The Cadherin-binding Specificities of B-Cadherin and LCAM

Cynthia Murphy-Erdosh,* Cathleen K. Yoshida,* Nancy Paradies,§ and Louis F. Reichardt*‡

*Neuroscience Program, Department of Physiology and ‡Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, California 94143-0724; and §Department of Anatomy and Cell Biology, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0521

Abstract. The cadherin family of calcium-dependent cell adhesion molecules plays an important part in the organization of cell adhesion and tissue segregation during development. The expression pattern and the binding specificity of each cadherin are of principal importance for its role in morphogenesis. B-Cadherin and LCAM, two chicken cadherins, have similar, but not identical, spatial and temporal patterns of expression. To examine the possibility that they might bind to one another in a heterophilic manner, we generated, by cDNA transfection, L-cell lines that express LCAM or B-cadherin. We then examined the abilities of these cells to coaggregate with each other and with other cadherin-expressing cells in short-term aggregation assays. The B-cadherin– and the LCAM-expressing cell lines segregate from P-, N-, or R-cadherin–expressing cells. B-cadherin– and LCAM-expressing cell lines, however, appear to be completely miscible, forming large mixed aggregates. Chick B-cadherin and murine E-cadherin also form mixed aggregates, indistinguishable from homophilic aggregates. Murine E-cadherin and chick LCAM coaggregate less completely, suggesting that the heterophilic interactions of these two cell lines are weak relative to homophilic interactions. These data suggest that heterophilic interactions between B-cadherin and LCAM are important during avian morphogenesis and help identify the amino acids in the binding domain that determine cadherin specificity.

For decades developmental biologists have understood the importance of the mechanisms of cell–cell adhesion for the complex processes of embryonic development (Townes and Holtfretter, 1955). When dissociated cells from different tissues are mixed together, cells of like types aggregate with one another and cells of different types segregate from one another, suggesting that the mechanisms involved in cell adhesion are necessary for cell sorting and tissue segregation. The cadherin family of cell adhesion molecules is an important component of the tissue segregation machinery (reviewed by Takeichi, 1991). In vivo experiments, in which cadherins are ectopically expressed (Detrick et al., 1990; Fujimori et al., 1990) or cadherin function is blocked by antibodies (Matsunaga et al., 1988; Bronner-Fraser et al., 1992), demonstrate that proper cadherin expression and function are necessary for normal tissue segregation during development.

The hallmark of this family of transmembrane glycoproteins is their ability to mediate homophilic calcium dependent cell aggregation. Fibroblast-like L-cell lines, which normally do not express cadherins, form aggregates in a calcium-dependent manner when transfected with cadherin cDNA's (Edelman et al., 1987; Nagafuchi et al., 1987; Nose et al., 1988; Miyatani et al., 1989; Inuzuka et al., 1991; Murphy-Erdosh et al., 1994). When different cadherin expressing cell lines are mixed, the resulting aggregate segregation is determined by cadherin expression, in much the same way as the segregation of heterotypic tissues (Nose et al., 1988). These data suggest that the homophilic binding mechanisms of cadherins are major determinants of tissue specificity and adhesion.

Nose et al. (1990) demonstrated that a specific sequence in the first extracellular domain of E-cadherin is important for the selective binding specificity of this cadherin. Experiments using cell lines expressing chimeric molecules of P- and E-cadherin suggest that the first 113 amino acids of the cadherins mediate homophilic binding. Many of the epitopes for the function blocking monoclonal antibodies against the cadherins also map to this domain, providing credence to its importance in intermolecular interactions. Synthetic peptides from specific regions of the 113–amino acid EC-1 domain block cadherin-mediated aggregation narrowing the binding domain to the region surrounding the highly conserved HAV tripeptide (Blaschuk et al., 1990). When the amino acids in positions 78 and 83 of E-cadherin are changed to those found in P-cadherin, the cadherin specificity also changes such that cells transfected with the mutated cadherin interact in a heterophilic manner with both E- and P-cadherin–transfected cells (Nose et al., 1990).

Address correspondence to L. F. Reichert, Howard Hughes Medical Institute-Room U426, University of California, San Francisco, CA 94143-0724. Tel.: (619) 476-3976. Fax: (415) 566-4969.

Heterophilic interactions among non-mutated cadherins have also been described. When N-cadherin–expressing lens cells and LCAM-expressing liver cells are cocultured, adherens junctions containing both N-cadherin and LCAM form at heterotypic cell contact sites (Volk et al., 1987). Relatively few heterotypic contacts are found suggesting that homotypic adherens junctions are more stable than heterotypic junctions. Another example of weak heterophilic interactions between cadherins has been described by Inuzuka et al. (1991). L-Cells transfected with R-cadherin coaggregate with N-cadherin–transfected L-cells. The aggregates generated under these conditions are not randomly mixed but appear to be conglomerates of smaller aggregates. After an extended period of time, N- and R-cadherin–expressing cells segregate more extensively from one another suggesting that the heterophilic interactions between N- and R-cadherin are weaker than their homophilic interactions.

The complementary expression patterns of cadherins supports the idea that cadherins help determine tissue specificity. The expression patterns of different cadherins rarely overlap; with different cadherins usually being expressed in separate tissues (Crossin et al., 1985; Hatta and Takeichi, 1986). Even functionally distinct regions of the same tissue often express different cadherins (Shimamura et al., 1992; Shimamura and Takeichi, 1992; Murphy-Erdosh et al., 1994). The weak heterophilic interactions between some of the cadherins may mediate the interactions of adjacent tissues maintaining the integrity of the whole embryo. It is clear that both the tissue distribution and the binding specificities of cadherins are important for determining their function during embryonic development.

B-Cadherin has an unusual pattern of expression compared to other known cadherins. The spatial and temporal expression pattern of B-cadherin greatly overlaps that of LCAM; there are few instances where one can find one of these two chicken cadherins without the other (Murphy-Erdosh et al., 1994). This suggested to us that they may interact with one another in a heterophilic manner. In this study, the binding specificities of both B-cadherin and LCAM were examined using L-cell lines transfected with these cadherins. Short-term coaggregation experiments suggest that chick B-cadherin and chick LCAM do specifically interact with one another in a way that is surprisingly indistinguishable from their individual homophilic interactions. In this assay, murine E-cadherin also interacts with both chick B-cadherin and chick LCAM. Both B-cadherin and LCAM transfected cells segregate from chick N-, chick R-, and murine P-cadherin–expressing cells.

Materials and Methods

Unless otherwise noted reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Cell Lines

L929 cells (American Type Culture Collection, Rockville, MD) were grown and maintained in DME media including 10% fetal calf serum and 100 U/ml penicillin/streptomycin in a humidified 8% CO_2 atmosphere. All cadherin-transfected cell lines were maintained in the above medium supplemented with 400 μg/ml G418 sulfate (geneticin; GIBCO BRL, Gaithersburg, MD).

Several B-cadherin expressing L929 cell lines were generated by calcium-phosphate transfection as described by Graham et al. (1973) with the "glycerol shock" modification suggested by Parker et al. (1979). Briefly, 20 μg of the B-cadherin construct, pBCADAct-1, (Murphy-Erdosh et al., 1994) containing 250 mM $CaCl_2$ were mixed with 2× Hepes-buffered saline (270 mM NaCl, 1.5 mM Na_2HPO_4/NaH_2PO_4, 50 mM Na-Hepes, pH 7.05) while vortexing. After incubation for 30 min at room temperature, the calcium phosphate-DNA precipitate was added dropwise to semi-confluent L929 cells in unmodified DME media. The cells were incubated for 4 h at 37°C, after which they were "shocked" with 2 ml 15% glycerol in 1× Hepes-buffered saline for 2 min. After 3 d in complete media they were split and placed in selection media with 600 μg/ml G418. Single colonies were isolated and expanded after 10 d growth. Of the many cell lines that were selected, two were subcloned and used in the experiments in this study, B1C5 C9, referred to as BL-1 for simplicity, and B2D2 G10, referred to as BL-2. As a control, a separate plate of L929 cells was transfected with N-cadherin cDNA driven by the β-actin promoter in the expression vector, pHβApr-2neo (Sarorelli et al., 1990). Several cell lines were selected that expressed N-cadherin. One clone, N1C2, was used only as a negative control for B-cadherin expression (see Results).

