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Gap Junction Formation between Cultured Embryonic Lens Cells Is Inhibited

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Intercellular communication mediated by gap junctions is important for tissue homeostasis in the avascular lens, and extensive areas of gap junctions form between fiber cells during fiber cell differentiation and lens development. We examined the role of the calcium-dependent cell adhesion molecule, N-cadherin, in the process of gap junction formation between fiber cells. Lentoids, multicellular structures with characteristics of differentiated fiber cells, were isolated from embryonic chick lens cultures and subsequently paired to provide an *in vitro* model of fiber cell interactions. Gap junction formation between cells of paired lentoids was monitored by observing the lentoid-to-lentoid transfer of fluorescent dyes, either calcein or Lucifer yellow, over a time course of up to 48 hr. Dye transfer between lentoids was inhibited upon the addition to the medium of Fab fragments (100–622 μg/ml) of a monoclonal antibody specific for N-cadherin, and also by the reduction of extracellular calcium in the incubation medium. However, the addition of Fab fragments (100–1500 μg/ml) of an antibody to a fiber-cell-specific integral membrane protein, MIP, did not change the time course nor extent of dye transfer between lentoids. Our results, using cultured embryonic cells, extend those from previous studies with cell lines and transfected cells. We conclude that cadherin interactions facilitate the formation of gap junctions between embryonic lens fiber cells, by the stabilization of membrane appositions and/or by the generation of an intracellular signal(s).

INTRODUCTION

Direct cell–cell communication mediated by gap junctions is one type of cell interaction that is thought to be important for controlling the precise spatial and temporal events which occur during embryonic development (Caveney, 1985; Warner, 1992; Wolpert, 1978). The presence of gap junctions in early embryonic systems was first indicated by the electrical coupling detected within these systems (Lo and Gilula, 1979; Potter *et al.*, 1966). Reports of the coincidence of communication compartments in embryos with patterns of coordinated differentiation further suggested that gap junctions play a role in embryogenesis (Fraser and Bryant, 1985; Warner and Lawrence, 1982; Weir and Lo, 1982). Perturbation of junctional communication with antibodies to gap junction proteins demonstrated that cell differentiation and tissue patterning were affected by intercellular communication (Fraser *et al.*, 1987; Warner *et al.*, 1984). More recently, studies in transgenic mice, in which connexin 43 was deleted, indicated that this gap junction protein was needed for normal heart development (Reaume *et al.*, 1995). Thus, the transmission of small molecules, including regulatory substances, via gap junction channels appears to be a significant aspect of development (Warner, 1992).

Factors that regulate gap junction communication, therefore, can have an impact on developmental processes. One level of regulation of intercellular communication involves the assembly of gap junction channels between cells. The assembly of junctions is controlled by events directly affecting gap junction proteins, such as posttranslational modifications and trafficking within the cell (Lampe, 1994; Musil *et al.*, 1990; Puranam *et al.*, 1993). However, the establishment of functional cell–cell communication also appears to depend on the interactions of cellular components other than gap junction proteins, e.g., adhesion proteins.

The importance of specific cell adhesion molecules, the cadherins, for the formation of communicating junctions has been demonstrated in a variety of cultured and trans-

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fected cells. Members of the cadherin family mediate cell adhesion via calcium-dependent, homophilic interactions (Takeichi, 1988). Prior to the identification of cadherin molecules, a connection between a divalent cation-dependent event and intercellular communication was suggested (Davidson *et al.*, 1984; Loewenstein, 1967). In addition, it was postulated that membrane proteins other than gap junction proteins were responsible for creating stable membrane associations that preceded the formation of gap junctions (Loewenstein, 1981). Recent studies using cell lines and primary cell cultures revealed a relationship between the expression of cadherins and cell communication mediated by gap junctions. Cells that were deficient in the expression of cadherins displayed limited functional and morphological evidence for the presence of gap junctions (Matsuzaki *et al.*, 1990; Mege *et al.*, 1988; Musil *et al.*, 1990). However, following transfection of cells with cDNA encoding various cadherins, increases in dye coupling and in gap junction structures were observed. The dependence of gap junction communication on external calcium was also correlated with the expression of E-cadherin in mouse epidermal cells (Jongen *et al.*, 1991).

The focus of the present study was on the role of cadherins in the assembly of gap junctions between embryonic cells, specifically chick lens fiber cells. The differentiation of lens fiber cells in the embryonic chick is a well-characterized, developmental process (Piatigorsky, 1981). Distinctive changes in cell shape, in membrane interactions, and in the composition of the cell cytoplasm and plasma membrane occur during the differentiation of fiber cells (Benedetti *et al.*, 1976; Piatigorsky, 1981). Gap junctional plaques increase substantially during differentiation to cover more than 60% of the area of plasma membranes (Kuszak *et al.*, 1978; Lo and Harding, 1986), but little is known about the regulation of the formation of gap junctions between fiber cells. Because chick lens fiber cells are known to express N-cadherin (aka ACAM, see Methods) (Hatta and Takeichi, 1986; Volk and Geiger, 1984, 1986b), the relationship between gap junction formation and cadherin-mediated cell adhesion was examined using differentiated lens fiber cells.

The analysis was performed with lentoids, multicellular structures that developed in cultures of embryonic chick lens cells and that displayed many characteristics of differentiated fiber cells (Menko *et al.*, 1984, 1987; Patek *et al.*, 1986; Piatigorsky *et al.*, 1972). Lentoids were isolated and subsequently paired to provide a well-defined *in vitro* model for gap junction assembly between fiber cells. The formation of gap junctions between cells of paired lentoids was detected by the transfer of calcein, following the uptake of the esterified dye by cells in one lentoid of the pair. Conventional dye injection was also performed in some experiments to detect gap junction assembly.

In order to interfere with cadherin interactions between the cells of adjacent lentoids, Fab fragments of an antibody specific for an extracellular domain on N-cadherin (Volk *et al.*, 1990) were included in the medium during lentoid pairing experiments. In a second approach to interfere with cadherin interactions between cells of lentoids, the concentration of extracellular calcium was decreased, a method known to disrupt cadherin-mediated adhesion between cells (Duband *et al.*, 1987; Kartenbeck *et al.*, 1991; Volberg *et al.*, 1986). Results from these experiments demonstrated that cadherin-mediated interactions play an important role in the establishment of gap junction communication between embryonic cells.

METHODS

Cell Culture

Embryonic chick lens cells were cultured as previously described (Menko *et al.*, 1984). Briefly, lenses were dissected from 10-day-old embryonic White Leghorn/Rhode Island Red chicks and digested with 0.08% trypsin (Gibco BRL, Grand Island, NY) at 37°C for 20–30 min with agitation. Cells from the digested lenses were plated at a density of $2–5 \times 10^5$ cells per dish onto 35-mm tissue culture dishes that were previously coated with Vitrogen 100, bovine dermal collagen (Celtrix Laboratories, Palo Alto, CA). Lens cell cultures were maintained for up to 9 weeks in a 37°C, 5% CO_2 humidified incubator. The culture medium, Medium 199 (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), was replaced every 2 to 3 days.

Lentoid Isolation

Multilayered groups of cells termed ''lentoids'' developed during a process of *in vitro* differentiation in the lens cell cultures. Clearly delineated lentoids (lentoid islands) were isolated from the periphery of the culture dishes in 2- to 9-week cultures, using gentle pressure near the base with a pulled glass needle. Intact lentoid islands were lifted away from the surrounding, less-differentiated cells and transferred from the culture dish into Vitrogen 100-coated 10-mm tissue culture inserts (Nunc, Naperville, IL). The inserts, 10-mm-diameter plastic cylinders with a microporous filter at one end, were placed inside wells of a 24-well plate. The wells with the inserts contained 1 ml Medium 199 with 10% FBS and the plates were incubated at 37°C and 5% CO_2 for 24 to 48 hr to allow the lentoids to adhere to the surface of the inserts.

Calcein Dye Transfer

The retention of calcein within cells is partially determined by dye concentration and incubation temperature (information provided by the manufacturer, Molecular Probes, Inc., Eugene, Oregon) (Thomas, 1986). In the present study, a range of calcein-AM concentrations (2, 4, 8, and 10 μM) and several temperatures (30, 33, and 37°C) were used to determine the optimum conditions for long-term retention and detection of calcein in lentoids. Calcein fluorescence in lentoids was visible for at least 50 hr using a calcein-AM concentration of 8 μM to load cells. Calcein-containing lentoids incubated at 30 and 33°C were more intensely fluorescent for a longer time than lentoids incubated at 37°C. Therefore, all dye loading was carried out at 33°C using 8 μM calcein-AM.

Inserts containing adhering lentoids were rinsed with 0.5 ml Medium 199 prior to loading lentoids with calcein-AM. In this step and subsequent labeling, serum-free medium was used. An 8 μM

solution of calcein-AM was prepared immediately before use from a stock solution of 1 mg/ml calcein-AM in DMSO. Lentoids were then incubated with 0.3 ml 8 μM calcein-AM for 13 min at 33°C and 5% CO_2. Medium 199 plus 10% FBS was used to rinse inserts two times, and inserts were moved to wells with fresh Medium 199 plus 10% FBS. In the initial stages of experimental design, calcein-AM-loaded lentoids were maintained at 33°C and 5% CO_2 for 4 to 24 hr prior to pairing with nonloaded lentoids. As the details of the procedure became refined, loaded lentoids were paired with nonloaded lentoids 4 to 6 hr after loading. Therefore, in all Fab fragment and EGTA experiments, lentoids, whether incubated with Fab fragments, PBS, or EGTA, were paired 4 to 6 hr after loading. Inserts were rinsed several more times with Medium 199 plus 10% FBS during this period. Residual calcein-AM was removed from the inserts by repeated washes and by the action of endogenous esterases contained in the serum.

Nonloaded lentoids for pairing with the calcein-containing lentoids were removed from lens culture dishes and were placed with the adherent, calcein-containing lentoids. A pulled glass needle was used to manipulate the lentoids into contact to form pairs. In most cases, paired lentoids were of approximately similar size. Occasionally, a larger lentoid was paired with more than one smaller lentoid. A dissecting microscope with a magnification of 50× was used to ensure that the lentoids were in physical contact. Unpaired, nonloaded lentoids in the inserts served as controls for nonspecific uptake of calcein.

