S180 cells, and observed the appearance of functional GJs. However, since the study was carried out in fibroblasts which usually do not express L-CAM, whether and how such a regulatory relationship between cell adhesion molecules and GJIC exists under normal physiological conditions are not known. The present study was undertaken first to identify the molecular species involved in cell-cell adhesion and GJIC of mouse epidermal cells and then to study their regulatory relationship.

## Materials and Methods

### Cell Culture

The base medium used for primary and established cell line cultures was Eagle's minimal essential medium without  $\text{CaCl}_2$ . To this medium were added: (Sigma Chemical Co., St. Louis, MO) insulin, 5  $\mu\text{g}/\text{ml}$ ; epidermal growth factor, 5 ng/ml; transferrin, 10  $\mu\text{g}/\text{ml}$ ; *O*-phosphorylethanolamine, 10  $\mu\text{M}$ ; 2-aminoethanol, 10  $\mu\text{M}$ ; hydrocortisone, 1  $\mu\text{M}$ ; gentamycin sulfate, 50  $\mu\text{g}/\text{ml}$  and glutamine (Gibco Laboratories, Grand Island, NY), 2 mM.

This medium was then supplemented with 10% FBS treated with Chelex (Chelex 100 resin; BioRad Laboratories, Richmond, CA) to remove calcium (Brennan et al., 1975). As measured by atomic absorption analysis, the  $\text{Ca}^{2+}$  concentration in this medium was  $\sim 0.03$  mM and  $\text{CaCl}_2$  was then added to give a final concentration of 0.05 mM. When high-calcium medium was required,  $\text{CaCl}_2$  was added.

### Primary Cultures

Cultures of primary keratinocytes were obtained from 6- to 9-wk-old SEN-CAR mice following the procedure described by Miller et al. (1987). Typical cell yield and viability, determined by trypan blue exclusion, were  $15\text{--}30 \times 10^6$  cells/dorsal skin with 60–80% viability.

Dishes (Falcon Plastics, Cockeysville, MD) were precoated with fibronectin plus albumin plus vitrogen (FAV) solution according to Kawamura et al. (1985). To each 60-mm dish were added 2.0 ml of a solution of bovine fibronectin (10  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co.), BSA (0.1 mg/ml; Sigma Chemical Co.), collagen (30  $\mu\text{g}/\text{ml}$ ; Collagen Corp., Palo Alto, CA), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free PBS. After aspiration, the dishes were air dried for 15 min and  $20 \times 10^6$  viable cells were seeded in 4 ml of medium. After 24 h, medium was replaced and 2 or 3 d later cultures were used for experiments.

### Mouse Epidermal Cell Lines

The cell lines used in this study represent early, intermediate and late stages of mouse skin carcinogenesis (Klann et al., 1989). The 3PC cell line was obtained after exposure of adult primary keratinocytes in vitro to 7,12-dimethylbenz[*a*]anthracene (DMBA; 0.1  $\mu\text{g}/\text{ml}$  for 24 h) and a shift to high- $\text{Ca}^{2+}$  medium to select for  $\text{Ca}^{2+}$ -resistant cells. Such cells are thought to be "initiated" cells which are promoted to form tumors by tumor promoters (Yuspa et al., 1989).

Papilloma-derived cell lines were obtained from tumors appearing after DMBA initiation of dorsal skin of 7-wk-old female SENCAR mice followed by brief (P1/17) or extended (P2/15; P3/22) promotion with TPA. The CA 3/7 cell line was obtained from a carcinoma 16 wk after completion of the DMBA/TPA regimen. The characteristics of these cell lines have been described in detail elsewhere (Klann et al., 1989). These cell lines were maintained in the low- $\text{Ca}^{2+}$  medium.

### Immunostaining

Cells were grown on microscope slides. At confluence, cultures were washed twice with PBS containing 1 mM  $\text{CaCl}_2$ , pH 7.5, for 5 min and then fixed in ice-cold pure ethanol for 30 min. After air drying at room temperature, slides were kept at  $-20^\circ\text{C}$ . All subsequent treatments were done at room temperature. Slides were washed three times for 5 min with PBS containing 5% (wt/vol) skim milk and then incubated for 60 min at room temperature with rat E-cadherin antibody ECCD-2 (Shirayoshi et al., 1986) diluted 1:100 with PBS containing 5% skim milk. After washing three times with PBS for 5 min, slides were incubated for 30 min in FITC anti-rat IgG (Sigma Chemical Co.) diluted 1:16 in PBS. Then the slides were washed three times with PBS for 5 min and mounted in 90% glycerol-10% PBS (pH 8.0) containing 0.1% paraphenylenediamine to prevent bleaching (Johnson and Nogueira-Aranjo, 1981). Photographs were taken on Tri-X films (Eastman Kodak Co., Rochester NY) with a microscope (VANOX T; Olympus Corporation of America, New Hyde Park, NY).

### Connexin 43 Immunofluorescence

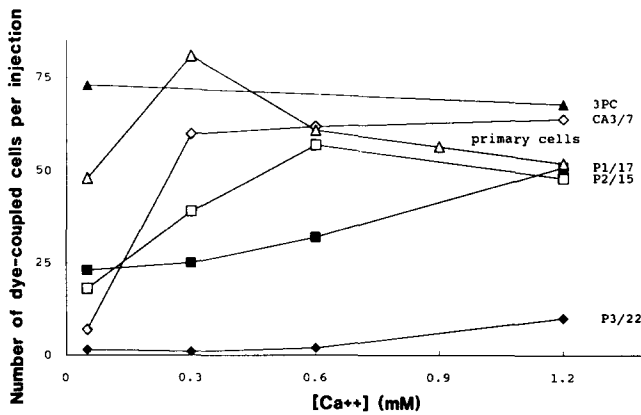
The series of cell lines were cultured in chamber slide (Lab-Tek, Nunc, Roskilde, Denmark) and then fixed with acetone at  $-20^\circ\text{C}$  for 5 min. The cells were incubated with the affinity-purified antibody raised against connexin 43 peptide (El Aoumari et al., 1990) for 1 h (1:20), and for 1 h with biotinylated anti-rabbit antibodies (Vector Laboratories Inc., Burlingame, CA; 1:200), and then with FITC-labeled streptavidin for 1 h (Vector Laboratories Inc., 1:50). Preparations were examined with a fluorescent microscope (Olympus Corporation of America).