LCAM-expressing cells were produced by transfecting L-cells (clone 2071; American Type Culture Collection) with pEC1312 vector containing an L-CAM cDNA driven by the SV-40 T-antigen early promoter (Edelman et al., 1987). Co-transfection (Wigler et al., 1979) was done by the calcium-phosphate method similar to that described above with the following modifications. 5 h after addition of the DNA calcium phosphate precipitate (19 μg L-CAM cDNA expression vector plus 1 μg pSV2neo, pH 7.9), the media was removed and cells were "shocked" with 25% glycerol in serum free medium for 2 min. Glycerol was rinsed away and the cells were cultured in growth media for 48 h. Transfectants were then selected by incubation in media containing 400 μg/ml G418 and the resulting clones were isolated by ring cloning. The clone LE6-2, referred to as LL, was the only LCAM-expressing cell clone used in this study.

Other cell lines used in the mixed aggregation assays include the P-cadherin expressing L-cell line, PLβ2, the E-cadherin expressing L-cell line, ELβ1 (Nose et al., 1988), the N-cadherin expressing cell line, CNLβ1, (Miyatani et al., 1989) and R-cadherin expressing cell line, RLβ2, (Inuzuka et al., 1991) and were all generously provided by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). To simplify the terminology, these lines are referred to as PL, EL, NL, and RL, respectively.

Antibodies and Immunoblot Analysis

The characterization and preparation of the monoclonal antibody against B-cadherin, 5A6, was described previously (Murphy-Erdosh et al., 1994). In the mixed aggregation experiments, 5A6 was used at 40–60 μg/ml. The allotype-matched monoclonal 1H6 against chick integrin α_3 was used as a negative control at 40 μg/ml. The anti-LCAM polyclonal antibody, generously donated by Drs. Cheng-Ming Chuong (USC, Los Angeles, CA) and Bruce Cunningham, (Scripps Research Institute, La Jolla, CA) was used for function blocking at 80 μg/ml.

For immunoblot analysis, confluent monolayers of transfected and untransfected L-cells were trypsinized in the presence of calcium as described below for the aggregation experiments. After the trypsin was thoroughly washed out, the cell pellets were resuspended in extraction buffer and immunoblot analysis was performed as previously described (Murphy-Erdosh et al., 1994). Two pan-cadherin antibodies were used, the Marrs pan-cadherin rabbit antiserum (Marrs et al., 1993) was generously donated by Drs. James Marrs and James Nelson (Stanford University School of Medicine, Stanford, CA) this antiserum was used for immunoblotting at a dilution of 1:500.

Immunofluorescence of Cultured Cells

A detailed description of the methods used for the immunofluorescence of the B-cadherin expressing cell lines was described previously (Murphy-Erdosh et al., 1994). Briefly, living cells plated at a density of 50,000 cells/well in a Lab-Tek 16-well slide (Nunc, Naperville, IL) were incubated for 1 h with 2 μg/ml of 5A6 in growth media. The cells were washed briefly, fixed in 4% paraformaldehyde in PBS and incubated with a fluorescein conjugated donkey anti-mouse antibody (Jackson Laboratories, West Grove, PA; 1:200). After washing and mounting with gelvatol, photographs were taken using a Nikon epifluorescence microscope with a 40× objective (NA = 1.0) and TMAX 400 film.

Table I. Long-Term Co-aggregation of LCAM and B-cadherin–expressing Cells

Cell type 1	Cell type 2	Fraction cell type 1	Standard deviation	*n*
LCAM	LCAM	.533	.038	11
LCAM	BCAD	.459	.026	15
BCAD	BCAD	.514	.014	10
L929	LCAM	.012	.013	12
L929	BCAD	.006	.009	11

Approximately equal numbers of single cell suspensions of parent L929 cells or LCAM-expressing, or B-cadherin–expressing cells, labeled with diI or diO, were mixed in growth medium and shaken in a humidified, 8% CO_2 atmosphere for 20 h (see Materials and Methods). Aggregates were sampled randomly. Results show that the mixed aggregates of LCAM and B-cadherin–expressing cells are quite homogeneous in composition. The standard deviation of aggregate composition does not differ significantly between homophilic and heterophilic aggregates. Cells were also interspersed within aggregates (data not shown). Since almost all cells aggregated, the average fraction of each cell type in a population of aggregates reflects its relative concentration in the suspension. In contrast, very low numbers of L929 cells were included in either LCAM or B-cadherin cell aggregates. *n* = Number of aggregates counted.

Cell Aggregation Assay

To determine the time course of aggregation and antibody specificities, short-term aggregation (0–60 min) experiments were performed as described by Takeichi (1977). Monolayer cultures were treated with 0.01% trypsin (Worthington Biochemical Corporation, Freehold, NJ) in the presence of 1 mM calcium (TC), or with trypsin and EDTA (TE)[1] for 15 min at 37°C. In the presence of calcium (TC), the calcium-dependent cell adhesion system, mediated by cadherins, is preserved, whereas, the calcium-independent cell adhesion system is destroyed. Under TE conditions, both adhesion systems are proteolyzed. The trypsinized cells were washed gently in Hepes-buffered HBSS containing calcium and 1% BSA, and then dissociated by washing extensively in calcium-free Hepes-buffered HBSS containing 1% BSA. After the cells were thoroughly dissociated, 1–5 × 10^5 cells per well were transferred to 24-well dishes (Costar Corp., Cambridge, MA) for a final volume of 0.5 ml Hepes-buffered HBSS containing 1% BSA with or without 1 mM $CaCl_2$. The plates were rotated at 80–90 rpm at 37°C for the specified length of time. Cells were fixed with an equal volume of 8% paraformaldehyde in PBS, pH 7.5.

The number of particles in each sample was determined using a Coulter counter. For the time course experiments, the amount of aggregation was calculated using the formula Nt/No (Nt is the number of single cells at time = t, and No is the number of single cells at time = 0). For the antibody experiments, the amount of aggregation was determined by the formula Nt/N_{noCa}, where N_{noCa} is the number of particles after 60 min in the absence of calcium. Significant differences between conditions were determined using the paired *t*-test before normalization with To or T_{noCa} control (*Statview*, Abacus, Berkeley, CA). Differences were considered significant only if the p value was less than 0.05. Photographs were taken with Nikon phase contrast microscope after fixation while the cells were in 24 well plates.

For the mixed aggregation experiments, cadherin transfected cells or L929 cells were labeled overnight in monolayers with either 1:2,500 of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [Molecular Probes, Eugene, OR] in saturated ethanol solution) or 1:250 of DiO (3,3′-dioctadecyloxacarbocyanine perchlorate [Molecular Probes, Eugene, OR] in saturated ethanol solution). The cells were washed extensively with Hepes-buffered HBSS containing calcium to prevent cross contamination of the dyes. After single cell suspensions were obtained as described above, in short term assays (see Figs. 2–8), 1 × 10^5 cells per well of each of two types were transferred to a 24-well dish and rotated at 80–90 rpm at 37°C for 1 h. For the longer assays summarized in Table I (20 h) dissociated single cells were resuspended in growth medium (10% fetal calf serum, DME, 100 U/ml penicillin/streptomycin) 0.02 M Na-Hepes, pH 7.4, and reaggregated at the same density and rotation speed as used in short term assays.

For analysis, 50 μl of the fixed aggregates were removed, placed on a slide, and covered with a coverslip. Aggregates were photographed under epifluorescence optics using the 6.3× objective on a Nikon Microphot-FXA. The numbers of cells of each type in the aggregates were counted from the projected slides. Cells appearing yellow were counted as superimposed red and green cells. In short term assays, only aggregates with more than 10 cells were counted. When aggregates were attached to one another, they were counted as separate aggregates if they were bigger than 20 cells and had less than one quarter of the surface of the smaller aggregate attached. If one of the attached aggregates was smaller than 20 cells, or had a larger attached region, then the two aggregates were counted as a single aggregate. The cells at the interface of the attached aggregates were counted for each aggregate. In long-term assays, aggregates were larger and only those with at least 40 cells were counted. Significant differences between populations of aggregates were determined by analysis of variance of the data, normalized by the arcsin transformation, using the Bonferroni/Dunn post-hoc procedure for multiple comparisons (*Statview*, Abacus). Single sample *t*-tests on untransformed data were used to determine differences from complete mixing (50%), or no mixing (100%).

1. *Abbreviation used in this paper*: TE, trypsin with EDTA.

Results

Characterization of the Cell Lines

B-Cadherin expressing lines were generated by introducing a B-cadherin construct (Murphy-Erdosh et al., 1994), containing the entire mature sequence of B-cadherin driven by the β-actin promoter, into L929 cells, which do not express significant levels of cadherins and do not exhibit calcium-dependent aggregating activity. Colonies were selected both by G418 resistance and visually by an observed increase in the number of intercellular contacts. Out of the 11 colonies picked, 9 expressed some level of B-cadherin. Two of these lines, BL-1 and BL-2, were subcloned and used in the experiments described.