After various incubation times in a 33°C, 5% CO_2 humidified incubator, lentoid pairs were viewed with both phase-contrast and fluorescence optics on a Zeiss IM35 inverted microscope. A standard fluorescein filter set (excitation 490 nm, emission 525 nm) was used to view calcein. The lentoid pairs were photographed at each time point with T-Max 400 film (Eastman Kodak Company, New Haven CT). The number of lentoid pairs and the number of pairs with dye transfer at specific times were later analyzed and scored blindly from the photographs by two individual investigators.

Lucifer Yellow Microinjection

The preparation of lentoid pairs for Lucifer yellow microinjection was the same as for the calcein approach described above, without the dye-loading step. At various times after pairing, an insert with lentoid pairs was lifted out of the well and placed in a 35-mm dish. A cell in one lentoid of a lentoid pair was microinjected with 2% Lucifer yellow (dilithium salt, Sigma Chemical Co., St. Louis, MO) in PBS (136 mM NaCl, 1.48 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$, 2.7 mM KCl, pH 7.3–7.5) using a General Valve picospritzer set at 30 psi. The microinjected lentoid pair was monitored for up to three hours, and photographed at various time points with T-Max 400 film. The number of lentoid pairs displaying dye transfer was later tabulated from the photographs.

Fab Fragment and EGTA Treatments

The calcium-dependent cell adhesion molecule referred to as N-cadherin (Hatta and Takeichi, 1986) or ACAM (Volk and Geiger, 1984, 1986b) is expressed by lens fiber cells. It is generally recognized that N-cadherin and ACAM are identical (Duband *et al.*, 1988; Hatta *et al.*, 1988), and for clarity the term N-cadherin will be used throughout this paper.

Fab fragments of antibody were generated from two sources: (1) ascites fluid containing monoclonal antibody to N-cadherin (clone GC-4, Sigma Chemical Co.), and (2) rabbit antiserum containing antibodies generated to a peptide sequence corresponding to the predicted third extracellular loop of bovine MIP (generous gift of B. Yancey) (Gorin *et al.*, 1984). The rabbit antiserum cross-reacted with chicken MIP as determined by Western immunoblot. The ascites fluid and antiserum were dialyzed against 100 mM Tris/2.7 mM EDTA (pH 8.0) overnight at 4°C. To obtain the IgG portion, the dialyzed material was run over a protein A agarose column, and the bound material was eluted with low pH Elution buffer supplied in an ImmunoPure Fab Preparation Kit (Pierce, Rockford IL). Fractions were monitored by absorbance at 280 nm and pooled. The pooled antibody fractions were then dialyzed against 20 mM phosphate/10 mM EDTA (pH 7.0) overnight at 4°C, and concentrated (Centricon-30 or -100 microconcentrator, Amicon, Beverly, MA).

Fab fragments were prepared according to the procedures contained in the Fab Preparation Kit. Briefly, antibody was digested with papain at 37°C for 13 to 16 hr, and the digested material was run over a protein A agarose column to separate the Fab fragments from the Fc fragments and the undigested antibody. The nonbound Fab fragment fractions were dialyzed against PBS overnight at 4°C, concentrated with a Centricon-10 microconcentrator, and filter sterilized.

The purity of the Fab preparations was determined with silver-stained SDS–polyacrylamide gels of the preparation under reduced conditions. A doublet with an apparent electrophoretic mobility in the range of 25 kDa, the appropriate size for Fab fragments, was observed. The protein content was determined either by absorbance at 280 nm or by bicinchoninic acid (BCA) protein assay (Pierce). The reactivity of the Fab fragment fractions with membranes from embryonic chick lens cells and cultured lens cells was determined by Western immunoblot analysis. Immunofluorescence experiments with lens cell cultures indicated the reactivity of the Fab fragment preparations with cells in lentoid islands.

Various amounts of the Fab fragment preparations were then added to the Medium 199 with 10% FBS used during the incubation of lentoids prior to (45–120 min), during, and after lentoid pairing. Parallel control wells of lentoids contained Medium 199 with 10% FBS to which an equivalent volume of PBS was added.

In some experiments, extracellular calcium was decreased in the lentoid medium by the addition of various amounts of a 0.5 M buffered stock solution of ethylene glycol bis-N,N,N',N'-tetraacetic acid (EGTA, Sigma Chemical Co.). The free calcium concentrations of the 1.0, 1.1, 1.25, and 1.5 mM EGTA solutions were determined by published methods (Brooks and Storey, 1992). Lentoids were incubated in EGTA-containing medium prior to (1–2 hr), during, and after lentoid pairing.

Dot Blots

Using dot blots, media samples from wells containing Fab fragments were tested for degradation of antibody over the course of the experiments. Samples were serially diluted with 0.1 M $NaHCO_3$, pH 9.65. Then, 5 μl of each sample was dotted onto nitrocellulose (Gelman Biotrace NT, Ann Arbor, MI) and allowed to air dry for 40 min. The dot blot was blocked with 2% BSA in TBS (25 mM Tris base, 150 mM NaCl, pH 7.3–7.6) for 1 to 2 hr prior to incubation with ^{125}I-labeled goat anti-mouse or anti-rabbit IgG antibody (ICN, Costa Mesa, CA) for $1\frac{1}{2}$ hr at room temperature. The dot blot was then processed for autoradiography with Kodak X-OMAT film.

Western Immunoblots

Lenses were dissected from 10- to 14-day embryonic White Leghorn/Rhode Island Red chicks and stored in liquid nitrogen until processed. Embryonic chick lens cell cultures were rinsed once with TBS plus protease inhibitors (10 m*M* EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1–2 m*M* PMSF), and cells were scraped off the dish and processed immediately. Crude membrane samples were then prepared in a similar fashion from both embryonic lens cells and cultured lens cells. All samples were kept on ice and in the presence of protease inhibitors during the following processing steps. Tissue was sonicated at intervals with a probe sonicator for a total of 3 to 5 min. Cellular material was pelleted in a Beckman Microfuge E and resuspended with TBS plus protease inhibitors. Protein content was determined by BCA protein assay, and samples were aliquoted and stored at −70°C.

The crude membrane samples were electrophoresed on 8 or 12% SDS–polyacrylamide gels according to Laemmli (Laemmli, 1970). Separated proteins were transferred to nitrocellulose (Gelman Biotrace NT) in Towbin buffer (Towbin *et al.,* 1979) with the addition of 0.01% SDS. The blot was treated for 1 to 3 hr with blocking buffer (2% BSA, 0.05% Tween 20 in TBS), and then incubated overnight at 4°C with primary antibody. The Fab fragment preparation or the intact monoclonal antibody for N-cadherin was used at 10 μg/ml. The rabbit antiserum prepared to MIP was diluted 1:400 and the Fab fragment preparation was diluted to 10 μg/ml. After washes in blocking buffer, the blot was incubated with ^{125}I-labeled goat anti-mouse or anti-rabbit IgG antibody for $1\frac{1}{2}$ hr at room temperature. The radiolabeled proteins were detected by autoradiography with Kodak X-OMAT film.

Immunofluorescence

Embryonic chick lens cell cultures were rinsed three times with PBS (68 m*M* NaCl, 0.74 m*M* KH_2PO_4, 4.05 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.35 m*M* KCl, pH 7.3–7.5), fixed at room temperature for 10 min with 4% formaldehyde, and rinsed again three times with PBS. In experiments with the monoclonal antibody to N-cadherin, cells were optionally permeabilized for 10 min at room temperature with 0.1% Triton X-100 and rinsed three times with PBS. Experiments with antiserum to MIP were always performed with nonpermeabilized cells. Cells in the dishes were incubated with 10 m*M* glycine and 2% BSA in PBS overnight at 4°C to quench reactive aldehydes and block nonspecific protein binding sites. The monoclonal antibody for N-cadherin or the Fab fragment preparation was diluted to a final specific antibody concentration of 20–28 μg/ml in 2% BSA. Antiserum with reactivity to MIP was diluted 1:400 and the Fab fragment preparation was diluted to a concentration of 100 μg/ml in 2% BSA. Cultures were incubated for $1\frac{1}{2}$ hr at room temperature with primary antibody solutions. Dishes without primary antibody served as negative controls. After several washes with 2% BSA, rhodamine-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added for 1 hr at room temperature. Samples were viewed with epifluorescence on a Zeiss IM35 inverted microscope with a rhodamine filter (excitation 546 nm, emission 590 nm), and photographed with T-Max 400 film.

RESULTS

Paired Lentoids as a Model of Fiber Cell Interactions

Multicellular lentoids developed during a reproducible process of differentiation in the cultures of embryonic chick lens cells (Menko *et al.,* 1984; Okada *et al.,* 1971; Patek *et al.,* 1986). By Day 9, initial lentoid development was evident when clusters of cells with a swollen appearance arose among a monolayer of cells. The lentoid cells continued to increase in height compared to the surrounding undifferentiated or pavement-packed epithelial cells. By Weeks 2–3, lentoids were evident on the periphery of the culture dish as raised, roughly spherical entities, lentoid islands, surrounded by flatter cells. The central area of the culture exhibited more extensive ridges and networks of lentoids. As described previously, lentoid cells displayed numerous characteristics of differentiated lens fiber cells, including increased cell volume and the expression of a fiber cell-specific protein, MIP (Menko *et al.,* 1984, 1987; Okada *et al.,* 1971; Patek *et al.,* 1986). Morphological and functional evidence for the presence of gap junctions in lentoids has previously been reported (Berthoud *et al.,* 1994; Menko *et al.,* 1987).

Lentoid islands were an integral part of the complex culture system, and were carefully removed from the surrounding less well differentiated cells. The isolated lentoids, composed of approximately 100 cells, were highly coherent groups of cells that remained intact during and after transfer to 10-mm tissue culture inserts. After the lentoids adhered to the inserts, additional lentoids were added and paired with the adherent lentoids. The insert size facilitated successful pairing of lentoids. The paired lentoid system provided a lens fiber cell model with which to study the formation of gap junctions.

Calcein Dye Transfer Is an Effective Method for the Detection of Gap Junction Formation

Calcein, a fluorescein derivative, was used in a noninvasive method to detect gap junction formation between paired lentoids (Fig. 1). Other fluorogenic substances, such as carboxyfluorescein and BCECF [2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein)], have also been used to detect intercellular communication (El-Sabban and Pauli, 1991; Goodall and Johnson, 1982; Kidder *et al.,* 1987). These dyes are retained only by viable cells (Rotman and Papermaster, 1966) and are of appropriate molecular mass to pass through gap junction channels. A population of single, unpaired, multicellular lentoids was incubated with the acetoxymethyl ester of calcein (calcein-AM), which is a nonfluorescent, electrically neutral, membrane-permeable compound. Upon entrance into lentoid cells, calcein-AM was cleaved by intracellular esterases to produce the fluorescent, negatively charged molecule calcein (M_r 623 Da). Numerous lentoids in an insert were bulk loaded with calcein simultaneously using this approach. As seen with confocal microscopy, calcein was present within all cells of a lentoid structure, not just within peripheral cells (data not shown).