### Inhibition of Protein Synthesis

Inhibition of protein synthesis was measured using the [ $^3\text{H}$ ]Leucine incorporation assay. Confluent monolayers (6-cm dishes) were treated with different concentrations of cycloheximide for 30 min at  $37^\circ\text{C}$ . Immediately after start of the treatment, [ $^3\text{H}$ ]Leucine was added to each dish (1  $\mu\text{Ci}/\text{ml}$ ). After 30 min, cells were washed three times with PBS and lysed with 2 ml NaOH solution (0.5 M). Protein was precipitated with TCA (final concentration 10%) and the amount of acid-precipitable radioactive material was determined by filtration (Millipore Continental Water Systems, Bedford, MA) (Yamasaki et al., 1975). To determine nonspecific binding of radioactivity, [ $^3\text{H}$ ]Leucine was added to cells kept at  $0^\circ\text{C}$  for 30 min. Experimental points were carried out in triplicate and inhibition of protein synthesis was calculated after subtraction of nonspecific binding.

### Gap Junctional Intercellular Communication

GJIC was measured by means of dye coupling. In brief, glass capillaries prepared with a dual-step puller (Narishige Scientific Laboratory, Tokyo, Japan) were filled with a 10% solution (wt/vol) of Lucifer yellow CH dissolved in 0.33 M lithium chloride and fixed just beneath the condenser of an inverted microscope (Injectoscope IMT-2SYF; Olympus Corporation of America). Individual cells in monolayer cultures were injected with the aid of an Eppendorf automatic microinjector (model 5242; Brinkman Instruments Inc., Westbury, NY). The extent of intercellular communication was determined by the number of fluorescent neighboring cells scored 20 min after injection. Experiments were carried out in duplicate and in each dish, at least 20 individual injections were done.



**Figure 1.** Ca<sup>2+</sup>-dependent GJIC in mouse primary keratinocytes and epidermal cell lines. Cells were cultured in low-Ca<sup>2+</sup> medium (0.05 mM) as described in Materials and Methods. When dishes were confluent, different concentrations of Ca<sup>2+</sup> were added and 6 h later GJIC was determined with the dye-transfer method. Data represent average values of two dishes and in each dish at least 20 injections were done. When the mean number of dye-coupled cells per injection was <10, SD were 10–20% and when the mean number was >10, SD were 5–8%. For reasons of clarity SDs are not indicated in the figure.

### Transfection

Confluent P3/22 cells were trypsinized, centrifuged (300 g for 5 min), and suspended in PBS (pH 7.5) at a density of  $2 \times 10^6$ – $10^7$  cells/ml. The plasmids PSV2 neo to give G418 resistance (Southern and Berg, 1982) and p-BATEM2 containing the E-cadherin cDNA (Nose et al., 1988) at a ratio of 1:10 were co-transfected into P3/22 cells by electroporation. An aliquot (0.8 ml) of the cell suspension was added to the cuvette together with 10  $\mu$ g pBATEM2, 1  $\mu$ g PSV2 neo, and 10  $\mu$ g herring sperm DNA which served as carrier. The suspension was mixed and left on ice for 15 min, mixed again, and electroporated at 1,200–1,600 V (Genepulser; BioRad Laboratories). After electroporation, cuvettes were placed on ice for 15 min, then cells from one cuvette were placed in two separate dishes (6 cm) to which 4 ml of culture medium were added. After 48 h, medium was replaced and G418 (Sigma Chemical Co.; 50  $\mu$ g/ml) was added to select resistant clones. Clones were selected by the morphological appearance of tightly connected cell clusters and were maintained in low Ca<sup>2+</sup> medium.

### Northern Blot Analysis

Cell cultures were washed with PBS (pH 7.5) and RNA was extracted using a single-step thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). RNA concentration in samples was determined

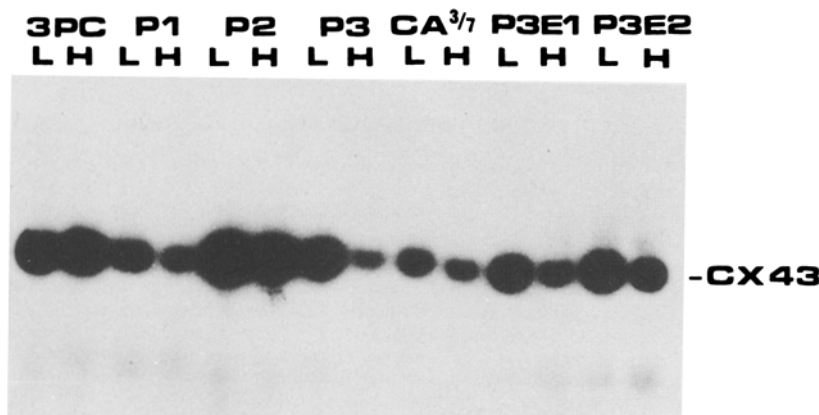
spectrophotometrically at 260 nm and by the intensity of RNA-ethidium bromide staining on the gels. RNA samples (10–40  $\mu$ g) were subjected to electrophoresis on 1% agarose/formaldehyde gels, and subsequent ethidium bromide staining confirmed the loading of similar RNA amount for each sample. Gels were capillary-blotted in  $10\times$  SSC onto nylon membranes (Gene Screen Plus; New England Nuclear, Boston, MA) and baked. Radio-labeled cDNA probes of connexin 32 (Paul, 1986), connexin 43 (Beyer et al., 1987), connexin 26 (Zhang and Nicholson, 1989), E-cadherin (Yoshida-Noro et al., 1984), and P-cadherin (Nose et al., 1987) were prepared using multiprime DNA-labeling systems (Amersham Corp., Arlington Heights, IL). Blots were prehybridized in 50% formamide, 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4), 1% SDS, 10  $\mu$ g herring sperm DNA,  $5\times$  Denhart's solution for 3 h at 42°C, and then hybridized overnight in the same buffer with the labeled probes and 10% dextran sulfate at 42°C. Blots were washed twice in  $2\times$  SSC, 7% SDS at 65°C for 30 min, and twice in  $0.1\times$  SSC for 30 min at room temperature before exposure at  $-70^\circ\text{C}$  to hyperfilm MP (Amersham Corp.) with an intensifying screen. Northern blot results presented here were reproduced at least twice with independent experimental samples.