BL-2 and BL-1 both express B-cadherin on their surface. Immunoblot analysis of the cell extracts, using a pan-cadherin antibody made against the cytoplasmic domain of E-cadherin (Marrs et al., 1993), demonstrates that both BL-1 and BL-2 express a protein of 120 kD, recognized by

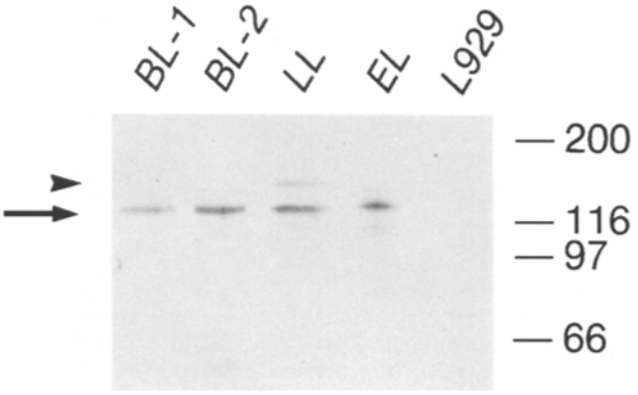

Figure 1. An immunoblot of the cadherin transfected L-cells with a pan-cadherin antiserum demonstrates that these cells express equivalent levels of cadherins. NP-40 cell extracts of each of the cell lines were made after the cells had been treated with trypsin in the presence of $CaCl_2$, B-cadherin–expressing L-cell-1, BL-1, extract (lane *1*), B-cadherin–expressing L-cell-2, BL-2, extract (lane *2*), LCAM-expressing L-cell, LL, extract (lane *3*), E-cadherin expressing L-cell, EL, extract (lane *4*), and L929 cell extract (lane *5*). The pan-cadherin antibody recognizes a cadherin in each of the transfected cell lines with molecular weights between 120 to 130 kD (*arrow*). The arrowhead marks the position of the unprocessed recombinant LCAM expressed in the LL-cells.

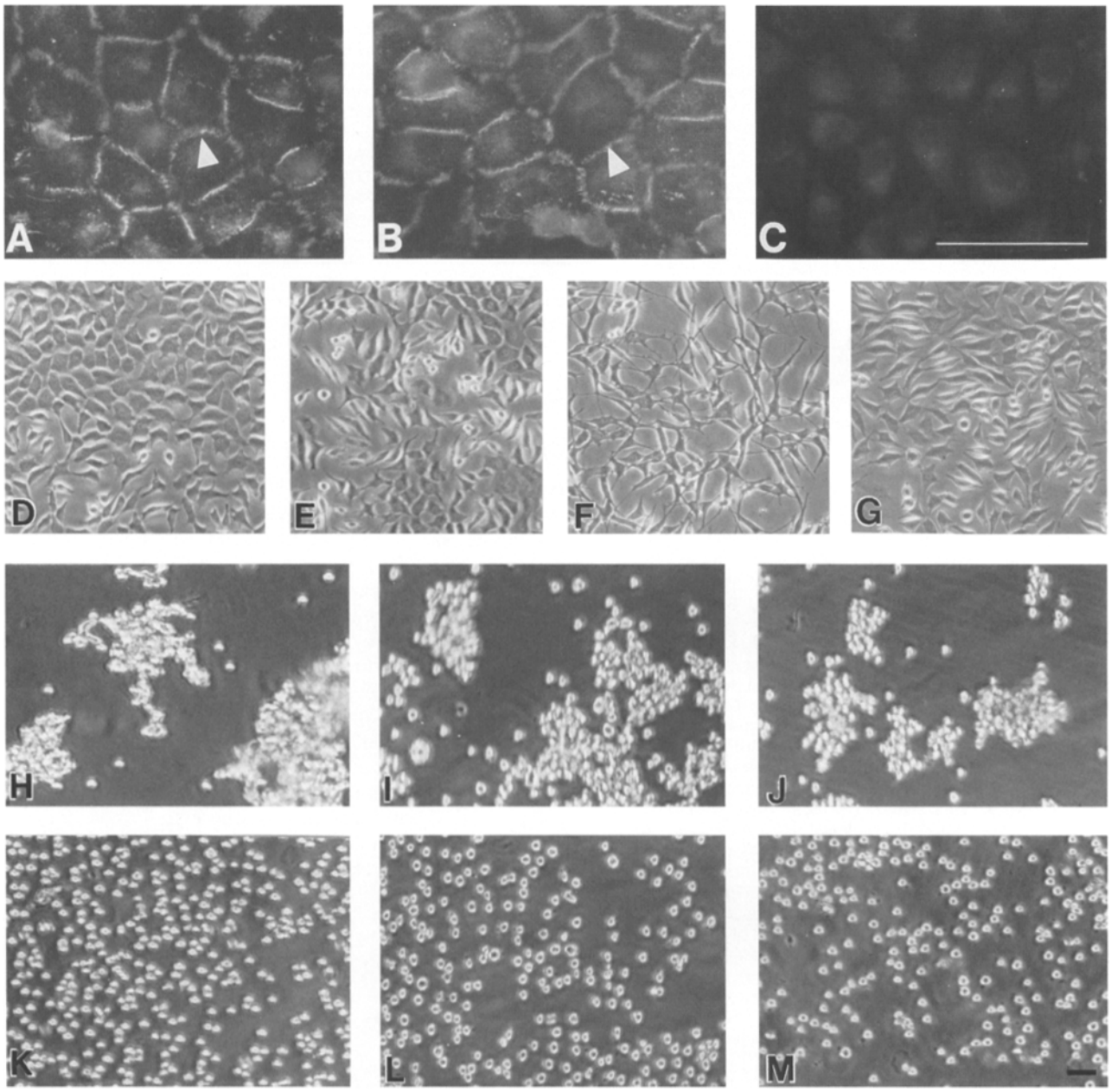

Figure 2. Characterization of the B-cadherin and LCAM-transfected cells demonstrates that they express the appropriate, functional cadherins on their surfaces; BL-2 (*A*, *D*, *H*, and *K*), BL-1 (*B*, *E*, *I*, and *L*), LL (*F*, *J*, and *M*), L929 (*G*), N1C2 (*C*). Immunofluorescence with the mAb 5A6, demonstrates that B-cadherin immunoreactivity is most prominant at sites of cell contact (*arrowheads*) in the B-cadherin–transfected cells, BL-2 and BL-1 (*A* and *B*), but is not present in the N-cadherin–transfected cells, N1C2 (*C*). The morphologies of each of the cell lines, BL-2 (*D*), BL-1 (*E*), LL (*F*), and L929 (*G*), are different as seen with phase contrast optics. In aggregation assays, each of the cadherin transfected lines, BL-2 (*H*), BL-1 (*I*), and LL (*J*), forms aggregates in the presence of calcium. This aggregation can be blocked by specific antibodies; BL-2 (*K*) and BL-1 (*L*) aggregation is blocked by mAb 5A6. LL cell aggregation (*M*) is blocked by the anti-LCAM polyclonal antibody.

this antibody (Fig. 1, lanes *1* and *2*, respectively). Another pan-cadherin antibody, CADCYTO2, and the B-cadherin polyclonal antibody, αB-EC5, (Napolitano et al., 1991) also recognize a 120-kD protein in these two cell lines (data not shown), demonstrating that they express B-cadherin. Immunofluorescence with 2 μg/ml of the B-cadherin specific mAb 5A6 localizes B-cadherin to points of cell–cell contact in both cell lines (Fig. 2, *A* and *B*). N1C2 cells, transfected with an N-cadherin construct, do not express B-cadherin on their surface as evidenced by the lack of mAb 5A6 labeling (Fig. 2 *C*).

In addition to G418 selection, the B-cadherin–transfected cell lines were selected based on their morphology. Both of the cell lines used in this study, BL-2 and BL-1, make many more intercellular connections than the parental L-cell line (Fig. 2, *D*, *E*, and *G*). Their morphology is much like that of the 5-1 B-cadherin-expressing line previously described (Murphy-Erdosh et al., 1994); they tend to

grow in epitheliod colonies of cells which form many intercellular connections. The morphology of the LCAM-expressing cell line, LL, is quite different from all of the other cadherin-transfected cell lines used in this study. LL cells are spindle-like and have many connections to one another by thin processes (Fig. 2 *F*). The LL cell line was not selected on the basis of morphology. The difference in morphology could either be due to their different parental backgrounds, L-cell clone 2071 for the LL cells and clone 929 for the other cadherin-transfected cells, or random variation in the morphology of L-cell clones.