At 4 to 24 hr subsequent to bulk loading lentoids with calcein, nonloaded lentoids were paired with the calcein-containing lentoids. The lentoid pairs were monitored for

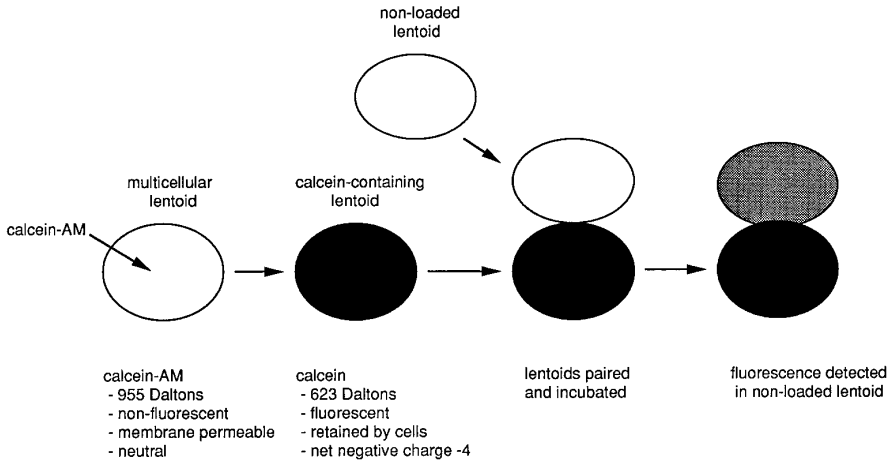

FIG. 1. Calcein dye transfer method. Isolated lentoids were incubated with 8 μM calcein-AM at 33°C for 13 min. Calcein-AM entered the cells and was cleaved by endogenous esterases to form calcein. Calcein-containing multicellular lentoids were paired with nonloaded multicellular lentoids, and the lentoid pairs were incubated at 33°C for a period of time. The detection of fluorescence in the nonloaded lentoid of the lentoid pair was interpreted to mean that calcein dye had transferred via gap junctions, which formed between the cells of the adjacent lentoids.

dye transfer over a period of time, and the detection of fluorescence in cells of the previously nonloaded lentoids indicated the movement of calcein between cells of adjacent lentoids via newly formed gap junctions (Fig. 2). The fluorescent image of a lentoid pair in Fig. 2B illustrates the detection of calcein in cells of the previously negative, nonloaded lentoid 28 hr after pairing.

Unpaired, nonloaded lentoids in the same inserts as the lentoid pairs served as controls to monitor uptake from the medium of any residual calcein-AM or leaked calcein. Nonloaded lentoids were placed close to, but not in contact with, calcein-containing lentoids at the same time lentoid pairs were formed (Fig. 3). Figure 3B depicts an example of an unpaired lentoid in which fluorescence was not detected after 26 hr of coincubation with calcein-containing lentoids. An evaluation of all experiments indicated that fewer than 3% ($n = 92$) of the unpaired control lentoids displayed fluorescence over the time course of the experiments, emphasizing the requirement for cell contact. In those that did fluoresce, the intensity was faint and rapidly decreased over the first 30 hr of incubation, rather than increase as in the paired lentoids which displayed dye transfer.

With this method, it was possible to monitor each lentoid pair at various times, and a time course of junction formation for each pair was determined. The earliest time calcein fluorescence was detected in cells of the recipient lentoid of a lentoid pair was 17 hr postpairing. Sixty-three percent of the lentoid pairs were positive for calcein dye transfer by 26 hr postpairing, and 89% of lentoid pairs displayed transfer of calcein to cells of the nonloaded lentoid of the pair when observed by 48 hr after pairing (Table 1). Calcein was also detected in lentoids observed more than 48 hr following pairing, although the fluorescence was fainter by this time due to fading and some leakage of calcein (Thomas, 1986). Data were not routinely collected from these later time points.

The method of monitoring calcein dye transfer to detect the formation of gap junctions was validated by parallel microinjection studies with Lucifer yellow. Paired lentoids were incubated for various lengths of time similar to the times used in the calcein dye transfer experiments. A 2% Lucifer yellow solution was then microinjected into a cell of one lentoid of a lentoid pair. The noninjected lentoid was monitored for the appearance of dye, interpreted as transfer via gap junctions. In previous work in which rhodamine-conjugated dextran was coinjected with Lucifer yellow into lentoid cells, no spread of dextran to adjacent cells was observed (personal observation, R. Johnson), emphasizing that dye transfer occurred via gap junctions not cytoplasmic bridges.

The fluorescent images of the lentoid pair in Fig. 4 illustrate the progressive transfer of dye from cell to cell within the microinjected lentoid, as well as the transfer of dye to cells of the recipient lentoid. Typically, within the first 45 min, dye spread throughout cells of the donor lentoid, with decreased intensity seen with increased distance from the site of microinjection. Beginning at variable times during this 45-min time period, dye was also detected in the recipient lentoid, with a gradient of dye intensity related to the distance from the lentoid–lentoid interface. However, a distinct border between the donor and recipient lentoids was always evident. In observations made up to 1.5 hr after the start of the microinjection, the dye intensities within the two lentoids approached equilibrium, with no obvious gradient visible within a lentoid. However, a clear difference in fluorescence intensity was routinely observed between

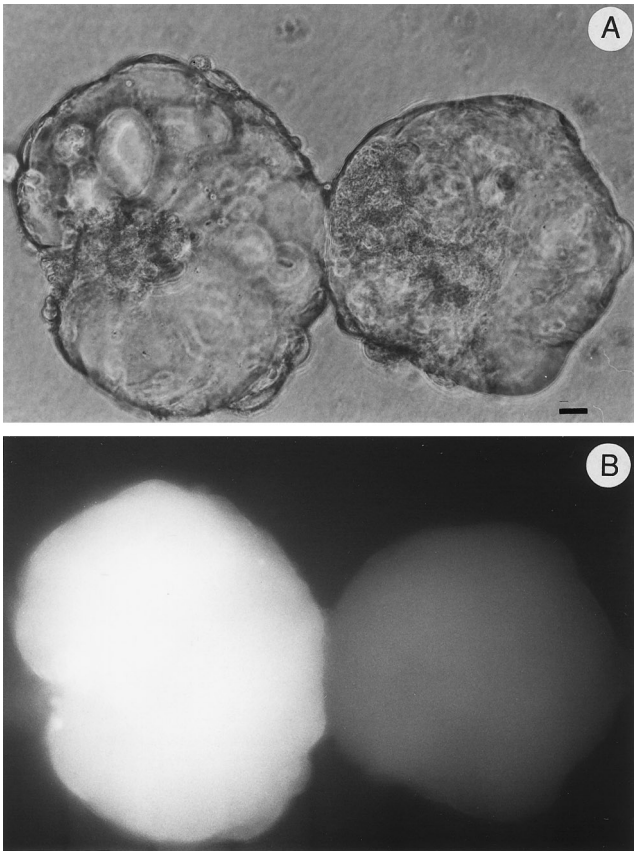

FIG. 2. Demonstration of calcein dye transfer between lentoids paired for 28 hours. Phase-contrast (A) and fluorescent (B) images of a lentoid pair composed of a calcein-containing lentoid on the left and a nonloaded lentoid on the right. At 28 hr postpairing, fluorescence was detected in the previously negative lentoid on the right. Bar, 60 μm.

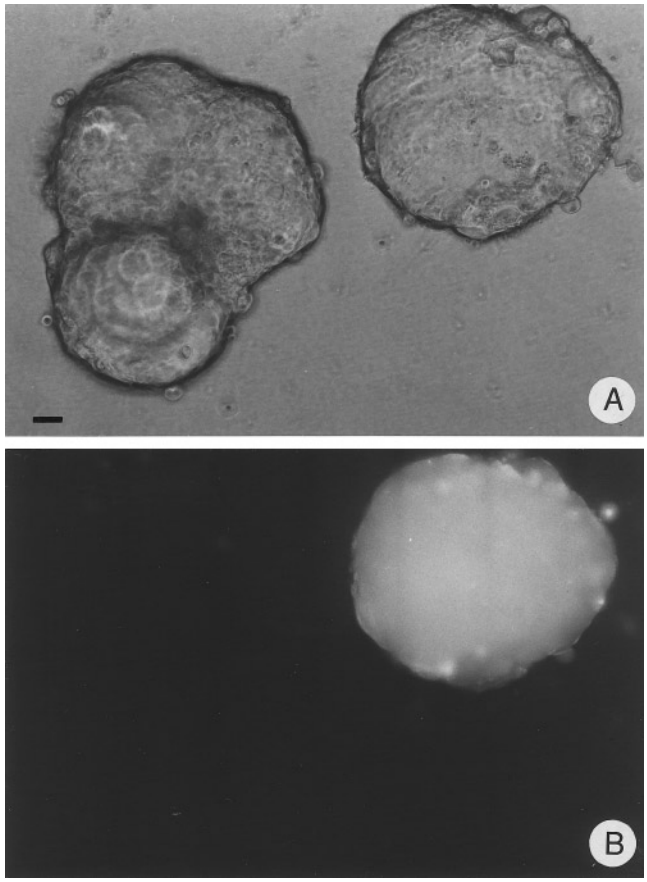

FIG. 3. Unpaired lentoids monitor nonspecific uptake of calcein. Phase-contrast (A) and fluorescent (B) images of a lentoid close to, but not in contact with, a calcein-containing lentoid. Calcein dye was not detected in the nonloaded lentoid on the left after 26 hr of coculture with the calcein-containing lentoid on the right. Bar, 100 μm.

donor and recipient lentoids within a pair, with the donor lentoid appearing more bright, even 2 to 3 hr after the microinjection. A possibility is that some dye became bound within the cells of the donor lentoids.