## Results

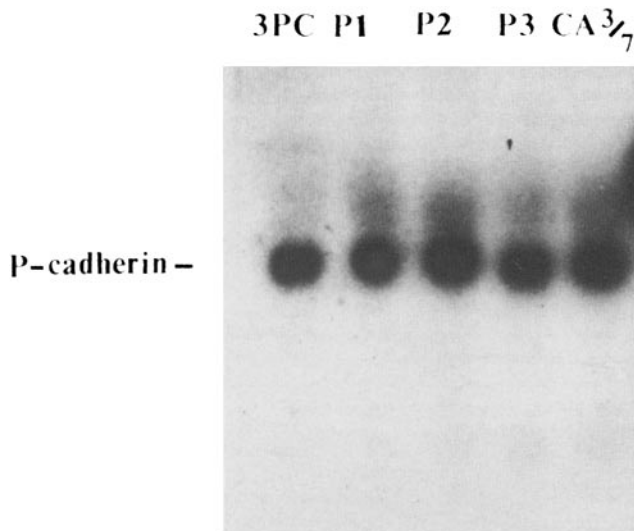
### Ca<sup>2+</sup> Dependence of Gap Junctional Intercellular Communication of Mouse Epidermal Cells

Calcium dependence of mouse epidermal cell morphology and function is well documented (Hennings et al., 1980). In the present study, we first examined the effect of Ca<sup>2+</sup> on GJIC in primary mouse keratinocytes and the panel of cell lines derived from different stages of mouse skin multistage carcinogenesis. The origin of the lines and their characterization have been described in detail (Klann et al., 1989). In low-Ca<sup>2+</sup> medium (0.05 mM), GJIC capacity was inversely proportional to the stage of tumorigenesis (Fig. 1). Primary keratinocytes and the “initiated” cells (3PC) showed highest GJIC; two of the papilloma-derived cell lines had intermediate GJIC (P1/17 and P2/15), whereas a third papilloma-derived cell line (P3/22) and the carcinoma-derived cell line (CA 3/7) were poor communicators under these conditions. This is generally in agreement with results previously obtained with these cell lines (Klann et al., 1989), although some differences in absolute GJIC values were apparent.

Fig. 1 also illustrates how GJIC changed as a function of Ca<sup>2+</sup> concentration in the medium. In this experiment, GJIC was measured 6 h after addition of Ca<sup>2+</sup>. This time point was chosen since a preliminary experiment had shown that extended incubation of primary keratinocytes (24 h) in high-Ca<sup>2+</sup> medium resulted in reduced GJIC, probably because



**Figure 2.** Northern analysis of connexin 43 mRNA in mouse epidermal cells. Cell lines were cultured in low-Ca<sup>2+</sup> medium (0.05 mM) and, at confluence, half of the cultures were switched to high Ca<sup>2+</sup> for 3 h. Cells were lysed, mRNA was extracted and analyzed on Northern blots for the presence of connexin 43. Each lane contained 20  $\mu$ g total RNA. H, high Ca<sup>2+</sup>; L, low Ca<sup>2+</sup>.



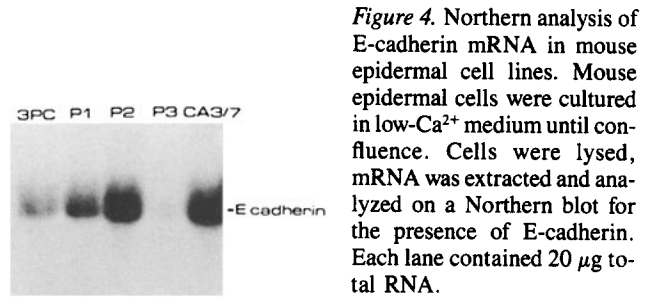
**Figure 3.** Northern analysis of P-cadherin mRNA in mouse epidermal cell lines. Mouse epidermal cells were cultured in low-Ca<sup>2+</sup> medium and at confluence cells were lysed, mRNA was extracted and analyzed on a Northern blot for the presence of P-cadherin. Each lane contained 20  $\mu$ g total RNA.

of terminal differentiation. In the primary keratinocytes, 0.3 mM Ca<sup>2+</sup> caused a considerable increase in GJIC but higher concentrations did not cause significant changes. No major change in GJIC was observed for the cell line 3PC. In contrast, two of the papilloma-derived cell lines (P1/17 and P2/15) and the carcinoma-derived cell line CA 3/7 showed considerable increases in GJIC, and, in 1.2 mM Ca<sup>2+</sup>, reached the same levels as the primary and 3PC cells. However, the GJIC of P3/22 cells in 1.2 mM Ca<sup>2+</sup> showed a relatively small increase, achieving only ~15% of the GJIC of the other lines.

#### **Evidence that Connexin 43 Is Responsible for Mouse Epidermal GJIC**

When gene expression of various connexins (gap-junction proteins) was determined by Northern blot analysis, hybridization with radiolabeled connexin 32, and connexin 26 probes gave consistently negative results with primary mouse keratinocytes and the mouse epidermal cells lines (data not shown). On the other hand, connexin 43 was clearly expressed in all cell lines tested (Fig. 2). The levels of mRNA varied considerably among the cell lines, with P2/15 having the highest level and P1/17 the lowest. When these mRNA levels were compared with GJIC for cells in low- or high-Ca<sup>2+</sup> condition, no relationship could be observed (Fig. 2); thus, the low level of mRNA found in line P1/17 was apparently sufficient to establish good GJIC under high-Ca<sup>2+</sup> conditions.

Immunohistological staining of connexin 43 proteins in these cell lines reveals a good correlation between the appearance of immunospots on cell contact areas and their GJIC level measured by dye transfer assay. When GJIC was increased in cell lines P1/17, P2/15, and CA 3/7 by transferring them to high-calcium medium, there was a parallel increase in the appearance of connexin 43 protein. Furthermore, when GJIC of P1/17 and P3E1 (E-cadherin transfected clone, see below) was inhibited by a tumor-promoting phorbol ester,



**Figure 4.** Northern analysis of E-cadherin mRNA in mouse epidermal cell lines. Mouse epidermal cells were cultured in low-Ca<sup>2+</sup> medium until confluence. Cells were lysed, mRNA was extracted and analyzed on a Northern blot for the presence of E-cadherin. Each lane contained 20  $\mu$ g total RNA.