LL cells express LCAM homogeneously on their surface as determined by immunofluorescence with the polyclonal antibody against LCAM (data not shown). In immunoblot analysis, the Marrs pan-cadherin antibody recognizes a protein of the correct molecular weight for LCAM in LL cells (Fig. 1, lane *3*). The larger molecular weight protein recognized by the pan-cadherin antibody in LL cells is likely to be a preprocessed form of the recombinant LCAM which includes 103 amino acids of the amino-terminal region of NCAM. This preprocessed form is unlikely to be expressed on the surface because it is not degraded when the cells are treated with TE trypsin (data not shown). The level of the mature form of LCAM expressed in LL cells is similar to that of B-cadherin in BL-1 and BL-2 cells.

Aggregating Activity of B-cadherin and LCAM-expressing Cells

The extent to which these cell lines have calcium-dependent aggregating activity, mediated by the cadherins expressed on their surfaces, was determined by the classical methods of Takeichi (1977). When cells are treated with trypsin in the presence of calcium so that the cadherin integrity is maintained, BL-2, BL-1, and LL cells aggregate extensively in calcium containing media (Fig. 2, *H*, *I*, and *J*, respectively), but not at all in media free of calcium (data not shown). This demonstrates that each line expresses functional cadherin at sufficient levels to induce and maintain stable cell contacts. The calcium-dependent aggregating activity of these cells is rapid, with significant aggregation taking place within 15 min and continuing for at least one hour (Fig. 3). BL-2 cells aggregate more quickly and more extensively than either BL-1 or LL cells, which appear to have similar kinetics. The extent of aggregation of BL-1 and BL-2 cells is signficantly different ($P < 0.05$, Student's *t*-test) at both 45 and 60 min, the time used for the mixed aggregation assays, but, the extent of aggregation of BL-1 and LL cells is not statistically different at any time point. EL, PL, NL, and RL cells aggregate with rates similar to that of BL-2 (data not shown). When treated with trypsin and EDTA, neither BL-2, BL-1, or LL cells are able to recover their calcium-dependent aggregation within an hour (data not shown).

The other cadherin transfected cells, EL, NL, PL, and RL, behave in a cadherin-dependent manner as described previously (Nose et al., 1988; Miyatani et al., 1989; Inuzuka et al., 1991). The kinetics of aggregation of all of the lines fall within the range of BL-2 and BL-1, suggesting that all of these lines have similar expression levels of cadherins on their surfaces. Immunoblot analyses with the

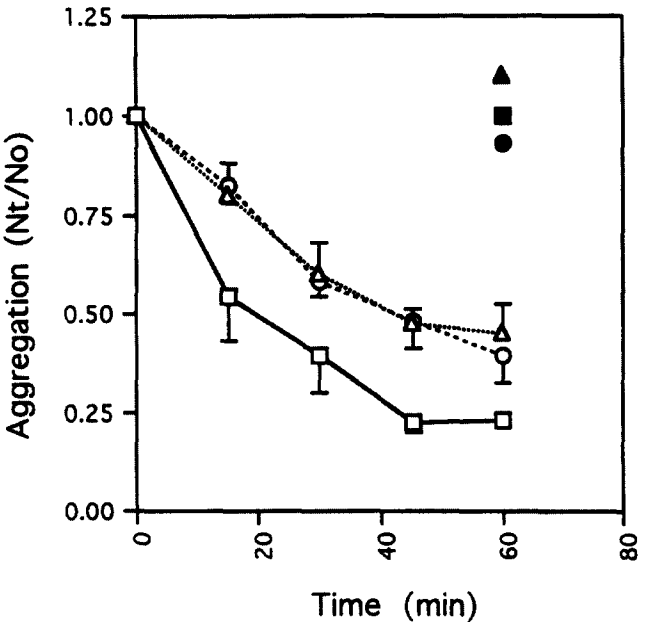

Figure 3. The calcium-dependent aggregation of the transfected cell lines increases over the course of an hour. Single cell suspensions of TC-treated transfected cells were placed on a rotary shaker at a concentration of 2×10^5 cells/ml. At the indicated times, the cells were fixed and the extent of aggregation was analyzed on a Coulter counter. BL-2 cells (□) aggregate more quickly and completely than BL-1 (△) or LL (○) which aggregate in a similar time-dependent fashion. At 60 min, BL-1 and BL-2 cell aggregation is significantly different ($P < 0.05$, Student's *t*-test), however, there is no statistical difference between the aggregation of BL-1 and LL cells. The low levels of aggregation in the absence of calcium for BL-2 cells (■), BL-1 cells (▲), and LL cells (●) are not significantly different from the aggregation at time = 0. Points represent the mean aggregation (± SEM) of three or more experiments. The error bars are shown in only one direction for aesthetic reasons.

Marrs' pan-cadherin antibody and the CADCYTO2 antibody (data not shown) support this hypothesis, demonstrating that BL-1, BL-2, LL, and EL have similar levels of cadherin expression (Fig. 1, lanes *2*, *3*, and *4*, respectively). The expression levels of NL, PL, and RL are similar to the expression levels of EL (Inuzuka et al., 1991). The parental cell line, L929, does not show any detectable aggregation under any of the experimental conditions (data not shown), nor does it express cadherins as evidenced by the absence of protein bands in immunoblot analysis with the pan-cadherin antibodies (Fig. 1, lane *5*).

The aggregation of BL-2 and BL-1 seen in the presence of calcium is dependent upon functional B-cadherin because it can be significantly blocked by the anti-B–cadherin mAb 5A6 in a dose dependent manner (Figs. 2, *K* and *L* and 4 *A*). At high antibody concentrations (60 μg/ml), mAb 5A6 blocks both BL-1 and BL-2 cell aggregation ($P < 0.001$, ANOVA), however, BL-1 cell aggregation is blocked more completely than BL-2 aggregation. The difference between BL-1 and BL-2 cell aggregation at mAb 5A6 concentrations of 60 μg/ml is significant ($n = 5$, $P < 0.001$, ANOVA). Although the reason for this is not clear, it could be due to the significant difference in the amount

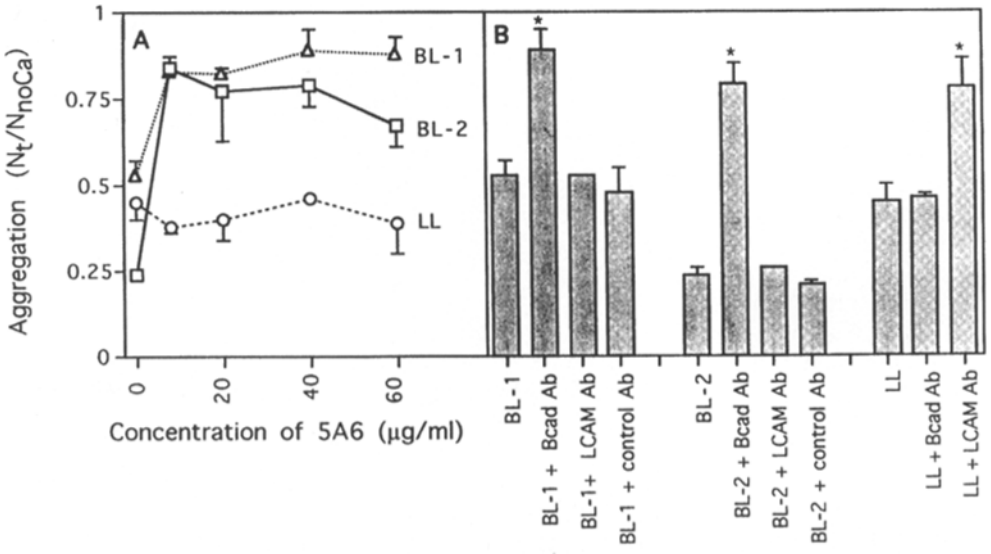

Figure 4. (*A*) The aggregation of B-cadherin–transfected cells, BL-2 (□) and BL-1 (△), is blocked by the antibody against B-cadherin, 5A6. LL (○) cell aggregation is not affected by 5A6. (*B*) Although the B-cadherin antibody 5A6 (60 μg/ml) blocks both BL-1 and BL-2 cell aggregation, neither BL-2 or BL-1 aggregation are affected by the anti-LCAM antibody or the control mAb 1H6. LL aggregation, however, is inhibited by an anti–LCAM antibody where as mAb 1H6 has no effect. Each point represents the mean aggregation + or − the SEM ($n = 4$). The asterisks indicate that differences from the control situation are significant at $P < 0.001$, Student's *t*-test.

of baseline aggregation. It is also possible, at higher antibody concentrations, that the whole IgG may increase aggregation by crosslinking cells together. The allotype-matched control antibody, anti–integrin α_3 mAb 1H6 (40 μg/ml), did not have any effect on the aggregation of BL-2 or BL-1 cells (Fig. 4 *B*) nor did the anti–B-cadherin mAb 5A6 have any effect on the aggregation of LL cells (Fig. 4 *B*), or any of the other cadherin expressing cells used (data not shown).