With the dye injection method it was possible to microinject a lentoid pair at a single time point and observe the pair for dye transfer for only several hours. In 40% of the lentoid pairs microinjected 17–26 hr after pairing, the transfer of Lucifer yellow to cells of the noninjected lentoid of the pair was detected, while 63% of lentoids paired for 28–48 hr prior to microinjection displayed transfer of dye (Table 1). Lentoids paired for much longer periods of time (3 to 8 days) displayed transfer of dye in a greater percentage of pairs.

In the calcein experiments, lentoid pairs observed up to 48 hr postpairing displayed a fluorescence pattern similar to that for Lucifer yellow, i.e., the intensity of the fluorescence was uniform throughout a donor lentoid and throughout a recipient lentoid, yet dye intensity did not reach equilibrium between donor and recipient lentoids within the time frame of the experiments. Thus, as revealed by both the calcein and Lucifer yellow experi-

TABLE 1
Comparison of Dye Transfer Methods

Time observed postpairing	Lentoid pairs with dye transfer	
	Calcein	LY
17–26 hr	63% (n = 123)	40% (n = 10)
28–48 hr	89% (n = 103)	63% (n = 19)
>48 hr	ND	95% (n = 21)
All time points	86% (n = 161)	72% (n = 50)

Note. Comparison of the percentage of lentoid pairs which demonstrated dye transfer using the calcein dye approach and Lucifer yellow microinjection. ND, not determined.

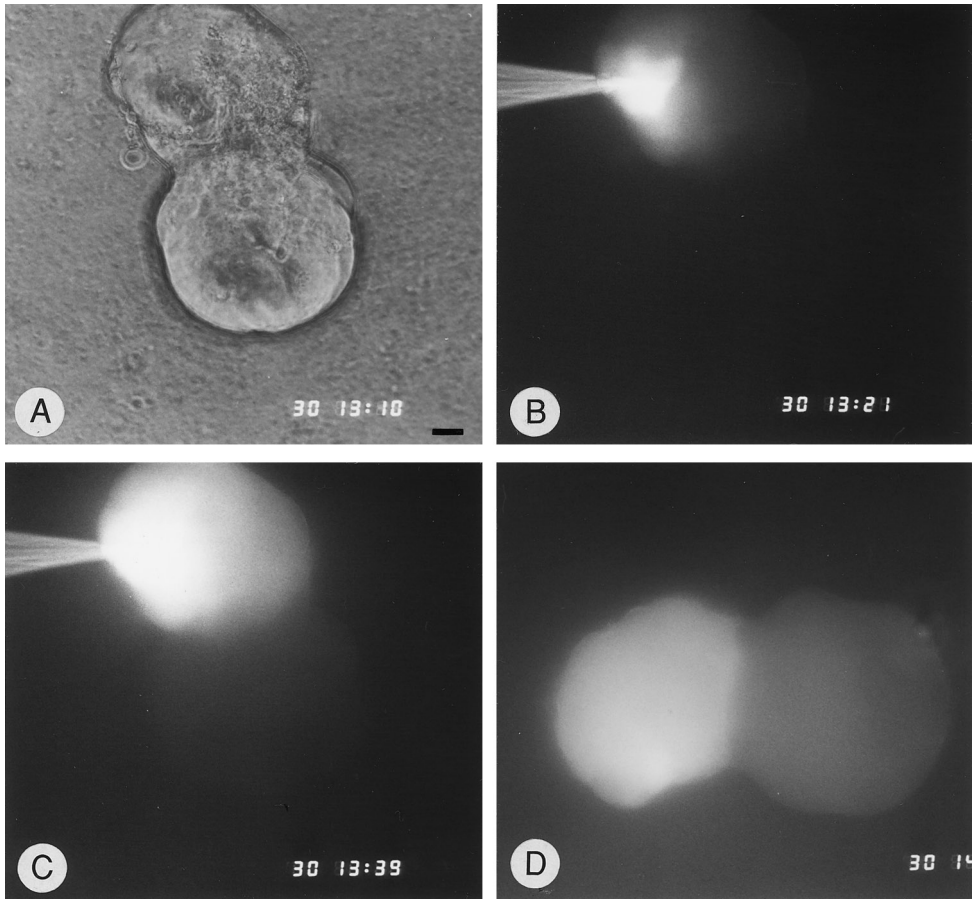

FIG. 4. Lucifer yellow dye transfer between paired lentoids. (A) Phase-contrast micrograph of lentoids prior to microinjection. (B–D) Fluorescent images of the lentoid pair 10, 28, and 66 min, respectively, after the initiation of the microinjection of a 2% Lucifer yellow solution into the upper lentoid. (In D, the photograph was shifted 90° with respect to A, B, and C.) With increased time, fluorescence was detected in the noninjected lentoid. Bar, 60 μm.

ments, the permeability of junctions assembled within lentoids was greater than the permeability of junctions assembled between paired lentoids. As a result, the junctional permeability between lentoids was the limiting factor in the assays for gap junction assembly. This was not unexpected due to the fact that donor and recipient lentoids developed for 2 to 9 weeks in lens cell cultures prior to being manipulated, while lentoids in pairs were in contact for no more than 48 hr.

The detection of gap junction formation between cells of paired lentoids with calcein dye transfer was in agreement with results obtained with Lucifer yellow transfer (Table 1). In fact, calcein dye transfer was a more sensitive method for detecting gap junction formation in observations made within 48 hr of lentoid pairing. Calcein has been reported to be nontoxic to cells (Radcliff *et al.*, 1991), and lentoids loaded with calcein appeared healthy by phase microscopy observations and as determined by trypan blue exclusion 3 to 4 days after loading (data not shown). We concluded that the calcein dye transfer method was a valid, effective, even advantageous, approach to monitor the formation of cell–cell communication between cells of paired lentoids.

N-Cadherin Is Expressed by Cells in Lens Cell Cultures

A monoclonal antibody, GC-4, specific for an epitope on the extracellular portion of N-cadherin (Volk *et al.*, 1990) was used to determine the expression of N-cadherin in cultured lens cells. N-cadherin was previously identified in embryonic chick lens cells and was shown to migrate with an apparent electrophoretic mobility of 135 kDa in SDS–polyacrylamide gels (Hatta and Takeichi, 1986; Volk and Geiger, 1984). Similarly, the Western immunoblot in Fig. 5 shows that a single reactive band with an apparent molecular migration of 132 kDa was detected in membranes from

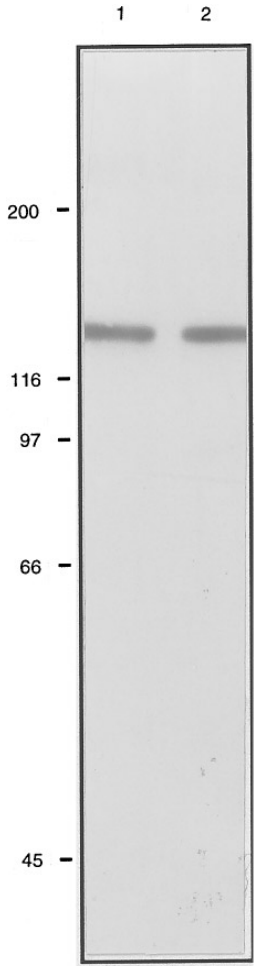

FIG. 5. Detection of N-cadherin by Western immunoblot analysis. Proteins in membrane preparations from 22-day lens cell cultures (lane 1, 69 μg protein) and 10- to 14-day embryonic chick lenses (lane 2, 76 μg protein) were separated on an 8% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with a monoclonal antibody to N-cadherin. A single band, which migrated with an apparent molecular mass of 132 kDa, was detected in both samples. Molecular weight standards are indicated on the left in kDa.

embryonic chick lens cells (lane 2) and in membranes from cultured lens cells (lane 1).

In immunofluorescence experiments with the monoclonal antibody to N-cadherin, bright fluorescent staining was detected along cell membranes of all cell types in lens cell cultures, lentoid cells, undifferentiated, and pavement-packed epithelial cells. As reported previously, labeling of contacts between cultured chick lens cells by an antibody to N-cadherin (ID-7.2.3) was extensive when the cells were treated with Triton X-100 (Volk and Geiger, 1984, 1986b). A comparison of fluorescent labeling of nonpermeabilized cells and permeabilized cells in lens cell cultures with the monoclonal antibody GC-4 for N-cadherin indicated that a similar labeling pattern was observed in either case (data not shown). However, the fluorescence in permeabilized cells was more distinct and less hazy than in nonpermeabilized cells, possibly due to a change in the conformation or accessibility of the antigenic site in the presence of the detergent. Therefore, immunofluorescence studies with the monoclonal antibody for N-cadherin were performed primarily on permeabilized cells. The phase-contrast image in Fig. 6A shows a lentoid island surrounded by a monolayer of undifferentiated cells and pavement-packed epithelial cells. As seen in the fluorescent image in Fig. 6B, lentoid cells were intensely outlined after incubation with antibody to N-cadherin. Results from these experiments indicated that N-cadherin was expressed in cultured embryonic chick

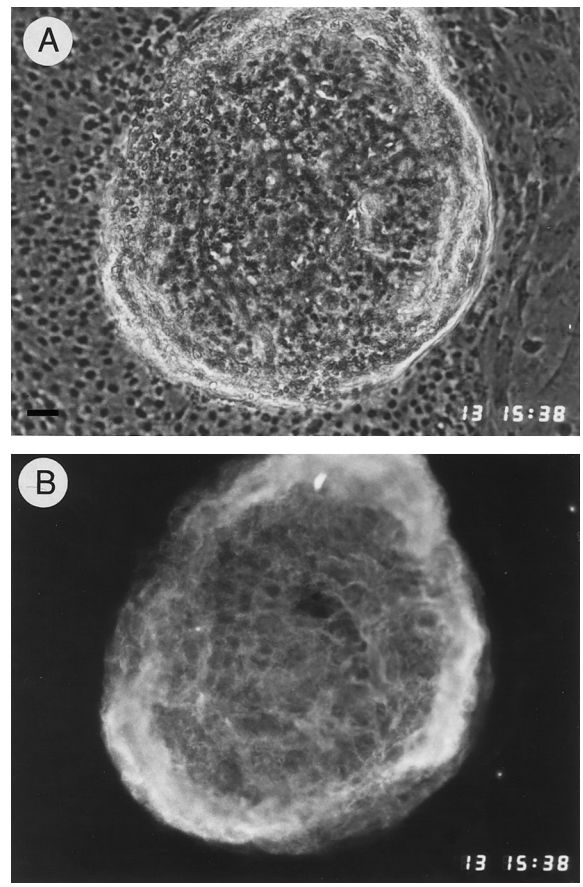

FIG. 6. Detection of N-cadherin in embryonic lens cell cultures by immunofluorescence. A fixed and permeabilized 34-day lens culture was incubated with monoclonal antibody to N-cadherin, followed by rhodamine-labeled anti-mouse secondary antibody. A lentoid surrounded by less-differentiated cells is shown in the phase-contrast micrograph (A). The corresponding fluorescent image (B) shows the bright cell outlines displayed by the lentoids. Due to the increased height of the lentoid cells, the cells surrounding the lentoid are out of the plane of focus. Bar, 60 μm.

lens cells and, importantly for the present study, by lentoid cells.