12-*O*-tetradecanoylphorbol 13-acetate, connexin 43 protein expression was also decreased. On the other hand, connexin 43 protein expression of P3/22 cells was not affected by the calcium concentration of the culture medium, which is in line with their GJIC ability (see below).

These results suggest that connexin 43 is responsible for mouse epidermal cell GJIC. It is, however, possible that connexins other than connexin 32 and connexin 26 are also expressed in these cells and contribute to the level of GJIC observed.

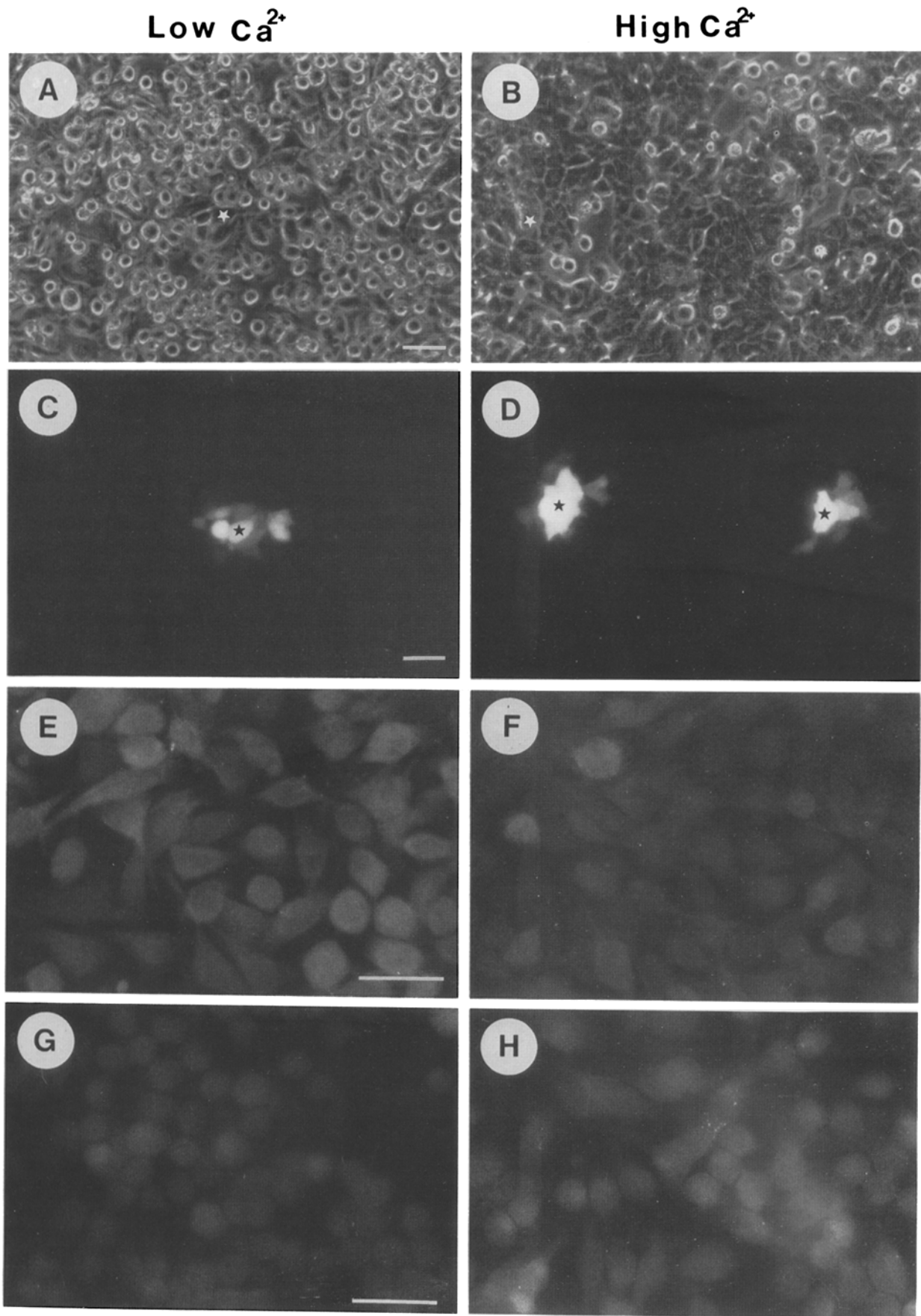
#### **E-cadherin but not P-cadherin Expression Correlates with GJIC in Mouse Epidermal Cells**

Northern analysis for the presence of mRNA hybridizing with a radiolabeled probe of P-cadherin showed that all cell lines were positive and had comparable levels of mRNA (Fig. 3). However, no immunohistochemically positive staining was observed in any cell line in high- or low-Ca<sup>2+</sup> medium (results not shown). Also, mRNA levels of E-cadherin were examined. The cell lines 3PC, P1/17, P2/15, and CA 3/7 showed a positive response with a tendency towards higher expression levels with advancing tumorigenesis (Fig. 4). However, the line P3/22 had a very weak band when compared with the other cell lines (Fig. 4). Since the levels of E-cadherin mRNA, obtained from cells cultured in low-Ca<sup>2+</sup> medium, were consistent with GJIC levels under the same conditions, we decided to follow this further. E-cadherin is known to be Ca<sup>2+</sup> dependent and GJIC in the papilloma-derived cell lines and the carcinoma-derived cell line was also Ca<sup>2+</sup> dependent. Therefore, cells were cultured in

**Table I. Immunocytochemical Staining of Mouse Epidermal Cells with E-cadherin Antibody**

Cells	Low Ca <sup>2+</sup>	High Ca <sup>2+</sup>
Primary keratinocytes	+	++
3PC	+	+
P1/17	+/-	+++
P2/15	+/-	++
P3/22	-	-
CA3/7	-	+++
P3E1	-	+++
P3E2	-	+++