The aggregation of the LCAM expressing LL cells is blocked by the anti-LCAM antibody ($P < 0.05$, Student's *t*-test) (Figs. 2 *M* and 4 *B*). Neither of the B-cadherin transfected lines (Fig. 4 *B*) nor the E-cadherin–transfected line (see Fig. 7 *F*) were affected by this LCAM specific antibody.

The Binding Specificity of the B-Cadherin Transfectants

To test the binding specificity of B-cadherin, we mixed the B-cadherin–expressing cell lines with each of the other cadherin expressing lines. The two types of cells in each assay were distinguished by the dye with which they were labeled, either DiI, which fluoresces red, or DiO, which fluoresces green. Fig. 5 shows fluorescent photographs of representative aggregates for each mixing conditions. Fig. 6 shows the quantitative analysis of the aggregates of the B-cadherin–transfected cells mixed with each of the other cadherin-transfected cell lines. The type of dye used to label the cells does not effect the amount of mixing (data not shown). In the coaggregation experiments, the BL-2 cell line and the BL-1 cell line behave similarly (Fig. 6). For this reason, we will discuss the results of the two B-cadherin–transfected lines collectively. Both B-cadherin–expressing cell lines mix completely with one another and neither cell line coaggregates with control L929 cells (Figs. 5 and 6). As previously demonstrated (Nose et al., 1988; Miyatani et al., 1989; Inuzuka et al., 1991), EL cells segregate from PL, NL, and RL cells; NL cells segregate from PL cells, but coaggregate with RL cells; and, only occasional L929 cells can be seen mixed in the homophilic aggregates of E-, P-, N-, or R-cadherin transfected cells (data not shown).

When B-cadherin cells are mixed with LCAM-expressing LL cells heterophilic aggregates are produced that cannot be distinguished from homophilic aggregates of either BL-1, BL-2, or LL cells alone (compare Fig. 5, *A* and *C*). The B-cadherin antibody, mAb 5A6, completely blocks the association of B-cadherin cells with LL cells ($P < 0.005$) (Figs. 5 *D* and 6). The anti–LCAM antibody also blocks the interaction of B-cadherin–transfected cells with LCAM-transfected cells ($P < 0.005$) (Figs. 7 *D* and 8). The anti-B–cadherin mAb 5A6 has no detectable effect on the homophilic LL cell aggregation, nor does the anti–LCAM antibody have any effect on B-cadherin–mediated aggregation (Fig. 4 *B*). These data demonstrate that the B-cadherin–expressing cells and the LCAM-expressing cells interact with one another through a heterophilic B-cadherin/LCAM-interaction that is strong enough to induce cell aggregation indistinguishable from homophilic cell aggregation.

Since B-cadherin and LCAM are often co-expressed in chick embryos, the behaviors of these cells in longer term (20 h) aggregation assays were also examined. Results in Table I show that the composition of LCAM-B–cadherin aggregates was very similar to that of LCAM–LCAM aggregates. In neither case was there evidence for segregation of two classes of aggregates consisting primarily of single cell types. Within aggregates, the two cell types were also interspersed (not shown). No evidence for segregation of B-cadherin and LCAM-expressing cells was seen in these experiments, but segregation may occur in different conditions.

B-Cadherin expressing cells also aggregate with the mu-

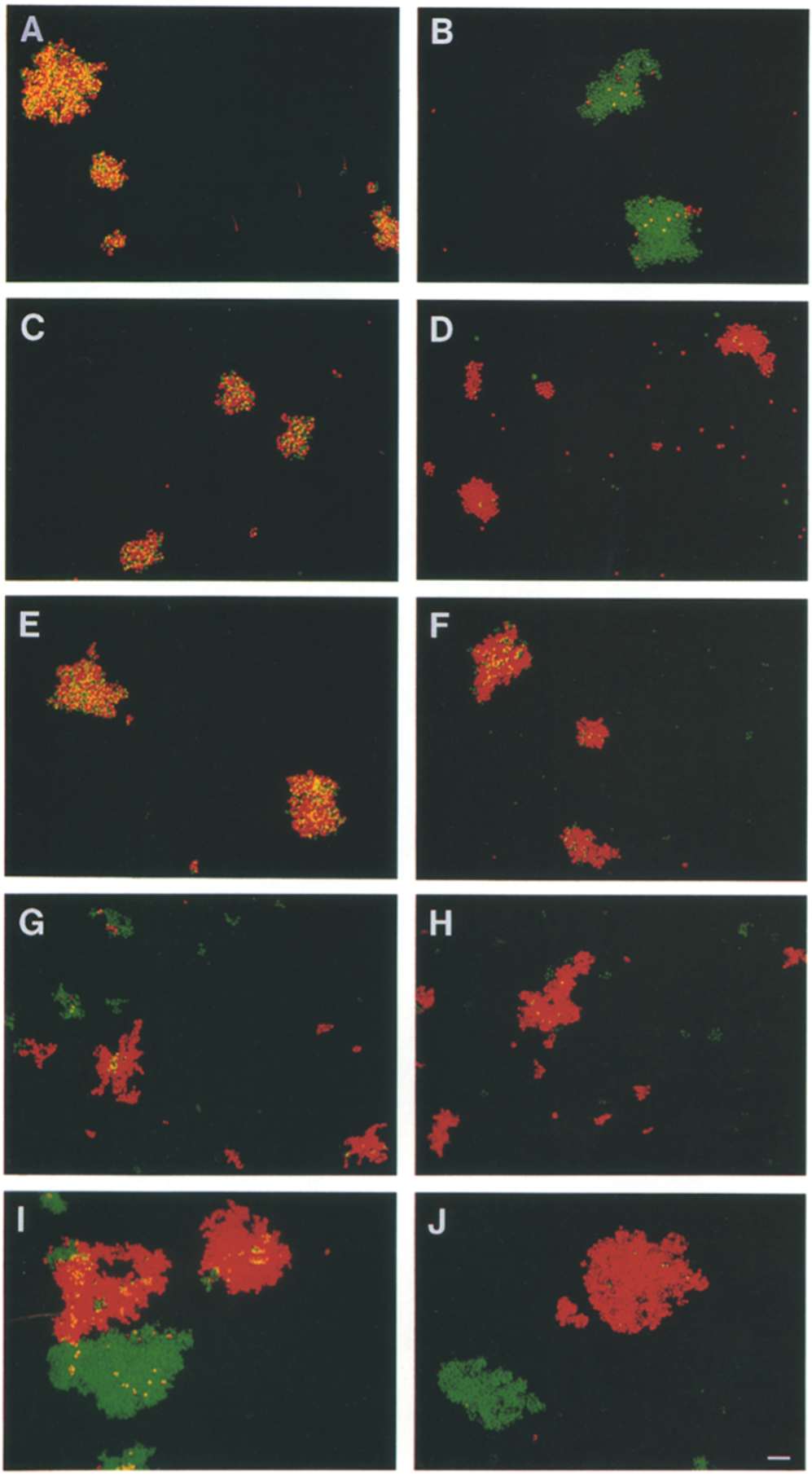

Figure 5. Photographic images of the aggregates formed when mixing two cell types. In all cases, B-cadherin–transfected cells are labeled with DiO and appear green where as the other cells are labeled with DiI and appear red. Yellow cells are the superimposition of red cells with green cells in different planes of focus. (*A*) DiI and DiO labeled B-cadherin–transfected cells form completely mixed aggregates. (*B*) Few L929 cells are mixed in with the BL cell aggregates. (*C*) BL cells and LL cells completely coaggregate. (*D*) The mAb 5A6 blocks the inclusion of B-cadherin–expressing cells in LL cell aggregates. (*E*) BL and EL cells, are completely miscible. (*F*) mAb 5A6 inhibits B-cadherin–transfected cell aggregation and contamination of these cells in the E-cadherin cell aggregates. (*G*) PL and BL cells form separate aggregates. (*H*) mAb 5A6 decreases the number of B-cadherin–expressing cells in the P-cadherin cell aggregates. (*I*) NL cells form aggregates that segregate from the BL cell aggregates. (*J*) RL cells completely segregate from BL cells.