Dye Transfer Is Inhibited by Treatment of Lentoid Pairs with Fab Fragments of Antibody to N-Cadherin

In order to investigate the role of N-cadherin-mediated cell adhesion in the establishment of gap junctions between cells of paired lentoids, Fab fragments of antibody to N-cadherin were added to the incubation medium in lentoid-pairing experiments. Results from Western immunoblot analysis and immunofluorescence experiments indicated that the Fab fragment preparation of N-cadherin-specific antibody retained activity to N-cadherin expressed in lens cells and in cultured lens cells (data not shown). Fab fragment concentrations of 100, 125, 200, 250, and 622 μg/ml in Medium 199 with 10% FBS were used to treat lentoids under experimental conditions. Each experiment also included control lentoid pairs which were incubated in standard culture medium to which PBS was added (a volume equivalent to that of the Fab fragment preparation). Prior to pairing, calcein-containing lentoids and nonloaded lentoids to be paired were pretreated with Fab fragment or PBS control solutions for 45 to 120 min. Lentoids were then paired, and the pairs remained in the appropriate solutions during subsequent timed incubations. In order to detect the effect of the Fab fragments on the extent or time course of junction formation, calcein dye transfer between paired lentoids was monitored at several time points throughout an experiment.

Calcein dye transfer between lentoids was inhibited at all concentrations of N-cadherin-specific Fab fragments tested when compared to the dye transfer observed in the matched control pairs. The paired lentoids shown in Fig. 7A were incubated with 250 μg/ml Fab fragments, and are representative of pairs incubated at all Fab fragment concentrations used. Fluorescence was not detected in cells of the nonloaded lentoid 26 hr postpairing, as seen in the fluorescence image in Fig. 7B, nor at the end point of the experiment at 43 hr postpairing.

The data from all Fab fragment and PBS control experiments were compiled and grouped into several time categories (Fig. 8). Seven percent of the lentoid pairs treated with Fab fragments of antibody to N-cadherin and observed 20–22 hr postpairing displayed transfer of calcein to cells of nonloaded lentoids. Thirty-three percent of the control lentoid pairs displayed dye transfer during the same time period. The calcein dye transfer between cells of control lentoid pairs then reached and maintained a level of approximately 90% in observations made through 34 hr postpairing. In contrast, dye transfer between cells of Fab fragment-treated pairs gradually rose and reached a maximum level of 60%. The detectable level of calcein within lentoids in both Fab fragment- and PBS control-treated pairs decreased with longer incubation times due to some calcein fading and leakage, as described previously (Thomas, 1986).

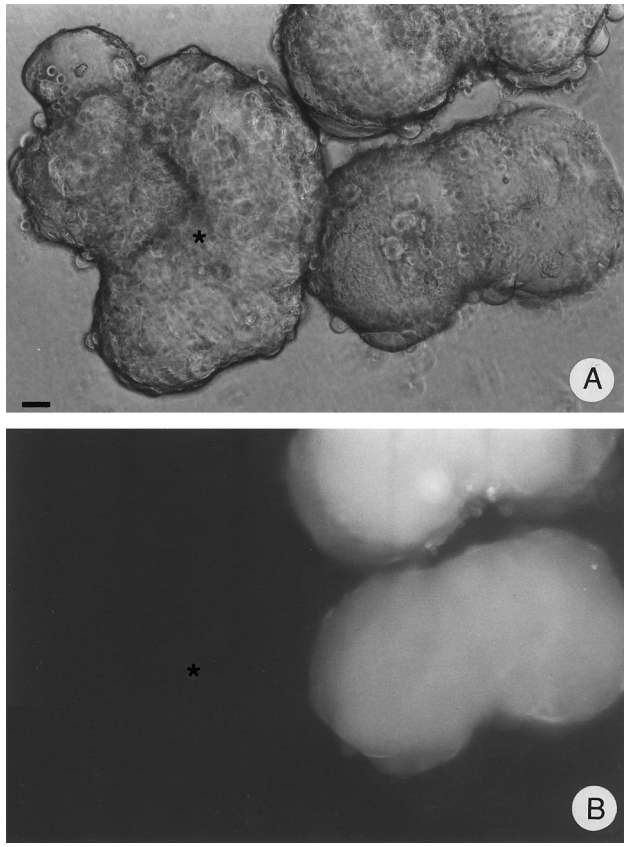

FIG. 7. Inhibition of calcein dye transfer between lentoids treated with Fab fragments of N-cadherin-specific antibody. Calcein-containing lentoids and nonloaded lentoids were incubated with 250 μg/ml Fab fragments of antibody to N-cadherin prior to, during, and after pairing. The phase-contrast image (A) shows two calcein-containing lentoids on the right paired with a single nonloaded lentoid on the left (*). As seen in the fluorescent image of the lentoids (B), calcein dye was not detected in the nonloaded lentoid 26 hr after pairing. Bar, 100 μm.

The results were analyzed by a χ^2 test to determine the statistical significance of the observed differences in the numbers of lentoid pairs which displayed dye transfer after treatment with Fab fragments of antibody to N-cadherin or with PBS. Data for controls and experimentals were grouped according to length of treatment and data were pooled for different Fab concentrations studied during the same time period. Experimental results were significant to a level of 0.0068 at the earliest times studied (20–22 hr). The level of significance increased to 0.000002 and 0.0004 at the next two time periods (24–26 and 28–34 hr). Therefore, gap junction formation between cells of paired lentoids was markedly inhibited by the Fab fragments of antibody to N-cadherin during these time periods. At the latest evaluation time period (41–46 hr), when dye transfer was detected in 71% of the controls and 49% of the experimentals, the level of significance was 0.1072 based on χ^2 analysis.

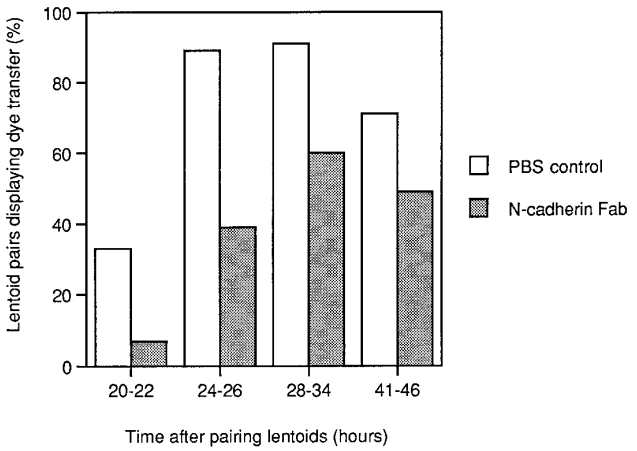

FIG. 8. Histogram of the percent of lentoid pairs that demonstrated calcein dye transfer after incubation with Fab fragments of antibody to N-cadherin or with PBS in the standard culture medium. Data from several concentrations of antibody (100–600 μg/ml) were pooled. Each lentoid pair (n = 53 for N-cadherin, n = 45 for PBS control) was observed at each time point. Statistical significance as determined by χ^2 test; 0.0068 at 20–22 hr, 0.000002 at 24–26 hr, 0.0004 at 28–34 hr, 0.1072 at 41–46 hr.

Dot blot analysis was used to test for the possible degradation of antibody Fab fragments in the standard culture medium during the incubations with lentoid pairs at 33°C. For each experiment, Fab fragment- and PBS-containing media were analyzed for reactivity with ^{125}I-labeled anti-mouse antibody. All Fab fragment-containing samples (at concentrations of 100–622 μg/ml) reacted with the labeled antibody at all time points, even when diluted to 1:20. Within an experiment, the reactivity of the Fab fragment solutions displayed no detectable changes with increased incubation times, from 0 to 46 hr (data not shown). All PBS-containing samples were negative at all time points, indicating that none of the components of the Medium 199 containing 10% FBS were reactive with the anti-mouse antibody. The labeled anti-mouse antibody, a polyclonal antibody, most likely recognized several epitopes within the Fab fragments. While it is unknown whether the region of the Fab fragment which reacted with N-cadherin contained an epitope(s) recognized by the labeled anti-mouse antibody, no quantitative change in label intensity was observed in the dot blots over the time course of the experiments. Therefore, we conclude that it was unlikely that significant degradation of the Fab fragments occurred during the incubation times involving lentoid pairs.

Inhibition Is Specific to Interference with N-Cadherin

The specificity of the inhibition of gap junction formation by antibody to N-cadherin was analyzed with two different approaches. The goal of the first approach was to determine whether the inhibition was the result of nonspecific antibody effects and/or the result of simply binding antibody over the surface of lentoid cells. For these studies, the effect on gap junction formation of binding Fab fragments of an antibody to MIP was determined. MIP is the major integral membrane protein of lens fiber cells (Waggoner and Maisel, 1978), and is known to be expressed on lentoid cell surfaces (Menko *et al.*, 1984; Patek *et al.*, 1986). MIP is unrelated to cadherins and to the other two major families of adhesion molecules.

The topological model for MIP includes six transmembrane regions (Gorin *et al.*, 1984), and Fab fragments were made from antiserum generated to a peptide sequence corresponding to the predicted third extracellular loop of MIP. The reactivity of the Fab fragment preparation with membrane proteins from embryonic chick lens cell cultures was determined by Western immunoblot analysis. A single, immunoreactive band with an apparent molecular migration of 28 kDa, indicative of chicken MIP, was observed. The Fab fragment preparation was also used in immunofluorescence experiments in which the lens cell cultures were fixed, but not permeabilized. Without intentional permeabilization, the Fab fragments should have access only to antigens exposed on the surfaces of cells. Distinct membrane outlines were observed, specifically in lentoid regions, with little or no staining evident in areas of pavement-packed epithelial or undifferentiated cells (Frenzel, 1994).