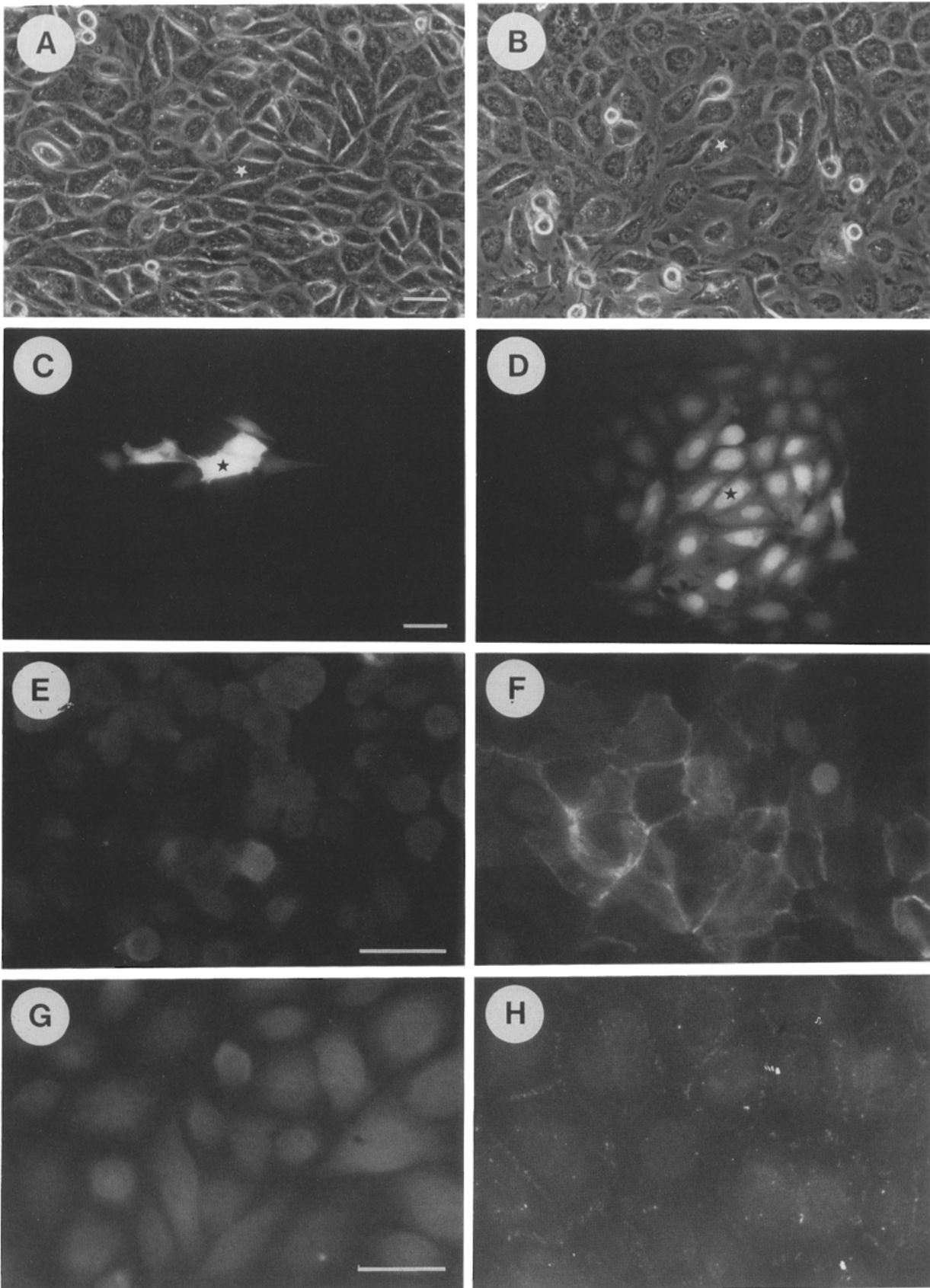
Primary cells cultured in FAV-coated dishes and cell lines cultured on microscope slides were maintained in low-Ca<sup>2+</sup> (0.05 mM) medium. At time zero, half of the cultures were switched to high-Ca<sup>2+</sup> (1.2 mM) medium and 6 h later all cultures were fixed and stained as described in Materials and Methods. Semiquantitation of results was conducted using the level of staining in 3PC cells as the baseline. Five grades were used for this purpose from nondetectable to high expression; -, +/-, +, ++, and +++. Low Ca<sup>2+</sup> = 0.05 mM; high Ca<sup>2+</sup> = 1.2 mM.



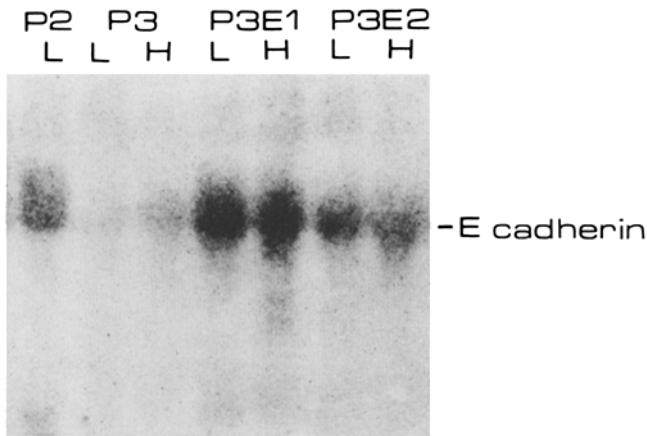
**Figure 5.** Morphology, GJIC ability and immunostaining of E-cadherin and connexin 43 in P3/22 cells grown in low- (left) and high- (right)  $Ca^{2+}$  medium. *A* and *B*, phase-contrast micrograph; *C* and *D*, GJIC measured by dye transfer. Cells were microinjected with Lucifer yellow CH solution and the dye transfer was monitored by fluorescence microscopy. (★) Microinjected cells: *E* and *F*, immunocytochemical staining of E-cadherin; *G* and *H*, immunocytochemical staining of connexin 43. Bar, 50  $\mu$ m.

Low  $Ca^{2+}$

High  $Ca^{2+}$



**Figure 6.** Morphology, GJIC ability and immunostaining of E-cadherin and connexin 43 in an E-cadherin transfectant, P3E1, grown in low- (*left*) and high- (*right*)  $Ca^{2+}$  medium. *A-H*, the same as in Fig. 5. Bar, 50  $\mu m$ .