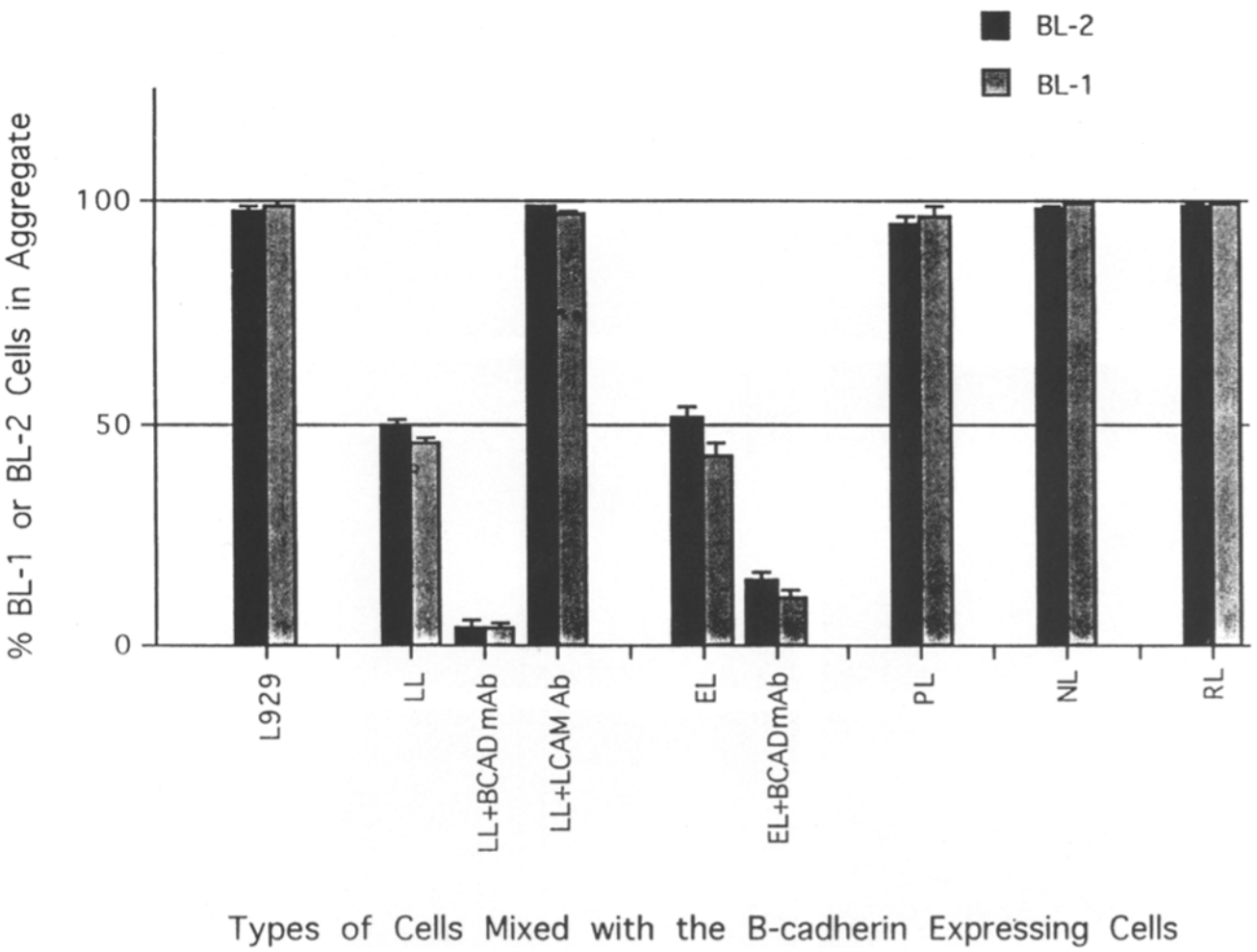

Figure 6. In these coaggregation experiments, two cell populations, one labeled with DiI and another labeled with DiO, were mixed and allowed to aggregate. The DiO and DiI labeled cells in each aggregate were counted. The graph presents the mean percentage of B-cadherin–expressing cells in the aggregates (±SEM). The line at 50% represents the complete coaggregation of a mixture of two populations of B-cadherin–expressing cells, one population labeled with DiI and the other with DiO. Under these conditions, the percentage of one cell population in the aggregate is 50% ± 1%. When two cell populations completely coaggregate, the percentage of B-cadherin–expressing cells in the aggregates is 50%. When the two cell populations segregate, two distinct aggregate populations result; only the data representing the predominantly B-cadherin aggregates are shown.

rine E-cadherin expressing cell line, EL, in short term assays (Fig. 6). Complete mixing can be seen between the B-cadherin and E-cadherin cell lines (Fig. 5 *E*). Again, this is indistinguishable for homophilic cell aggregation. The anti-B–cadherin mAb 5A6 antibody significantly blocks the mixing of B-cadherin cells in EL aggregates ($P < 0.01$) (Figs. 5 *F* and 6).

B-Cadherin expressing cells do not significantly mix with PL, NL, or RL cells in short term assays. There may, however, be weak interactions between B-cadherin and the other cadherins that cause aggregates to attach to one another as seen in Fig. 5, *G* and *I*. Because we generally count attached aggregates as separate aggregates (see Materials and Methods), the contribution of these weak interactions is minimized in the quantitative analysis of the data. Qualitatively, however, in short term assays we observe that B-cadherin cell aggregates often associate with P- and N-cadherin cell aggregates, but rarely with R-cadherin cell aggregates. In longer term (20 h) assays, some mixed aggregates between B- and N-cadherin–expressing cells were seen, in which the individual cell types remained almost entirely segregated within the aggregates (not shown). In these longer term assays, such mixed aggregates were not seen when B-cadherin–expressing cells were mixed with the parent L cells (Table I) or when LCAM and N-cadherin–expressing cells were mixed (not shown). Longer term interactions between B- and P- or E-cadherin–expressing cells were not examined.

The Binding Specificity of LCAM-expressing Cells

Although LCAM expressing LL cells randomly associated with B-cadherin–transfected cells in a cadherin dependent manner, these LCAM expressing cells did not associate randomly with E-cadherin–expressing EL cells in short term assays (Fig. 8). The intermediate mixing seen with LL and EL cells is significantly different from both complete mixing, as seen with homophilic interactions ($P < 0.01$),