Lentoid-pairing experiments were performed with the Fab fragment preparation using the same methodology as described for the experiments with antibody to N-cadherin. No inhibition of gap junction formation between cells of paired lentoids was observed at any time point when 100 to 1500 μg/ml of the Fab fragment preparation was added to the incubation medium. For example, the level of significance of dye transfer detected between 36 lentoid pairs treated with 1200 μg/ml of Fab fragments of antibody to MIP and 36 control lentoid pairs was 0.3–1.0 for all time categories. Results from these experiments demonstrated that high concentrations of Fab fragments of an antibody to a membrane protein were not sufficient to inhibit the formation of gap junctions between cells of paired lentoids. Therefore, the inhibition of the assembly of junctions between cells of paired lentoids was interpreted as a specific affect of the N-cadherin antibody.

Because cadherin-mediated cell adhesion is known to be a calcium-dependent process (Takeichi, 1988), a second approach to determine the specificity of the inhibition of gap junction formation involved lentoid pairing experiments with decreased levels of extracellular calcium. The free calcium concentration of standard Medium 199 with 10% FBS was approximately 1.8 mM, and the level of free calcium was adjusted by the addition of EGTA according to published methods (see Methods). Cell adhesion between cultured chick lens cells was previously reported to be rapidly reduced when calcium concentrations were decreased to 0.3 and 0.5 mM (Volk and Geiger, 1986b). Even at an extracellular calcium concentration of 0.7 mM, cell adhesion was

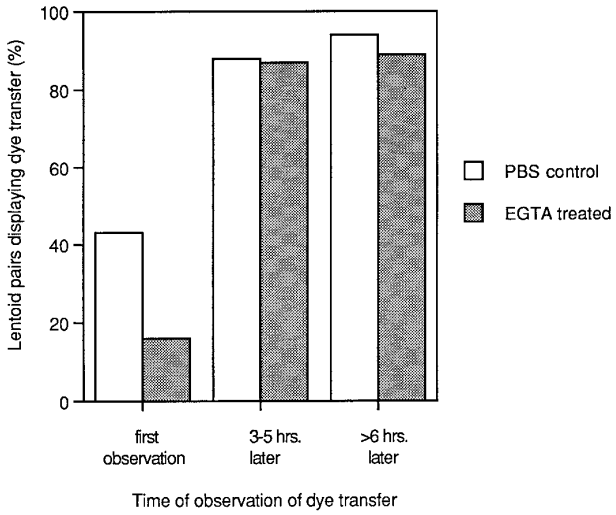

FIG. 9. Histogram of the percent of lentoid pairs that displayed dye transfer after treatment with EGTA. Data were grouped according to the time after the initial observation for dye transfer. Results from experiments with free calcium concentrations of 0.30, 0.55, 0.70, and 0.80 m*M* (EGTA, $n = 39$) were pooled and compared to results from the experiments with a free calcium concentration of 1.8 m*M* (PBS control, $n = 37$). Statistical significance as determined by χ^2 test: 0.0307 at time of initial observation, 0.7592 at second observation time, and 0.7010 at third observation time.

disrupted after 10 min, while incubation in an extracellular calcium concentration of 1.0 m*M* did not alter existing cellular junctions as observed at the level of light microscopy (Volk and Geiger, 1986b). Therefore, lentoid-pairing experiments were performed with free, external calcium concentrations of 0.30, 0.55, 0.70, and 0.8 m*M*. Lentoids were incubated in the EGTA-containing medium prior to, during, and after pairing. Experimental controls consisted of lentoids incubated in standard medium (1.8 m*M* calcium), with an appropriate amount of PBS added as a solvent control. Dye transfer was monitored as previously.

The pattern of dye transfer between cells of paired lentoids was consistent from experiment to experiment in the EGTA study, although the initial time of postpairing observations varied. Results from experiments with the different reduced external calcium concentrations were pooled and are shown in Fig. 9. Calcein dye transfer was detected at the first observation time point (15–22 hr postpairing) in 16% of the lentoid pairs treated with EGTA and in 43% of the PBS control lentoid pairs, a level of significance of 0.0307 as determined by χ^2 analysis. In observations made either 3 to 5 hr later or greater than 6 hr later, the percentage of lentoid pairs which displayed dye transfer was 87 and 89% in the EGTA-treated lentoids, and 88 and 94% in the PBS control lentoids ($\chi^2 = 0.7592$ and 0.7010, respectively). Therefore, at the first observation time, dye transfer between lentoids was inhibited when lentoids were incubated in decreased concentrations of extracellular calcium. However, the inhibition was not maintained, and EGTA-treated lentoid pairs subsequently attained control levels of gap junction formation.

In experiments in which extracellular calcium was decreased below 0.3 m*M*, the integrity of lentoid cell membranes appeared to be compromised. To confirm this possibility, lentoids were incubated in medium with free calcium concentrations of 0.05, 0.1, 0.3, and 1.8 m*M* for 1 hr, followed by the addition of Lucifer yellow to the incubation medium for an additional 2 hr. A limited number of surface cells of lentoids incubated in 1.8 m*M* free calcium took up Lucifer yellow (data not shown). Some lentoids incubated in 0.3 m*M* calcium displayed a fluorescent image similar to lentoids incubated in 1.8 m*M* calcium, while other lentoids incubated in 0.3 m*M* calcium displayed more of an overall glow. However, in lentoids incubated in 0.05 m*M* free calcium, dye appeared to entirely fill all lentoids, and the fluorescent images of the lentoids were more intense than the images of lentoids incubated with higher levels of extracellular calcium. In general, the lower the extracellular calcium concentration, the greater the extent of uptake of Lucifer yellow from the medium by the lentoid cells (data not shown). The observed leakiness of lentoid cells in decreasing extracellular calcium concentrations could be due to nonspecific cell damage. Alternatively, the leakiness could be the result of the opening of gap junction hemichannels, as suggested for retinal horizontal cells in reduced extracellular calcium (DeVries and Schwartz, 1992), and, more specifically, as demonstrated with hemichannels composed of a chicken connexin (Cx56), a lens fiber gap junction protein (Rup *et al.*, 1993), under conditions of low external calcium (Ebihara *et al.*, 1995).

DISCUSSION

In this study, the biological importance of cadherin interactions for the assembly of gap junctions was demonstrated using primary cultures of embryonic cells. These findings extended the previous work on cultured cell lines to a developing, embryonic system. An immunologic probe, specific for N-cadherin and known to interfere with cadherin function, inhibited the formation of communicating gap junctions between the lens fiber-like cells of lentoids. The inhibition was attributed to specific interference with N-cadherin interactions because no effect on junction formation was detected in the presence of high levels of Fab fragments of an antibody for another fiber cell membrane protein. This interpretation was corroborated by experiments with reduced levels of extracellular calcium, known to disrupt cadherin interactions, in which the time course of junction formation was affected. Thus, the involvement of a calcium-dependent system which regulates the process of gap junction assembly was suggested. We conclude that N-cadherin interactions facilitate the process of gap junction assembly. Because lentoids used in this study are an *in vitro* model for lens development and for terminally differenti-

ated lens fiber cells, these results are directly applicable to our understanding of events occurring in the developing lens. Therefore, the formation of extensive areas of gap junctions between differentiating fiber cells *in vivo* likely depends on the prior establishment of cadherin-based interactions between the cells.

Inhibition of Gap Junction Formation in Embryonic Cells and Correlations with Studies on Cell Lines

Embryonic cells have been analyzed in only a few studies for a relationship between cell adhesion and gap junction formation. Utilizing mouse preimplantation embryos, for example, dye transfer and ionic coupling between blastomeres were reduced upon treatment with either low calcium-containing medium or with an antiserum that included reactivity to uvomorulin or a related calcium-dependent cell adhesion molecule (Goodall, 1986). A role for a calcium-dependent, cell adhesion event in the establishment of cell communication in an embryonic system was indicated. However, the inclusion of intact antibody specific for E-cadherin in the same system had no effect on gap junction communication (Goodall, 1986). The potential problems associated with the use of intact antibody are discussed below. In another study, the role of NCAM-mediated cell adhesion in the formation of gap junctions between cells of cultured chick neuroectoderm was examined (Keane *et al.,* 1988). The application of Fab fragments of antibody to NCAM resulted in a decrease in the extent of dye transfer to neighboring cells. In addition, the expression of neuronal markers of differentiation was inhibited, suggesting a role for adhesion and/or gap junctions in the differentiation process. The establishment of gap junction-mediated cell communication was linked temporally to the expression of NCAM. Therefore, in embryonic cells the level of intercellular communication was affected by interference with calcium-dependent and calcium-independent cell adhesion interactions.

In our study, the importance of cadherin interactions for the establishment of cell communication in cultured embryonic lens cells was demonstrated by the inclusion of cadherin-specific antibody and by the reduction of extracellular calcium. Our findings are in agreement with findings from studies with cell lines and transfected cells in which the same methods were used to perturb cadherin interactions (Duband *et al.,* 1987; Hatta *et al.,* 1988; Meyer *et al.,* 1992; Volberg *et al.,* 1986; Wheelock and Jensen, 1992). For example, when Novikoff hepatoma cells were reaggregated in the presence of Fab fragments of an antibody to N-cadherin, only limited dye transfer and gap junction assembly were detected (Meyer *et al.,* 1992). Also, a decrease in the assembly of gap junction structures between reaggregating Novikoff cells was observed when the extracellular concentration of calcium was lowered (Miner *et al.,* 1995). In S180 cells transfected with cDNA for specific cadherins, increases in dye coupling between cells and in gap junction structures observed in cell membranes were reported (Matsuzaki *et al.,* 1990; Mege *et al.,* 1988). S180 cells normally do not express known cadherin molecules and display few gap junctions. However, upon treatment of the cadherin transfected S180 cells with antibody to L-CAM or to N-cadherin, the cells displayed the loss of an epithelial-like morphology and a decrease in the number of gap junctions structures (Matsuzaki *et al.,* 1990; Mege *et al.,* 1988). A difference in the phosphorylation state of the gap junction protein connexin 43 was also detected and was linked to the ability of the cells to communicate (Musil *et al.,* 1990). Therefore, the concept that gap junction communication between cells is dependent on the presence of functional cadherin molecules is well supported by the present study of embryonic cells and by a variety of earlier studies on cell lines.