**Figure 7.** E-cadherin mRNA expression in P3/22 transfectants. Cells were cultured in low- $\text{Ca}^{2+}$  medium. At confluence, half of the cultures were switched to high  $\text{Ca}^{2+}$  for 3 h. Cells were lysed, mRNA extracted and analyzed on a Northern blot. Each lane contained 10  $\mu\text{g}$  total RNA. H, high  $\text{Ca}^{2+}$ ; L, low  $\text{Ca}^{2+}$ .

both low- and high- $\text{Ca}^{2+}$  media and stained with ECCD-2, an antibody against E-cadherin (Shirayoshi et al., 1986).

The data on immunocytochemical staining represent relative levels of the E-cadherin antibody (ECCD-2) binding using the cell line 3PC as the standard (Table I). Cell line P3/22 was the only cell line which was negative for E-cadherin antibody staining in high- $\text{Ca}^{2+}$  as well as in low- $\text{Ca}^{2+}$  culture media. The pattern of E-cadherin immunocytochemical staining was consistent with patterns of GJIC under different  $\text{Ca}^{2+}$  conditions (compare Table I and Fig. 1).

#### Stable Transfection of E-cadherin cDNA

The foregoing results suggested a role for E-cadherin in controlling GJIC. To further examine this, P3/22 cells were transfected with the pBATEM2 plasmid carrying the E-cadherin cDNA together with the pSV2-neo plasmid carrying the neomycin-resistance gene.

Transfected cells were cultured for 3 wk in the presence of G418 (50  $\mu\text{g}/\text{ml}$ ) and transfectants were selected on the appearance of epithelioid structures. Parental P3/22 cells in culture are loosely arranged with many rounded cells easily detaching from the culture dish (Fig. 5, A and B). In stark contrast, transfectants had a very different morphology. At subconfluence, the cells formed closely packed colonies, and

in the confluent state, epithelioid sheets were formed (Fig. 6, A and B). After selection of the clones, they were further subcultured without addition of G418 and were stable for at least 20 passages.

#### Characterization of Transfectants

The presence of E-cadherin in transfectants was confirmed by Northern blot analysis of mRNA obtained from two clones in low- and high- $\text{Ca}^{2+}$  media. Two cell lines tested, designated P3E1 and P3E2, expressed E-cadherin mRNA and no difference in mRNA levels was seen between low- and high- $\text{Ca}^{2+}$  conditions (Fig. 7).

Expression of connexin 43 and E-cadherin proteins in the parent cell line, P3/22, and its transfectant, P3E1, in low- and high- $\text{Ca}^{2+}$  media were compared by immunocytochemical staining with the corresponding antibodies (Figs. 5 and 6). The levels of GJIC, measured by dye transfer, in these cell lines are also presented in Figs. 5 and 6. In low- $\text{Ca}^{2+}$  medium, GJIC levels as well as the expression of E-cadherin and connexin 43 were low. In high- $\text{Ca}^{2+}$  medium, the transfectant P3E1, but not the parent P3/22, showed higher GJIC and expression of E-cadherin and connexin 43. Thus, the levels of expression of connexin 43 and E-cadherin and the level of GJIC appear to be controlled by  $\text{Ca}^{2+}$  in a concerted fashion.

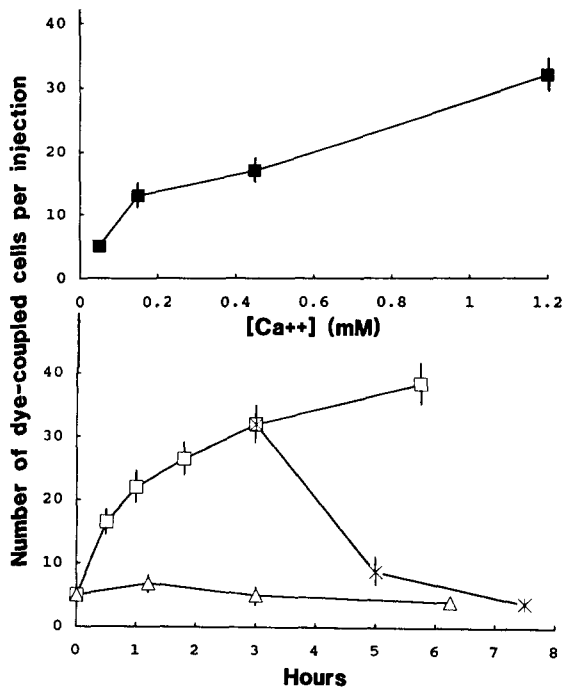
GJIC was determined in five different transfectant clones (Table II). Among these clones, P3E1 and P3E4 showed slightly elevated GJIC in low- $\text{Ca}^{2+}$  medium compared to the parental cell line P3/22. When cultured in high- $\text{Ca}^{2+}$  medium, all transfectants showed marked increases in GJIC, although the kinetics were different. As shown in Table II, the increase of GJIC noted 3 h after the  $\text{Ca}^{2+}$  switch ranged from 2.7- (P3E4) to 65.7-fold (P3E3). The increase continued at 24 h for P3E1, P3E2, and P3E5, but GJIC was markedly reduced for P3E3 in high- $\text{Ca}^{2+}$  medium at this time. In this latter line, the decreased GJIC and also the morphological changes observed resembled those of primary keratinocytes in high- $\text{Ca}^{2+}$  medium. Cell line P3E1 was selected for further characterization of the  $\text{Ca}^{2+}$ -dependence of GJIC (Fig. 8). Addition of  $\text{Ca}^{2+}$  to confluent cultures caused a concentration-dependent increase in GJIC (Fig. 8, top). This increase appeared to be fast. As little as 30 min after a switch to high  $\text{Ca}^{2+}$  (1.2 mM), a threefold increase in GJIC was observed (Fig. 8, bottom). Upon return to low- $\text{Ca}^{2+}$  medium, cells rapidly resumed low GJIC (Fig. 8, bottom).

**Table II.** Calcium-dependent Gap Junctional Intercellular Communication in Cloned Cell Lines Selected from E-cadherin Transfected P3/22 Mouse Epidermal Cells

Clone	3 h*		24 h*	
	Low $\text{Ca}^{2+}$	High $\text{Ca}^{2+}$	Low $\text{Ca}^{2+}$	High $\text{Ca}^{2+}$
P3E1	6 $\pm$ 1	29 $\pm$ 3	6 $\pm$ 1	45 $\pm$ 5
P3E2	4 $\pm$ 0.3	37 $\pm$ 4	2 $\pm$ 0.5	30 $\pm$ 2
P3E3	0.7 $\pm$ 0.2	46 $\pm$ 5	0.8 $\pm$ 0.3	4 $\pm$ 0.7
P3E4	9 $\pm$ 2	24 $\pm$ 2	nt	nt
P3E5	3 $\pm$ 2	13 $\pm$ 2	2 $\pm$ 0.7	19 $\pm$ 2

\* Time after switch to high  $\text{Ca}^{2+}$ .