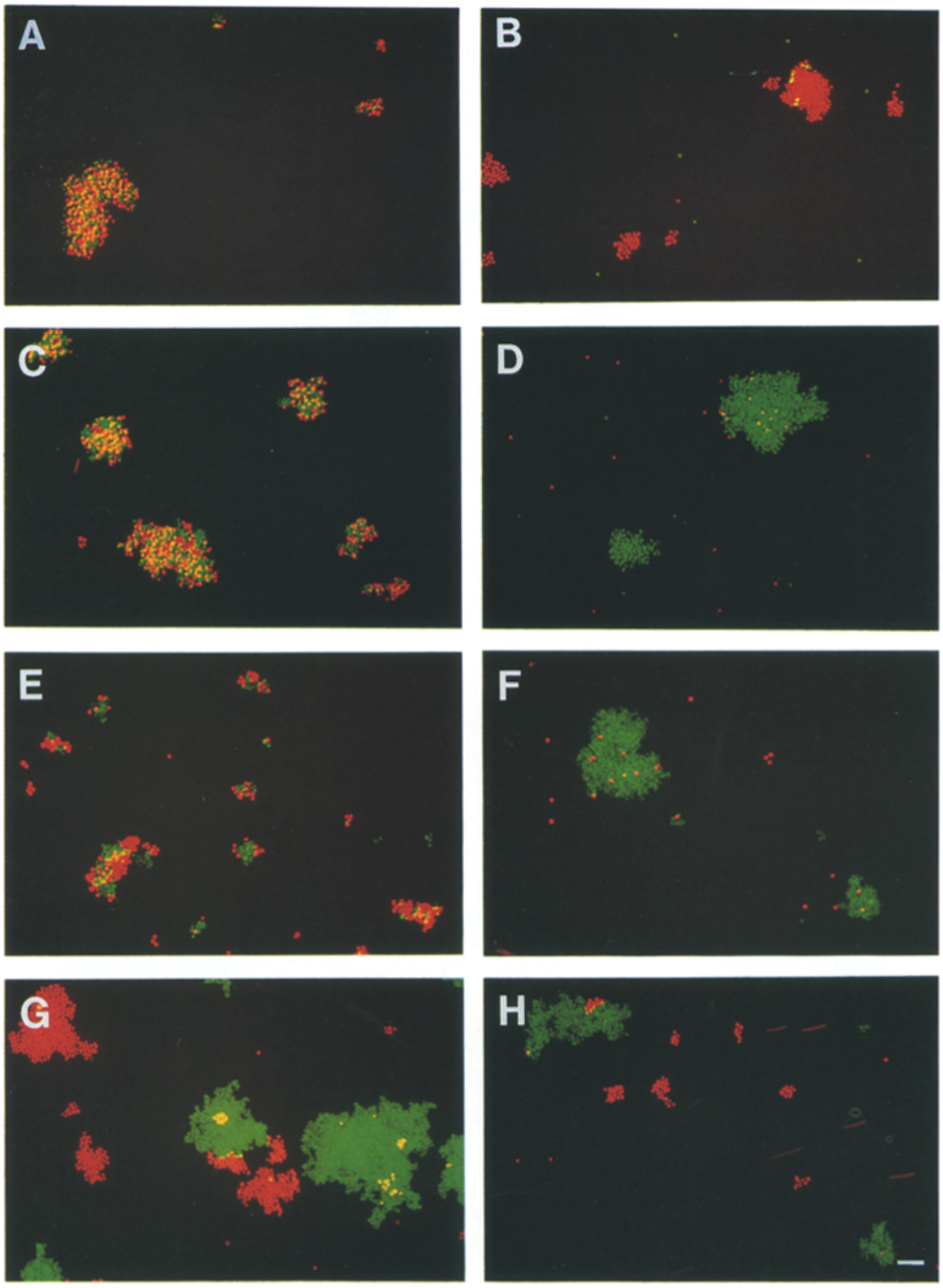

Figure 7. LL cells coaggregate with EL and B-cadherin transfected cells but not with any of the other cell lines tested. LL cells are labeled with DiI and appear red. Other cell lines are labeled with DiO and appear green. When a red and a green cell are above or below one another they will appear yellow. (*A*) DiI and DiO labeled LL cells coaggregate completely. (*B*) L929 cells do not mix into the LL cell aggregates. (*C*) LL and BL-2 cells coaggregate randomly. (*D*) Because the anti-LCAM antibody blocks LL cell aggregation, it inhibits the formation of heterotypic aggregates between BL-2 cells and LL cells. (*E*) LL and EL cells mix with one another partially, forming clusters of aggregates of the two cell types. (*F*) Anti-LCAM antibody blocks the coaggregation of LL and EL cells, so that only EL cell aggregates are present. (*G*) LL and PL cells form separate aggregates. (*H*) LL and NL cells are also immiscible.

and no mixing, as seen with control L929 cells ($P < 0.05$) (Figs. 7, *A*, *B* and *E* and 8). This suggests that the heterophilic interaction of LCAM and E-cadherin is weaker than homophilic interactions or the heterophilic interactions of B-cadherin with either LCAM or E-cadherin. The E-cadherin/LCAM heterophilic interaction appears to be stronger, however, than the tentative interactions seen when homogeneous aggregates attach to one another. The interactions of the EL and LL cells are LCAM dependent; they can be blocked by the LCAM antibody ($P < 0.05$) (Figs. 7 *F* and 8).

Although LL cells do not significantly coaggregate with N-, R-, or P-cadherin–expressing cell lines in short term assays, aggregates of these cell lines do attach to one another, suggesting that weak heterophilic interactions exist between these cadherins. Similar weak interactions between LCAM and N-cadherin were described previously (Volk et al., 1987). As described above, mixed aggregates were not seen in longer term assays in which LCAM-expressing cells were mixed with either parent L cells or N-cadherin–expressing L cells. Longer term assays of possible interactions between LCAM and E-, P-, and R-cadherins were not performed.

Discussion

The data presented here indicate that B-cadherin, not only

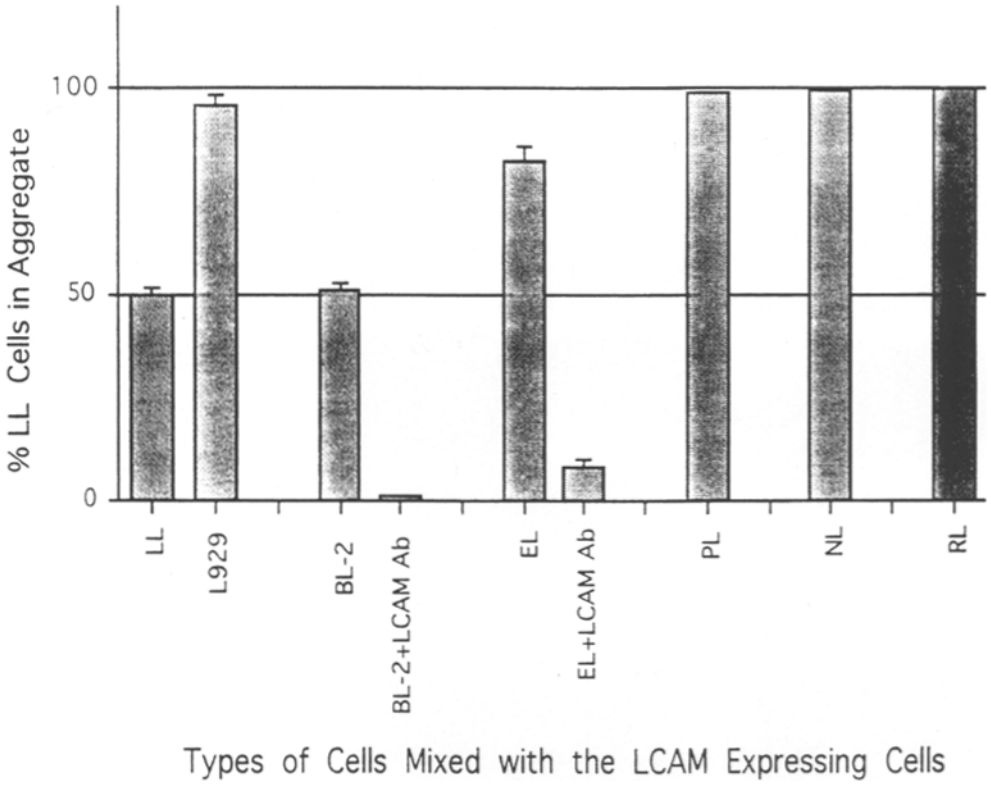

Figure 8. The mean percentage of LL cells in aggregates made up of predominantly LL cells. LL cells form randomly mixed aggregates with themselves and BL cells, but segregate from L929, NL, RL, or PL cells. LL cell coaggregation with BL-2 cells is blocked by the LCAM antibody. LL cells significantly, but incompletely, coaggregate with EL cells; this intermediate level of coaggregation is blocked by the anti-LCAM antibody.

interacts in a homophilic manner, but also binds strongly in a heterophilic manner to LCAM and E-cadherin and associates weakly with P-, N-, and R-cadherin. Strong heterophilic interactions between LCAM and B-cadherin were seen in both short and long term assays. As discussed below these findings have significant implications for possible cadherin/cadherin interactions during development and for identifying the structural basis for cadherin specificity.

The extent of coaggregation of two different cell types depends on the strength of adhesion between the two types (heterophilic adhesion) relative to the strength of cohesion within a type (homophilic adhesion) (Steinberg, 1963). If the strength of heterophilic and homophilic adhesion are approximately equal, then the two cell types will mix randomly, as seen with B-cadherin and LCAM expressing cells or B- and E-cadherin–expressing cells. If homophilic interactions are much stronger than heterophilic interactions, then one would get separation of the two cell types as seen with B-cadherin and P-, N-, or R-cadherin. Intermediate degrees of coaggregation, as observed between E-cadherin and LCAM, would be expected to occur if the strength of the heterophilic adhesion is less than, but not greatly different from, one of the two homophilic interactions.