Utilization of Fab Fragments

Fab fragments were used in the lentoid-pairing experiments to avoid potential problems associated with the use of intact antibody. The bivalency of intact antibody is known to cause the agglutination of cells, the patching of cell surface antigens, and the internalization of antibody/antigen complexes (Gumbiner *et al.,* 1988; Knudsen and Wheelock, 1992; Matsuzaki *et al.,* 1990). While these actions of intact antibody were utilized in some studies of cadherins (Knudsen and Wheelock, 1992), the patching of antigens or the removal of cell surface proteins was not desirable in the lentoid pairing experiments. In addition, the generation of false-positive or false-negative results was possible with the use of intact antibody. The association of single antibody molecules with epitopes located on different cells could artificially link cells in a manner resembling cadherin-mediated adhesion (Volk and Geiger, 1986a). Similarly, the agglutination of S180 cells with WGA led to an increase in the level of dye transfer that was comparable to the level in cadherin-transfected cells (Mege *et al.,* 1988). Further, the binding of intact antibody can crosslink epitopes and can activate cellular responses, such as the generation of intracellular signals (Kornberg *et al.,* 1991). Alternatively, due to the size of intact antibody, an inhibitory effect on gap junction communication could simply be the result of steric hindrance, rather than a specific effect on cadherin interactions. These potential problems were eliminated or minimized by the use of Fab fragments of antibody to examine the role of N-cadherin in gap junction formation.

Interference with Cadherin Interactions: Loss of Membrane Stabilization? Lack of an Intracellular Signal?

The inhibition of gap junction formation by antibody specific for N-cadherin could be explained in several ways. The simplest explanation correlates the distance between apposed cell membranes with the ability of gap junction proteins to interact and form intercellular channels. Cadherin-mediated cell adhesion reduces the space between cells and stabilizes

membrane associations, which would then facilitate the formation of gap junctions or other intercellular junctions. The theory that stable membrane associations are established via cell adhesion molecules prior to the formation of intercellular junctional complexes was proposed as part of a general morphoregulatory hypothesis, the ''precedence theory'' (Edelman, 1988). Cadherin interactions have previously been shown to be required for the formation of adherens junctions, tight junctions, and desmosomes between Madin–Darby canine kidney cells (Gumbiner *et al.*, 1988). In our study, the potential loss of membrane stabilization by the inclusion of Fab fragments of antibody specific for N-cadherin is different, however, from a general steric hindrance mechanism. An illustration of an inherent physical barrier to membrane associations is seen in the inverse relationship of the level of NCAM interactions with the PSA content of the molecule (Rutishauser *et al.*, 1988; Yang *et al.*, 1992). While the binding of Fab fragments to the cell surface may be a slight barrier to cell interactions, a general steric hindrance effect does not appear to be a sole explanation for the results from our experiments since Fab fragments of an antibody to the lens fiber cell membrane protein MIP did not inhibit gap junction formation (see Results) (Meyer *et al.*, 1992). Rather, the inhibition of the formation of communicating junctions by Fab fragments of antibody appears to be a more specific response to the lack of cadherin interactions.

Interference with cadherin interactions could result in the deficiency of an intracellular signal and thus prevent a cascade of events necessary for junction formation. Cadherin molecules are proposed to be morphoregulatory molecules (Edelman, 1988; Takeichi, 1988), and a number of changes in cell morphology and behavior are observed upon the establishment of adhesion between cells. These changes imply the generation of intracellular signals. For example, alterations in cadherin expression occur during cell segregation in tissue formation (Hatta and Takeichi, 1986), and the polarization of membrane proteins, along with the reorganization of the cytoskeleton, coincided with the expression of cadherins in transfected cells (Marrs *et al.*, 1995; McNeill *et al.*, 1990). Several investigators have also reported the opening of calcium channels upon the binding of cell adhesion molecules (Doherty *et al.*, 1991; Saffell *et al.*, 1992). The transmembrane signal itself, not the actual cell adhesion event, was proposed to be required for the resulting cellular responses and morphological changes which follow cadherin interactions (Doherty *et al.*, 1991; Saffell *et al.*, 1992). The association of cadherins with catenin molecules could represent yet another means of signal transduction. Signaling activity mediated by β-catenin has recently been proposed to influence gap junction communication and axis formation in frog embryos (Funayama *et al.*, 1995; Guger and Gumbiner, 1995; Heasman *et al.*, 1994). Thus, a signaling pathway(s) was potentially perturbed by the binding of Fab fragments to lentoid cells.

Incomplete Inhibition

It should be noted that complete inhibition of gap junction formation was not achieved as a result of treatment with Fab fragments of antibody to N-cadherin, nor by decreased extracellular calcium concentrations. As indicated by the results from the dot blot analysis (see Results), the lack of complete inhibition of dye transfer between lentoids treated with Fab fragments was likely not due to antibody degradation. It is likely, however, that additional cadherin molecules or other adhesion molecules are coexpressed on lentoid cell surfaces. Different cadherins can be coexpressed in a single tissue, such as N- and R-cadherin in embryonic chick retina (Inuzuka *et al.*, 1991) and E- and P-cadherin in embryonic lung (Hirai *et al.*, 1989). Therefore, it is possible that an additional known or unknown member of the cadherin family is expressed on lentoid cells. Also, NCAM and Thy-1, two members of the Ig-superfamily with known or potential adhesion roles, have been identified in lens fiber cells (Terkelsen *et al.*, 1989; Watanabe *et al.*, 1989), and Thy-1 was detected in lentoid cell membranes by immunofluorescence (data not shown). Therefore, some stabilization of membrane contacts between cells and/or signal generation could occur via molecules other than N-cadherin and such interactions might allow for limited assembly of gap junctions. The possible involvement of steric hindrance has already been discussed, but the other potential adhesion molecules may have been more involved in the experiments with EGTA, given the absence of added Fab fragments.

Results from other studies in which cadherin interactions were perturbed also indicated incomplete inhibition of the cell behavior being investigated. Cell aggregation, migration, and adhesion, as well as the translocation of membrane associated molecules, were partially prevented or delayed, but not eliminated, by the inclusion of antibodies to E- or N-cadherin (Balsamo *et al.*, 1991; Gumbiner *et al.*, 1988; Hatta and Takeichi, 1986; Hirai *et al.*, 1989; Wheelock and Jensen, 1992). Therefore, total inhibition of gap junction formation between lentoids was not necessarily expected.

SUMMARY

In summary, utilizing the method of calcein dye transfer to study gap junction-mediated communication, we have demonstrated that gap junction formation between cells of paired lentoids is inhibited by Fab fragments of antibody specific for N-cadherin. We conclude that cadherin interactions are important for the assembly of gap junctions between cells of differentiated lentoids. Because lentoids represent terminally differentiated lens fiber cells, we propose that the cadherin adhesion system is also an important component of gap junction formation in the developing lens. Cadherin-mediated cell adhesion reduces the distance between cell membranes and stabilizes membrane associations. Cadherin interactions have also been linked to the initiation of cellular responses and to the generation of intracellular signals. Both of these aspects of cadherin-mediated cell adhesion may play a role in the establishment and maintenance of the large network of gap junctions that exists in the lens. The variable expression of cadherins

throughout embryos most likely impacts the establishment of intercellular junctions and developmental processes in other tissues as well.

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REFERENCES

Balsamo, J., Thiboldeaux, R., Swaminathan, N., and Lilien, J. (1991). Antibodies to the retinal *N*-acetylglactosaminylphosphotransferase modulate N-cadherin-mediated adhesion and uncouple the *N*-cadherin transferase complex from the actin-containing cytoskeleton. *J. Cell Biol.* 113, 429–436.

Benedetti, E. C., Dunia, I., Bentzel, C. J., Vermorken, A. J. M., Kibbelaar, M., and Bloemendal, H. (1976). A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochim. Biophys. Acta* 457, 353–384.

Berthoud, V. M., Cook, A. J., and Beyer, E. C. (1994). Characterization of the gap junction protein connexin56 in the chicken lens by immunofluorescence and immunoblotting. *Invest. Ophthalmol. Visual Sci.* 35, 4109–4117.

Brooks, S. P. J., and Storey, K. B. (1992). Bound and determined: A computer program for making buffers of defined ion concentrations. *Anal. Biochem.* 201, 119–126.

Caveney, S. (1985). The role of gap junctions in development. *Annu. Rev. Physiol.* 47, 319–335.

Davidson, J. S., Baumgarten, I. M., and Harley, E. H. (1984). Effects of extracellular calcium and magnesium on junctional intercellular communication in human fibroblasts. *Exp. Cell Res.* 155, 406–412.

DeVries, S. H., and Schwartz, E. A. (1992). Hemi-gap-junction channels in solitary horizontal cells of the catfish retina. *J. Physiol.* 445, 201–230.

Doherty, P., Ashton, S. V., Moore, S. E., and Walsh, F. S. (1991). Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G-protein-dependent activation of L- and N-type neuronal calcium channels. *Cell* 67, 21–33.

Duband, J., Nuckolls, G., Ishihara, A., Hasegawa, T., Yamada, K., Thiery, J., and Jacobson, K. (1988). Spatial and temperal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *J. Cell Biol.* 107, 1385–1396.

Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M., and Thiery, J. P. (1987). Adhesion molecules during somitogensis in the avian embryo. *J. Cell Biol.* 104, 1361–1374.

Ebihara, L., Berthoud, V. M., and Beyer, E. C. (1995). Distinct behavior of connexin56 and connexin46 gap junctional channels can be predicted from the behavior of their hemi-gap-functional channels. *Biophys. J.* 68, 1796–1803.

Edelman, G. M. (1988). Morphoregulatory molecules. *Perspect. Biochem.* 27, 3533–3543.

El-Sabban, M. E., and Pauli, B. U. (1991). Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. *J. Cell Biol.* 115, 1375–1382.

Fraser, S. E. and Bryant, P. J. (1985). Patterns of dye coupling in the imaginal wing disc of *Drosphila melanogaster. Nature* 317, 533–536.

Fraser, S. E., Green, C. R., Bode, H. R., and Gilula, N. B. (1987). Selective disruption of gap junctional communication interferes with a patterning process in hydra. *Science* 237, 49–55.

Frenzel, E. M. (1994). ''Lens Fiber Cell Gap Junctions: Formation Studies with Lentoids.'' Ph.D. Thesis, University of Minnesota, St. Paul.

Funayama, N., Fagotto, F., McCrea, P., and M., G. B. (1995). Embryonic axis induction by the Armadillo repeat domain of β-catenin: Evidence for intracellular signaling. *J. Cell Biol.* 128, 959–968.