Cells were cultured in low- $\text{Ca}^{2+}$  medium until confluence. At time zero, half of the dishes were switched to high- $\text{Ca}^{2+}$  (1.2 mM) medium and 3 h later or the next day GJIC was determined by the dye transfer method as described in Materials and Methods. Data represent average value of two dishes and in each dish at least 20 injections were performed. Low  $\text{Ca}^{2+}$  = 0.05 mM; high  $\text{Ca}^{2+}$  = 1.2 mM. nt, not tested.



**Figure 8.** Ca<sup>++</sup>-dependent GJIC in P3E1 cells. (Top) Confluent cultures of P3E1 cells were treated with different Ca<sup>2+</sup> concentrations and after 3 h, GJIC was determined with the dye-transfer method (see Materials and Methods). (Bottom) Confluent cultures of P3E1 cells were treated with 1.2 mM Ca<sup>2+</sup> and GJIC was followed in time (□). After 3 h in high Ca<sup>2+</sup> some cultures were switched to 0.05 mM Ca<sup>2+</sup> (X). Control dishes were kept in 0.05 mM Ca<sup>2+</sup> (Δ). Data are average values of two dishes and in each dish 20 cells were injected. Bars represent SD.

**Evidence that E-cadherin Controls the Function, but not Synthesis, of Connexin 43**

Since the foregoing results strongly indicate that E-cadherin regulates GJIC, and since connexin 43 appears to be the channel protein responsible for GJIC of mouse epidermal cells, we tested whether E-cadherin controls the synthesis of connexin 43 molecules or their function. The results presented above suggested that connexin 43 mRNA levels among various cell lines are relatively stable, regardless of variance in GJIC levels (Fig. 2), and are not dependent on calcium con-

centration, suggesting that calcium-dependent control of connexin 43 is posttranscriptional.

To determine whether the Ca<sup>2+</sup>-induced increase of GJIC in the E-cadherin transfectants requires protein synthesis, P3E1 and P3E2 cells were cultured in low-Ca<sup>2+</sup> medium until confluence and then switched to high Ca<sup>2+</sup> in the presence of cycloheximide. As shown in Table III, there was a clear enhancement of GJIC in the high-Ca<sup>2+</sup> medium even in the presence of cycloheximide, indicating that protein synthesis is not required for establishing GJIC in these transfectants. Similarly, when P3E cells were transferred from high to low-Ca<sup>2+</sup> medium, E-cadherin expression at cell contact areas started to disappear even in the presence of cycloheximide, which inhibited protein synthesis by >90% (Table III). Moreover, immunohistochemical appearance of connexin 43 in high-Ca<sup>2+</sup> medium was also not affected by the presence of cycloheximide. These results again suggest that calcium-dependent regulation of GJIC by E-cadherin is because of posttranslational control of connexin 43.

**Discussion**

Our data indicate that calcium-dependent regulation of GJIC in mouse epidermal cells involves the function of a calcium-dependent cell adhesion molecule, E-cadherin. Two main lines of evidence support this conclusion: (a) E-cadherin expression and GJIC in the mouse epidermal cell lines examined were Ca<sup>2+</sup>-dependent and the -induced increases in GJIC were accompanied by enhanced levels of E-cadherin molecule expression; and (b) transfection of E-cadherin cDNA into P3/22 cells, which otherwise had very low E-cadherin expression and GJIC in low- and high-Ca<sup>2+</sup> media, resulted in a stable Ca<sup>2+</sup>-dependent expression of E-cadherin accompanied by Ca<sup>2+</sup>-dependent regulation of GJIC. Involvement of other cell adhesion molecules in the regulation of mouse epidermal cell GJIC cannot be excluded; nevertheless, our results strongly suggest an important role of E-cadherin.

This is the first report to directly demonstrate that Ca<sup>2+</sup>-dependent regulation of GJIC involves Ca<sup>2+</sup>-dependent cell adhesion molecules. Several previous observations have, however, suggested an important role of cell adhesion molecules in the regulation of GJIC. For example, Keane et al. (1988) have shown that in developing chick neuroectoderm, expression of neural cell adhesion molecule (N-CAM) co-

**Table III. Calcium-dependent Gap Junctional Intercellular Communication in E-cadherin Transfectant Clones during Treatment with Cycloheximide**

Cycloheximide (μg/ml)	P3E1				P3E2	
	[ <sup>3</sup> H]Leucine incorporation inhibition %	Number of dye-coupled cells/injection		[ <sup>3</sup> H]Leucine incorporation inhibition %	Number of dye-coupled cells/injection	
		low Ca <sup>2+</sup>	high Ca <sup>2+</sup>		low Ca <sup>2+</sup>	high Ca <sup>2+</sup>
Control		2 ± 1	40 ± 4		3 ± 2	33 ± 4
0.5	88		43 ± 4	82		nt
1.0	96		51 ± 5	91		31 ± 4

Cells were cultured in low-Ca<sup>2+</sup> medium (0.05 mM) until confluence. At time zero, GJIC was determined in control dishes and then cycloheximide was added just before cultures were switched to high-Ca<sup>2+</sup> medium (1.2 mM). GJIC was again determined 3–4 h later. In parallel dishes, protein synthesis was measured by the [<sup>3</sup>H]Leucine incorporation method as described in Materials and Methods.



incides with communication fields; blockage of N-CAM function by antibodies inhibited GJIC. E-cadherin antibody was also reported to block GJIC of cultured teratocarcinoma PCC3 cells (Kanno et al., 1984). More recently, Mege et al. (1988) have transfected L-CAM cDNA into otherwise L-CAM-deficient and GJIC-incompetent mouse sarcoma S180 cells and observed the morphological appearance of functional gap junctions. These results support the idea that, when cells are in contact, they need to recognize each other as homologous partners through cell adhesion (recognition) molecules, before they can form functional gap junctions.