The relatively strong heterophilic interactions of B-cadherin with LCAM and E-cadherin have not been described for cadherins before. However, weak heterophilic interactions similar to those seen in our experiments have been described (Volk et al., 1987; Inuzuka et al., 1991). The attachment of aggregates of LCAM and N-cadherin–expressing cells is likely to be similar to the heterophilic association of N-cadherin and LCAM necessary for the formation of heterotypic junctions between lens and liver cells described by Volk et al. (1987). Similar weak interactions are seen between many of the cell types used in the coaggregation experiments. Aggregates of R-cadherin–expressing cells rarely interact with LCAM, P-, E-, or B-cadherin–expressing cells. The reason for this difference is not clear.

R-cadherin does, however, interact with N-cadherin (Inuzuka et al., 1991) in a way that resembles the interaction that we observe between LCAM and E-cadherin. The heterogeneous aggregates of N- and R-cadherin expressing cells are composed of clusters of cells of each type, suggesting that this heterophilic interaction is stronger than the interaction between B- and N-cadherin but weaker than homophilic interactions of either cadherin. Although both N- and R-cadherin are present in the nervous system, their precise temporal and spatial patterns of expression have very little overlap (Inuzuka et al., 1991; Redies et al., 1992), consistent with the hypothesis that these cadherins generally function to segregate distinct regions of tissues.

Based on sequence homologies, LCAM has often been identified as the chicken homologue of E-cadherin (Gallin et al., 1987; Ringwald et al., 1987; Crossin et al., 1990; Geiger and Ayalon, 1992). If this were true then one would expect that E-cadherin and LCAM-expressing cells would be completely miscible with one another. However, the LCAM/E-cadherin heterophilic interactions appear to be relatively weak, suggesting that these two molecules are not functional homologues. Others have also questioned the assignment of E-cadherin and LCAM as homologues (Nose et al., 1988; Miyatani et al., 1989; Pouliot, 1992). The species-specific selectivity of liver cells suggests that there may be species-specific cadherins in this tissue. Mouse

liver cells which express E-cadherin and chick liver cells which express LCAM do not adhere well to one another when mixed. On the other hand, mouse and chick brain cells, which both express N-cadherin, do adhere well to one another (Grady and McGuire, 1976). Although LCAM is the closest relative of E-cadherin according to sequence homology, 65%, these two cadherins are not nearly as closely related as the N-cadherins across species, 92 to 94% (Pouliot, 1992).

One might suggest that B-cadherin is the homologue of E-cadherin given the strong heterophilic interactions between B- and E-cadherin and the similar expression patterns of these two adhesion molecules. This too, however, seems unlikely because the sequence homology between B- and E-cadherin is 64%, less than the sequence homology between B-cadherin and the clearly different cadherin, LCAM, 65%. Taken together, these data suggest that either the chicken homologue of E-cadherin has not yet been identified or, as Pouliot (1992) suggested, there are species-specific cadherins and, therefore, not every cadherin has a closely related homologue in other vertebrate species.

It is possible, however, that the differences that we see in mixing between LCAM and E-cadherin–transfected cells could be due to different levels of cadherin expression on their surfaces. Friedlander et al. (1989) and Steinberg and Takeichi (1994) have demonstrated that cadherin-expressing cells sort from one another not only according to the affinity of the cadherin–cadherin interaction, but also according to the amount of cadherin expression on their cell surfaces. Like most other groups using these techniques, we have been unable to determine conclusively the relative amounts of cadherins expressed on the surfaces of the different cell types, however, we detect no differences in overall expression levels in immunoblots with pan-cadherin antibodies. BL-2 and BL-1, which both express B-cadherin, differ in the extent to which they aggregate (Fig. 3), indicating that they may have different levels of expression of B-cadherin on their surface. Even so, when mixed, these two cell lines form completely mixed aggregates, showing no segregation. The two B-cadherin expressing lines also coaggregate equally well with each of the other transfected cell lines, including LCAM-expressing LL cells which have aggregation kinetics similar to BL-1 cells and E-cadherin–expressing EL cells which have kinetics of aggregation similar to those of BL-2 cells. Because the small difference in kinetics between the BL-2 and BL-1 has no effect on the coaggregation of the B-cadherin cell lines, there is no reason to expect this same level of difference to effect EL and LL heterophilic interactions. The amount of cadherin expression, therefore, is not likely to be the cause of the intermediate degree of interaction seen between LCAM and E-cadherin.

The fact that we do not see segregation between the two B-cadherin cell lines that have different aggregation kinetics is not necessarily inconsistent with the results of Friedlander et al. (1989) or Steinberg and Takeichi (1994). The cell sorting assays of these two groups differ from the coaggregation assay presented in this study in many respects. Most significantly our coaggregation experiments are short term relative to the sorting assays of these other groups, which take place over the course of 6 h to 4 d. The long-term, cell sorting assays may favor the establishment of an equilibrium state in which the cells have time to freely migrate to preferred partners. In the coaggregation experiments, the cells do not have this opportunity. This assay therefore is not as sensitive to cell preferences as the sorting assay which detects smaller differences in adhesion.

The heterophilic interactions of B-cadherin described in this study have helped identify the amino acids in the binding region that determine cadherin specificity. Several lines of evidence suggest that the first 113 amino acids of the EC-1 domain of the cadherins are important for the cadherin recognition site. When Nose et al. (1990) analyzed the coaggregation of L-cells transfected with mutant forms of E-cadherin, they found that when they simultaneously mutated the amino acids in positions 78 and 83, flanking the highly conserved HAV tripeptide, the specificity of the cadherin was altered. Our results are consistent with their hypothesis that amino acid 78, is important to the binding specificity of the classical cadherins. However, our results do not support the hypothesis that identity in amino acid 83 is essential for specificity. B-, E-cadherin, and LCAM, which form heterophilic interactions with one another, all have serine in position 78, whereas they differ in the amino acid expressed in position 83 (Fig. 9). P- and B-cadherin have the same amino acid in position 83, but do not interact with one another in aggregation assays. N- and R-cadherin, which have some affinity for one another, also have the same amino acid in position 78 and different amino acids in position 83. These data suggest that the amino acid in position 78, but not 83, is important for conferring specificity of the cadherins. Other amino acids that have not yet been mutated are also likely to play important roles in cadherin specificity.

Tissue segregation and morphogenesis during development are thought to be mediated, in part, by the differential adhesive properties of embryonic cells. It has been hypothesized that the homophilic binding properties of the cadherins confer tissue specificity and mediate tissue seg-

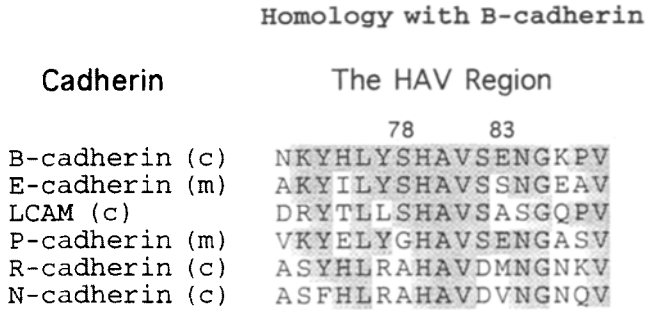

Figure 9. The regions flanking the conserved HAV tripeptide, in particular the amino acids in positions 78 and 83, have been suggested to be important for determining cadherin binding specificity. B-cadherin, E-cadherin, and LCAM have identical amino acids in position 78, confirming that the identity of the amino acid expressed in position 78 could be important for cadherin binding specificities. The identity of the amino acid in position 83, however, does not correlate with the binding specificities of the cadherins, suggesting that this amino acid is not involved in determining specificity.

regation (Takeichi, 1991; Geiger and Ayalon, 1992). In general the tissue distribution of the cadherins supports this hypothesis, however, there are many examples of tissues that express multiple cadherins at the same time. Most notably, LCAM and B-cadherin are often coexpressed in the same tissues during development (Murphy-Erdosh et al., 1994). These two cadherins have the homophilic binding properties of classical cadherins. However, they also bind to each other in a heterophilic manner that appears to be indistinguishable from homophilic adhesion in both short term (1 h) and long term (20 h) assays. If the affinities of the heterophilic and homophilic interactions of B-cadherin and LCAM are in fact similar, as the coaggregation data suggest, then these two adhesion molecules are not likely to be involved in tissue segregation. It is unclear, however, what the function of these two cadherins could be in adjacent regions of functionally distinct tissues where their expression patterns are disparate. In vivo experiments with function-blocking antibodies, and longer-term assays in more stringent conditions, to determine if cells expressing these two cadherins ever segregate from one another, would provide answers to this complex developmental question.

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