Goodall, H. (1986). Manipulation of gap junctional communication during compaction of the mouse early embryo. *J. Embryol. Exp. Morphol.* 91, 283–296.

Goodall, H., and Johnson, M. H., (1982). Use of carboxyfluorescein diacetate to study formation of permeable channels between mouse blastomeres. *Nature* 295, 524–526.

Gorin, M. B., Yancey, S. B., Cline, J., Revel, J. P., and Horwitz, J. (1984). The major intrinsic protein (MIP) of the bovine fiber membrane characterization and structure based on cDNA cloning. *Cell* 39, 49–59.

Guger, K. A., and Gumbiner, B. M. (1995). β-catenin has Wnt-like activity and mimics the Niewkoop signalling center in *Xenopus* dorsal–ventral patterning. *Dev. Biol.* 172, 115–125.

Gumbiner, B., Stevenson, B., and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* 107, 1575–1587.

Hatta, K., Nose, A., Nagafuchi, A., and Takeichi, M. (1988). Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: Its identity in the cadherin gene family. *J. Cell Biol.* 106, 873–881.

Hatta, K., and Takeichi, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* 320, 447–449.

Heasman,J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 79, 791–803.

Hirai, Y., Nose, A., Kobayashi, S., and Takeichi, M. (1989). Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. *Development* 105, 262–270.

Inuzuka, H., Redies, C., and Takeichi, M. (1991). Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development. *Development* 113, 959–967.

Jongen, W. M., F., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M., and Yamasaki, H. (1991). Regulation of connexin43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* 114, 545–555.

Kartenbeck, J., Schmelz, M., W. W., F., and Geiger, B. (1991). Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured inlow Ca^{2+} ion medium. *J. Cell Biol.* 113, 881–892.

Keane, R. W., Mehta, P., Rose, B., Honig, L. S., Loewenstein, W. R., and Rutishauser, U. (1988). Neural differentiation, NCAM-mediated adhesion and gap junctional communication in neuroectoderm: A study *in vitro. J. Cell Biol.* 106, 1307–1319.

Kidder, G. M., Rains, J., and McKeon, J. (1987). Gap junction assembly in the preimplantation mouse conceptus is independent of

microtubules, microfilaments, cell flattening, cytokinesis. *Proc. Natl. Acad. Sci. USA* 84, 3718–3722.

Knudsen, K. A., and Wheelock, M. J. (1992). Plakoglobin, or an 83-kD homologue distinct from β-catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol.* 118, 671–679.

Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991). Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β1 integrins. *Proc. Natl. Acad. Sci. USA* 88, 8392–8396.

Kuszak, J., Maisel, H., and Harding, C. V. (1978). Gap junctions of chick lens fiber cells. *Exp. Eye Res.* 27, 495–498.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Lampe, P. D. (1994). Analyzing phorbal ester effects on gap jutional communication: A dramatic inhibition of assembly. *J. Cell Biol.* 127, 1895–1905.

Lo, C. W., and Gilula, N. B. (1979). Gap junctional communication in the preimplantation mouse embryo. *Cell* 18, 399–409.

Lo, W.-K., and Harding, C. V. (1986). Structure and distribution of gap junctions in lens epithelium and fiber cells. *Cell Tissue Res.* 244, 253–263.

Loewenstein, W. R. (1967). On the genesis of cellular communication. *Dev. Biol.* 15, 503–520.

Loewenstein, W. R. (1981). Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* 61, 829–913.

Marrs, J. A., Andersson-Fisone, C., Jeong, M. C., Cohen-Gould, L., Zurzolo, C., Nabi, I. R., Rodriguez-Boulan, E., and Nelson, W. J. (1995). Plasticity in epithelial cell phenotype: Modulation by expression of different cadherin cell adhesion molecules. *J. Cell Biol.* 129, 507–519.

Matsuzaki, F., Mege, R. M., Jaffe, S. H., Friendlander, D. R., Gallin, W. J., Goldberg, J. I., Cunningham, B. A., and Edelman, G. M. (1990). cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in cultured S180 cells. *J. Cell Biol.* 110, 12391–1252.

McNeill, H., Ozawa, M., Kemler, R., and Nelson, W. J. (1990). Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell* 62, 309–316.

Mege, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. I., Cunningham, B. A., and Edelman, G. M. (1988). Construction of epitheloid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* 85, 7274–7278.

Menko, A. S., Klukas, K. A., and Johnson, R. G. (1984). Chicken embryo lens cultures mimic differentiation in the lens. *Dev. Biol.* 103, 129–141.

Menko, A. S., Klukas, K. A., Liu, T.-F., Quade, B., Sas, D. F., Preus, D. M., and Johnson, R. G. (1987). Junctions between lens cells in differentiating cultures: Structure, formation, intercellular permeability and junctional protein expression. *Dev. Biol.* 123, 307–320.

Meyer, R. A., Laird, D. W., Revel, J.-P., and Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell Biol.* 119, 179–189.

Miner, P., Lampe, P., Atkinson, M., and Johnson, R. (1995). Extracellular calcium and cadherins regulate the process of gap junction assembly between cells in culture. *In* ''Intercellular Communication through Gap Junctions'' (Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, and T. Shimazu, Eds.), Vol. 4, pp. 331–334. Elsevier Science B. V., Amsterdam.

Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990). Differential phosphorylation of gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111, 2077–2088.

Okada, T. S., Eguchi, G., and Takeichi, M. (1971). The expression of differentiation by chick lens epithelium in *in vitro* cell culture. *Dev. Growth Different.* 13, 323–335.

Patek, C. E., Vornhagen, R., Rink, H., and Clayton, R. M. (1986). Developmental changes in membrane protein expression by chick lens cells in vivo and in vitro and the detection of main intrinsic polypeptide (MIP). *Exp. Eye Res.* 43, 29–40.

Piatigorsky, J. (1981). Lens differentiation in vertebrates: A review of cellular and molecular features. *Differentiation* 19, 134–153.

Piatigorsky, J., Webster, H., and Wollberg, M. (1972). Cell elongation in the cultured embryonic chick lens epithelium with and without protein synthesis. Involvement of microtubules. *J. Cell Biol.* 55, 82–92.

Potter, D. D., Furshpan, E. J., and Lennox, E. S. (1966). Connections between cells of the developing squid as revealed by electrophysiological methods. *Proc. Natl. Acad. Sci. USA* 55, 328–335.

Puranam, K. L., Laird, D. W., and Revel, J.-P. (1993). Trapping an intermediate form of connexin43 in the golgi. *Exp. Cell Res.* 206, 85–92.

Radcliff, G., Waite, R., LeFevre, J., Paulik, M. D., and Callewaert, D. M. (1991). Quantification of effector/target conjugation involving natural killer (NK) or lymphokine activated killer (LAK) cells by two-color flow cytometry. *J. Immunol. Methods* 139, 281–292.

Reaume, A. G., de Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M., and J., R. (1995). Cardiac malformation in neonatal mice lacking connexin 43. *Science* 267, 1831–1834.

Rotman, B., and Papermaster, B. (1966). Menbrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. USA* 55, 134–141.

Rup, D. M., Veenstra, R. D., Wang, H. Z., Brink, P. R., and Beyer, E. C. (1993). Chick connexin-56, a novel lens gap junction protein. *J. Biol. Chem.* 268, 706–712.

Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M., and Sunshine, J. (1988). The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science* 240, 53–57.

Saffell, J. L., Walsh, F. S., and Doherty, P. (1992). Direct activation of second messenger pathways mimics cell adhesion molecule-dependent neurite outgrowth. *J. Cell Biol.* 118, 663–670.

Takeichi, M. (1988). The cadherins: Cell–cell adhesion molecules controlling animal morphogenesis. *Development* 102, 639–655.

Terkelsen, O. B. F., Bock, E., and Møllgard, K. (1989). NCAM and Thy-1 in special sense organs of the developing mouse. *Anat. Embryol.* 179, 311–318.

Thomas, J. A. (1986). Intracellularly trapped pH indicators. *In* ''Optical Methods in Cell Physiology'' (P. DeWeer and B. M. Salzberg, Eds.). Wiley-Interscience, New York.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.

Volberg, T., Geiger, B., Kartenbeck, J., and Franke, W. W. (1986). Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca^{2+} ions. *J. Cell Biol.* 102, 1832–1842.

Volk, T., and Geiger, B. (1984). A 135-kd membrane protein of intercellular adherens junctions. *EMBO J.* 3, 2249–2260.

Volk, T., and Geiger, B. (1986a). A-CAM: A 135-kD receptor of

intercellular adherens junctions. II. Antibody-mediated modulation of junction formation. *J. Cell Biol.* 103, 1451–1464.

Volk, T., and Geiger, B. (1986b). A-CAM: A 135-kD receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. *J. Cell Biol.* 103, 1441–1450.

Volk, T., Volberg, T., Sabanay, I., and Geiger, B. (1990). Cleavage of A-CAM by endogenous proteinases in cultured lens cells and in developing chick embryos. *Dev. Biol.* 139, 314–326.

Waggoner, P. T., and Maisel, H. (1978). Immunofluorescent study of a chick lens fiber cell membrane polypeptide. *Exp. Eye Res.* 27, 151–157.

Warner, A. (1992). Gap junctions in development-a perspective. *Semin. Cell Biol.* 3, 81–91.

Warner, A. E., Guthrie, S. C., and Gilula, N. B. (1984). Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* 311, 127–131.

Warner, A. E., and Lawrence, P. A. (1982). Permeability of gap junctions at the segmental border in insect epidermis. *Cell* 28, 243–252.

Watanabe, M., Kobayashi, H., Rutishauser, U., Kata, M., Alcala, J., and Maisel, H. (1989). NCAM in the differentiation of embryonic lens tissue. *Dev. Biol.* 135, 414–423.

Weir, M. P., and Lo, C. W. (1982). Gap junctional communication compartments in the *Drosophila* wing disk. *Proc. Natl. Acad. Sci. USA* 79, 3232–3235.

Wheelock, M. J., and Jensen, P. J. (1992). Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. *J. Cell Biol.* 117, 415–425.

Wolpert, L. (1978). Gap junctions: Channels for communication in development. *In* ''Intercellular Junctions and Synapses'' (J. Feldman, N. B. Gilula, and J. Pitts, Eds.), pp. 83–94. Chapman and Hall, London.

Yang, P., Yin, X., and Rutishauser, U. (1992). Intercellular space is affected by the polysialic acid content of NCAM. *J. Cell Biol.* 116, 1487–1496.

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