In addition to regulation of GJIC, it is evident that E-cadherin has another function in the epidermal cells. The E-cadherin transfectants had a markedly altered morphology as compared to P3/22 cells and appeared as closely linked epithelioid sheets, even in low-Ca<sup>2+</sup> medium in which E-cadherin molecules were not expressed at cell-cell contact areas. This apparent Ca<sup>2+</sup> independence of morphological change in E-cadherin transfectants is in contrast to the strict Ca<sup>2+</sup> dependence of GJIC of these cells and therefore it is likely that these processes are regulated by E-cadherin by different mechanisms. In all other parent cell lines which expressed E-cadherin mRNA, morphology was epithelioid regardless of the Ca<sup>2+</sup> concentration in the culture medium, while the GJIC was Ca<sup>2+</sup> dependent, supporting the idea that morphology and GJIC ability are differently controlled in epidermal cells. Concerning the morphological alterations of the transfectants, it has been shown that both L-CAM (Mege et al., 1988) and E-cadherin (Nagafuchi and Takeichi, 1988) interact specifically with the cytoskeleton. It has been proposed that catenins mediate E-cadherin clustering by making complex with the cytoplasmic domain of E-cadherin and which link E-cadherin with actin (Ozawa et al., 1990a). Furthermore, it has been demonstrated that the association of E-cadherin and catenin is calcium independent (Ozawa et al., 1990b). Thus, such an association may play an important role in calcium-independent morphological changes observed in our E-cadherin transfectants.

Regulatory mechanisms of GJIC involving cell adhesion molecules may explain previously observed phenomena such as selective GJIC. When BALB/c 3T3 cells were transformed by a variety of carcinogens, we always observed selective GJIC between transformed and nontransformed cells; there was a similar level of GJIC among homologous cells, but no GJIC between heterologous cells (Enomoto and Yamasaki, 1984; Yamasaki et al., 1987; Hamel et al., 1988). Subsequently, we found that both transformed and nontransformed cells express similar levels of connexin 43 mRNA (Yamasaki et al., unpublished observation). Similar results were also obtained between tumorigenic and nontumorigenic rat liver epithelial cells (Mesnil and Yamasaki, 1988). Since the same connexin genes are expressed in transformed and nontransformed cells, alterations in cell recognition between these two types of cell may be a mechanism for such selective GJIC. In fact, subtle alteration in cell adhesion molecule expression in certain types of tumor cell has been reported (Eidelman et al., 1989; Hashimoto et al., 1989; Shimoyama et al., 1989). Recently, Frixen et al. (1991) have reported that the selective loss of E-cadherin expression is associated with dedifferentiation and invasiveness of human carcinoma cells.

Modulation of GJIC by extracellular Ca<sup>2+</sup> concentration has been observed not only in epidermal cells (Dotto et al.,

1989; and this study), but also in fibroblasts (Davidson et al., 1984). Since cadherin is a family of calcium-dependent cell adhesion molecules, it will be important to see whether other cadherins play an important role in calcium-dependent regulation of GJIC in other types of cell.

The present results also suggest that connexin 43 is the gap junction protein responsible for GJIC in mouse epidermal cells. Our results further suggest that E-cadherin regulation of GJIC in these cells involves calcium-dependent posttranslational control of connexin 43; the increase in GJIC and appearance of connexin 43 proteins at cell-cell boundaries occur rapidly after shifting Ca<sup>2+</sup> concentration in the presence of cycloheximide. It is now important to determine whether E-cadherin interacts directly or indirectly with gap junction protein (presumably connexin 43 in the present study) to establish GJIC.

Previously, we suggested that a progressive decrease in GJIC observed in cells derived from progressively malignant stages of skin carcinogenesis represents a good biological marker of malignancy (Klann et al., 1989). This suggestion was based on data on GJIC measured only in low-Ca<sup>2+</sup> media. Our present results, however, indicate that levels of GJIC in high Ca<sup>2+</sup> are similar in all cell lines except P3/22, which had virtually no E-cadherin expression. The net difference of GJIC between high- and low-Ca<sup>2+</sup> media, therefore, increased as the malignancy of the cells increased. These results suggest that the process of regulation of GJIC, rather than the mere level of GJIC per se, is involved in carcinogenesis. One relevant regulatory mechanism may involve cell adhesion molecules, such as E-cadherin, as suggested in the present study. In fact, several reports demonstrate that during cell transformation, changes in cell adhesion molecules occur. For example, CAM 105, which is present in normal liver, is not expressed in liver cell carcinomas nor after partial hepatectomy (Odin and Obrink, 1986). Likewise, N-CAM is no longer present in cerebella cell lines (Greenberg et al., 1984) nor in primary retina cells transformed by Rous sarcoma virus (Brackenbury et al., 1984). Furthermore, a recent study suggested that a tumor-suppressor gene of human colon cancer has a sequence homology to N-CAM (Fearson et al., 1990). These results are consistent with the idea that unimpaired cell-cell interaction is an important tumor-suppressive element (Yamasaki, 1990b).

Mouse epidermal cell differentiation in culture is regulated by extracellular Ca<sup>2+</sup> (Yuspa et al., 1989). The presence of a Ca<sup>2+</sup> gradient in epidermis in vivo (Menon et al., 1985) suggests that the Ca<sup>2+</sup> level may also play an important role as a physiological regulator of epidermal differentiation in vivo. On the other hand, the possible importance of GJIC in the maintenance of homeostasis between mouse epidermal cells in vivo has been proposed based on the observation that GJIC of epidermal cells is compartmentalized into cylindrical units which correspond to their differentiating units (Kam et al., 1986). Taken together with our observations that Ca<sup>2+</sup>-dependent cell adhesion molecules regulate GJIC of epidermal cells, it is tempting to postulate a sequence of events which regulate mouse epidermal cell differentiation, i.e., extracellular Ca<sup>2+</sup>→cell adhesion (recognition)→GJIC→cell differentiation control. Interruption of such a sequence may disturb the normal pathway of differentiation and contribute to malignant transformation of epidermal cells. Consistent with such a postulate are the findings

that the early stage of skin carcinogenesis involves the alteration of gene(s) controlling the Ca<sup>2+</sup>-dependent induction of epidermal cell differentiation (Yuspa and Morgan, 1981), and that the GJIC level per se as well as the Ca<sup>2+</sup> dependence of GJIC are altered in malignantly transformed epidermal cells (Klann et al., 1989; and this study).

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